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Membrane Lipids and Enzymes of Cultured High- and Low-Metastatic B16 Melanoma Variants[1]

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ABSTRACT

B16 melanoma cell variants were used to determine if the metastatic properties of these cells could be correlated to distinct plasma membrane, microsome, and mitochondrial membrane lipid compositions and membrane-bound enzyme activities in high- and low-metastatic cell variants, respectively. The high-metastatic B16-F10 melanoma cell membranes had lower cholesterol/phospholipid ratios, lower arachidonic acid content, lower polyunsaturated fatty acid content, higher phosphatidylcholine/phosphatidylethanolamine ratios, and higher succinate cytochrome *c* reductase activity than those of B16-F1 melanoma cell membranes. No differences in cholesterol/phospholipid ratio were noted in the mitochondria. Na^+-K^+-adenosinetriphosphatase activity and solubility of 5′-nucleotidase activity were also similar. The data indicate that the membrane lipid composition of B16-F10 melanoma cells is distinct from that of B16-F1 melanoma cells and may help to elucidate the molecular basis for the different metastatic properties of these cell lines *in vivo*.

INTRODUCTION

In recent years, considerable advances in our understanding of the process of tumor cell metastasis have been derived from the availability of malignant cell sublines of differing metastatic potential (13, 34). Increasingly, the cell surface membrane has been implicated as the site for phenotypic expression of metastatic potential (33). There are numerous examples where tumor cell sublines, selected *in vivo* or *in vitro* for altered metastatic properties, expressed cell surface differences in display, structure, amount, or response of cell surface proteins-glycoproteins. Increased shedding of membrane proteins (31), loss of hormonal regulation (30), nonadherence to immobilized lectins (35, 37), exposure of membrane glycoproteins (38), and exposure of membrane anionic sites (36) all indicate that alterations in membrane composition and/or structure may be present. One molecular basis for such differences may be alterations in membrane lipid composition. Membrane fatty acid composition can affect the specific activity of cell enzymes (55), the susceptibility to complement-mediated cytolysis (55), the phagocytic activity (26, 43, 44), the exposure of membrane proteins (46), and the lateral mobility of receptors (20). Altered membrane cholesterol can affect endocytosis (17), lymphocyte activation by lectins (39), exposure of receptors to hormones (25), and membrane protein exposure (4, 5). Altered phospholipid polar head group composition modulates membrane enzymes (18), receptors (12), endocytosis (42, 43), and agglutinability of cells (41). Despite these correlations, a detailed comparison of membrane lipid composition and distribution in malignant cells of different metastatic potential has not been reported. Herein, we describe the subcellular fractionation and lipid characterization of membranes from high-metastatic B16-F10 and low-metastatic B16-F1 melanoma variants.

MATERIALS AND METHODS

Cells and Cell Culture. B16 melanoma cell variant F10 (high-metastatic) and F1 (low-metastatic) lines were obtained through the generosity of Dr. E. M. Jensen, E. G. & G. Mason Research Institute, Worcester, MA, and Dr. I. J. Fidler, Frederick Cancer Research Center, Frederick, MD. The cells were cultured in monolayer with Eagle's minimum essential medium (500 ml), heat-inactivated fetal calf serum (50 ml), 100× nonessential amino acids (5 ml), 200 mM L-glutamine (10 ml), 100× minimum essential medium vitamin solution (5 ml), penicillin/streptomycin (5 ml; 10,000 units each), Fungizone (5 ml; 250 μg/ml), gentamicin (2.5 ml; 10 mg/ml), 7.5% sodium bicarbonate (14 ml), 100 mM sodium pyruvate (5 ml, and 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (2 ml). The atmosphere was maintained at 98% humidity and 5% CO_2, and the incubation temperature was 37°. The cells were routinely tested for bacterial, fungal, and *Mycoplasma* contamination.

Cell Fractionation. Plasma membranes, microsomes, and mitochondria were isolated basically as described earlier (45) with the following difference: (*a*) the 33,000 × *g* supernatant from 1 g, wet weight, of cells was layered on one sucrose gradient tube to avoid overloading of the gradient; and (*b*) 40 up-and-down strokes were used with the Dounce homogenizer to break the cells.

Enzyme Determinations. The specific activity of ouabain-sensitive Na^+-K^+-ATPase was determined as described previously (45) with the following modifications. The final concentration of reagents in the reaction mixture was 30 mM imidazole-HCl (pH 7.5), 110 mM NaCl, 15 mM KCl, 50 mM NaN_3, 0.5 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, 4 mM $MgCl_2$, 3 mM ATP, with or without 1 mM ouabain, and 10 to 60 μg membrane protein per 150 μl total reaction volume. The reaction mixture was incubated for 45 min at 37° and was terminated with an equal volume of 1% sodium dodecyl sulfate. A color reagent for determination of free phosphate (1 part 10% ascorbic acid + 6 parts 0.42% ammonium molybdate·$4H_2O$ in 1 N H_2SO_4) was added at 5.3 times the original reaction volume, the mixture was incubated at 45° for 5 min, and the phosphomolybdate complex was measured spectrophotometrically at 660 nm (1).

The specific activity of 5′-nucleotidase was determined in a 0.3-ml incubation mixture containing 50 mM glycine-HCl (pH 8.7), 0.2 mM EDTA, 6 mM AMP, 10 mM $MgCl_2$, and 15 to 60 μg of protein. The reaction was carried out at 37° for 30 to 60 min. The incubation was terminated with an equal volume of 1% sodium dodecyl sulfate, and the liberated phosphate was determined with the color reagent described above for Na^+-K^+-ATPase.

NADPH-dependent and succinate-dependent cytochrome *c* reductase were assayed as described by Sottocasa *et al.* (48). Protein was determined by the method of Lowry *et al.* (24).

Lipid Determinations. All organic solvents were glass distilled and all glassware was washed with sulfuric acid/dichromate before use. Whole cells or membrane fractions were suspended in 1.0 ml of the phosphate-buffered saline solution mentioned above. A 0.2-ml aliquot was removed

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for protein determination (24). The remaining 0.8-ml aliquot was extracted by the method of Bligh and Dyer (3) as described by Ames (2). Before and after silicic acid chromatography, total lipid and total lipid phosphate were determined by the methods of Marzo *et al.* (27) and Ames (1), respectively. Neutral lipids and phospholipids were separated by silicic acid chromatography (45). The phospholipid composition of whole cells and membrane fractions was determined by exposing the cells to 2.0 μCi of ^{32}P/ml of medium (New England Nuclear; carrier free) as described by Schroeder *et al.* (45).

Fatty acid methyl esters were prepared from phospholipids by using BF_3/methanol (Supelco, Inc., Bellefonte, PA). The samples were dissolved in hexane and analyzed by gas-liquid chromatography using temperature programming in a 6-foot-high glass column of 15% Silar 10C on 100/120 Gas-Chrom R (Applied Science Laboratories, Inc., State College, PA). The methyl ester derivatives were identified by comparison of their retention times with standards obtained from Supelco (NIH-D, QUALMIX-S, QUALMIX-M, and QUALMIX-L) and also by comparison of their retention times relative to methyl esters prepared from dieicosylphosphatidylcholine, kindly provided by Dr. Craig Jackson, Department of Biological Chemistry, Washington University School of Medicine, St. Louis, MO. A Sigma 2 gas chromatograph (Perkin Elmer, Inc., Norwalk, CT) equipped with dual-flame ionization detectors and a 3390A Reporting Integrator (Hewlett-Packard, Avondale, PA) was used to quantitate peak areas. Cholesterol content was determined by the method of Sokoloff and Rothblatt (47) using stigmasterol (Steraloids, Inc., Wilton, NH) as an internal standard on a 6-foot-high glass column of 3% OV-17 on 80/100 Chromosorb W (HP) (Pierce Chemical Co., Rockford, IL).

RESULTS

Subcellular Fractionation of B16 Melanoma Cell Lines. In order to determine if membrane differences may exist between B16-F1 and B16-F10 cell lines, subcellular fractionations were performed. Specific marker enzymes for plasma membranes (Na^+-K^+-ATPase and Mg^{2+}-ATPase), microsomes (NADPH-dependent cytochrome *c* reductase), and mitochondria (succinate-dependent cytochrome *c* reductase) were used to monitor the fractionation (Tables 1 and 2) of the 2 cell lines. The specific activity of Na^+-K^+-ATPase was enriched 4.3- and 5.1-fold in Fraction S_1 for B16-F10 and B16-F1 cells, respectively. Similarly, Mg^{2+}-ATPase was enriched 4.1- and 3.5-fold, respectively. The S_1 membrane fraction was reduced 13.9- and 42.7-fold, respectively, in the mitochondrial enzyme marker, succinate-dependent cytochrome *c* reductase. However, some microsomal marker copurification was indicated by NADPH-cytochrome *c* reductase. Consequently, membrane Fraction S_1 was designated as the plasma membrane fraction. Based on its enrichment of succinate-dependent cytochrome *c* reductase activity, Fraction S_5 was designated as the mitochondrial membrane fraction (enrichment of 6.4- and 3.7-fold for B16-F10 and B16-F1 cells, respectively). Last, Fraction S_2 was enriched 2.7- and 1.9-fold, respectively, in NADPH-cytochrome *c* reductase and was designated as the microsomal fraction. In summary, plasma membrane, microsome, and mitochondrial subfractions enriched approximately 4- to 5-fold, 2-fold, and 4- to 6-fold in respective markers were obtained from B16 melanoma cell lines. Significant differences in the fold purification were not noted between the cell lines.

A comparison of membrane enzyme activities between the

Table 1

Membrane fractionation scheme for B16-F10 melanoma cells

		Membrane enzyme specific activity (nmol/min/mg protein)			
Cell fraction	Protein (mg)	Na^+-K^+-ATPase	Mg^{2+}-ATPase	NADPH-dependent cytochrome *c* reductase	Succinate-dependent cytochrome *c* reductase
Cell homogenate	10.1 ± 1.7[a]	27.7 ± 3.9	16.2 ± 6.0	68.6 ± 7.4	162.4 ± 16.9
33,000 × *g* supernatant	6.5 ± 2.1	24.4 ± 5.9	15.8 ± 7.7	57.9 ± 10.2	1.0 ± 0.9
33,000 × *g* pellet	2.1 ± 0.2	57.2 ± 6.7	24.3 ± 12.3	187.0 ± 19.8	455.9 ± 56.2
S_0[b]	0.50 ± 0.08	10.0 ± 6.1	71.8 ± 10.7	83.2 ± 9.2	5.5 ± 5.5
S_1	0.65 ± 0.19	118.3 ± 21.1	65.1 ± 12.9	118.5 ± 30.1	11.7 ± 7.0
S_2	0.42 ± 0.11	61.2 ± 13.7	17.9 ± 10.4	187.5 ± 31.8	1.0 ± 0.9
S_3	0.53 ± 0.13	70.3 ± 16.1	47.5 ± 20.5	101.3 ± 8.2	53.1 ± 12.1
S_4	0.92 ± 0.18	28.7 ± 5.1	28.2 ± 17.2	157.2 ± 31.8	92.4 ± 39.7
S_5	1.28 ± 0.36	29.2 ± 9.4	20.5 ± 11.9	180.5 ± 14.6	1051.5 ± 206.0
S_6	1.08 ± 0.28	36.9 ± 13.8	11.3 ± 5.4	179.2 ± 26.0	149.0 ± 57.9

[a] Mean ± S.E. (n = 4 to 7).
[b] S_0 to S_6, fractions taken from sucrose gradient.

Table 2

Membrane fractionation scheme for B16-F1 melanoma cells

		Membrane enzyme specific activity (nmol/min/mg protein)			
Cell fraction	Protein (mg)	Na^+-K^+-ATPase	Mg^{2+}-ATPase	NADPH-dependent cytochrome *c* reductase	Succinate-dependent cytochrome *c* reductase
Cell homogenate	7.5 ± 1.0[a]	23.3 ± 5.7	18.3 ± 7.3	87.0 ± 3.3	89.6 ± 11.6
33,000 × *g* supernatant	4.8 ± 0.8	15.5 ± 3.8	26.4 ± 4.7	55.1 ± 9.9	2.9 ± 2.9
33,000 × *g* pellet	1.8 ± 0.4	58.8 ± 9.7	15.9 ± 4.8	157.3 ± 15.1	279.1 ± 31.2
S_0[b]	0.27 ± 0.09	59.3 ± 11.1	22.0 ± 11.0	125.8 ± 11.3	0.55 ± 0.55
S_1	0.33 ± 0.12	118.2 ± 12.9	64.8 ± 42.0	165.0 ± 28.1	2.1 ± 2.1
S_2	0.35 ± 0.13	75.7 ± 7.5	38.2 ± 18.7	195.2 ± 40.3	2.6 ± 2.6
S_3	0.48 ± 0.14	59.6 ± 13.1	18.6 ± 13.3	161.0 ± 19.1	1.0 ± 0.8
S_4	0.49 ± 0.11	67.6 ± 9.0	2.5 ± 2.1	171.2 ± 21.5	135.0 ± 6.8
S_5	1.13 ± 0.32	30.3 ± 1.7	22.8 ± 22.7	177.7 ± 9.0	330.4 ± 38.4
S_6	1.37 ± 0.21	21.6 ± 11.3	36.1 ± 16.4	144.1 ± 10.2	36.0 ± 16.3

[a] Mean ± S.E. (n = 4 to 7).
[b] S_0 to S_6, fraction taken from the sucrose gradient.

B16-F10 and B16-F1 melanoma lines indicated that the specific activity of the mitochondrial enzyme, succinate-cytochrome *c* reductase, was 1.8- and 3.2-fold higher in the crude homogenate and mitochondrial membrane fraction, respectively, of B16-F10 melanoma cells as compared to B16-F1 melanoma cells (Table 3). No other significant differences were noted.

Exfoliation of 5′-Nucleotidase. The plasma membrane ectoenzyme 5′-nucleotidase has been used as a biochemical indicator of the metastasizing capability of transformed cells (6). Both the activity of the enzyme in cell homogenates and purified plasma membranes and the appearance of the enzyme in the serum have been utilized as markers of degree of tumor metastasis. As indicated in Table 4, no significant difference in 5′-nucleotidase specific activity was noted between B16-F1 and B16-F10 cell lines in any step of the cell fractionation. In addition, the 5′-nucleotidase activity was consistently enriched in the soluble supernatant. Even in the plasma membrane fraction, where 5′-nucleotidase has been used as a marker enzyme, there was only a modest 70 and 32% increase in specific activity over the cell homogenate. In contrast, the Na^+-K^+-ATPase activity was consistently enriched in the particulate membrane fractions, being enriched 4.3- and 5.1-fold, respectively, in the purified S_1 sucrose gradient fraction. Thus, 5′-nucleotidase appears to be solubilized from both B16 melanoma variants to an equal extent. This solubilization appears to be in the form of soluble enzyme and as microvesicles (9, 35, 50). The latter may represent specific plasma membrane domains (Table 5). The microvesicles are sedimented at 196,000 × *g*. The vesicles contain cholesterol and phospholipid at a molar ratio of 0.18 to 0.24 for B16-F1 and B16-F10 cells, respectively. No significant differences in lipid content (Table 5), fatty acid composition (Table 6), or phospholipid composition (Table 7) were noted in microvesicles of B16-F1 as compared to B16-F10 melanoma cells. The lipid composition of these vesicles was the same as that of vesicles recovered from the culture medium of the respective cell lines.

Lipid Composition of B16 Melanoma Cell Membranes. Lipid analysis of B16-F1 cell homogenate and plasma membranes indicated a 2.6- and 1.7-fold enrichment, respectively, in cholesterol as compared to B16-F10 cell homogenate and plasma membrane (Table 8). A comparison of the cell lines indicated that the B16-F10 plasma membrane did not significantly differ from that of the B16-F1 in phospholipid content per mg protein. The microsomes of B16-F10 cells contained more phospholipid than did those of the B16-F1 cell line. Mitochondrial lipid contents were not significantly different. In summary, the cholesterol/phospholipid ratio was greater in cell homogenate, plasma mem-

Table 3

Membrane enzymes of high- and low-metastatic B16 lines

Membrane fractions were obtained from cultured B16-F10 and B16-F1 melanoma variants as described in "Materials and Methods."

Cell fraction	Enzyme	Enzyme activity (nmol/min/mg protein) B16-F1	B16-F10
Cell homogenate	Na^+-K^+-ATPase	23.3 ± 5.7[a]	27.7 ± 3.9
	Mg^{2+}-ATPase	18.3 ± 7.3	16.2 ± 6.0
	Succinate-dependent cytochrome *c* reductase	89.6 ± 11.6[b]	162.4 ± 16.9[b]
	NADPH-dependent cytochrome *c* reductase	87.0 ± 3.3	68.6 ± 7.4
Plasma membrane	Na^+-K^+-ATPase	118.2 ± 12.9	118.3 ± 31.1
	Mg^{2+}-ATPase	64.8 ± 42.0	65.1 ± 12.9
Microsome	NADPH-dependent cytochrome *c* reductase	171.2 ± 21.5	187.5 ± 31.8
Mitochondria	Succinate-dependent cytochrome *c* reductase	330.4 ± 38.4[b]	1051.5 ± 206.0[b]

[a] Mean ± S.E. (n = 4 to 7).
[b] $p < 0.025$, as determined by Student's t test.

Table 4

Subcellular fractionation of 5′-nucleotidase in B16 melanoma cell lines

All procedures for cell fractions were carried out as described in "Materials and Methods" except that aliquots for enzyme assays were removed from the 33,000 × *g* supernatant (1 hr) followed by an additional 18 hr centrifugation at 196,000 × *g*.

Cell fraction	Specific activity (nmol/min/mg protein) B16-F10 5′-Nucleotidase	B16-F10 Na^+-K^+-ATPase	B16-F1 5′-Nucleotidase	B16-F1 Na^+-K^+-ATPase
Cell homogenate	13.6 ± 2.1[a]	27.7 ± 3.9	16.1 ± 2.5	23.3 ± 5.7
33,000 × *g* supernatant	35.8 ± 1.0	24.4 ± 5.9	25.6 ± 3.3	15.5 ± 3.8
33,000 × *g* pellet	17.8 ± 3.3	57.2 ± 6.7	21.4 ± 3.6	58.8 ± 9.7
33,000 × *g* supernatant/33,000 × *g* pellet	2.0 ± 0.2	0.4 ± 0.1	1.2 ± 0.2	0.3 ± 0.1
196,000 × *g* supernatant	26.1 ± 3.1	0.6 ± 0.3	19.6 ± 2.9	1.2 ± 0.9
196,000 × *g* pellet	9.7 ± 2.6	16.9 ± 2.7	7.1 ± 3.1	13.8 ± 2.0
196,000 × *g* supernatant/196,000 × *g* pellet	2.7 ± 0.6	0.04 ± 0.01	2.8 ± 0.4	0.08 ± 0.02
Sucrose gradient Fraction S_1	23.2 ± 3.1	118.3 ± 31.1	22.9 ± 4.4	118.2 ± 12.9

[a] Mean ± S.E.

brane, and microsome of B16-F1 as compared to B16-F10 cells.

Phospholipid Fatty Acid Composition of B16 Melanoma Variants. The degree of fatty acid unsaturation of membrane phospholipids is an important factor determining membrane structure and function (55). In addition, polyunsaturated fatty acids are important prostaglandin precursors which may be involved in the metastatic process (15, 40, 49). The B16-F1 melanoma cell homogenate and plasma membrane phospholipids were enriched in arachidonic acid (20:4) and other polyunsaturated fatty acids (Table 9). The 20:4 content of B16-F1 cell homogenate and plasma membranes were 1.7- and 1.5-fold, respectively, higher than those of B16-F10 melanoma cells. Likewise, the other polyunsaturated fatty acids were elevated 2.5- and 5.0-fold, respectively, in the B16-F1 as compared to B16-F10 cell line. These changes were also reflected in the ratio of unsaturated to saturated fatty acids which was significantly higher in the cell homogenate and plasma membrane of B16-F1 melanoma cells.

Table 5

Lipid composition of microvesicles from B16 melanoma cell lines

Lipid analysis on the 196,000 × *g* pellet from Table 4 was determined as described in "Materials and Methods."

Cell line	Cholesterol/protein (nmol/mg)	Phospholipid/protein (nmol/mg)	Cholesterol/phospholipid (nmol/nmol)
B16-F1	22.9 ± 2.1[a]	133.4 ± 15.5	0.18 ± 0.03
B16-F10	26.8 ± 5.9	109.2 ± 14.7	0.24 ± 0.03

[a] Mean ± S.E. (n = 3).

Table 6

Phospholipid fatty acid composition of microvesicles from B16 melanoma cell lines

Phospholipid fatty acid composition was determined as described in "Materials and Methods." Other polyunsaturated fatty acids consist of 18:3, 20:3, 20:5, 22:4, 22:5, and 22:6.

Fatty acid	% composition (wt %)	
	B16-F1	B16-F10
14:0	2.1 ± 0.6[a]	2.1 ± 0.4
16:0	14.6 ± 2.1	16.6 ± 3.4
16:1	2.1 ± 1.3	5.6 ± 2.5
18:0	1.4 ± 0.3	1.4 ± 0.2
18:1	14.5 ± 2.4	14.6 ± 1.5
18:2	43.0 ± 2.6	45.3 ± 2.8
20:4	8.4 ± 2.1	6.1 ± 1.5
Other polyunsaturated	13.9 ± 4.6	8.3 ± 3.4
Unsaturated/saturated	4.5 ± 0.4	4.0 ± 0.3

[a] Mean ± S.E. (n = 3).

Table 7

Phospholipid composition of microvesicles from B16 melanoma cell lines

The phospholipid composition of microvesicles obtained as described in the legend to Table 5 was determined as described in "Materials and Methods."

Phospholipid	Mole % composition	
	B16-F1	B16-F10
Phosphatidylcholine	47.6 ± 1.9[a]	44.5 ± 2.5
Phosphatidylethanolamine	12.6 ± 1.7	14.5 ± 1.5
Sphingomyelin + lysophosphatidylcholine	7.0 ± 0.9	8.0 ± 1.2
Phosphatidylinositol	12.3 ± 1.8	15.0 ± 3.4
Phosphatidylserine	6.8 ± 1.0	6.7 ± 0.6
Other	13.7 ± 2.2	11.3 ± 4.1
Phosphatidylcholine/phosphatidylethanolamine	3.7 ± 0.3	3.1 ± 0.4

[a] Mean ± S.E. (n = 3).

Phospholipid Composition of B16 Melanoma Cell Membranes. Alterations in phospholipid composition and in phosphatidylethanolamine methylation may be associated with the malignancy of neoplastic cells (14, 28, 29). The primary phospholipid species present in all membranes of B16-F1 melanoma cells were phosphatidylcholine and phosphatidylethanolamine (Table 10), comprising 75% of the cellular lipids. The ratios of phosphatidylcholine to phosphatidylethanolamine were 1.62, 1.10, 0.93, and 0.78 in the cell homogenate, plasma membrane, microsomes, and mitochondria of B16-F1 melanoma cells. Minor phospholipids such as phosphatidylinositol, phosphatidylserine, phosphatidylglycerol, cardiolipin, sphingomyelin, with lysophospholipids comprised the remainder. The presence of small amounts of phosphatidyl-*N*,*N*′-dimethylethanolamine [1.7 ± 0.2% (S.E.)] and phosphatidyl-*N*-monomethylethanolamine in the cell homogenate as well as in individual membrane fractions indicated the presence of the phosphatidylethanolamine methylation pathway for synthesis of phosphatidylcholine (11).

A comparison of the B16-F1 melanoma phospholipid composition (Table 10) with that of the B16-F10 melanoma (Table 11) indicated a qualitative similarity in phospholipid composition. However, significant quantitative differences were evident. The B16-F10 cell line had less phosphatidylethanolamine, phosphatidyl-*N*,*N*′-dimethylethanolamine, and phosphatidyl-*N*-monomethylethanolamine but more phosphatidylserine than did the B16-F1 cell line. Significantly higher ratios of phosphatidylcholine to phosphatidylethanolamine were present in the microsomes and mitochondria of B16-F1 melanoma cells.

Table 8

Lipid composition of B16 melanoma variants

Protein, total lipid, total phospholipid, and cholesterol were determined as explained in "Materials and Methods."

Cell fraction	B16 melanoma variant	Total lipid (mg/mg protein)	Phospholipid (nmol/mg protein)	Cholesterol (nmol/mg protein)	Cholesterol/phospholipid (molar ratio)
Cell homogenate	F1	0.14 ± 0.01[a, b]	160 ± 12	67 ± 5[c]	0.42 ± 0.03[c]
	F10	0.09 ± 0.01[b]	108 ± 11	25 ± 4[c]	0.23 ± 0.02[c]
Plasma membrane	F1	0.22 ± 0.04	238 ± 23	111 ± 6[b]	0.47 ± 0.04[b]
	F10	0.17 ± 0.02	194 ± 27	67 ± 8[b]	0.34 ± 0.02[b]
Microsomes	F1	0.12 ± 0.03	129 ± 15[b]	53 ± 9	0.41 ± 0.03[b]
	F10	0.20 ± 0.03	251 ± 22[b]	47 ± 11	0.18 ± 0.04[b]
Mitochondria	F1	0.17 ± 0.02	216 ± 26	17 ± 5	0.08 ± 0.04
	F10	0.17 ± 0.02	205 ± 21	26 ± 8	0.12 ± 0.03

[a] Mean ± S.E. (n = 3).
[b] $p < 0.05$, determined by Student's t test.
[c] $p < 0.01$, determined by Student's t test.

Table 9

Phospholipid fatty acid composition of B16 melanoma membranes

Phospholipids were separated by silicic acid chromatography, and fatty acid methyl esters were prepared and analyzed as described in "Materials and Methods."

Fatty acid	B16 melanoma variant	Fatty acid composition (wt %)			
		Cell homogenate	Plasma membrane	Microsomes	Mitochondria
14:0	F1	2.9 ± 0.8[a]	3.0 ± 0.1	0.8 ± 0.6	2.0 ± 1.0
	F10	0.1 ± 0.1	1.0 ± 1.7	2.0 ± 0.7	1.2 ± 0.7
15:0	F1	3.2 ± 0.2	3.6 ± 0.5	4.1 ± 3.6	5.8 ± 1.5
	F10	6.7 ± 2.2	5.0 ± 3.7	4.6 ± 1.2	4.4 ± 2.2
16:0	F1	14.9 ± 1.9	16.4 ± 1.2	12.9 ± 0.5	16.3 ± 1.1
	F10	17.6 ± 0.6	13.2 ± 5.3	13.9 ± 0.5	16.7 ± 0.7
16:1	F1	6.6 ± 0.7	5.4 ± 0.3	3.9 ± 0.4	6.4 ± 1.0
	F10	8.6 ± 0.6	14.2 ± 7.8	7.7 ± 0.2	9.6 ± 0.2
18:0	F1	11.5 ± 0.4	15.2 ± 0.3	12.4 ± 1.6	12.2 ± 0.8
	F10	14.5 ± 1.2	14.3 ± 2.8	11.5 ± 1.0	11.0 ± 0.2
18:1	F1	37.1 ± 3.5	39.7 ± 1.1	30.6 ± 3.7	39.2 ± 1.4
	F10	41.1 ± 0.7	46.7 ± 4.5	39.1 ± 1.2	43.6 ± 1.1
20:4	F1	8.7 ± 0.9[b]	4.0 ± 0.9[b]	24.5 ± 7.2[b]	7.9 ± 1.0
	F10	3.6 ± 1.0[b]	3.3 ± 0.2[b]	3.7 ± 0.8[b]	5.2 ± 1.2
Other polyunsaturated[c]	F1	15.5 ± 4.1[b]	10.6 ± 2.7[b]	11.6 ± 1.2	9.3 ± 0.8
	F10	6.2 ± 1.3[b]	2.1 ± 2.8[b]	16.5 ± 1.1	7.1 ± 0.5
Unsaturated/saturated	F1	2.12 ± 0.11[b]	2.11 ± 0.03[b]	2.40 ± 0.18	2.21 ± 0.11
	F10	1.71 ± 0.09[b]	1.96 ± 0.04[b]	2.22 ± 0.09	2.38 ± 0.06

[a] Mean ± S.E. (n = 3 to 7).
[b] $p < 0.05$, determined by Student's t test.
[c] Other polyunsaturated fatty acids consist primarily of 18:2 and 20:3 with smaller amounts of 20:5, 22:4, 22:5, and 22:6. Therefore, these are included in the calculation of the unsaturated to saturated ratio.

Table 10

Phospholipid composition of B16-F1 melanoma membranes

The phospholipid composition was determined by thin-layer chromatography as described in "Materials and Methods." Data were compared with those of Table 11 by Student's t test.

Phospholipid	Phospholipid composition (mol %)			
	Cell homogenate	Plasma membrane	Microsomes	Mitochondria
Phosphatidylcholine	46.5 ± 1.5[a]	38.4 ± 3.1	33.9 ± 3.8	31.0 ± 4.8
Lysophosphatidylcholine	1.0 ± 0.1	1.5 ± 0.1	1.2 ± 0.2	0.6 ± 0.1
Phosphatidylethanolamine	28.7 ± 2.8	34.9 ± 3.1	36.6 ± 2.8[b]	40.0 ± 2.1[b]
Phosphatidyl-*N,N'*-dimethylethanolamine	1.7 ± 0.2[b]	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1
Phosphatidyl-*N*-monomethylethanolamine	1.8 ± 0.3[b]	1.2 ± 0.2	0.4 ± 0.1	0.1 ± 0.1
Phosphatidylinositol	4.9 ± 1.9	1.7 ± 0.3	2.7 ± 0.6	5.0 ± 0.9
Phosphatidylserine	5.4 ± 1.7	9.2 ± 1.7	7.9 ± 1.1[b]	4.1 ± 1.2[b]
Phosphatidylglycerol	3.6 ± 0.3	4.0 ± 0.4	6.0 ± 1.3	4.0 ± 0.8
Cardiolipin	2.8 ± 0.2	0.1 ± 0.1	2.1 ± 0.4	3.1 ± 0.8
Sphingomyelin	1.2 ± 0.7	1.5 ± 0.3	1.2 ± 0.4	0.8 ± 0.2
Diphosphatidylinositol	0.7 ± 0.3	0.5 ± 0.2	0.7 ± 0.3	1.6 ± 0.7
Lysophosphatidylethanolamine	0.7 ± 0.3	0.1 ± 0.1	3.7 ± 0.2	0.1 ± 0.1
Other	3.7 ± 0.5	8.5 ± 2.9	5.8 ± 1.2	6.4 ± 1.5
Phosphatidylcholine/phosphatidylethanolamine	1.7 ± 0.2	1.1 ± 0.2	1.0 ± 0.2[b]	0.8 ± 0.2[c]

[a] Mean ± S.E. (n = 4 to 7).
[b] $p < 0.05$, as determined by Student's t test.
[c] $p < 0.10$, as determined by Student's t test.

DISCUSSION

Membrane Enzymes of B16 Melanoma Cells. Cellular membranes of normal and tumor cells differ significantly in the activity of many membrane-bound enzymes (32, 54). Consequently, if the activity of such enzymes can be correlated to the degree of malignancy of the cell, it might be expected that highly malignant, metastasizing cells might differ to a greater extent from less malignant, poorly metastasizing cells in the activities of the same membrane-bound enzymes. The specific activity of Na^+-K^+-ATPase in isolated hepatoma plasma membranes was reduced 2- to 30-fold and that of 5′-nucleotidase was also diminished as compared to normal or regenerating liver (6, 53, 54). In fact, the specific activity of 5′-nucleotidase decreased in the cell homog-

enate and plasma membrane in metastasizing tumors as compared to nonmetastasizing tumors. However, no significant differences in the specific activities of these membrane-bound enzymes were found between B16-F1 and B16-F10 melanoma cells.

Malignant tumors also have fewer mitochondria and demonstrate loss of many mitochondrial enzymes (53, 54). The data presented here indicated that the cell homogenate and mitochondria of the highly metastatic B16-F10 melanoma cell line had a 1.8- and a 3.2-fold higher specific activity of succinate-cytochrome *c* reductase, an inner mitochondrial membrane enzyme, respectively, than those of B16-F1 melanoma cells. Since the mitochondrial fraction of B16-F10 cells was 1.7-fold more pure than that of the B16-F1 cells, the actual difference in succinate-cytochrome *c* reductase was probably no more than 2-fold (instead of 3.2-fold). Thus, unlike the decreased mitochondrial function noted when tumor cells were compared to normal cells, highly metastatic B16-F10 melanoma cells either had similar or slightly enhanced activity of at least one mitochondrial enzyme, succinate-dependent cytochrome *c* reductase.

Exfoliated 5′-Nucleotidase-containing Microvesicles. Since B16 melanoma cells shed microvesicles (35), it is possible that differences in exfoliated 5′-nucleotidase (either soluble or as microvesicles) exist between the 2 melanoma cell lines. Shedding of 5′-nucleotidase and appearance of the enzyme in the serum of cancer patients were used as an *in vivo* marker for metastasis (6, 7, 22). The data presented here indicated that both B16-F1 and B16-F10 melanoma cells released microvesicles distinct from plasma membranes during the cell fractionation procedure. These vesicles were enriched in 5′-nucleotidase but deficient in Na^+-K^+-ATPase. Other investigators determined a similar relationship in exfoliated vesicles from glioma and neuroblastoma cells (50). These cells preferentially shed membrane vesicles deficient in Na^+-K^+-ATPase but enriched in 5′-nucleotidase. However, the latter investigators were unable to detect any 5′-nucleotidase activity in either the whole B16 melanoma cells or in cell superfusates. The reason for this discrepancy is not known but may be due to differences in the assay method used for 5′-nucleotidase. Differences in the soluble/microvesicle or [soluble + microvesicle]/particulate membrane 5′-nucleotidase activity ratios were not noted between the B16-F1 and B16-F10 melanoma cell lines. Thus, from extrapolating these data obtained *in vitro*, it seems unlikely that the appearance of 5′-nucleotidase in serum of mice carrying this melanoma would be indicative of the degree of tumor metastasis unless other factors are operating *in vivo* to influence the rate of microvesicle shedding or of 5′-nucleotidase release as a soluble protein.

The microvesicles obtained from B16-F1 and B16-F10 melanoma cells appear to represent select membrane domains not only with respect to enzyme activities but also with regard to lipid composition. As demonstrated in Table 5, the cholesterol/phospholipid ratio of microvesicles from B16-F1 and B16-F10 melanoma cells was 1.5- to 2.5-fold lower than that of the corresponding plasma membrane (Table 8). Likewise, the unsaturated/saturated fatty acid ratio in the microvesicle phospholipids was approximately 2-fold greater than that of the primary plasma membranes (Table 6 *versus* Table 9). Lastly, the phosphatidylcholine/phosphatidylethanolamine ratio in the microvesicles was 3-fold greater than in the primary plasma membranes (Table 7 *versus* Tables 10 and 11). Thus, it appears that the microvesicles may be shed from specialized membrane lipid domains, a concept supported by earlier data indicating greater sphingomyelin and polyunsaturated fatty acid content in exfoliated vesicles than in corresponding plasma membranes from glioma and neuroblastoma cells (50). These data would also indicate that the exfoliated membrane vesicles of B16 melanoma cells obtained herein might be expected to be more fluid than the parent plasma membrane especially because of the low cholesterol/phospholipid ratio. Normal thymocytes shed vesicles that have a cholesterol/phospholipid ratio similar to that of the thymocyte plasma membranes (51). In contrast, leukemic GRSL cells shed vesicles that were 3.5-fold enriched in cholesterol/phospholipid ratio, as compared to GRSL plasma membranes, and they were consequently much more rigid than the corresponding plasma membrane (51). Since the exfoliated vesicles of highly metastatic B16-F10 melanoma cells as well as those of low-metastatic B16-F1 melanoma cells have a low cholesterol/phospholipid ratio, it seems likely that they represent a completely different membrane domain than that shed by thymocytes or GRSL cells.

Lipid Composition of B16 Melanoma Variants. Several sig-

Table 11

Phospholipid composition of B16-F10 melanoma membranes

All conditions were as described in the legend to Table 10.

Phospholipid	Phospholipid composition (mol %)			
	Cell homogenate	Plasma membrane	Microsomes	Mitochondria
Phosphatidylcholine	46.4 ± 0.9[a]	42.5 ± 3.0	42.3 ± 2.4	40.4 ± 2.0
Lysophosphatidylcholine	1.2 ± 0.1	1.4 ± 0.2	1.0 ± 0.1	0.6 ± 0.2
Phosphatidylethanolamine	24.7 ± 2.1	30.5 ± 2.4	24.3 ± 2.4[b]	32.3 ± 1.5[b]
Phosphatidyl-*N,N′*-dimethylethanolamine	0.7 ± 0.1[b]	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1
Phosphatidylmonomethylethanolamine	0.7 ± 0.2[b]	1.6 ± 0.3	0.1 ± 0.1	0.1 ± 0.1
Phosphatidylinositol	3.6 ± 1.4	3.5 ± 1.0	3.4 ± 0.5	4.5 ± 1.0
Phosphatidylserine	6.9 ± 2.6	10.0 ± 1.9	11.8 ± 0.6[b]	9.6 ± 0.5[b]
Phosphatidylglycerol	3.0 ± 0.2	4.2 ± 0.5	4.3 ± 0.5	3.1 ± 0.1
Cardiolipin	3.6 ± 0.2	0.7	1.7 ± 0.7	4.1 ± 1.3
Sphingomyelin	1.1 ± 0.2	1.6 ± 0.3	1.2 ± 0.2	0.9 ± 0.2
Diphosphatidylinositol	1.3 ± 0.6	0.6 ± 0.1	0.7 ± 0.2	0.4 ± 0.1
Lysophosphatidylethanolamine	0.6 ± 0.2	1.5 ± 0.4	0.1 ± 0.1	0.1 ± 0.1
Other	5.9 ± 1.8	6.5 ± 0.3	2.1 ± 0.7	2.7 ± 0.7
Phosphatidylcholine/phosphatidylethanolamine	1.9 ± 0.2	1.4 ± 0.2	1.8 ± 0.2[b]	1.2 ± 0.1[c]

[a] Mean ± S.E. (n = 4 to 7).
[b] $p < 0.05$, as determined by Student's t test.
[c] $p < 0.10$, as determined by Student's t test.

nificant differences in lipid composition of the B16 melanoma membranes were obtained when the B16-F1 and B16-F10 cell lines were compared. First, the B16-F10 melanoma cell homogenate and plasma membrane phospholipids contained 55 and 37% less arachidonic acid (20:4), respectively, than did those of the B16-F1 melanoma cells. Arachidonic acid is an important prostaglandin precursor. Although, in general, increased prostaglandin content has been found in the blood, urine, and tumor cells of cancer patients (23), decreased prostaglandin formation by highly metastatic tumor cell lines indicates a potential relationship between prostaglandins and the metastatic process (15, 40, 49). These investigators found that the highly metastatic B16-F10 melanoma formed less prostaglandin D_2 compared to the low-metastatic B16-F1 melanoma. Prostaglandin D_2 inhibits platelet aggregation *in vivo* and may therefore prevent the formation of platelet-B16 melanoma cell emboli. Formation of such emboli may protect the highly metastatic melanoma cell from destruction in the systemic circulation until it can form metastatic sites in the lung. *In vitro* blockade of prostaglandin D_2 production by indomethacin increased the metastatic rate of the B16 melanoma cells *in vivo* (49). Thus, the decreased prostaglandin D_2 levels and higher metastatic properties of the B16-B10 melanoma cell lines as compared to the B16-F1 melanoma cell lines may be due to the decreased availability of arachidonic acid, the prostaglandin D_2 precursor. In addition, decreased polyunsaturated fatty acid content decreased the susceptibility of tumor cells to complement-mediated cytolysis (55). The tumorigenicity of transformed FK3T3 cell lines was enhanced by decreased polyunsaturated fatty acid content while the tumorigenicity of a poorly tumorigenic derivative, AK 3T3 cells, was decreased by decreased polyunsaturated fatty acid content (10).

Not only was the level of arachidonic acid decreased in the plasma membranes and cell homogenate of B16-F10 cells, but the other polyunsaturated fatty acids were also decreased 5- and 2.5-fold, respectively, as compared to B16-F1 cells. This decrease was reflected in the unsaturated/saturated fatty acid ratio and indicates that the B16-F10 plasma membranes may be less fluid than those of the B16-F1 cells. In contrast, another finding was the opposite effect, that the 28% lower cholesterol/phospholipid molar ratio would be expected to increase the fluidity of the B16-F10 plasma membrane. Since the effects of these 2 parameters on fluidity were opposed, structural analysis to determine whether the unsaturated/saturated fatty acid ratio or the cholesterol/phospholipid ratio predominated were performed and described elsewhere.[3] In general, plasma membranes of tumors such as hepatomas have higher cholesterol/phospholipid ratios than normal liver (53). This difference between normal and tumor cells was largely due to defective feedback regulation of cholesterol biosynthesis in tumor cells (8, 10, 53), B16-F10 cells have less sterol carrier protein than do B16-F1 cells.[4] If higher cholesterol/phospholipid ratios are associated with degree of malignancy and, by extension, metastatic potential, then the B16-F10 melanoma plasma membrane should have had a cholesterol/phospholipid ratio higher than that of the B16-F1 melanoma cells. The data presented herein indicated the opposite trend. Thus, it appears that metastatic potential may not be predicted from simple extension of the membrane properties of cells that do or do not form primary tumors.

The cholesterol/phospholipid ratio of mitochondrial membranes of B16-F10 cells was not significantly different from that of the B16-F1 cells. Hepatoma mitochondria have a 4- to 7-fold higher content of cholesterol than normal liver mitochondria (32, 53). The elevated cholesterol/phospholipid ratio is associated with a decreased ability of the hepatoma mitochondria to undergo P_i-induced swelling and other membrane functions (32, 53). Elevated cholesterol/phospholipid ratios in normal liver mitochondria increased the activity of succinate-cytochrome *c* reductase. Since the cholesterol/phospholipid ratio was not significantly different between the 2 cell lines, it seems unlikely that the same mechanism for regulation of mitochondrial enzyme activity is operative in B16 melanoma mitochondria as in normal liver mitochondria.

Phospholipid Composition. The phospholipid composition of B16 melanoma cells indicated few differences between B16-F10 and B16-F1 cell lines except for: (*a*) the phosphatidylcholine/phosphatidylethanolamine ratio was higher in all membrane fractions of B16-F10 melanoma; and (*b*) the content of phosphatidylserine was higher in B16-F10 microsomes and mitochondria of B16-F10 melanoma cells. Neoplastic cell membranes compared to normal cell membranes showed decreased phosphatidylcholine/phosphatidylethanolamine ratios and increased phosphatidylserine content (52). The phosphatidylcholine/phosphatidylethanolamine ratio is a determinant of cell-cell interactions (16) or cell-substrate adherence (41), endocytosis (42, 43), and receptor binding (12, 42). A decreased phosphatidylcholine/phosphatidylethanolamine ratio in tumorigenic LM fibroblast membranes resulted in increased homotypic concanavalin A-mediated agglutination (42), increased concanavalin A-mediated hemagglutination, and decreased metastatic rate of LM tumors in nude mice (21). Although the phosphatidylcholine/phosphatidylethanolamine ratio was higher in B16-F10 melanoma cells than in B16-F1 melanoma cells, the content of plasma membrane-methylated phosphatidylethanolamines (phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N*,*N'*-dimethylethanolamine) was significantly lower in B16-F10 than in B16-F1 melanoma cells (Tables 9 and 10). Decreased methylation of phosphatidylethanolamine was also noted in mouse ascites cells and rat hepatomas (14) as compared to normal liver and blood cells. This observation may be highly significant because in rat mast cells a pool of phosphatidylcholine, synthesized from phosphatidylethanolamine by the methylation pathway, is preferentially attacked by phospholipase A_2. Arachidonic acid is released from this phosphatidylcholine and is subsequently converted to prostaglandins (19). Thus, not only did the B16-F10 melanoma plasma membranes have less arachidonic acid content but they may also have less phosphatidylcholine arising via methylation of phosphatidylethanolamine than do the B16-F1 melanoma plasma membranes. Both of these factors may account for the decreased prostaglandin D_2 content and increased metastatic potential of the B16-F10 melanoma cells.

In summary, the data described here represent a first attempt to isolate and biochemically characterize subcellular membranes from B16 melanoma cell variants. The comparison of membrane enzyme and lipid compositional properties of the high-metastatic B16-F10 and low-metastatic B16-F1 cell lines may help to understand the molecular basis for the differences in metastatic properties between the 2 cell lines. As such, to our knowledge, the work represents the first isolated membrane characterization

[3] F. Schroeder, Florescence probes in metastatic B16 melanoma membranes. Biochim. Biophys. Acta, in press, 1984.

[4] F. Schroeder, A. B. Kier, and M. E. Dempsey, unpublished observation.

and comprehensive comparison of lipids and membrane enzymes from high- and low-metastatic cell lines.

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