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Docosahexaenoic acid (DHA) alters the phospholipid molecular species composition of membranous vesicles exfoliated from the surface of a murine leukemia cell line

E. Eugene Williams *, Benjamin D. May, William Stillwell, Laura J. Jenski

Department of Biology, Indiana University/Purdue University at Indianapolis, 723 West Michigan Street, Indianapolis, IN 46202, USA

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Abstract

Previously, we presented evidence that the vesicles routinely exfoliated from the surface of T27A tumor cells arise from vesicle-forming regions of the plasma membrane and possess a set of lateral microdomains distinct from those of the plasma membrane as a whole. We also showed that docosahexaenoic acid (DHA, or 22:6n-3), a fatty acyl chain known to alter microdomain structure in model membranes, also alters the structure and composition of exfoliated vesicles, implying a DHA-induced change in microdomain structure on the cell surface. In this report we show that enrichment of the cells with DHA reverses some of the characteristic differences in composition between the parent plasma membrane and shed microdomain vesicles, but does not alter their phospholipid class composition. In untreated cells, DHA-containing species were found to be a much greater proportion of the total phosphatidylethanolamine (PE) pool than the total phosphatidylcholine (PC) pool in both the plasma membrane and the shed vesicles. After DHA treatment, the proportion of DHA-containing species in the PE and PC pools of the plasma membrane were elevated, and unlike in untreated cells, their proportions were equal in the two pools. In the vesicles shed from DHA-loaded cells, the proportion of DHA-containing species of PE was the same as in the plasma membrane. However, the proportion of DHA-containing species of PC in the vesicles (0.089) was much lower than that found in the plasma membrane (0.194), and was relatively devoid of species with 16-carbon acyl components. These data suggested that DHA-containing species of PC, particularly those having a 16-carbon chain in the *sn*-1 position, were preferentially retained in the plasma membrane. The data can be interpreted as indicating that DHA induces a restructuring of lateral microdomains on the surface of living cells similar to that predicted by its behavior in model membranes.

Keywords: Membrane microdomain; Membrane exfoliation; Microdomain composition; Docosahexaenoic acid; Shed vesicle; Lipid microdomain; Phospholipid class; Phospholipid molecular species

1. Introduction

An increasing body of evidence indicates that the lipids of biological membranes are not distributed evenly throughout the bilayer. The spatial irregularity of membrane lipid distribution occurs both laterally and across the bilayer (for reviews see [1–7]). Though both types of asymmetry have been docu-

Abbreviations: DHA, docosahexaenoic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; HPLC, high-performance liquid chromatography

* Corresponding author. Fax: +1 (317) 274-2846; E-mail: ewilliam@iupui.edu

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mented, their physiological consequences, and the forces involved in producing and maintaining them, are not well understood. Transbilayer asymmetry of membrane phospholipids is known to be important for certain cell-cell communication events involving macrophages and red blood cells [8–11] and in platelet activation [12–14]. Transbilayer asymmetry of membrane phospholipids is maintained in part by an ATP-utilizing aminophospholipid translocase that transports aminophospholipids from the outer leaflet of the membrane to the inner leaflet [15–17].

Our understanding of lateral asymmetry in cell membranes is less well developed. Lateral asymmetry is believed to include the non-covalent clustering of membrane lipids into a complex patchwork of microdomains [2,3,18,19]. The physiological significance of these lipid microdomains is unclear, but they are probably important structural features of the membrane, and may be important in a number of cellular processes [3,20]. For example, the structure of lipid microdomains is known to influence the activity of protein kinase C [21,22], and is thought to be a key regulator of a variety of other enzymes [21,23–27]. Phospholipid lateral microdomains may also be involved in membrane fusion events, intracellular lipid and protein trafficking, and the modulation of membrane permeability (references in [20]).

Docosahexaenoic acid (DHA, 22:6n-3), common in fish oil, is a member of the n-3 family of fatty acids (i.e. fatty acids having three carbons between the methyl terminus and the last double bond) that is also the longest and most unsaturated fatty acid commonly found in the phospholipids of biological membrane [28]. As a component of cell membranes, DHA influences a variety of membrane functions including the activity of membrane bound enzymes [24,25,29], cell recognition phenomena [30,31], membrane molecular order [29,32], the expression of membrane-bound growth factors [33], membrane permeability to both electrolytes and non-electrolytes [29,34,35], and it promotes membrane fusion [36]. DHA has also proven to be an effective anti-cancer agent [37,38]. It reduces the rate of tumor cell proliferation [39–41], alters the recognition and cytolysis of cancerous cells by the immune system [30,31,34,42], and prolongs life in tumor-bearing animals [38]. DHA also appears to be importantly involved in development [43] and in the adaptation of the membranes of poikilotherms to cold [29]. Furthermore, these effects seem to be characteristic of DHA alone. Other fatty acids, including palmitic, stearic, linoleic, α-linolenic, arachidonic, and eicosapentaenoic acids, are either ineffective or much less effective than DHA at influencing membrane properties and membrane-associated cellular events [30,34,36,38,41,44,45]. Despite these well-documented and apparently unique effects of DHA, their underlying biochemical mechanisms are far from clear.

Though there are a number of ways DHA might influence membrane-associated functions (e.g. [46–49]), it is becoming apparent that many of the actions of DHA are related to its ability to alter membrane lateral microdomain structure [44,50–54]. We recently documented evidence that the small membranous vesicles released from a murine tumor cell line possess a subset of microdomains distinct from those of the plasma membrane as a whole [54]. We also showed that their composition and structure could be significantly altered by DHA [54]. In the present communication we have determined into which phospholipid classes and molecular species DHA is incorporated, and how these different molecular species are distributed between the different subsets of microdomains.

2. Materials and methods

2.1. T27A cell culture

T27A is a non-T, non-B leukemia cell line first isolated from a BALB/c mouse after infection with the Friend murine leukemia virus [55]. These cells normally, and continuously, exfoliate small vesicles into their culture medium. Cells were cultured in spinner flasks containing RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 2 mM glutamine, 25 mM HEPES, 15% bovine calf serum (HyClone Laboratories, Logan, UT), penicillin (100 units/ml), and streptomycin (100 μg/ml). Fatty acid-enriched medium was prepared exactly as described previously [54] with DHA or oleic acid purchased from Nu-Check-Prep (Elysian, MN). Cultures in normal or fatty acid-enriched medium were maintained at 37°C in humidified incubation chambers

flushed with 5% CO_2 in air. Cell density was closely monitored and kept below 2×10^6 cells/ml. For collection of exfoliated vesicles, cells were pelleted by a low speed centrifugation (15 min at $500\times g$), resuspended in fresh medium (normal or fatty acid-enriched), then allowed to exfoliate vesicles for 15 h. In the experiments designed to determine protein exfoliation rates the same procedure was followed except that the cells were allowed to exfoliate for the time indicated in Fig. 1.

2.2. Isolation of plasma membranes and exfoliated vesicles

Plasma membrane and exfoliated vesicles were collected by differential and density gradient centrifugation as described [54]. Briefly, cultures were centrifuged twice at low speed to remove cells before the vesicles were collected from the resulting conditioned medium by centrifugation for either 3 h at $11\,000\times g$ or 45 min at $32\,000\times g$ depending on culture volume. Plasma membranes were isolated [54] using the buffers described by Molnar et al.[56] and the centrifugation method of Kaduce et al. [57].

2.3. Protein and cholesterol assays

The protein content of isolated membrane fractions was assessed using Pierce's (Rockford, IL) Micro BCA protein assay kit and bovine serum albumin as the standard. Cholesterol was assayed using the methods of Contreras et al. [58] exactly as described previously [54]. Briefly, samples were treated with cholesterol oxidase and the area of the peak corresponding to cholestenone (4-cholesten-3-one) was quantified after it eluted from a C-18 high-performance liquid chromatography (HPLC) column.

2.4. Total lipid extraction and phospholipid class separation

Membrane lipids were extracted using chloroform/methanol [59] and quantified by determining total phosphorous according to Chen et al. [60]. The lipid extract was dried under nitrogen and taken up in a minimal volume of normal phase sample solvent (2-propanol/hexane/ethanol [4.9:3.7:1.1, v/v]) [61]. Samples were resolved into neutral lipids and individual phospholipid classes by HPLC on a 250 mm×10 mm Ultrasphere silica column (Beckman, Fullerton, CA) using Beckman System Gold Nouveau hardware and software and the mobile phase solvent gradient system of Wiley et al. [61] (i.e. 100% isopropanol/hexane/ethanol/1 mM ammonium phosphate/acetic acid [490:370:115:25:0.4, v/v] to 100% isopropanol/hexane/ethanol/1 mM ammonium phosphate/acetic acid [490:370:60:80:0.4, v/v]). Detection was at 205 nm. The peaks corresponding to phosphatidylethanolamine (PE) and phosphatidylcholine (PC), which together made up 89.6% of the phospholipids of the plasma membrane (see Section 3), were collected and quantified as above. The mass of the remaining phospholipids was calculated as the difference between the sum of the masses of PE and PC masses and the mass of the total membrane phospholipids.

2.5. Separation of DHA-containing molecular species of PE and PC

Purified PE and PC were dried, taken up in reverse phase sample solvent (methanol/water/acetonitrile [90.5:7.0:2.5, v/v]), and resolved into their constituent molecular species by HPLC on a 4.6 mm×250 mm Ultrasphere ODS (C-18) column using a mobile phase of methanol/286 mM choline chloride/acetonitrile (90.5:7.0:2.5, v/v) [61]. The advantages of using this method include rapid turn around time and minimal sample loss because the lipids are not derivatized before separation. Also, detection at 205 nm is sensitive to the number of double bonds present, making changes in DHA content easy to identify. Peaks corresponding to DHA-containing species were identified by their large increase in absorbance after culture in DHA-enriched medium. These and the other peaks were also identified by (a) comparison to authentic standards [synthesized by us and purchased from Avanti Polar Lipids (Alabaster, AL) and Sigma (St. Louis, MO)], (b) comparison of observed retention times to published values [61], and (c) gas chromatography as described elsewhere [54]. Peak areas were converted into mass by pre-determined correction factors specific to each phospholipid species [61].

2.6. Statistics

Paired data were compared using a Student's *t*-test. Multiple comparisons were made with one-way, two-tailed analysis of variance and Bonferoni's post-hoc test using Instat software (GraphPAD Software, San Diego, CA). Percentage data were arcsine transformed before statistical analysis [62].

3. Results

DHA is known to be an effective anti-cancer agent against a number of tumor types (e.g. [38,63–65]), including T27A murine leukemia [38,41]. In the experiments described here, cells of the treatment group were cultured in medium that had a concentration of DHA (0.152 mM, bound to albumin) low enough to allow the cells to survive and proliferate at a rate indistinguishable from that of cells cultured in normal growth medium, but which resulted in substantial incorporation of the fatty acid into the phospholipids of their membranes [41,54].

The amounts of cholesterol and total phospholipid found in these membrane fractions were indistinguishable from those determined previously [54]. Treatment of T27A cells with DHA resulted in similar reductions in the ratios of phospholipid to protein and cholesterol to protein in the plasma membrane, yet did not influence the ratio of cholesterol to phospholipid there (Table 1). Cells treated with DHA also exfoliated significantly less protein in vesicles than cells in normal culture (Fig. 1). The same treatment using oleic acid (18:1) instead of DHA did not result in a reduced rate of protein exfoliation after 9 h (Fig. 1). DHA treatment reduced both the initial rapid phase of protein shedding (0–1.5 h) and the subsequent, less rapid phase of release (1.5–48 h).

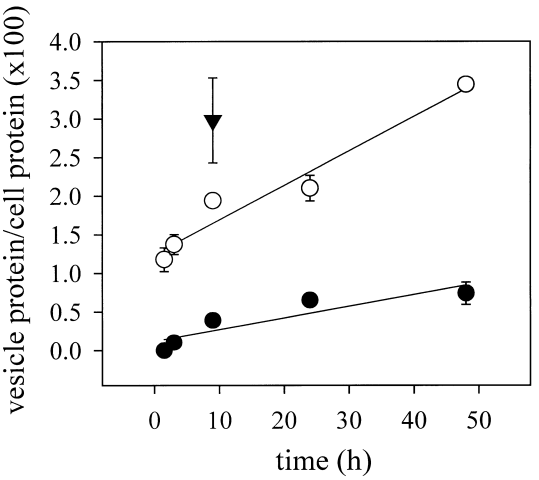

Fig. 1. The rate of protein exfoliation in the form of vesicles from T27A murine leukemia cells. The data are expressed as the ratio of protein in vesicles to protein in whole cells in order to account for minor differences in cell proliferation rates in the different cultures. Cells cultured in DHA-enriched medium (filled circles) exfoliated protein at a significantly lower rate than cells cultured in normal medium (open circles). Cells treated with oleic acid (triangle) did not exhibit a reduced rate of vesicle protein exfoliation. Each point represents the mean and S.E.M. of three independent cultures.

After 15 h of culture, the pooled vesicles from untreated cells (i.e. cells cultured in normal medium) had significantly different phospholipid to protein,

Table 1
The composition of plasma membrane and exfoliated vesicles of T27A murine leukemia cells cultured in normal (−DHA) and DHA-enriched (+DHA) media

	Plasma membrane		Exfoliated vesicles	
	−DHA	+DHA	−DHA	+DHA
PL[c]/protein (mol/μg)	54.2 ± 13.65^{a}	20.0 ± 3.94	6.0 ± 0.52^{a}	14.0 ± 1.31
Cholesterol/protein (mol/μg)	$7.7 \pm 1.22^{a,b}$	2.6 ± 0.14^{b}	3.2 ± 0.55^{a}	2.6 ± 0.61
Cholesterol/PL (mol/mol)	0.14 ± 0.020^{a}	0.13 ± 0.022	$0.53 \pm 0.138^{a,b}$	0.19 ± 0.053^{b}
PE/PC[d] (mol/mol)	0.63 ± 0.007	0.65 ± 0.104	0.37 ± 0.067	0.61 ± 0.130

Values are the mean ± S.E.M. for three or more independent cell cultures. Values in a row with the same superscript letter are significantly different ($P < 0.05$). Statistical analyses were not performed between the comparisons (plasma membrane −DHA and exfoliated vesicles +DHA) and (plasma membrane +DHA and exfoliated vesicles −DHA).

[c]Phospholipid.

[d]The ratio of phosphatidylethanolamine to phosphatidylcholine.

Table 2
The molecular species composition of the PC pool in plasma membrane and exfoliated vesicles of T27A cells[a]

Total chain length	Molecular species	Plasma membranes		Exfoliated vesicles	
		−DHA	+DHA	−DHA	+DHA
34	16:0/18:1n-9[b]	35.6	22.7	22.4	22.0
	16:0e/18:1n-9[c,d]	2.7	0.0	18.6	10.1
	16:0/18:2n-6[b]	16.2	10.7	10.9	7.5
36	18:0/18:1n-9[b]	7.3	1.1	4.5	16.7
	18:0/18:2n-6[d]	17.2	9.1	23.0	16.0
	18:1n-9/18:2n-6[b]	6.2	3.7	3.2	2.5
38	16:0/22:6n-3[b]	0.4	5.2	0.2	1.7
	16:1n-7/22:6n-3[d]	0.2	2.6	0.1	0.5
	18:0/20:4n-6[d]	1.3	1.1	3.0	2.1
40	18:0/22:6n-3[b]	1.0	6.7	1.2	4.7
	18:1n-9/22:5n-6[d]	0.7	1.1	1.1	1.0
	18:1n-9/22:6n-3[d]	0.7	2.5	0.7	1.6
	18:2n-6/22:6n-3[d]	0.4	2.4	0.0	0.4
	unidentified[e]	0.0	17.7	1.4	0.0
Total		89.9	86.6	90.3	86.8

[a]The data represent the average values of two independent cell cultures for each membrane preparation.
[b]Molecular species with this superscript were identified by comparison to authentic standards and/or by gas chromatography of their fatty acid methyl esters.
[c]e indicates an ether linkage (i.e. plasmalogen).
[d]The identity of these species was inferred by comparison of their retention times to those in [61] using 16:0/16:1n-7, 16:0/18:1n-9, 16:0/18:2n-6, 16:0/20:4n-6, 16:0/22:6n-3, 16:1n-7/16:1n-7, 18:0/18:1n-9, 18:0/22:6n-3, 18:1n-9/18:1n-9, and 18:2n-6/18:2n-6-PCs as reference points.
[e]The identity of this molecular species could not be verified. Its retention time was closest to that of 16:1/18:1-PC.

cholesterol to protein, and cholesterol to phospholipid ratios than the plasma membrane from which they arose (Table 1). In contrast, cells enriched with DHA released vesicles in which all of these ratios were statistically indistinguishable from those of the plasma membrane (Table 1). DHA treatment significantly reduced the cholesterol to protein ratio in the plasma membrane, but the other ratios remained unchanged (i.e. Bonferroni-corrected $P \geq 0.05$, Table 1).

The plasma membrane of untreated cells was composed of 55.0% PC, 34.6% PE, and 10.4% of the remaining phospholipid classes, mostly phosphatidylserine, phosphatidylinositol, and phosphatidic acid. Because PC and PE together made up almost 90% of the total phospholipids, the ratio of PE to PC provided a sensitive indicator of changes in the phospholipid class composition of these membranes. Comparison of PE to PC ratios (Table 1) revealed that normally cultured cells exfoliated vesicles that had the same PE to PC ratio as the parent plasma membrane. Though this ratio tended to be lower in vesicles compared to plasma membrane, the difference was not statistically significant. Culture in DHA-enriched medium did not significantly alter the PE to PC ratio in either the plasma membrane or the shed vesicles (Table 1).

The molecular species composition of the PC and PE pools of plasma membrane and exfoliated vesicles from untreated and DHA treated cells are shown in Tables 2 and 3. Each membrane preparation was characterized by a unique assortment of molecular species of both PC and PE. In all membrane preparations, the three most abundant species of PC made up more than 50% of the total PC pool, and approximately 90% of this pool was divided among the 14

Table 3
The molecular species composition of the PE pool in plasma membrane and exfoliated vesicles of T27A cells[a]

Total chain length	Molecular species	Plasma membranes		Exfoliated vesicles	
		−DHA	+DHA	−DHA	+DHA
34	16:0/18:1n-9[b]	10.8	5.7	25.7	14.1
	16:0/18:2n-6[c]	6.5	18.7	4.3	9.9
36	16:0/20:3n-6[c]	8.7	10.5	9.6	11.3
	18:0/18:1n-9[b]	4.4	0.0	1.5	0.0
	18:0/18:2n-6[b]	18.1	26.5	5.8	24.9
	18:1n-9/18:2n-6[c]	4.6	1.8	4.2	2.7
38	16:0e/22:5n-3[c,d]	4.5	2.0	4.1	1.6
	16:0/22:6n-3[c]	0.4	0.2	0.1	0.0
	16:1n-7/22:6n-3[c]	0.0	0.8	0.3	0.0
	18:0/20:3n-9[c]	7.1	2.3	0.2	2.7
	18:0/20:4n-6[c]	8.3	3.4	11.7	2.4
	18:0/20:5n-3[c]	3.1	3.1	2.6	3.5
40	18:0/22:5n-3[c]	4.4	0.1	4.3	0.0
	18:0/22:5n-6[c]	2.3	1.1	5.2	1.8
	18:0/22:6n-3[b]	4.8	17.8	4.5	17.6
	18:1n-9/22:6n-3[c]	1.0	2.5	0.0	2.5
	18:2n-6/22:6n-3[c]	0.2	0.6	0.0	0.0
Total	89.2	97.1	84.1	95.0	

[a]The data represent the average values of two independent cell cultures for each membrane preparation.
[b]Molecular species with this superscript were identified by comparison to authentic standards and/or by gas chromatography of their fatty acid methyl esters.
[c]The identity of these species was inferred by comparison of their retention times to those in [61] using 16:0/18:1, 18:0/18:1n-9, 18:0/22:6n-3, 18:1n-9/18:1/n-9, 18:3n-6/18:3n-6, and 22:6n-3/22:6n-3-PEs as reference points.
[d]e indicates an ether linkage (i.e. plasmalogen).

species listed in Table 2. The remaining PC was dispersed among 15 other molecular species that occurred in much smaller proportions. DHA was associated with five molecular species of PC (16:0/22:6n-3-PC, 16:1n-7/22:6n-3-PC, 18:0/22:6n-3-PC, 18:1n-9/22:6n-3-PC, and 18:2n-6/22:6n-3-PC, Table 2), most of which were present in all four membrane preparations (Table 2). Combined, the DHA-containing species of PC made up 2.7% of the PC pool in the plasma membrane of untreated cells and 2.2% in the vesicles exfoliated from them. After DHA treatment, these values increased to 19.4% in the plasma membrane and 8.9% in exfoliated vesicles (Fig. 2).

In the PE pools, the 17 molecular species shown in Table 3 accounted for 84.1 to 97.1% of the total. DHA associated with the PE pools was found incorporated into the same five acyl chain combinations that were found in the PC pool (i.e. 16:0/22:6n-3-PE, 16:1n-7/22:6n-3-PE, 18:0/22:6n-3-PE, 18:1n-9/22:6n-3-PE, and 18:2n-6/22:6n-3-PE, Table 3). DHA appeared to be preferentially incorporated into PE over PC as PE contained it in larger proportions both under normal and DHA-enriched culture conditions. DHA-containing species of PE made up 6.4% of the total PE pool in the plasma membrane of untreated cells, and 4.9% in exfoliated vesicles. DHA treatment increased these values to 21.9 and 20.1%, respectively (Fig. 2).

Fig. 2 reveals that in the plasma membrane of untreated cells, PE contained more than twice the proportion of DHA-containing molecular species as did PC. This was also true in the vesicles exfoliated from untreated cells. After DHA treatment, the propor-

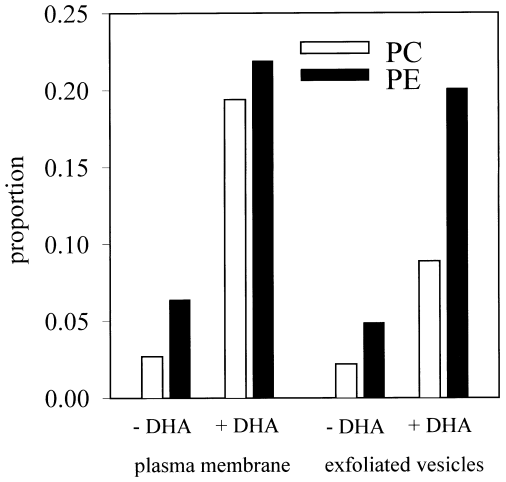

Fig. 2. The proportion of DHA-containing molecular species of PC and PE in the plasma membrane and exfoliated vesicles from T27A cells cultured in normal (−DHA) and DHA-enriched (+DHA) media.

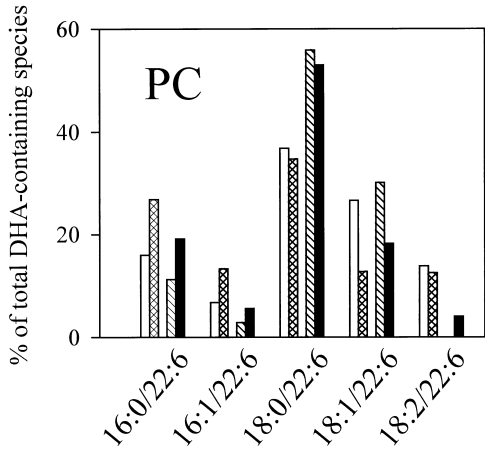

Fig. 3. The distribution of DHA among the five molecular species of PC in which it was found. The data are expressed as the percentage of the total DHA-containing species represented by each indicated species in the plasma membrane and exfoliated vesicles of untreated T27A cells (plasma membrane, open bars; exfoliated vesicles, hatched bars) and cells cultured in DHA-enriched medium (plasma membrane, cross-hatched bars; exfoliated vesicles, filled bars). The sum of the values for each membrane preparation equals 100.

tion of DHA-containing species in the plasma membrane increased dramatically in both the PE and PC pools, and unlike the control cells, the proportions of DHA-containing species present in the two pools were the same. While the plasma membrane and exfoliated vesicles from DHA treated cells contained similar proportions of DHA-containing species of PE, exfoliated vesicles contained sharply lower proportions of DHA-containing species of PC than the plasma membrane. These data suggest that DHA-containing species of PC were preferentially excluded from the vesicle forming regions of the plasma membrane, or stated differently, were preferentially retained by the plasma membrane.

Culturing T27A cells in DHA-supplemented medium did not result in the formation of any additional species of PC or PE; i.e. only those five species detected in the untreated cells were found in the DHA treated cells. However, in both untreated and DHA-treated cells, DHA was not distributed randomly among those species. In the PC pool of the plasma membrane of untreated cells, DHA was found predominantly paired with 18:0 and 18:1 (Fig. 3). In the vesicles shed from untreated cells, this predominance was even more conspicuous as 18:0/22:6n-3-PC and 18:1n-9/22:6n-3-PC together made up 86% of the DHA-containing molecular species of PC (compared to 63% in the plasma membrane). This suggests that 16-carbon species of PC were preferentially retained in the plasma membrane. After culture in DHA-enriched medium, the distribution of DHA among the molecular species of PC in the plasma membrane was shifted towards species

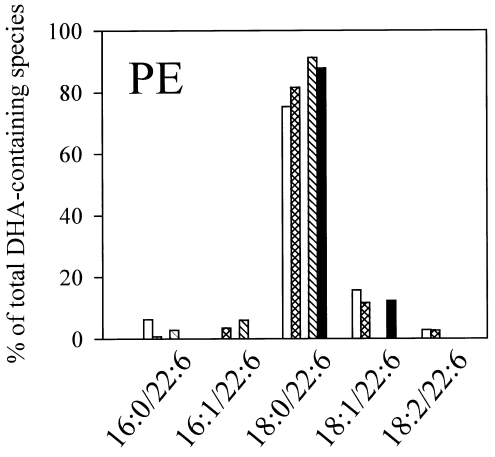

Fig. 4. The distribution of DHA among the five molecular species of PE in which it was found. The data are expressed as the percentage of the total DHA-containing species represented by each indicated species in the plasma membrane and exfoliated vesicles of untreated T27A cells (plasma membrane, open bars; exfoliated vesicles, hatched bars) and cells cultured in DHA-enriched medium (plasma membrane, cross-hatched bars; exfoliated vesicles, filled bars). The sum of the values for each membrane preparation equals 100.

having a 16 carbon chain in the *sn*-1 position (Fig. 3). The predominance of 18:0/22:6n-3-PC remained, but 16:0/22:6n-3-PC and 16:1/22:6n-3-PC replaced 18:1/22:6n-3-PC as the next most abundant species. This same shift in the distribution of DHA was observed in the vesicles released from DHA-loaded cells (Fig. 3).

The distribution of DHA among the PE molecular species was markedly different from that seen in the PC pools (Fig. 4). Seventy-five to 91% the DHA-containing species of PE in all membrane preparations was represented by a single species: 18:0/22:6-PE. Notably, the proportion of this species was lowest (75%) in the plasma membrane of untreated cells, and highest (91%) in the vesicles shed from those cells. Thus, cell culture in DHA-enriched medium resulted in slightly higher proportions of 18:0/22:6n-3 in the plasma membrane and slightly lower proportions in the exfoliated vesicles.

4. Discussion

Recently, we showed that the vesicles released from the surface of T27A murine leukemia cells arise from non-random regions of the plasma membrane that are distinct in both structure and lipid composition from the plasma membrane as a whole [54]. Culturing these cells in DHA-enriched medium, using the same methods described here, resulted in a 10-fold increase in the DHA content of the plasma membrane, and a change in the lipid composition and structure of the exfoliated vesicles that was consistent with the vesicles being made up of a distinct subset of lateral domains [54]. Here, we have extended our analysis to included the phospholipid class and molecular species composition of these membranes.

The nearly identical reduction in the phospholipid to protein and cholesterol to protein ratios in the plasma membrane of cells treated with DHA, along with the observation that this treatment does not change the cholesterol to phospholipid or PE to PC ratios (Table 1), suggests that incorporation of DHA into phospholipids of the plasma membrane increases the protein content of that membrane. While it is well known that membrane lipid composition can influence the activity of membrane-associated enzymes [66–68], and the lateral distribution of membrane proteins [2–4], total protein content is less frequently considered. Peck et al. [69] found that modification of cell lipid composition using the same technique we used in the present study caused an increase in the protein content of the plasma membrane of murine lymphocytes. They suggested that this effect was due to either increased expression of protein or decreased shedding of protein-rich vesicles. Our data confirm the involvement of the latter pathway, but do not exclude the possibility of increased expression.

Incorporation of DHA renders the ratios of phospholipid to protein, cholesterol to protein, and cholesterol to phospholipid in exfoliated vesicles indistinguishable from those in the parent plasma membrane. It also changes the phospholipid molecular species composition of the putative microdomains. The non-random distribution of these membrane components between exfoliated vesicles and their source membrane suggests that there is some mechanism responsible for partitioning membrane components into different regions of the membrane. Though the nature of this mechanism is unknown, the data presented here and elsewhere [53,54,70] suggest that it is readily influenced by the DHA content of the membrane. To date, no enzyme has been discovered that segregates individual phospholipids into patches. Instead, it is thought that microdomains are formed by passive, non-ideal lipid-protein and lipid-lipid interactions [2,4,20,54,71]. For example, in model membrane systems containing no protein, even the simplest mixtures of phospholipids can form microdomains. Membranes composed of a single phospholipid exhibit cluster formation [72,73], and when two lipid species are mixed, even those with the same head group and fully saturated acyl chains do not mix ideally [74]. Additional complexity in phospholipid structure leads to additional complexity in non-ideal mixing [75,76]. DHA-containing phospholipids appear to be among those particularly effective at inducing microdomain formation [53,54,70].

In the plasma membrane of untreated T27A cells, DHA makes up a larger proportion of the PE pool than the PC pool (Fig. 2). A similar disparity in DHA distribution between phospholipid classes has been observed in the total cell membranes of these

cells [41]. After culture in DHA-enriched medium, the plasma membrane has equal proportions of DHA in the PE and PC pools (Fig. 2), and DHA-containing species of PC appear to be preferentially retained in the plasma membrane while DHA-containing species of PE are exfoliated in proportion to their content in the plasma membrane. It is interesting to note that when these cells are fused with liposomes made of DHA-containing species of PE they grow normally, but when fused with liposomes made of DHA-containing species of PC they show a dose-dependent decrease in cell viability [41]. Those and other data [30,38] suggest that these tumor cells can accumulate large quantities of DHA in PE with no harmful effects, but modest quantities of DHA in PC are toxic. We do not know the distribution of DHA among the phospholipid classes of the plasma membrane when DHA is present in the culture medium at high enough concentrations to be toxic, but if it similar to that shown here, the anti-tumor effects of DHA [30,34,41,63] may involve the retention of DHA-containing species of PC in the plasma membrane.

The phospholipid class into which DHA is incorporated can have a significant influence on the functional properties of the resulting phospholipid. For example, DHA-containing species of PC influence thromboxane and prostaglandin receptors, but DHA-containing species of PE do not [77]. The microdomain-inducing effects of DHA probably also depend on the phospholipid class in which it occurs. Cholesterol tends to preferentially interact with PC over PE, and thus microdomains of PC preferentially sequester cholesterol, while microdomains of PE do so much less effectively [78–80]. The vesicles exfoliated from T27A cells after culture in DHA-enriched medium contain dramatically less cholesterol than those released from untreated cells [54]. Cholesterol is known to interact unfavorably with polyunsaturated species of phospholipid [81–83], including those containing DHA [53,84]. Thus, it can be predicted that DHA-containing species of PE are among those least likely to intermix with cholesterol. The data presented here can, therefore, be interpreted as reflecting DHA-induced alterations in the microdomain structure of the plasma membrane of these cells.

It is also noteworthy that the selective retention of DHA-containing species of PC in the plasma membrane of untreated cells seems to favor those species with 16-carbon chains in the *sn*-1 position, and that 18:0/22:6n-3-PE overwhelmingly predominates the PE pools of both plasma membrane and exfoliated vesicles. The significance of these observations is unclear because there is little known about the relative effects of different DHA-containing species of phospholipid on membrane-associated cell functions. For example, while it is known that 18:0/22:6n-3-PC is highly cytotoxic and 18:0/22:6n-3-PE is not [30,41], and that the cytotoxicity of 18:0/22:6n-3-PC is unique among species of PC having 18:0 in the *sn*-1 position and other n-3 and n-6 fatty acids in the *sn*-2 position [85], the effects of DHA paired with PCs having other fatty acyl groups at the *sn*-1 position are unknown. The microdomain-inducing properties of these other DHA-containing species are also unknown. It has been suggested that specific DHA-containing species of phospholipid are involved in the low temperature acclimation of membrane function in poikilotherms [29], and low temperature acclimation may involve the restructuring of membrane microdomains [20], however, no direct evidence of this has accumulated so far.

In summary, we have found that the vesicles exfoliated from the surface of T27A murine leukemia cells arise from non-random regions of the plasma membrane, and that they probably represent a different subset of membrane lateral microdomains than is present on the parent plasma membrane as a whole. Culturing the cells in DHA-enriched medium results in a large increase in the DHA content of the phospholipids of the plasma membrane and exfoliated vesicles, but does not alter their phospholipid class composition. DHA does have a profound effect on the cholesterol, protein, and phospholipid molecular species composition of exfoliated vesicles. These results are consistent with DHA inducing a reorganization of the microdomain structure of the plasma membrane in living cells.

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