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CHANGES IN LIPID COMPOSITION OF NEUROBLASTOMA C1300 N18 CELL DURING DIFFERENTIATION

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Abstract—Phospholipids and cholesterol were found to be the main lipids in mature and immature neuroblastoma cells. The ratios for the total cholesterol/phospholipids in these undifferentiated and differentiated cells were 0.33 and 0.52, respectively. The ratios of 0.45 and 0.62 were obtained with corresponding plasma membrane fractions. Individual fatty acid contents in the loosely bound lipid fraction were higher than in tightly bound lipids. The total levels of saturated fatty acids increased in both of these fractions. While arachidonic acid content significantly decreased, it increased simultaneously (600%) in the free fatty acid fraction during differentiation. The amount of cholesterol esters increased three-fold as a result of maturation.

For the first time it was possible to detect, in neuroblastoma cells, several lipids, namely *N*-acylphosphatidylethanolamine, *N*-acylethanolamine and semilysobisphosphatidic acid. They all changed during maturation. Total *N*-acylphosphatidylethanolamine content decreased by 50%, disappearing completely from membrane fractions. *N*-Acylethanolamine disappeared from the cell as well as from membrane fractions. On the other hand the total cellular content of semilysobisphosphatidic acid increased without any alterations in its membrane content.

Functional implications of our investigations are discussed.

Neuroblastoma is a tumor which may spontaneously regress.[7,19] This is an interesting process but very little is known about its mechanism. That is why neuroblastoma cells adapted to the *in vitro* conditions are widely used as a model for studying malignant cell differentiation.

Generally there are a few per cent of spontaneously differentiating cells in cell culture of mouse or human neuroblastoma. These cells possess certain morphological, electrophysiological and biochemical properties of a mature neuron. In order to induce morphological and biochemical changes within the same cell population, inhibitory agents of cell proliferation are used. Many reports indicate that cAMP plays a key role in neuroblastoma maturation. An elevation of the intracellular level of cAMP by dibutyryl cAMP, prostaglandin E or by inhibitor of cAMP phosphodiesterase induces many differentiated functions of mature neurons.[40,42,43]

Differentiation of mouse neuroblastoma cells can also be induced by 5′-bromodeoxyuridine,[8,59] dimethylsulfoxide,[24] serum-free medium,[51] medium supplemented with delipidated serum,[32] X-ray-irradiation,[41] nerve growth factors,[26,54] gangliosides[16] and many other agents.[27,31,38,44,48]

The properties of neuroblastoma cells differentiated by these agents have been described.[23,42] However, we have little information until now about lipid composition of mature and immature neuroblastoma cell.[4,32,62] Comprehensive data are available for other cell lines.[22,47] Some papers deal with lipid composition and lipid metabolism in neurons and neuroblastoma cells.[5,6,9,60–65] However, there is no correlative information about changes in lipid content during differentiation.

The aim of the present investigation was to perform detailed studies on neuroblastoma cell lipid composition during maturation. We endeavored to determine the content of phospholipids, cholesterol, cholesterol esters, the content of glycerols, free fatty acids and acyl chains in the individual lipids. The fraction of lipids tightly and loosely bound with proteins in mature and immature cells were also characterized.

Experiments from many laboratories have reported that different agents which caused morphological cell differentiation induced unidentical biochemical changes in cells.[17,30,52] From this we studied the effect of some agents on the main lipid cell component, phospholipids, in comparative aspect. In these experiments the dibutyryl cAMP, 5′-bromodeoxyuridine, dimethylsulfoxide, serum-free medium and gangliosides were used. All other experiments were performed with 5′-bromodeoxyuridine as a differentiating agent.

EXPERIMENTAL PROCEDURES

Cell culture

Neuroblastoma C1300 N18 cells were cultured in the presence of 10% bovine serum at 37°C in Eagle's medium. To induce cell differentiation, 5′-bromodeoxyuridine, di-

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methylsulfoxide and dibutyryl cAMP were added to the culture medium in concentrations of 4×10^{-5} M, 2% and 1×10^{-3} M, respectively. Serum-free medium supplemented with 50–100 μg/4 ml bovine brain gangliosides was also used. The cells were used for the experiment on the fourth day of cultivation. They were suspended in 0.9% sodium chloride, centrifuged at 2000 r.p.m. at 4°C and homogenized.

Assay methods

Microthin layer chromatography. Lipid extracts were prepared from cell homogenates by the method of Bligh and Dyer.[3] Phospholipids were analysed by thin layer two-dimensional chromatography on silica gel plates (60×60 mm^2) by using the chloroform–methanol–benzene–28% ammonia (65:30:10:6) and the chloroform–methanol–benzene–actone–acetic acid–water (70:30:10:4:5:1)[2,55] as solvent systems.

To detect small amounts of phospholipids, molybdate and malachite green spray reagents were used.[56,57] Detection and identification of *N*-acylphosphotidylethanolamine was done in special chromatographic solvent systems.[21,58]

Gas–liquid chromatography. Quantitative determination of *N*-acylethanolamine was performed on gas chromatograph GC-9A (Schimadzu, Japan) with synthetic standards using FQ-capillary column 0.2×25 (mm i.d. × m) with chemically bound OV-101. Column temperature was 285°C; the temperature of detector and of injector was 295°C. The rate of flow of carrier-gas (Helium) was 65 ml/min, and that of hydrogen was 30 ml/min.

Cholesterol, cholesterol esters, free fatty acids and glycerols were quantitatively determined on gas chromatograph "Chrom-5" (CSSR) with dual flow system DIP. Column of 3×0.5 (mm i.d. × m) containing OV-1, 1.5% on Shimalite 80–100 mesh (Shimadzu, Japan) was used. The column temperature programme was between 100 and 350°C with a rate increase of 2°C/min. Injector and detector temperature was 350°C, the rate of flow of carrier gas (helium) was 100/min, the rate of flow of hydrogen was 28 ml/min.

The delipidized protein pellets were extracted twice with chloroform–methanol (1:1, v/v) and three times with methanol in order to obtain a fraction of loosely bound lipids with protein. The last methanol extract had no detectable fatty acids. These extracts were combined with the extract obtained by the method of Bligh and Dyer[3] and designated as a fraction of loosely bound lipids. The protein pellets were suspended, homogenized and hydrolysed for 45 min at 100°C with 2% sodium methylate to obtain the tightly bound lipid fraction. In order to obtain methyl esters of fatty acids, the probes were treated with 3 M hydrochloric acid in methanol for 40 min at 100°C.[29]

The methyl esters of fatty acids of the tightly and loosely bound lipids were analysed by gas chromatograph "Chrome-5". Glass column 3×2.4 (mm i.d. × m), 10% Silar 10CP on Chromosorb W/HP, 80–100 mesh was used. Temperature programme was 140–250°C, at the rate of 2°C/min.

Internal lipid standards

Fluorocholesterol and triglycerol of lauric acid were used as internal standards for gas chromatography. Before lipid extraction, exact amounts of these compounds were added to the cells. Fluorocholesterol was not modified after all analytical procedures while trilaurate degradated with formation of methyl esters of lauric acid. The amount of cholesterol and fatty acids was calculated from the contents of internal standards in the probe.

Membrane fractions

Plasma membrane and microsomal fractions were isolated by the method of differential centrifugation in Percoll and Ficoll. Plasma membrane banded in Percoll zone 1.04–1.05 density, while the microsomal fraction banded in the Ficoll zone 1.17–1.18 density. All procedures for membrane isolation and the testing of the purity of membrane fractions were performed as described.[4]

Acetylcholinesterase activity was analysed by the method of Ellman *et al.*[10] Protein content was determined by the method of Lowry *et al.*[28]

The function of sodium channels was examined as described.[39]

RESULTS

Characterization of cell differentiation

Cell differentiation was monitored by morphological features and by protein content and acetylcholinesterase. Undifferentiated cells were small (40 μm D), round with or without short neurites. Differentiated cells were two–three times larger, of polygonal form with long (more than 50 μm) neurites. The protein content of undifferentiated cells in logarithmic and stationary growth phases was 140–160 μg/10^6 and 180–190 μg/10^6 cells, respectively. Protein quantity increased to 250–280 μg/10^6 cells in differentiated cells and activity of acetylcholinesterase increased almost 10 times. The velocity of ion efflux through veratridine-activated sodium channels of mature cells was five–six times higher than in immature culture. These data show that the cells possess distinct characteristics of mature neurons.[39,50]

Changes in phospholipid contents of cells differentiated by different agents

The total and individual phospholipid contents were examined in mature and immature cells (Table 1). The content of total phospholipids was nearly twice as high as that of immature ones. The main part of phospholipids was represented by phosphatidylcholine. Its relative quantity did not change as a result of differentiation in all studied cases. Lysophosphatidylcholine was undetectable in immature cell and in cells differentiated in the presence of 5′-bromodeoxyuridine, dimethylsulfoxide and dibutiryl cAMP. However, this phospholipid appeared in cells cultured in serum-free medium. The addition of gangliosides increased the lysophosphatidylcholine level two-fold.

Phosphatidylinositol level in cells differentiated in serum-free medium and in the presence of gangliosides was lower than in undifferentiated cells and cells treated by other agents.

It is of interest to note that phosphatidylethanolamine content in cells cultured in serum-free medium, in medium with gangliosides and in the presence of dibutyryl cAMP increased during differentiation. However, it did not change in cells treated by 5′-bromodeoxyuridine and dimethylsulfoxide when compared to immature cells.

In the presence of 5′-bromodeoxyuridine, dimethylsulfoxide and serum-free medium diphosphatidylglycerol content was constant during differentiation. Its level decreased in cells treated by dibutyryl cAMP

Table 1. The phospholipid content in undifferentiated cells and cells differentiated by different agents of neuroblastoma C1300 N18

Phospholipids	Undifferentiated cells n = 6	Differentiated cells 5′-bromodeoxyuridine, $n = 8$	dimethylsulfoxide, $n = 6$	dibutiryl cAMP, $n = 4$	serum-free medium, $n = 6$	gangliosides, $n = 6$
Total phospholipids nmol/10^6 cells	21.43 ± 0.68	35.60 ± 0.66***	41.31 ± 2.84***	49.09 ± 11.14***	42.44 ± 2.2***	37.26 ± 0.47***
Phosphatidylcholine	62.23 ± 2.17	62.51 ± 2.34	62.92 ± 1.22	60.00 ± 1.04	61.02 ± 1.11	61.94 ± 1.17
Phosphatidylethanolamine	14.68 ± 0.41	15.60 ± 0.97	16.06 ± 1.77	20.65 ± 1.08**	19.02 ± 0.33***	17.24 ± 0.34***
Lysophosphatidylcholine	—	—	—	—	1.43 ± 0.12***	3.60 ± 0.28***
Phosphatidylinositol	4.62 ± 0.30	5.22 ± 0.48	4.68 ± 0.59	4.66 ± 0.57	3.54 ± 0.24*	3.26 ± 0.04*
Phosphatidylserine	3.00 ± 0.29	4.20 ± 0.23***	3.86 ± 0.45	4.12 ± 0.19**	2.97 ± 0.12	3.09 ± 0.16
Sphingomyelin	4.54 ± 0.43	4.14 ± 0.34	3.45 ± 0.64	3.51 ± 0.59	2.27 ± 0.30**	3.45 ± 0.02
Phosphatidylglycerol	2.48 ± 0.13	1.79 ± 0.72	1.67 ± 0.34	1.59 ± 0.59	2.24 ± 0.30	1.84 ± 0.19
Diphosphatidylglycerol	3.66 ± 0.18	2.84 ± 0.61	3.35 ± 0.59	2.60 ± 0.15*	3.01 ± 0.17	2.37 ± 0.10***
Unidentified phospholipids + start zone	1.82 ± 0.06	3.84 ± 0.93	2.47 ± 0.71	0.92 ± 0.14	3.53 ± 0.30***	6.25 ± 0.28***

Values are expressed as % of total phospholipids. M ± m.
$*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

and gangliosides. Differentiated and undifferentiated cells did not contain phosphatidic acid.

Phospholipid content in plasma membrane and microsomal fraction of cells differentiated by 5′-bromodeoxyuridine

Phospholipid content per milligram of protein in both fractions remained constant during differentiation. Similarly, small amounts of lysophosphatidylcholine detected in plasma membrane and microsomal fractions did not change during cell maturation (Table 2).

Phosphatidylcholine, sphingomyelin and phosphatidylinositol levels were equal in plasma membranes of differentiated and undifferentiated cells. Phosphatidylethanolamine and phosphatidylserine levels increased in plasma membrane of mature cells. The total amount of phospholipids with high chromatographic mobility decreased in plasma membrane of differentiated cells. The level of phosphatidylserine increased in microsomal fractions, but phosphatidylcholine, phosphatidylethanolamine, sphingomyelin and phosphatidylinositol were found to be constant during maturation.

N-*Acylphosphatidylethanolamine and* N-*acylethanolamine content in neuroblastoma cells during maturation*

The levels of semilysobisphosphatidic acid and lyso-*N*-acylphosphatidylethanolamine increased in the differentiated cell (Table 3). Lyso-*N*-acylphosphatidylethanolamine was located mainly in membrane fractions. The amount of semilysobisphosphatidic acid in membrane fractions represented only part of the total cell semilysobisphosphatidic acid. Thus it was also present in other cell compartments.

More than half of the *N*-acylphosphatidylethanolamine content was located in membranes. The total quantity of the *N*-acylphosphatidylethanolamine decreased by nearly 50% in the mature cell and lowered to trace values in membrane fractions during differentiation.

The small amount of *N*-acylethanolamine was determined in the whole undifferentiated cell and in its plasma membrane. In differentiated cells only traces of *N*-acylethanolamine were found.

Composition of tightly and loosely bound lipid fractions in differentiated and undifferentiated cells

The tightly bound fatty acids represented a small fraction (nearly 1–5%) of total fatty acids. Six unsaturated fatty acids were found only in the loosely bound lipid fraction. (Tables 4 and 5). The amount of individual fatty acids notably differed in fractions of tightly and loosely bound lipids of undifferentiated cells. Neuroblastoma cell maturation led to significant changes of fatty acid composition in tightly and loosely bound lipids.

Table 2. The content of phospholipids in plasma membrane and microsomal fraction of undifferentiated cells and cells differentiated by 5′-bromodeoxyuridine neuroblastoma C1300 N18

Lipids	Undifferentiated cells		Differentiated cells	
	Plasma membrane 1	Microsomal fraction 2	Plasma membrane 3	Microsomal fraction 4
Total phospholipids nmol/mg protein	400 ± 11	299 ± 17	433 ± 17	312 ± 66
Phosphatidylcholine	51.43 ± 3.06	34.09 ± 1.95	45.11 ± 2.90	30.88 ± 2.41
Lysophosphatidylcholine	0.08 ± 0.01	0.47 ± 0.02	0.23 ± 0.07	0.64 ± 0.13
Phosphatidylethanolamine	16.24 ± 1.12	31.01 ± 2.83	22.13 ± 0.85***	36.40 ± 2.07
Sphingomyelin	7.13 ± 0.45	6.85 ± 0.51	6.98 ± 0.57	6.19 ± 0.72
Phosphatidylserine	4.96 ± 0.33	1.26 ± 0.07	8.69 ± 0.59***	3.73 ± 0.19††
Phosphatidylinositol	10.32 ± 0.87	16.82 ± 1.04	9.57 ± 1.03	14.00 ± 0.96
Total phospholipids with high mobility	8.44 ± 0.41	7.97 ± 0.69	6.01 ± 0.85	7.43 ± 0.52
Start zone	1.40 ± 0.06	1.53 ± 0.12	1.28 ± 0.22	1.36 ± 0.14

Values are expressed as percentage of total phospholipids, M ± m, $n = 8$.
1 compared to 3: $***P < 0.001$. 2 compared to 4: $††P < 0.001$.

Table 3. The content of phospholipids with high chromatographic mobility and *N*-acylethanolamines in undifferentiated and differentiated neuroblastoma C1300 N18 cells

Lipid	Undifferentiated cells			Differentiated cells		
	Whole cell	Plasma membrane	Microsomal fraction	Whole cell	Plasma membrane	Microsomal fraction
Semilysobisphosphatidic acid	295 ± 2	72 ± 3	155 ± 14	445 ± 6***	71 ± 5	128 ± 19
N-Acylphosphatidylethanolamine	244 ± 10	152 ± 20	9 ± 1	133 ± 10***	trace	trace
Lyso-*N*-acylphosphatidylethanolamine	411 ± 23	297 ± 17	161 ± 8	502 ± 5**	388 ± 16**	153 ± 15
N-Palmitoylethanolamine	1.03 ± 0.05	1.13 ± 0.04	trace	trace	trace	trace
N-Stearoylethanolamine	0.30 ± 0.04	trace	trace	trace	trace	trace

nmol/10^9 cells, M ± m, $n = 4$.
$**P < 01$; $***P < 0.001$.

The content of many polyunsaturated fatty acids decreased in loosely bound lipids during differentiation. Simultaneously two fatty acids (16:0 and 18:0) increased by 50 and 100%, respectively. We also found decreased levels of some unsaturated fatty acids in tightly bound fatty acids after maturation, while the contents of 16:0, 18:0 and 18:1 ω9 fatty acids increased.

All data described above show that cell differentiation caused an increase of the total amount of saturated fatty acids in both the fractions. Simultaneously the cholesterol content increased only in the fraction of loosely bound lipids (by 40%) and it did not change in the fraction of tightly bound lipids (per milligram of protein). The data given in Table 6 demonstrate the increased quantity of cholesterol, cholesterol esters and free fatty acids as a result of cell differentiation (calculated in 10^6 cells). The ratio of cholesterol/phospholipids changed from 0.33 in undifferentiated cells to 0.52 in differentiated ones. In comparison with the whole cell, the plasma membrane was enriched in cholesterol. Plasma membrane cholesterol/phospholipid ratios in undifferentiated and differentiated cells were 0.45 and 0.62, respectively, indicating an increase both in the absolute content and in the relative proportions of cholesterol in neuroblastoma cells during maturation.

Table 4. The changes of fatty acid content in lipids loosely bound with proteins in neuroblastoma C1300 N18 cells during differentiation (percentage of total amount)

Fatty acid chains	Undifferentiated cells, $n = 7$	Differentiated cells, $n = 6$
14:0	1.60 ± 0.06	0.90 ± 0.22*
ai15:0	0.25 ± 0.01	0.23 ± 0.01
15:0	0.60 ± 0.12	0.50 ± 0.33
15:1ω8	0.14 ± 0.02	0.11 ± 0.01
i16:0	0.20 ± 0.02	0.20 ± 0.03
ai16:0	0.20 ± 0.02	0.13 ± 0.01
16:0	26.20 ± 0.90	39.00 ± 2.68***
16:1ω9	2.00 ± 0.12	2.40 ± 0.10
16:1ω5	0.40 ± 0.09	Trace
17:0	1.10 ± 0.16	1.61 ± 0.20
17:1ω10	0.44 ± 0.02	0.47 ± 0.04
17:1ω8	0.40 ± 0.04	0.11 ± 0.02***
18:0	11.00 ± 0.67	22.81 ± 0.96***
18:1ω9	25.30 ± 1.10	22.40 ± 1.95
18:2ω6	5.20 ± 0.21	2.20 ± 0.21***
18:3ω3	0.33 ± 0.01	0.12 ± 0.01***
19:1	0.51 ± 0.01	0.13 ± 0.01***
20:0	0.51 ± 0.01	0.39 ± 0.01***
20:1ω11	1.14 ± 0.01	0.68 ± 0.01***
20:2ω6	1.16 ± 0.02	1.11 ± 0.01*
20:3ω9	0.17 ± 0.01	0.14 ± 0.01
20:3ω6	0.94 ± 0.01	1.06 ± 0.01***
20:4ω6	11.40 ± 0.03	2.52 ± 0.49***
20:5ω3	0.13 ± 0.01	0.15 ± 0.08
22:0	1.26 ± 0.04	0.23 ± 0.04***
22:1ω11	1.91 ± 0.17	0.65 ± 0.17***
22:3ω6	0.41 ± 0.02	0.64 ± 0.02***
22:4ω6	2.10 ± 0.03	2.50 ± 0.03***
22:5ω3	3.06 ± 0.04	3.60 ± 0.07***
22:6ω3	0.31 ± 0.01	0.50 ± 0.01***
Total saturated	42.90%	65.90%
Total unsaturated	57.47%	40.99%

$*P < 0.05$; $***P < 0.001$.

Table 5. Fatty acid content in lipid fractions tightly bound with proteins in undifferentiated and differentiated neuroblastoma C1300 N18 cells (percentage of total amount)

Fatty acid chains	Undifferentiated cells, $n = 7$	Differentiated cells, $n = 6$
14:0	1.49 ± 0.01	0.51 ± 0.01***
ai 15:0	0.17 ± 0.01	0.13 ± 0.01*
15:0	1.09 ± 0.02	0.22 ± 0.02***
15:1ω8	0.24 ± 0.01	0.17 ± 0.01***
i 16:0	0.42 ± 0.02	0.34 ± 0.04
ai 16:0	0.39 ± 0.01	0.18 ± 0.01***
16:0	20.50 ± 3.38	42.20 ± 1.09***
16:1ω9	4.80 ± 0.76	2.10 ± 0.28**
16:1ω5	none	none
17:0	2.20 ± 0.19	1.14 ± 0.15**
17:1ω10	0.57 ± 0.04	trace
17:1ω8	0.18 ± 0.01	0.23 ± 0.01**
18:0	22.60 ± 2.81	32.60 ± 0.95**
18:1ω9	13.60 ± 0.14	19.30 ± 0.95***
18:2ω6	2.10 ± 0.27	0.19 ± 0.03***
18:3ω3	none	none
19:1	0.39 ± 0.01	0.21 ± 0.03***
20:0	0.50 ± 0.03	0.31 ± 0.03**
20:1ω11	0.26 ± 0.01	trace
20:2ω6	0.12 ± 0.01	0.13 ± 0.01
20:3ω9	none	none
20:3ω6	0.45 ± 0.04	0.55 ± 0.02
20:4ω6	17.60 ± 1.86	1.27 ± 0.30***
20:5ω3	0.15 ± 0.01	0.12 ± 0.01
22:0	0.46 ± 0.02	0.16 ± 0.01*
22:1ω11	1.07 ± 0.06	0.11 ± 0.05*
22:3ω6	none	none
22:4ω6	2.34 ± 0.72	2.04 ± 0.51
22:5ω3	none	none
22:6ω3	none	none
Total saturated	49.82%	77.79%
Total unsaturated	43.87%	26.40%

$*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

The content of free fatty acids and fatty acyl chains in individual lipids of mature and immature cells

The analysis of results presented in Tables 7–9 showed that the total contents of saturated fatty acids in cholesterol esters, triglycerols, phosphatidylserine,

Table 6. Cholesterol, cholesterol ester, glycerol and free fatty acid contents in undifferentiated and differentiated neuroblastoma C1300 N18 cells

Lipids	Undifferentiated cells	Differentiated cells
	nmol/10^6 cells	
Cholesterol	7.09 ± 0.28	18.62 ± 0.51***
Free fatty acids	0.06 ± 0.01	0.54 ± 0.08***
Mono, -di- and triglycerols	1.67 ± 0.34	2.15 ± 0.14
	pmol/10^6 cells	
Cholesterol esters	8.43 ± 0.75	24.90 ± 2.22***
	μg/mg protein	
Cholesterol in loosely bound fraction	20.90 ± 1.95	27.20 ± 0.95**
Cholesterol in tightly bound fraction	1.40 ± 0.21	1.70 ± 0.61

$n = 10$; $**P < 0.01$; $***P < 0.001$.

Table 7. Free fatty acids and fatty acyl chains in the content of cholesterol esters and triglycerols of undifferentiated and differentiated cells of neuroblastoma C1300 N18 (percentage of total fatty acids)

Fatty acid chains	Cholesterol esters		Free fatty acids		Triglycerols	
	Undifferentiated cells	Differentiated cells	Undifferentiated cells	Differentiated cells	Undifferentiated cells	Differentiated cells
14:0	4.54 ± 0.11	3.07 ± 0.17**	0.10 ± 0.02	1.50 ± 0.24**	5.17 ± 0.64	2.12 ± 0.23*
15:0 (ai + n)	0.24 ± 0.02	0.27 ± 0.03	none	0.07 ± 0.01	0.67 ± 0.08	0.64 ± 0.08
15:1	0.53 ± 0.07	0.62 ± 0.08	none	0.09 ± 0.01	0.92 ± 0.08	1.03 ± 0.27
16:0 (ai + i + n)	17.66 ± 0.84	25.19 ± 1.13**	64.75 ± 2.19	46.54 ± 1.48**	20.43 ± 1.17	24.97 ± 0.98*
16:1 (ω9 + ω5)	8.53 ± 0.49	8.03 ± 0.87	16.94 ± 1.21	0.48 ± 0.09***	4.82 ± 0.63	4.14 ± 0.71
17:0	1.03 ± 0.12	0.94 ± 0.06	none	0.05 ± 0.01	0.96 ± 0.06	0.75 ± 0.12
17:1 (ω10 + ω8)	0.72 ± 0.07	0.65 ± 0.03	none	0.10 ± 0.03	1.45 ± 0.21	1.27 ± 0.19
18:0	6.89 ± 0.44	10.44 ± 0.61**	5.79 ± 0.73	14.32 ± 1.26**	7.91 ± 0.48	15.24 ± 0.83**
18:1ω9	24.56 ± 1.93	20.80 ± 2.47	10.41 ± 1.04	25.78 ± 1.83**	28.03 ± 1.32	21.39 ± 0.95*
18:2ω6	2.98 ± 0.32	2.34 ± 0.40	1.15 ± 0.32	2.06 ± 0.19	3.11 ± 0.28	2.38 ± 0.41
18.3ω3	9.07 ± 0.67	3.15 ± 0.41**	none	0.29 ± 0.06	4.82 ± 0.23	1.05 ± 0.13***
19.1	0.22 ± 0.03	0.34 ± 0.08	none	none	0.41 ± 0.05	0.84 ± 0.06**
20:0	0.65 ± 0.04	0.18 ± 0.02***	0.32 ± 0.06	1.07 ± 0.24*	0.72 ± 0.03	0.21 ± 0.01***
20:1ω11	1.63 ± 0.18	0.76 ± 0.08*	none	1.53 ± 0.45	1.29 ± 0.11	0.58 ± 0.07**
20:2ω6	1.74 ± 0.32	1.29 ± 0.21	none	2.01 ± 0.36	1.59 ± 0.21	1.07 ± 0.09
20:3ω9	0.12 ± 0.01	0.10 ± 0.03	none	none	0.17 ± 0.03	0.22 ± 0.06
20:3ω6	1.49 ± 0.07	1.82 ± 0.12	none	0.46 ± 0.07	0.88 ± 0.06	2.09 ± 0.13**
20:4ω6	8.25 ± 0.80	3.47 ± 0.49**	0.52 ± 0.09	3.01 ± 0.48**	10.73 ± 0.86	4.26 ± 0.23**
20:5ω3	0.27 ± 0.04	0.48 ± 0.06*	none	0.64 ± 0.09	0.19 ± 0.01	0.53 ± 0.04**
22:0	2.69 ± 0.13	4.93 ± 0.57*	none	none	0.55 ± 0.04	4.77 ± 0.57**
22:1ω11	3.03 ± 0.42	2.41 ± 0.28	none	none	0.32 ± 0.06	1.85 ± 0.22**
22:3ω6	0.35 ± 0.04	0.85 ± 0.05***	none	none	0.35 ± 0.04	0.91 ± 0.06**
22:4ω6	1.09 ± 0.17	2.91 ± 0.36*	none	none	1.84 ± 0.26	3.44 ± 0.47*
22:5ω3	1.51 ± 0.24	4.62 ± 0.27***	none	none	2.17 ± 0.13	4.17 ± 0.39**
22:6ω3	0.21 ± 0.03	0.34 ± 0.08	none	none	0.56 ± 0.24	0.08 ± 0.01

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 8. Fatty acyl chains in the content of phosphatidylethanolamine and phosphatidylcholine of neuroblastoma C1300 N18 cells (% of total amount)

	Phosphatidylethanolamine		Phosphatidylcholine	
Fatty acid chains	Undifferentiated cells	Differentiated cells	Undifferentiated cells	Differentiated cells
14:0	none	none	0.23 ± 0.04	0.25 ± 0.03
15:0 (ai + n)	none	none	0.28 ± 0.02	0.26 ± 0.04
15:1	0.12 ± 0.01	0.36 ± 0.04**	0.11 ± 0.01	0.12 ± 0.01
16:0 (ai + i + n)	3.34 ± 0.27	5.17 ± 0.43*	11.74 ± 0.82	19.37 ± 0.87**
16:1 (ω9 + ω5)	0.51 ± 0.04	0.86 ± 0.09*	2.13 ± 0.24	2.14 ± 0.13
17:0	0.96 ± 0.06	1.41 ± 0.11*	0.36 ± 0.02	0.63 ± 0.04**
17:1 (ω10 ± ω8)	0.77 ± 0.05	2.12 ± 0.34*	1.07 ± 0.12	2.09 ± 0.36
18:0	20.83 ± 1.16	23.61 ± 1.48	18.82 ± 1.09	19.03 ± 2.17
18:1ω9	15.04 ± 0.72	11.52 ± 0.43*	37.49 ± 2.47	31.18 ± 1.65
18:2ω6	2.78 ± 0.49	2.78 ± 0.63	4.86 ± 0.22	5.01 ± 0.74
18:3ω3	0.10 ± 0.02	0.12 ± 0.01	0.63 ± 0.04	0.74 ± 0.08
19:1	0.44 ± 0.03	0.21 ± 0.03**	0.42 ± 0.04	0.36 ± 0.06
20:0	0.41 ± 0.05	0.40 ± 0.05	0.86 ± 0.06	1.24 ± 0.10*
20:1ω11	2.36 ± 0.37	2.30 ± 0.42	2.69 ± 0.18	2.93 ± 0.37
20:2ω6	0.39 ± 0.04	0.42 ± 0.03	0.26 ± 0.02	0.34 ± 0.06
20:3ω9	1.16 ± 0.12	1.07 ± 0.08	1.34 ± 0.07	0.88 ± 0.04**
20:3ω6	1.47 ± 0.08	1.16 ± 0.09	1.57 ± 0.11	1.03 ± 0.04**
20:4ω6	23.63 ± 0.93	19.20 ± 0.79*	8.16 ± 0.36	4.72 ± 0.48**
20:5ω3	1.01 ± 0.06	0.63 ± 0.03**	0.56 ± 0.02	0.41 ± 0.04*
22:0	0.65 ± 0.05	0.24 ± 0.02**	0.43 ± 0.03	0.18 ± 0.01**
22:1ω11	0.83 ± 0.06	0.51 ± 0.03**	0.99 ± 0.10	1.48 ± 0.12*
22:3ω6	0.32 ± 0.03	0.37 ± 0.05	0.41 ± 0.02	0.27 ± 0.03*
22:4ω6	7.99 ± 0.48	9.12 ± 0.54	3.01 ± 0.30	2.99 ± 0.43
22:5ω3	12.56 ± 0.81	13.47 ± 0.97	1.38 ± 0.15	1.43 ± 0.24
22:6ω3	2.33 ± 0.43	2.96 ± 0.26	0.45 ± 0.03	0.76 ± 0.08*

$*P < 0.05$; $**P < 0.01$.

phosphatidylinositol, phosphatidylethanolamine and phosphatidylcholine increased on average by 25% during differentiation. Only in sphingomyelin were the same amounts of saturated fatty acids in mature and immature cells detected.

In all studied individual lipids of differentiated cells the decreased level (average by 50%) of arachidonic acid was found. Only in the fraction of free fatty acids did the quantity of arachidonic acid rise six times higher in differentiated cells, while the amount of saturated fatty acids decreased.

DISCUSSION

The cell membranes are considered to be partly responsible for the control of growth and differentiation.[33,62] Hence, it is of great importance to study structural and functional properties of membranes in differentiated and undifferentiated cells in order to clarify the mechanisms of cell maturation. Neuroblastoma C1300 N 18 clone can be induced to undergo differentiation by relatively simple procedures. Cells treated by 5′-bromodeoxyuridine, dimethylsulfoxide, dibutyryl cAMP, serum-free medium and exogenous gangliosides developed morphological, electrophysiological and biochemical characteristics of mature neurons. However, different agents caused different changes in cell lipid composition during maturation.

Phospholipids and cholesterol were found to be the main lipid components in mature and immature cells. Plasma membranes in particular were enriched in cholesterol as compared with the whole cell. This was suggestive of their decreased fluidity in comparison with undifferentiated cells.[1,15] Another result supported this suggestion. The total level of saturated fatty acids significantly increased (by nearly 25%) during differentiation. But at the same time we found an increased quantity of cholesterol esters. In some cases lysophosphatidylcholine appeared in differentiated cells. These two lipids are known to increase membrane fluidity and permeability. Hence we cannot conclude how plasma membrane fluidity became modified during maturation in neuroblastoma cell. It is known that microviscosity of different transformed cells can be increased or decreased depending on the cell line.[33] Therefore this question needs special investigation.

The main part of all phospholipids in neuroblastoma cells is represented by phosphatidylcholine. Plasma membrane is enriched in phosphatidylcholine as compared with the microsomal fraction. The relative proportion of phosphatidylcholine was constant during differentiation. Phosphatidylethanolamine and phosphatidylserine levels increased in plasma membrane of differentiated cells. Consequently, the membrane phosphatidylcholine/(phosphatidylethanolamine + phosphatidylserine) ratio lowered. These changes help to acquire different physicochemical properties in membranes of differing lipid composition.

It is considered that lysophosphatidylcholine is not

Table 9. Fatty acyl chains in the content of sphingomyelin, phosphatidylserine and phosphatidylinositol of neuroblastoma C1300 N18 cells (percentage of total amount)

Fatty acid chains	Sphingomyelin		Phosphatidylserine		Phosphatidylinositol	
	Undifferentiated cells	Differentiated cells	Undifferentiated cells	Differentiated cells	Undifferentiated cells	Differentiated cells
14.0	4.02 ± 0.25	2.48 ± 0.36*	1.64 ± 0.22	3.16 ± 0.41*	trace	2.57 ± 0.48
15:0 (ai + n)	none	none	0.18 + 0.03	0.14 ± 0.02	none	0.17 ± 0.01
15:1	none	none	0.10 ± 0.01	0.11 ± 0.03	none	0.11 ± 0.01
16:0 (ai + i + n)	68.16 ± 3.07	51.39 ± 2.83*	12.65 ± 1.09	21.51 ± 1.42**	30.73 ± 2.86	27.32 ± 1.94
16:1 (ω9 + ω5)	6.83 ± 0.74	9.35 ± 0.94	0.44 ± 0.07	2.19 ± 0.18***	2.12 ± 0.41	1.86 ± 0.58
17:0	0.11 ± 0.01	0.13 ± 0.03	0.13 ± 0.01	0.15 ± 0.03	none	none
17:1(ω10 + ω8)	0.34 ± 0.02	0.41 ± 0.06	0.34 ± 0.03	0.41 ± 0.05	none	none
18:0	5.93 ± 0.44	12.18 ± 0.47***	36.30 ± 1.92	44.16 ± 2.34	34.41 ± 2.17	45.64 ± 2.19*
18:1ω9	7.11 ± 0.56	4.92 ± 0.25*	40.04 ± 3.26	15.20 ± 0.96**	5.12 ± 0.62	2.06 ± 0.14**
18:2ω6	1.00 ± 0.21	0.92 ± 0.07	0.68 ± 0.05	0.38 ± 0.02*	0.51 ± 0.07	0.29 ± 0.02
18:3ω3	none	none	none	none	0.11 ± 0.01	0.08 ± 0.01
19:1	none	none	none	none	none	none
20:0	3.17 ± 0.48	6.87 ± 0.82*	0.11 ± 0.01	0.27 ± 0.02**	0.74 ± 0.08	0.62 ± 0.07
20:1ω11	none	none	none	none	none	none
20:2ω6	none	none	0.13 ± 0.02	0.13 ± 0.03	0.56 ± 0.07	0.41 ± 0.04
20:3ω9	none	none	none	none	none	none
20:3ω6	none	0.49 ± 0.06	0.52 ± 0.07	0.21 ± 0.02*	1.11 ± 0.24	0.53 ± 0.09
20:4ω6	2.84 ± 0.50	1.41 ± 0.03*	4.37 ± 0.53	1.12 ± 0.21**	20.07 ± 1.19	14.65 ± 0.83*
20:5ω3	none	none	0.10 ± 0.01	0.12 ± 0.01	none	none
22:0	0.49 ± 0.06	4.98 ± 0.62**	0.20 ± 0.03	0.47 ± 0.03**	0.06 ± 0.01	none
22:1ω11	none	none	none	0.22 ± 0.03	none	none
22:3ω6	none	none	0.13 ± 0.02	0.91 ± 0.10***	none	none
22:4ω6	trace	2.13 ± 0.34	0.37 ± 0.04	3.77 ± 0.71**	0.96 ± 0.07	1.13 ± 0.18
22:5ω3	trace	2.06 ± 0.18	1.31 ± 0.26	4.82 ± 0.67**	2.33 ± 0.38	2.56 ± 0.47
22:6ω3	trace	0.28 ± 0.04	0.27 ± 0.03	0.65 ± 0.07**	1.17 ± 0.24	1.05 ± 0.12

$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

the constant component of intact neuroblastoma cells. However, we detected small amounts of lysophosphatidylcholine in membrane fractions of mature and immature cells. This is in agreement with recent observations.[4]

The amount of lysophosphatidylcholine remained constant in membrane fractions of cells differentiated by 5′-bromodeoxyuridine. However, in cells differentiated in the presence of serum-free medium and in the same medium supplemented with gangliosides, the lysophosphatidylcholine amount significantly increased.

In our experiments phosphatidylglycerol and diphosphatidylglycerol were undetectable in membrane fractions. There are conflicting reports as regards their presence in membranes.[4,37]

Compared to the whole cell, plasma membranes were enriched in sphingomyelin and phosphatidylinositol. They were characterized by a high cholesterol/phospholipid ratio and by the presence of almost half of the total cell *N*-acylphosphatidylethanolamine. This unique lipid composition could contribute to the specialized and dynamic properties of the plasma membrane. In fact, the distinct changes in cell and cell membrane lipid composition during cell differentiation are shown to correlate with the appearance of specialized membrane properties.[40,42,43]

For example, the excitable membrane appears only in differentiated cells.[39] Recent reports demonstrate a striking increase (about 10 times) in insulin receptors in mature neuroblastoma cells.[2,18,44] There are some cell lines which differ in their competence to be induced to differentiate into mature cells. The plasma membranes of these lines have different physical properties which are correlated with their fatty acid acyl chain composition.[53] This shows that protein membrane functions depend on lipid membrane composition. Membrane proteins interact with membrane lipids through several types of non-covalent forces. Recently it has been published that fatty acids can be tightly (covalently) bound with membrane proteins.[29,49] These lipids cannot be extracted by organic solvents. In order to determine whether neuroblastoma C1300 N 18 cell proteins contain tightly bound lipids we examined the fatty acids which remained with proteins after exhaustive extraction with organic solvents. As a result, we found more than 20 fatty acids in each fraction. The quantity of individual fatty acids was higher in the fraction of loosely bound lipids. The difference in the composition of loosely and tightly bound lipids was suggestive of their different functional roles.

The content of fatty acids in both fractions changed in a different way during differentiation. However, the total amount of saturated fatty acids increased by nearly 25% in both the fractions. The content of unsaturated fatty acids decreased in mature cells.

The particularly significant decrease of arachidonic acid in tightly and loosely bound lipid fractions and in all studied individual lipids appears to have functional implications. As reported for other transformed cells[25,33] the level of arachidonic acid in our experiments increased in the free fatty acid fraction during differentiation.

Little information is available on the mechanisms of lipid composition changes during neuroblastoma cell differentiation. It is described that these cells in culture easily incorporate lipids from the culture medium.[46] However, the lipid uptake from culture medium is a selective process which depends on the functional characteristics of the cell. It is thus possible that the process of lipid uptake takes place through different ways in mature and immature cells. The observed difference in fatty acid composition can also be explained by the decreased desaturase activities in malignant cells.[25,45,46] As the neuroblastoma line is of tumor origin, the high quantity of saturated fatty acids may reflect the neoplastic nature of the cells.

For the first time we found two phospholipids with high chromatographic mobility in neuroblastoma cells. They were identified as *N*-acylphosphatidylethanolamine and semilysobisphosphatidic acid in special solvent systems.

It is necessary to stress the changes in *N*-acylphosphatidylethanolamine and *N*-acylethanolamine contents. The amount of cell *N*-acylphosphatidylethanolamine decreased by 50% as a result of cell maturation. While *N*-acylphosphatidylethanolamine disappeared from membrane fractions of mature cells, *N*-acylethanolamine was detectable neither in the whole cell nor in the membrane fraction of differentiated cells.

Recently it has been shown that *N*-acylphosphatidylethanolamine and *N*-acylethanolamine are the components of some normal and ischemic vertebrate tissue.[11,36,58] *N*-Acylphosphatidylethanolamine and *N*-acylethanolamine are known to accumulate in infarcted myocardium;[12] *N*-acylethanolamine can change membrane permeability.[13,14] *N*-Acylphosphatidylethanolamine was also found in some transformed cells. The enzyme systems for its biosynthesis and catabolism are found in dog brain preparations.[34,35]

In this work we have reported, for the first time, the presence of *N*-acylphosphatidylethanolamine and *N*-acylethanolamine content in neuroblastoma cells. The drastic changes in the quantity of these lipids during cell differentiation suggest that these could in fact be of physiological significance.

Nothing is known about the physiological role of semilysobisphosphatidic acid in neuroblastoma cells. However, the concentration of this compound changed notably during differentiation in whole cell and remained constant in plasma membrane and microsomal fractions. Even then one cannot exclude the possibility that this minor phospholipid with high chromatographic mobility is of some physiological significance. However, it remains to be investigated.

CONCLUSION

Thus we like to conclude that the investigated changes in lipid compositions of undifferentiated and differentiated neuroblastoma cells do contribute to characterizing the process of malignant cell differentiation.

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