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Membrane properties of metastatic and non-metastatic cells cultured from C_3H mice injected with LM fibroblasts

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Little is known regarding the membrane properties of metastatic cells as compared to non-metastatic tumor cells. In order to remove variables such as site of growth and nutrition, C_3H mice and LM fibroblasts were used as a model system to derive cell lines from local tumors and lung metastases. LM cells were injected subcutaneously into C_3H mice and local skin tumors and secondary lung tumors were isolated, cultured in vitro and analyzed. The activities of lipid-sensitive membrane enzymes, membrane lipid composition, and membrane structure were correlated with metastatic ability. Plasma membranes and microsomes of the cultured metastatic cells had 3.8 ± 0.5- and 5.4 ± 0.6-fold elevated 5′-nucleotidase activity, respectively, as compared to plasma membranes and microsomes of cultured non-metastatic cells. The mitochondria of cultured metastatic cells had 3.5 ± 0.5-fold decreased succinate-dependent cytochrome-*c* reductase activity as compared to mitochondria of the cultured non-metastatic cells. The lipids of plasma membranes from the metastatic cells had $30 \pm 2\%$ and $46 \pm 7\%$ lower phosphatidylinositol and sterol/phospholipid ratio, respectively, and $30 \pm 3\%$ increased unsaturated/saturated fatty acid as compared to cultured non-metastatic cells. The lower sterol/phospholipid ratio correlated with a $30 \pm 1\%$ lower level of cytosolic sterol carrier protein in the cultured metastatic cells as compared to cultured non-metastatic cells. Multifrequency phase and modulation fluorometry in conjunction with the fluorescence probe, 1,6-diphenyl-1,3,5-hexatriene, was used to determine the static and dynamic aspects of membrane fluidity. The plasma membranes and microsomes of cultured metastatic cells were more fluid than those of cultured non-metastatic cells as indicated by $24 \pm 3\%$ and $7 \pm 1\%$, respectively, lower limiting anisotropy of 1,6-diphenyl-1,3,5-hexatriene in the membranes of the metastatic as compared to non-metastatic cells.

Introduction

Metastatic properties of cells have been associated with differences in cell surface membrane fluidity and lipid composition [1–11]. The physiological determinants of membrane fluidity include amount and domain structure of cholesterol, cholesterol/phospholipid ratio, fatty acid composition, and phosphatidylcholine/phosphatidylethanolamine ratio, and these factors may be associated with degree of malignancy [12–21].

Cholesterol homeostasis in highly malignant cells may be less regulated as compared to local tumors or in normal tissue [14,15]. The abnormal regulation of membrane cholesterol content, fatty acid composition, cholesterol/phospholipid ratio, and feedback inhibition of cholesterol biosynthesis observed in highly malignant cells may be associated with differences in the function and/or quantity of a group of cytosolic lipid transfer proteins, namely the sterol carrier proteins or SCPs (also named fatty acid binding proteins or FABPs) [16].

It has been difficult to make consistent generalizations between lipid membrane properties and the degree of malignancy. Most comparisons have been made between high versus low metastatic cells in culture or more rarely between highly malignant versus less malignant tumors. In the former case metastatic ability of the cultured cell lines is often ascertained after intravenous injection of the cells, thereby possibly circumventing important in vivo steps in the metastatic spread of tumors from the primary site. Since many experimental cell lines form metastases only slowly or not at all in normal mice, metastatic ability has often been compared only in T cell-deficient athymic mice

Abbreviations: SCP, sterol carrier protein; FABP, fatty acid binding protein.

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[10,19,29] *, a useful system but lacking some of the immune regulation which may influence in vivo metastatic ability of tumor cells. In the comparisons of membrane properties from directly excised local tumors versus excised metastases [10,11], the presence of inflammatory cells and the site of growth may become important factors, since contamination of the membrane preparations with inflammatory cells and host tissue is possible [30].

In the present work an analysis is made of membrane lipid composition and structural properties of cultured local and metastatic tumor cells from immunocompetent C_3H mice injected subcutaneously with LM fibroblasts. The relationship to cytosolic SCP levels are examined.

Materials and Methods

Cell culture. LM cells were originally obtained from the American Type Culture Collection (CCL 1.2). Cells were removed from liquid nitrogen, cultured in Corning 75 cm^2 monolayer plastic tissue culture flasks with Higuchi medium containing 10% calf serum for one week. The cells were transferred to suspension cell culture in serum-free, chemically-defined Higuchi medium containing choline (40 mg/ml) [10,31]. Prior to injection into mice, log phase LM cells were sedimented and resuspended in vehicle (serum-free, lipid-free, choline-containing Higuchi medium).

Injection of mice and derivation of non-metastatic and metastatic cell lines. A 0.5-ml aliquot of LM cells ($1 \cdot 10^7$ cells/ml) maintained for 2 years in continuous suspension culture, as described above, was inoculated subcutaneously over the left thigh of 25 female C_3H/Hen, Mouse Mammary Tumor Virus negative (MMTV −) mice (six- to eight-weeks old) obtained from Charles River Laboratories, Wilmington, NJ. Mice were housed in filter-covered suspended polycarbonate cages with wood shavings and automatic watering system. They were fed commercial rodent chow. Local tumor volume was measured every two days as described elsewhere [10,29].

After 45 days, a 30 g local tumor was taken from a C_3H mouse and a portion of the tumor was placed in tissue culture. These cultured local tumor cells were injected into 25 C_3H/Hen (MMTV −) mice as described above. In this second passage, a lung metastasis was isolated from one mouse. Cells from this lung metastasis were placed in tissue culture. The resultant cell line was designated Met 0. Local tumor cells were cultured from the same mouse with the lung metastasis. These local tumor cells were injected into 37 C_3H/Hen (MMTV −) mice. After 45 days, local tumor cells were cultured from one mouse and designated as the non-metastatic cells. The Met 0 cell line was also injected into 37 C_3H/Hen (MMTV −) mice as described above. Lung metastases were isolated from two mice and cultured as described below. The resultant cell lines, Met 1.1, Met 1.2, Met 2.1, Met 2.2, and Met 2.3, were designated as metastatic cells. Parameters from the metastatic cell lines were initially measured individually, and when no differences between the lines were seen, the measurements were averaged.

Necropsy. All mice were killed using euthanasia with CO_2 on day 45 post-inoculation. External lesions, mouse weight, local tumor size, and gross necropsy observations were recorded. Local tumors were dissected under sterile conditions, weighed and divided: (a) a small portion of the tissue was fixed in formalin for histopathological evaluation; and (b) the rest of the tumor was placed on ice for cell culture. Individual, visible lung metastases appeared as raised, slightly opaque, whitish areas 0.5–2.5 mm in diameter, and were trimmed away from lung tissue under sterile conditions using a dissecting microscope and surgical iris scissors. Some metastases were fixed in formalin for histopathologic confirmation and the rest were minced and returned to cell culture as described in the next section. The remaining lung tissue and other organs were treated as previously described to evaluate for microscopic metastases [10,29]. Lung tissue from a mouse not injected with tumor cells was processed to serve as a background control for biochemical and biophysical assays.

Culture of local and metastatic tumors from mice. Local tumors and lung metastases were excised under sterile conditions. The tumors were rinsed three times with Higuchi medium described above, to which were added 50 ml fetal bovine serum, 5 ml fungizone (250 μg/ml), 2.5 ml gentamycin (10 mg/ml), and 0.1 ml kanamycin (200 mg/ml) per 500 ml medium. This medium was designated as primary culture medium. The tumors were minced with a sterile scalpel, sedimented, and resuspended in Higuchi primary culture medium. The suspended local tumor and lung metastasis cells were cultured for three weeks in 75 cm^2 Corning polystyrene tissue culture flasks. The medium was changed bi-weekly. At the end of 3 weeks cell culture with the Higuchi primary culture medium the cells were place on Higuchi medium [31] containing 10% fetal bovine serum. The cells were then subcultured weekly (1 : 3 dilution) with decreasing % fetal bovine serum such that at the end of 3 weeks the medium was serum-free. Thereafter, the cells were cultured in serum-free, lipid-free Higuchi medium as described above for LM cells [31]. There were no significant differences between cell lines with regard to growth

* Membrane properties of high and low metastatic L cell variants (Kier A.B. and Franklin, C. (1989) Invasion Metastasis, submitted).

rate. The cultured non-metastatic cells (from local tumors) and cultured metastatic cells (from lung metastatases) were maintained at least 6 weeks in this chemically-defined medium prior to membrane fractionation. All membrane fractionations were done with cells in exponential growth phase.

Quantitation of sterol carrier protein. Sterol carrier protein was quantitated as described previously [16].

Membrane fractionation. Plasma membranes, microsomes, and mitochondria were isolated from exponentially growing cell lines as described earlier [19,31]. The specific activity of membrane bound enzymes was monitored for two reasons: First, membrane enzymes are used as markers for membrane purity in order to assure that the membrane fractionation procedure will yield comparable purifications for the different cell lines. The following enzyme markers were used as markers for membrane purity: the specific activities of ouabain-sensitive Na^+/K^+ATPase and 5′-nucleotidase (determined as described previously [13]) were purified approximately 8-fold with respect to cell homogenate in all cell lines tested. NADPH-dependent and succinate-dependent cytochrome-*c* reductase activities (assayed as described by Sottocasa et al. [32]) were purified 3- and 4-fold, respectively in all cell lines examined. Significant differences in fold purification of membrane fractions between cell lines were not observed. Thus, observed differences between non-metastatic and metastatic cells do not originate from different levels of impurities. Second, elevated activities of some enzymes such as 5′-nucleotidase have been implicated as potential markers for metastasis. Herein, we examined the generality of this possibility in the LM cell line using cultured metastastatic versus non-metastatic cells. Protein was determined by the method of Lowry et al. [33].

Trinitrobenzenesulfonic acid labeling. Cultured cells were sedimented and divided into two aliquots: one was treated with buffer only and one was treated with buffer plus 4 mM trinitrobenzenesulfonic acid under non-penetrating conditions (4°C) for 80 min in order to trinitrophenylate outer monolayer amine moieties, or under penetrating conditions (37°C) in order to trinitrophenylate amine moieties in both monolayers [19,34–36]. The trinitrophenylation reaction was terminated by the addition of ice-cold Tris buffer (0.15 M, pH 7.0). Cells were then washed with phosphate-buffered saline (pH 7.4), resuspended in 0.25 M sucrose/1 mM triethanolamine (pH 7.2), homogenized, and plasma membranes, microsomes, and mitochondria were isolated by differential and sucrose gradient centrifugation as previously described [31]. Treatment of cells with buffer alone versus no treatment gave identical results. Treatment of cells with trinitrobenzenesulfonic acid under the above conditions did not alter plasma membrane order as determined by nitroxide-labelled fatty acids and ESR techniques [36,37].

Plasma membrane aminophospholipid and sterol distribution. The percentage of phosphatidylethanolamine and phosphatidylserine located in the exofacial leaflet of the plasma membrane was determined using the trinitrobenzenesulfonic acid chemical labelling procedure [34,37]. The plasma membrane transbilayer distribution of sterol was determined also as described previously [19,35,38].

Lipid analyses. All organic solvents were glass-distilled, and all glassware was washed with sulfuric acid/dichromate before use. Lipids were quantitated as described previously [10].

Fluorescence probe incorporation and biophysical characterization. All procedures were performed using fluorescent dehydroergosterol [19,35,38] or 1,6-diphenyl-1,3,5-hexatriene [36,37,39–43]. Fluorescence intensity, polarization, lifetime, fractional fluorescence, limiting anisotropy, order parameter, and rotational rate were obtained as described therein.

Arrhenius plots of fluorescence intensity or polarization of 1,6-diphenyl-1,3,5-hexatriene in membranes were accomplished with an Exocal 100, Endocal 850, and ETP-3 Temperature Programmer system (Neslab Inst., Portsmouth, NH). The temperature of samples was increased from 4 to 45°C and then decreased from 45 to 4°C at 1°C/min. Temperature in the sample cuvette was continuously monitored with a WR-700 Digital Thermometer (Kernco Instruments, El Paso, TX) and a thermocouple placed directly above the lightpath.

Statistics. All values represent the means ± S.E. with *n*, the number of experiments, given in parenthesis. Statistical comparisons were made by Student's *t*-test.

Results

Local and metastatic tumor growth of LM fibroblasts in C_3H/Hen mice

In the first passage, local tumors were observed in 2/25 mice. Tumors were first palpable (and measurable) in 2–3 weeks post-inoculation. The tumor volume doubling times were 6 and 8 days, respectively. Upon histologic examination, these tumors were fibrosarcomas. In both animals with local tumors, there were no metastases evident in any organ system. Both the time of local tumor onset and the tumor volume doubling time were longer than for LM fibroblasts injected subcutaneously in athymic (nude) mice, 4 to 5 days and 2.15 ± 0.21 days, respectively [29].

On second passage of the local tumor cells, on day 45, 2/25 mice had local tumors with metastasis to lung (Met 0) observed in one of them. On third passage of the local tumor cells, after 45 days, 24/37 mice had developed local tumors with no metastases observed in any organ system. On passage of the Met 0 metastasis cells, 37/37 mice had local tumors, and 29/37 mice had lung metastases.

Tissue culture of non-metastatic and metastatic cells

In the serum-free, lipid-free Higuchi medium used herein, only malignant LM cells could survive after several passages. This property was evidenced by two characteristics of the cultured cells: the absence of cholesterol and the nutritional requirement for choline. Instead of cholesterol, the cultured tumor cells, like the original LM cell line, contained desmosterol due to their inability to convert de novo synthesized desmosterol to cholesterol. Also, in a separate experiment, in the absence of choline the cells in culture died, due to their inability to methylate ethanolamine to a level required to sustain cell growth. These properties were similar to those observed in the parent LM fibroblast strain [31]. Normal cells, in contrast, can synthesize both cholesterol and choline de novo. Since no cholesterol was detected, the cultures contained only LM derived tumor cells. Growth curves of cultured metastatic and non-metastatic cells were not significantly different.

Enzyme activity of membranes

As stated in Materials and Methods, the fold purification of enzyme markers for plasma membranes, microsomes, and mitochondria was not significantly different between metastatic and non-metastatic cells in culture. Thus, differences in membrane enzyme activities, lipid composition, and structure are not ascribed to different impurities. However, the specific activities of several lipid sensitive membrane enzymes were quite different in membranes isolated from the metastatic as compared to non-metastatic cells. The specific activity of 5′-nucleotidase was significantly elevated (3.8 ± 0.5)- and (5.4 ± 0.6)-fold, respectively, in plasma membranes and microsomes of metastatic cells ($P < 0.01$) as compared to the plasma membranes of non-metastatic cells. Na^+/K^+-ATPase specific activity was not significantly different between plasma membranes of non-metastatic and metastatic cells. The specific activity of the microsomal enzyme, NADPH-dependent cytochrome-*c* reductase, was significantly decreased 30% in microsomes of cell lines derived from lung metastases. The specific activity of succinate-dependent cytochrome-*c* reductase was (3.5 ± 0.5)-fold lower in mitochondria from metastatic versus non-metastatic cells.

Membrane sterol content and correlation with cytosolic sterol carrier protein

Sterol carrier protein (SCP) was present in the cytosol of all cell lines tested, ranging from 2 to 3% of cytosolic protein (Fig. 1). However, concentration of SCP was significantly lower ($30 \pm 1\%$) in cultured metastatic as compared to cultured non-metastatic cells ($P < 0.01$, $n = 3$ per cell line).

The plasma membranes of cultured non-metastatic cells had a desmosterol/phospholipid ratio of 0.40 ± 0.03. The plasma membrane desmosterol/phospholipid ratio was $46 \pm 7\%$ lower in cultured metastatic cells ($P < 0.01$). The lower desmosterol/phospholipid ratio was due to lower desmosterol (130 ± 28 versus 260 ± 20 nmol/mg protein) rather than increased phospholipid content. Thus, the desmosterol/mg membrane protein correlated directly with cytosolic sterol carrier protein content, in that lower desmosterol/protein levels were associated with lower cytoplasmic sterol carrier protein levels. Similar relationships were also obtained in the desmosterol/phospholipid molar ratio of microsomes, i.e., lower desmosterol/phospholipid ratios correlated with lower cytoplasmic sterol carrier protein levels. However, due to a lower mitochondrial phospholipid/mg protein content in the mitochondria from metastatic cells as compared to non-metastatic cells, the mitochondrial desmosterol/phospholipid ratio did not change with increasing cytosolic sterol carrier protein levels.

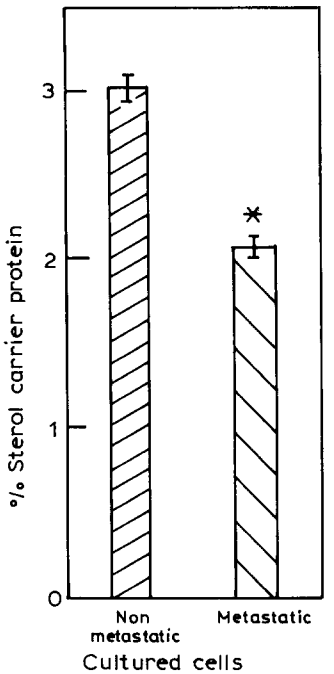

Fig. 1. Sterol carrier protein levels in cultured non-metastatic and metastatic tumor cells. All conditions were as described in Materials and Methods, except that sterol carrier protein, determined as described in Methods, was quantitated as a percent of total cytosolic protein. An asterisk refers to $P < 0.05$ ($n = 3$–5 per cell line).

Membrane phospholipid composition

The total phospholipids of plasma membranes from the local tumor cell line were comprised primarily of phosphatidylcholine ($45.8 \pm 2.5\%$), phosphatidylethanolamine ($27.6 \pm 1.0\%$), phosphatidylinositol ($6.3 \pm 0.6\%$), phosphatidylserine ($3.3 \pm 0.5\%$), and sphingomyelin ($3.2 \pm 0.9\%$). The phospholipid compositions of plasma membranes from metastatic cell lines were similar to those of the local tumor cell line, except for the content of phosphatidylinositol, which was $30 \pm 2\%$

TABLE I

Phospholipid fatty acid composition of membranes from cultured non-metastatic and metastatic tumor cells derived from C_3H mice injected with LM fibroblasts

Fatty acids were analyzed as described in Materials and Methods. Numbers refer to fatty acid nomenclature: the figure in front of the colon represents the number of carbon atoms while the figure after the colon refers to the number of double bonds. Values represent the means ± S.E. ($n > 5$). An asterisk (*) and plus (+) refer to $P < 0.01$ and $P < 0.05$, respectively, as compared to cultured non-metastatic tumor cells.

Cultured cell source	Fatty acid composition (%)					Ratio unsaturated/saturated
	16:0	16:1	18:0	18:1	>18	
Plasma membranes						
Non-metastatic	18.5 ± 0.9	6.9 ± 1.5	12.3 ± 1.1	49.4 ± 1.7	8.8 ± 1.2	1.54 ± 0.05
Metastatic	13.4 ± 1.0 *	2.3 ± 0.6 *	13.7 ± 0.5	58.3 ± 0.4 *	12.2 ± 1.8	2.02 ± 0.04 *
Microsomes						
Non-metastatic	15.6 ± 0.7	7.4 ± 1.3	13.1 ± 0.9	52.3 ± 0.9	1.4 ± 1.0	1.81 ± 0.03
Metastatic	15.0 ± 0.4	3.6 ± 1.0 +	15.2 ± 0.6	54.5 ± 1.0	12.5 ± 2.5 *	1.62 ± 0.07 +

lower (4.4 ± 0.1 versus 6.3 ± 0.6, $P < 0.01$) in plasma membranes from metastatic cells as compared to non-metastatic cells. Significant differences in microsomal phospholipid composition were not observed. However, the cardiolipin content of mitochondria from all metastatic cell lines was nearly 2-fold higher than in mitochondria from the local tumor cell line (10.1 ± 0.5 versus 5.2 ± 0.8, $P < 0.01$, $n = 5$). This difference was so pronounced that the ratio [anionic]/[neutral zwitterionic] phospholipids was 25% higher (0.30 ± 0.01 versus 0.24 ± 0.01, $P < 0.01$) in mitochondria from cultured metastatic as compared to cultured non-metastatic cells.

Transbilayer lipid distribution in plasma membranes

The transbilayer distribution of plasma membrane aminophospholipids was determined by chemical labelling with trinitrobenzenesulfonic acid under non-penetrating conditions (4°C). Under these conditions less than 1% and 0.1% of mitochondrial phosphatidylethanolamine and phosphatidylserine, respectively, were trinitrophenylated, indicating little leakage of the reagent into the cells. Only 3.6 ± 0.5% phosphatidylethanolamine and 1.8 ± 1.1% phosphatidylserine were exposed on the extracellular face of the plasma membranes from cultured non-metastatic cells. Under penetrating conditions (37°C) more than 95% of both these lipids were trinitrophenylated. The percent trinitrophenylated phosphatidylethanolamine or phosphatidylserine in plasma membranes from cultured metastases labeled with trinitrobenzenesulfonic acid did not differ significantly from that of the cultured local tumor cells.

The transbilayer distribution of sterol in plasma membranes of the cultured cells was determined using a fluorescent sterol, dehydroergosterol, and selective quenching of outer leaflet fluorescence by techniques previously described [19,35,38,41,42]. In the cultured non-metastatic cells, 18.3 ± 4.5% of the sterol was in the outer (exofacial) leaflet of the plasma membranes while the remainder was in the inner leaflet. There was no significant difference in the percent sterol in the outer leaflet of the plasma membrane in the cultured metastatic cells as compared to the cultured non-metastatic cells.

Phospholipid fatty acid composition of membranes from cultured local tumors and metastases

In addition to sterol/phospholipid ratio and phospholipid composition, the membrane phospholipid fatty acid composition is an important lipid characteristic that determines membrane structure. The most plentiful fatty acids found in phospholipids from both non-metastatic and metastatic cells were in the order 18:1 > 16:0 > 18:0 > 16:1 for plasma membranes, microsomes (Table I), and mitochondria (not shown). However, the ratio of unsaturated/saturated fatty acids of plasma membrane and microsomal phospholipids was 30 ± 3% higher and 11 ± 4% lower, respectively, in the cultured metastatic cells. In addition, the phospholipids of cultured metastatic cells were enriched in longer chain fatty acids (Table I). No significant differences in fatty acid composition of mitochondrial phospholipids between the non-metastatic and metastatic cells were observed.

Dynamic and structural properties of membranes

The plasma membrane structural properties of the metastatic cell lines differed significantly from those of the local tumor cell line. The structural static parameters (limiting anisotropy and order) of 1,6-diphenyl-1,3,5-hexatriene in plasma membranes from cultured metastatic cells were significantly lower ($P < 0.01$) than in plasma membranes from the non-metastatic cells (Table II). In contrast, significant differences in dynamic parameters (lifetime and rotational rate) were not observed. Fluorescence polarization is a composite of

both dynamic and static properties of the probe motion. Since polarization values of diphenylhexatriene were also lower in plasma membranes of the metastatic cells than in those of non-metastatic cells, polarization changes largely reflected differences in static rather than dynamic aspects of probe motion. Thus the motion of diphenylhexatriene in plasma membranes of cultured metastatic cells was significantly less restricted, indicating greater fluidity (less motional order). Basically similar results were obtained with the properties of diphenylhexatriene in microsomal (Table II) and mitochondrial membranes (not shown), in which the limiting anisotropy was lower in cultured metastatic cells as compared to the non-metastatic cells. Differences in lifetime or rotational rate were not observed. Since polarization was lower in microsomes (Table II) and mitochondria (not shown) of cultured metastatic cells, polarization changes again reflected differences in restriction to probe order rather than differences in dynamics.

Characteristic breakpoints in Arrhenius plots indicative of lipid phase, clustering or microdomain alteration of *trans*-parinaric acid but not diphenylhexatriene, were observed in plasma membranes, microsomes and mitochondria of the local tumor cell line (Fig. 2). Use of *trans*-parinaric acid detected breakpoints near $22 \pm 1°C$ and $37 \pm 1°C$. Similar breakpoints were observed with *cis*-parinaric acid near $23 \pm 1°C$ and $38 \pm 2°C$. The breakpoints were also observed with these fluorescence probe molecules in plasma membranes, microsomes, and mitochondria of the cultured metastatic cells, and did not differ significantly from those of the cultured non-metastatic cell line membranes.

TABLE II

Dynamic and structural properties of 1,6-diphenyl-1,3,5-hexatriene in membranes from cultured non-metastatic and metastatic tumor cells of C_3H mice injected with LM cells

Membrane dynamic and structural properties were determined as described in Materials and Methods. Values represent the means ± S.E. ($n > 5$). An asterisk (*) and plus (+) represent $P < 0.01$ and $P < 0.05$, respectively, by Student's *t*-test as compared to local tumors.

Parameter	Non-metastatic	Metastatic
Plasma membranes		
Polarization	0.292 ± 0.003	0.252 ± 0.005 *
Lifetime (ns)	8.4 ± 0.2	8.6 ± 0.2
Limiting anisotropy	0.174 ± 0.001	0.132 ± 0.005 *
Rotational rate (ns)	2.5 ± 0.1	2.4 ± 0.1
Order parameter	0.666 ± 0.006	0.580 ± 0.012 *
Microsomes		
Polarization	0.311 ± 0.002	0.297 ± 0.003 *
Lifetime (ns)	9.5 ± 0.1	9.9 ± 0.2
Limiting anisotropy	0.195 ± 0.003	0.180 ± 0.003 *
Rotation rate (ns)	2.2 ± 0.1	2.4 ± 0.1
Order parameter	0.71 ± 0.01	0.67 ± 0.01 +

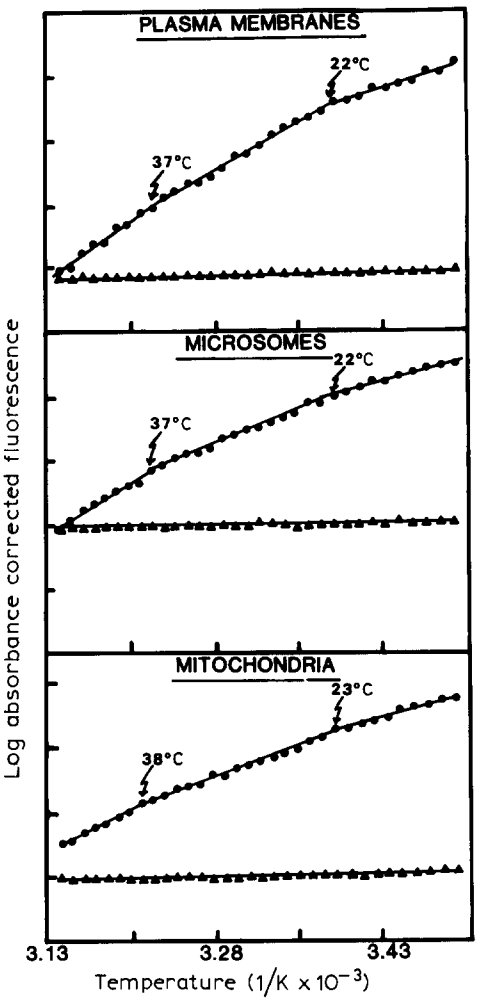

Fig. 2. Temperature dependence of *trans*-parinaric acid and 6-diphenyl-1,3,5-hexatriene fluorescence in cultured non-metastatic tumor cell membranes. Arrhenius plots of *trans*-parinaric acid (upper curves in each panel) and 1,6-diphenyl-1,3,5-hexatriene (lower curves in each panel) absorbance-corrected fluorescence were obtained on descending temperature scans as described in Materials and Methods.

Transbilayer structure of plasma membranes

The order parameter of 1,6-diphenyl-1,3,5-hexatriene in the inner leaflet of plasma membranes from cultured non-metastatic cells was higher than in the outer leaflet, 0.71 ± 0.01 vs. 0.53 ± 0.02 ($P < 0.01$). The outer leaflet of plasma membranes from the metastastic cells (0.47 ± 0.01) was significantly less ordered than that from the non-metastatic cells ($P < 0.01$). Significant differences in the inner leaflet fluidity of plasma membranes from cultured metastatic cells versus the non-metastatic cells were not observed. Thus, the decreased order of the plasma membrane 'bulk lipid' observed with diphenylhexatriene in the metastatic cells was due to a decreased ordering primarily of the outer leaflet.

Discussion

Direct comparisons of membrane properties from excised local tumors and excised metastases may be

obscured by several factors: (1) site of tumor growth [11,30]; (2) contamination of tumor by local (nontumorous) tissue, necrotic tissue, and host inflammatory cells [10,29]. Therefore, tumors in this study were placed into tissue culture before analysis. The cells were cultured for at least six weeks in the chemically-defined, serum-free, Higuchi medium to eliminate untransformed cells and inflammatory cells, which would be unable to survive this long without serum. Thus, it seems unlikely that the differences in membrane properties between non-metastatic and metastatic tumors were due to either inclusion of adjacent subcutaneous or lung tissue, or due to recruitment of host inflammatory cells. Neither metastatatic nor non-metastatic cell membrane enzymatic activity profiles resembled those of subcutaneous or lung tissue [10].

In earlier work from this laboratory [10], direct comparisons were made between membrane properties of excised (uncultured) local tumors and excised lung metastases [10], and the results presented herein are consistent with those studies: plasma membranes of cultured metastatic cells were more fluid than those of cultured non-metastatic cells.

Our previous membrane studies of non-metastatic and metastatic tumors were done with LM cell tumors derived from athymic (nude) mice. The results presented herein and by others [11] indicate that plasma membranes of metastases or cultured metastases from immunocompetent hosts were more fluid than those of local tumors or cultured non-metastatic tumor cells obtained from the same host. In investigating whether the LM cells could form tumors in immunocompetent mice, it was found that over 30 years ago LM fibroblasts were derived from L-929 cells [44,45], which in turn were derived from C_3H/Anf mouse fibroblasts over 45 years ago [46], when a primary strain of fibroblasts was cultured in the presence of 20-methylcholanthrene, establishing a tumorigenic fibroblast strain designated L-929. The incidence of local tumors formed by L-929 cells was 1/19 and 6/14 [46] and lung metastases were observed in 5 of 7 mice with local tumors [47]. The original substrain of C_3H/Anf mice is no longer available, so in the studies presented herein a closely related substrain, the C_3H/Hen (MMTV $-$) mouse was used. Local tumors, but no metastases, were observed in the first passage. Upon first passage of one of these local tumors, local tumors and metastases to the lungs were observed. Passage of metastatic cells resulted in an increased incidence of metastasis, while passage of the local tumor cells produced primarily local tumors in the time period of this study. Despite differences in the C_3H mouse substrain and other variables, LM fibroblasts formed local tumors in C_3H mice at roughly the same rate as their parent L-929 cell line, reported nearly half a century ago [44–47].

The amount of the ectoenzyme 5′-nucleotidase shed from plasma membranes has been used as a biochemical indicator of the metastasizing ability of transformed cells in cell homogenates, purified plasma membranes, and in serum [13,48,49]. The plasma membranes and microsomes of all metastatic cell lines had higher 5′-nucleotidase activity than those of the local tumor cell line. Whether the enzyme is shed into the plasma of C_3H mice in vivo is not known. However, as shown by the increased fluidity of the plasma membranes of the metastatic cell lines, especially of the outer leaflet, shedding of this ectoenzyme could be facilitated and will be a parameter of interest in future investigations.

Cell surface fluidity is controlled primarily by the ratio of sterol/phospholipid and degree of unsaturation of phospholipid fatty acids. Changes in both noted herein are consistent with the greater fluidity of cultured metastatic versus the local tumor cells. The metastatic cells had a lower plasma membrane sterol/phospholipid ratio and lower cytosolic sterol carrier protein levels than the cultured local tumor cells, and the resulting higher fluidity from the metastatic cell membranes was correlated with a decreased amount of sterol carrier protein. Highly metastatic B16 cell lines have also had lower sterol carrier protein levels than their less metastatic counterparts [16]. The molecular basis for the altered lipid composition is not known. Since sterol carrier protein can also stimulate activities of fatty acid esterification enzymes [25], the cytosolic levels of this protein may regulate plasma membrane sterol and unsaturated fatty acid content. These lipids in turn may influence plasma membrane fluidity and various proteins/receptors in the cell surface associated with malignancy.

Acknowledgements

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