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Cholesterol Levels and Plasma Membrane Fluidity in 3T3 and SV101-3T3 Cells

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Polyene antibiotics such as filipin selectively inhibit wheat germ agglutinin-induced agglutination of transformed and malignant cells compared to normal cells (Hatten ME, Burger MM: Biochemistry 18:739, 1979). Since filipin binds specifically to cholesterol, we measured cholesterol levels in 3T3 cells and SV101-3T3 cells. SV101-3T3 cells contained 50–100% more cholesterol per cell than 3T3 cells. Both cell types were starved for cholesterol by growth in lipid-depleted medium plus 25-hydroxycholesterol. The cholesterol level of SV101-3T3 cells decreased by 30–50%, while the level in 3T3 cells remained constant. Filipin-stained SV101-3T3 cells revealed bright patches of filipin under fluorescence microscopy. These patches were absent in 3T3 cells and in SV101-3T3 and 3T3 cells starved for cholesterol. We selectively labeled plasma membranes of these cells with a spin label analog of phosphatidylcholine. The spin label indicated differences in plasma membrane fluidity that may be related to the different cholesterol levels in 3T3 and SV101-3T3 cells.

Key words: virus transformation, membrane fluidity, plasma membrane, filipin, cholesterol, spin label, lectin agglutination

Hatten and Burger [1] found that polyene antibiotics such as filipin inhibit wheat germ agglutinin-induced agglutination of transformed or tumor cells but not normal cells. In addition, transformed cells are more sensitive to lysis by filipin. The polyene antibiotics bind specifically to membrane cholesterol [2]. The intrinsic fluorescence of filipin allows one to visualize bound filipin by fluorescence microscopy. Transformed or tumor cells stained with filipin reveal fluorescent patches, while normal cells stain diffusely. These observations prompted us to investigate cholesterol levels in mouse fibroblasts (Swiss 3T3) and their SV-40 virus-transformed derivative, SV101-3T3.

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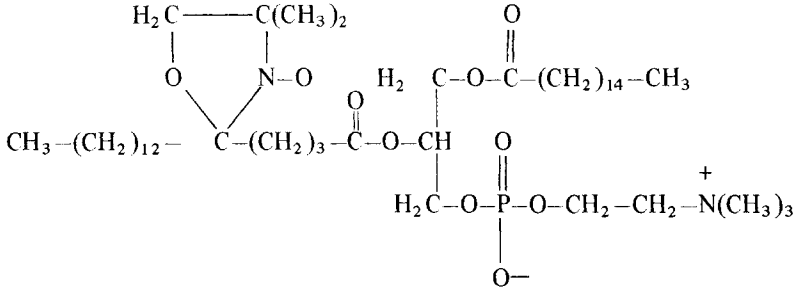
We find that SV101-3T3 cells have 50–100% more cholesterol per cell than 3T3 cells. The regulation of cholesterol levels in the transformed cell appears less stringent, as judged by the response of cells to cholesterol starvation. Cholesterol is mainly localized in the plasma membrane [3] and it alters membrane fluidity [4]. We measured plasma fluidity in intact, viable fibroblasts using a spin label phospholipid:



This spin label analog of phosphatidylcholine (SLPC) carries a 5-doxylstearate spin label in the β position. After incubating cells with a sonicated dispersion of SLPC and washing away unbound spin label, the SLPC was preferentially incorporated into the plasma membrane fraction. Plasma membrane fluidity measured with SLPC differed in 3T3 and SV101-3T3 cells. This difference in fluidity may be related to the elevated cholesterol levels in the transformed cell.

METHODS

Cells

Swiss 3T3 and SV101-3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) plus 10% calf serum or 10% lipid-depleted serum as described earlier [5].

Lipid Analysis

Lipids were extracted from cells by the method of Bligh and Dyer [6]. Phospholipid was measured by the method of Rouser et al [7]. Cholesterol was measured by gas-liquid chromatography with a Hewlett-Packard model 5830 gas chromatograph equipped with 1.8-m glass columns containing 3% OV-17 on 100/120 mesh Gas Chrom Q (Applied Science Laboratories, State College, Pennsylvania). Cholesterylacetate served as an internal standard.

Fluorescence Microscopy

Cells grown on collagen-coated coverslips were fixed with 0.1% osmium tetroxide in phosphate-buffered saline (PBS) (0.15 M NaCl–0.01 M sodium phosphate, pH 7.4), stained with filipin (20 μg/ml) in PBS for 30 min at 22°C, then washed with PBS. Filipin was the generous gift of Dr. G. B. Whitfield, Jr., Upjohn Co., Kalamazoo, Michigan. Stained cells were examined with a Zeiss Photomicroscope III fluorescence microscope equipped with epi-illumination.

Spin Labeling

A sonicated dispersion of SLPC was prepared as previously described [8]. Washed cells (5×10^8) were suspended in 0.01 M Tris-HCl (pH 7.5)–0.02 M NaCl–0.1% NaN_3 (0.2 ml). A sonicated dispersion of SLPC (0.4 ml) was added and the cells were incubated for 1 h at 22°C. Additional spin label (0.4 ml) was added and the cells were incubated for 30 min at 22°C. Cells were washed with PBS to remove unbound spin label, collected by centrifugation (600 rpm, 3 min, 22°C), and used for electron spin resonance (ESR) measurements or membrane fractionation.

Membrane Fractionation

Membrane fractions were isolated from spin-labeled cells by a modification of published procedures [9–11]. The details of our method will be published elsewhere. Marker enzymes used were 5′-nucleotidase (plasma membrane) [12], nicotinamide adenine dinucleotide dehydrogenase (NADH) (endoplasmic reticulum) [13], succinic dehydrogenase (mitochondria) [13]. After fractionation, spin label was extracted into chloroform [6] for quantitation by ESR spectroscopy.

RESULTS

Cholesterol Levels

Cholesterol and phospholipid levels in lipid extracts of whole cells are listed in Table I. The cholesterol content of SV101-3T3 cells was approximately twice that of 3T3 cells (15 fmoles/cell vs 7 fmoles/cell). SV101-3T3 had a correspondingly higher phospholipid content so the cholesterol: phospholipid ratio was the same (0.27) in these two cell types. Other investigators have reported values of 0.26–0.30 for the cholesterol: phospholipid ratio [1, 14].

Cells were starved for cholesterol by growth in lipid-depleted medium containing 25-hydroxycholesterol, a specific inhibitor of cholesterol biosynthesis [15]. Starvation for cholesterol reduced the cholesterol level in SV101-3T3 cells to 9.2 ± 2.3 fmoles/cell, while the level in 3T3 cells remained 9.0 ± 3.3 fmoles/cell (Table II).

Filipin Staining

We also observed the differential staining of SV101-3T3 cells reported by Hatten and Burger [1] (Fig. 1a, b). SV101-3T3 cells regularly displayed fluorescent patches, often near the perimeter of the nucleus. By focusing through the cell it was apparent that these patches reside near the upper or lower surface of the cell. 3T3 cells, in contrast, stained diffusely with filipin and rarely showed fluorescent patches. After cholesterol starvation 3T3 and SV101-3T3 cells rarely displayed fluorescent patches (a typical SV101-3T3 cell is shown in Fig. 1c).

TABLE I. Cholesterol and Phospholipid Levels in 3T3 and SV101-3T3

Cell type	Cholesterol per cell (fmoles)	Phospholipid per cell (fmoles)	Cholesterol:phospholipid molar ratio
SV101-3T3	15.1 ± 2.6	56 ± 10	0.27
3T3	6.9 ± 0.9	26 ± 3	0.27

Mean cholesterol and phospholipid levels (± SD) for cells grown in DMEM + 10% calf serum. The results of 12 trials for each cell type are represented.

TABLE II. Effect of Cholesterol Starvation on Growing Cells

	25-Hydroxycholesterol	Δ Cholesterol level, %	
Experiment	(nM)	3T3	SV101-3T3
1	50	23	-26
2	10	-13	-54
	50	-25	-48
3	10	24	-22
	50	-27	lysis

Cells were starved for cholesterol by growth in DMEM + 10% lipid-depleted serum + 25-dydroxycholesterol as indicated. The cholesterol level was compared to that of a parallel culture grown in DMEM + 10% calf serum. High levels of 25-hydroxycholesterol sometimes lysed SV101-3T3 cells.

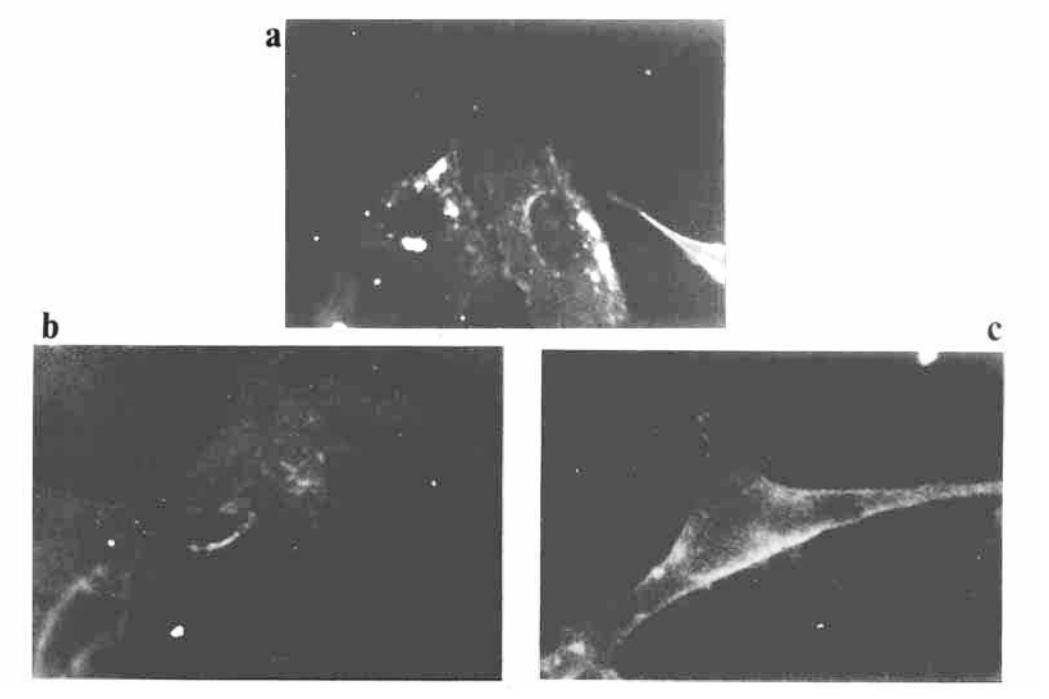


Fig. 1. Fluorescence micrographs of filipin-stained cells. a: SV101-3T3 cells grown in DMEM + 10% calf serum (× 1,600). b: 3T3 cells grown in DMEM + 10% calf serum (× 1,000). c: SV101-3T3 cells starved for cholesterol by growth in DMEM + 10% lipid-depleted serum + 50 nM 25-hydroxycholesterol (× 1,600).

Fluidity Measurements on Plasma Membranes of Intact Cells

We synthesized a spin label analog of phosphatidylcholine (SLPC) and found conditions that allowed us to incorporate this label into 3T3 and SV101-3T3 cells (see Methods). The cells were intact and viable after the labeling procedure, as judged by phase contrast

microscopy and trypan blue exclusion. The ESR spectra of labeled cells showed that the label was incorporated into membranes and not adsorbed to cells [8]. The phospholipid label was not degraded, as judged by extraction and thin-layer chromatography. Isolation of plasma membrane, mitochondria, and endoplasmic reticulum fractions from spin-labeled cells showed that the spin label is mainly localized in the plasma membrane fraction (Table III). The temperature dependence of the spectral parameter $2T_{\parallel}$ differed for 3T3 and SV101-3T3 cells (Fig. 2).

TABLE III. Membrane Fractions From Spin-Labeled SV101-3T3 Cells

	5'-Nucleotidase		Succinic dehydrogenase		NADH dehydrogenase		ESR signal intensity	
Fraction	Units/mg Protein	% of total	Units/mg protein	% of total	Units/mg protein	% of total	Units/mg protein	% of total
Homogenate	15	(100)	0.55	(100)	0.13	(100)	12	(100)
Mitochondria	9	3	3.6	45	0.19	1	13	5
Pellet	26	13	0.13	4	0.43	65	32	26
Plasma membrane	65	37	0.35	3	0.53	22	30	27

SV101-3T3 cells were spin-labeled and fractionated as described in Methods. Four fractions were analyzed: the crude homogenate, mitochondria, pellet (endoplasmic reticulum plus unbroken cells), and plasma membrane.

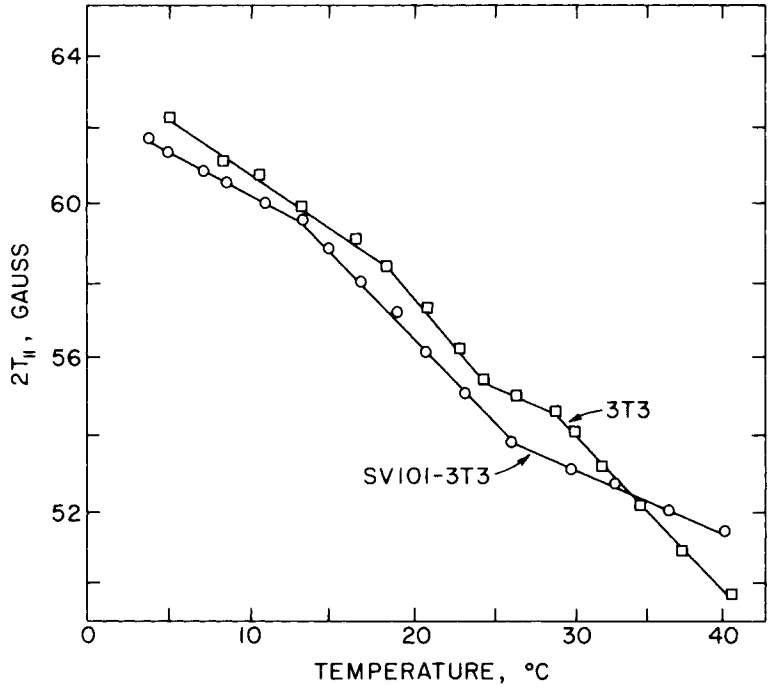


Fig. 2. Temperature dependence of $2T_{\parallel}$ in 3T3 cells and SV101-3T3 cells labeled with SLPC. Cells were spin-labeled as described in Methods. ESR measurements were carried out as described elsewhere [5].

DISCUSSION

We found consistently higher levels of cholesterol in SV40-transformed 3T3 cells compared to 3T3 cells. This result is particularly interesting in light of the alterations of cholesterol levels and cholesterol metabolism frequently observed in tumor cells [16] and the correlation of filipin staining with the transformed or tumor state [1]. From the response of SV101-3T3 cells to cholesterol starvation it seems clear that filipin staining is related to plasma membrane cholesterol levels.

SV101-3T3 cells are enriched for phospholipid as well as cholesterol. This result is surprising considering the smaller size of SV101-3T3 cells [14]. The excess membrane lipid may be related to the abundance of microvilli on the transformed cell surface [17] or to the elaboration of membrane vesicles [18].

Altering the fatty acyl composition of membrane phosphatides affects the temperature dependence of concanavalin A-induced and wheat germ agglutinin-induced agglutination of 3T3 and SV101-3T3 cells [19]. Spin label measurements revealed lipid structural changes occurring at temperatures corresponding to the agglutination effects [5]. The appearance of the cytoskeleton, as seen with immunofluorescent actin, myosin, and tubulin stains, was not altered (Buttrick, Hayward and Scandella, unpublished results), so we suspect that plasma membrane fluidity may regulate the function of lectin receptors directly. The previous measurements of membrane fluidity utilized fatty acid spin probes which partition into the lipid phase of cell membranes and probably label all cell membranes. These measurements indicated that bulk membrane fluidities in SV 101-3T3 and 3T3 cells are identical [5]. In contrast, plasma membrane fluidity measured here with SLPC differs. The difference in fluidity may be related to elevated cholesterol levels in the plasma membrane of the transformed cell. Recent work on cultured animal cells has shown that the sodium-potassium ATPase and adenyl cyclase activities are strongly influenced by cholesterol levels and membrane fluidity, as measured by lipid spin labels (M. Sinensky, personal communication).

Our studies of membrane fluidity in sea urchin eggs show that membrane fluidity changes after activation by sperm or parthenogenic agents [20]. The egg regulates plasma membrane fluidity and bulk membrane fluidity independently (Campisi, Galson, and Scandella, unpublished results). From the fibroblast agglutination studies it appears that the plasma membrane is heterogeneous with respect to fluidity. Taken together, these results show that membrane fluidity is complex. Changes in plasma membrane fluidity may have a crucial role in cell activation and transformation.

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