Cholesterol and Phospholipid Content of 3T3 Cells and Transformed Derivatives

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Cholesterol Surface Concentration, Proliferation Characteristics, Thermal Transition of Plasmamembrane

For 3T3, PY-3T3 and SV40-3T3 cells, the contents of total lipid, cholesterol and phospholipid were determined. Weight of total lipid per cell correlates closely with cellular volume as determined microscopically. This correlation is not observed for cholesterol or phospholipid content per cell.

Cholesterol content per cm^2 cellular surface area is found about fifty percent higher in the transformed cell lines compared to 3T3 cells. This result is discussed in relation to thermal transitions observed in physical properties of the plasma membrane of these cells as well as in lamellar phospholipid/water model systems containing cholesterol.

Introduction

Recent work on the mechanisms of regulation of cell proliferation has centered on the role of the cell surface membrane. In particular, studies using plant agglutinins [1–3], spin label probes [4] or electric surface charge of the cells [5, 6] as indicators of configurational membrane changes have suggested that the physical state of the lipid-protein matrix of the plasma membrane undergoes profound changes after stimulation of cell division in resting normal cells or after transformation. Thermal transitions observed in agglutination properties [7, 8] and in electric surface charge configuration [6] may reflect lateral phase separations in the plasma membrane, the latter being considered as a two-dimensional multicomponent system consisting of different species of membrane proteins and lipids. Using evidence both from agglutination [2] and from cell-electrophoresis studies, it has been suggested [6] that growth stimulation of resting normal cells by serum or protease treatment may be triggered by lateral phase separation due to the action of these agents and occuring at constant (growth) temperature.

From investigations of lamellar lipid model systems it is well-known that cholesterol affects profoundly the characteristics of thermal transitions in these systems [9].

Thus, the possibility arises that different characteristics of regulation of cell proliferation in normal and transformed cells are due to different contents of cholesterol in their surface membranes.

In animal cells, cholesterol may be considered to be located predominantly in the surface membrane [10, 11]. Therefore, it seemed worthwhile to determine total cholesterol content of normal and transformed cells in order to obtain evidence with regard to the above questions.

The data presented in the following show that indeed marked differences in the lipid composition exist between 3T3 cells and their transformed derivatives. In particular, cholesterol content per cm^2 cell surface turns out by about 50% larger in transformed cells than in 3T3 cells.

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Abbreviations: SV101-3T3, 3T3 cell line, transformed by simian virus 40 (SV 40); PY-3T3, 3T3 cells, transformed by polyoma virus; $\bar{d}$, average cell diameter, obtained by averaging over mean cell diameters of 14 independent cell preparations, each of which represents 30 to 50 microscopical observations of cell diameter; s, average standard deviation of cell diameters, obtained by averaging over standard deviations, each of which characterizes one of 14 independent cell populations; $\overline{d^2}$, average of cell diameter squared, corresponding to an average over 14 independent cell populations; $\bar{O}$, average surface of spherical cells, corresponding to an average over mean cellular surfaces of 14 independent cell populations.

Material and Methods

Stocks of the cells used in the present study (3T3, SV101-3T3, PY-3T3) were kindly provided by Dr. M. M. Burger, Basel. Cells were maintained at 37 °C on 94 mm plastic petri plates (Fa. Greiner, Nürtingen, W.-Germany) in antibiotica-free Dulbecco's modification of Eagle medium supplemented

with 10% heat-inactivated calf serum. Growth medium was changed three times weekly. Cells were tested repeatedly and found negative on mycoplasma contamination using the agar plate assay. Media and supplements were purchased from Flow Laboratories, Bonn, W.-Germany; the calf serum used throughout the study had the lot-number 40597. Cells were seeded at densities 2 to 5×10^3 per cm^2.

Saturation densities were $2-3 \times 10^4$ cells/cm^2 for 3T3 cells, $1.5-2 \times 10^5$ cells/cm^2 for PY-3T3 cells, and $2-3 \times 10^5$ cells/cm^2 for SV-101-3T3 cells. For each experiment, about 10^8 normal or transformed cells were removed from plates near saturation density by treatment at 37 °C with 0.25 per cent trypsin in Ca/Mg-free Earle's balanced salt solution. Cells were counted in a Neubauer-hemacytometer. Cell diameters were determined from averaging over 30 to 50 determinations of the size of (spherical) cells suspended in Ca-Mg-free Earle's balanced salt solution using a calibrated ocular-micrometer on the inverted microscope Zeiss UPI.

After washing the cells twice in Earle's balanced salt solution, the cell sediment usually was stored frozen at −80 °C until extraction. Some samples were extracted immediately after release from the plates; there was no significant difference in the results obtained for both types of preparation. The thawed (or fresh) cell sediment was extracted with chloroform/methanol (2:1, v/v) as described by Folch *et al.* [12], filtered and washed in the two phase system [12] obtained with water containing 0.05% $CaCl_2$. One extraction at room temperature was sufficient; repeated extraction with hot or cold chloroform/methanol (2:1, v/v) did not give any significant increase in the yield of total dry weight of the extract.

Dry weight of lipids was determined after evaporation of the extract to dryness and keeping the residue in vacuum over phosphorus pentoxide for 24 hours. The residue was redissolved in 0.5 ml chloroform/methanol/water (65:25:4, v/v), and aliquots taken for determination of total lipid phosphorus and free cholesterol.

Determination of total lipid phosphorus was done by the method of Bartlett [13]. Free cholesterol was determined by the ferric chloride procedure of Brown *et al.* [14], adapted to our purposes. Cholesterol was separated from cholesterol ester and other lipids by thin layer chromatography on precoated silicagel G plates from Merck, Darmstadt, W.-Germany, using chloroform as solvent. After visualizing with iodine, cholesterol spots were scraped off and eluted with 2 ml ethanol. Reaction with ferric chloride was performed in the presence of silicagel, which was sedimented by centrifugation before measuring absorbance at 531 nm. An equivalent area of silicagel was taken for a blanc. The amount of cholesterol was read from a calibration curve, which had been obtained with cholesterol standards under the same conditions as the analytical runs.

Results

Cellular contents of total lipid, free cholesterol and phospholipid, as obtained in our experiments on 3T3, PY-3T3 and SV101-3T3 cells are shown in Table I.

Cellular content of total lipid correlates closely with cell volume, as may be computed from average cell diameters given in line 1 of Table I.

Cell type	3T3	PY-3T3	SV101-3T3
Average cell diameter [μm]	18.59 ± 0.35 (14)	16.06 ± 0.26 (14)	15.05 ± 0.10 (14)
Average standard deviation of cell diameters [μm]	2.39 ± 0.13 (14)	2.21 ± 0.12 (14)	1.94 ± 0.15 (14)
Total lipids dry weight [pg/cell]	86.4 ± 9.2 (13)	63.8 ± 5.7 (10)	51.0 ± 5.9 (10)
Cholesterol [pg/cell]	4.90 ± 0.38 (13)	**5.53** ± 0.49 (10)	**4.69** ± 0.65 (10)
Phospholipid * [pg/cell]	33.9 ± 3.0 (13)	40.0 ± 3.9 (10)	31.9 ± 5.3 (10)
Molar ratio cholesterol/phospholipid	0.29	0.28	0.30

Table I. Cholesterol and phospholipid content of 3T3 cells and transformed derivatives (with standard error, and number of experiments on independent preparations in paranthesis).

* Obtained from lipid phosphorus determinations, using an average molar mass of phospholipid of 775 g mol^{-1}.

Densitometric evaluation of the thin layer-chromatograms showed only insignificant amounts of cholesteryl ester in our preparations. Cholesterol content per cell was found roughly equal for the three different cell lines tested. However, the weight percentage of cholesterol in total extracted lipids, as computed from lines 3 and 4 of Table I, turns out considerably lower in 3T3 cells (5.7%) than in PY-3T3 (8.7%) or on SV101-3T3 cells (9.2%). A very similar finding was reported [15] for WI 38 cells (10.2%) was compared to the SV40 transformed derivative WI 38 VI 13 A (14.4%). Similarly, phospholipid content per cell is roughly equal in our three cell lines, whereas weight percentage of phospholipid in total extracted lipids is lower in 3T3 cells (39.2%) as compared to PY-3T3 (62.6%) or SV101-3T3 cells (62.6%).

This is opposite to the finding of a lower weight fraction of phospholipid in total lipid of WI 38 cells compared to WI 38 VA 13 A cells [15].

From the numbers given above, it is evident that the sum of weight fractions of cholesterol and phospholipid in total lipid in 3T3 cells is considerably lower than in the two transformed lines. A very similar result was reported for rat and mouse lymphocytes as compared to leucaemic cells [16].

For cells growing in suspension, a correlation was reported between molar ratio of cholesterol to phospholipid and normal or transformed growth behaviour [16]. From our data on the three cell lines growing on solid substratum we have computed the corresponding ratio (see last line of Table I) which turns out to be equal within the precision of experiments for normal and transformed 3T3 cells. Thus, the molar ratio cholesterol to phospholipid in these cases does not correlate with transformed growth behaviour. This conclusion is supported by a survey on lipid analysis of 14 different cell lines grown *in vitro* [17–21], showing cholesterol to phospholipid ratios between 0.11 and 0.49 without any obvious correlation with the proliferation behaviour of the cells. The considerable variation of this ratio may be due mainly to the marked dependence of the fraction of phospholipid in total lipid on nutritional conditions, such as type of serum [17].

The numbers discussed above refer to cholesterol relative to total cellular lipid. More meaningful with respect to its possible effect on membrane-bound regulation processes is the surface concentration of cholesterol in the plasma-membrane.

Since cholesterol may be taken to be located mainly in the plasma-membrane [10, 11], we have computed from our data the molar content of cholesterol per cm^2 cell surface, as follows. The statistical distribution of cell diameters in a particular cell preparation may be characterized by the standard deviation of cell diameters determined microscopically. This quantity averaged over 14 independent preparations of each of the three cell lines investigated here, is given in line 2 of Table I. Using the statistical relation $\overline{d^2} = (\bar{d})^2 + s^2(n-1)/n$, we can calculate the average cellular surface area $\bar{O} = \pi \bar{d}^2$ from the average cell diameter $\bar{d}$ and the averaged standard deviations of cell diameters as $\bar{O} = \pi\{(\bar{d}) + s^2(n-1)/n\}$. The average cellular surface areas so obtained from the figures in lines 1 and 2 of Table I are given in line 1 of Table II. Standard errors given are calculated as usual from relations of error propagation. From line 4 of Table I and line 1 of Table II, the molar content of cholesterol per cm^2 cell surface is computed and given in line 2 of Table II. Again, standard error is calculated from Gauss'-relation of error propagation.

As is evident from these numbers, the surface concentration of cholesterol in virus-transformed derivatives of 3T3 cells is about 50 per cent higher than in the normal cells. Statistical evaluation by Student's t-test shows that the deviation of molar content of cholesterol per cm^2 surface between 3T3 cells and PY-3T3 cell is significant with $P > 0.99$. Similarly the deviation between 3T3 cells and SV101-3T3 cells is significant with $P > 0.97$.

Cell type	3T3	PY-3T3	SV101-3T3
Average cell surface [$\times 10^{-8}$ cm^2]	1104 ± 42 (14)	825 ± 26 (14)	724 ± 9 (14)
Cholesterol/cm^3 cell surface area [nmol cm^{-2}]	1.15 ± 0.10 (13)	1.73 ± 0.17 (10)	1.68 ± 0.24 (10)

Table II. Average cell surface area and molar cholesterol content per cm^2 cell surface for 3T3 cells and transformed derivatives (with standard error, and number of experiments in paranthesis).

This evaluation is based on cellular surface areas determined from suspended cells of spherical shape. Since 3T3 cells attached to petri plates are spread out much more than transformed cells under equivalent conditions, the above conclusion of a significant difference of cholesterol content per cm^2 cell surface between normal and transformed 3T3 cells is valid a forteriori for cells in cultivation.

This conclusion is further strengthened by scanning electron-microscopic observations on the surface topology of 3T3 cells and the virus-transformed derivatives. Comparing normal and virus-transformed cells growing on solid substratum [22] as well as those suspended after removal from the substratum [23], it has been found that 3T3 cells have more surface protrusions (such as microvilli) than SV40- or PY-virus-transformed derivatives, conferring an even larger surface area to the normal cells.

Discussion

The above result of an about 50% higher cholesterol surface concentration in transformed cells compared to normal is of considerable interest, because such a difference should be reflected by different physical properties of the plasma-membrane.

Increasing amounts of cholesterol in lamellar phospholipid/water model systems lead to a broadening of the gel to liquid crystalline transition. In fact, raising cholesterol content in 1,2-dipalmitoyl-L-lecithin/cholesterol/water systems from 20 mol% to 32 mol%, *i. e.* a relative increase by 60%, leads from a thermal transition spread over about 10 °C to virtual disappearance of this transition [9].

Using essentially the same cell preparations as in the present study, we have recently characterized the electric surface charge configuration of 3T3 and SV40-3T3 cells by micro-cellelectrophoresis and used as an indicator of changes of the physical state of the lipid-protein matrix of the plasma membrane [6]. In this work, for both cell lines thermal transitions have been observed, which turned out to be fairly narrow (<10 °C) for 3T3 cells, but rather broad (≈ 30 °C) for SV40-3T3 cells. As predicted from the studies on mixed lipid model systems, the broader transition in the transformed cell line is fully consistent with its higher cholesterol surface concentration determined above.

At present, the dependence of the physical state of the plasma membrane, for instance of the nature of its thermal transition(s), on the detailed conditions of their cultivation are not known. In particular the lipid composition might depend strongly on the type and amount of serum used. Thus, it might not generally be possible to provide for a very strict comparison of the results obtained under different conditions.

Nevertheless, it is of interest to discuss physical properties observed by other authors on these cell lines in the light of the data given above.

Using plant agglutinins such as Concanavalin A, fairly narrow transitions have been observed in agglutination of PY 3T3 and SV101-3T3 cells [7, 8]. However, these findings need not be contradictory to the above conclusions, because it has been shown by electron spin resonance and nuclear magnetic resonance techniques that plant lectins such as concanavalin A affect the physical state of plasma membrane lipids [24, 25]. In fact, secondary binding and lipophilic affinity of plant agglutinins to the plasma-membrane has been discussed in the context of the agglutination mechanism [26]. Thus, the plant lectins may not in each case be suitable to report the undisturbed state of the plasma-membrane.

Using a fatty acid spin label, the order parameter S pertaining to the lipid regions of 3T3 and several transformed derivatives has been determined and found by 10 to 15% higher in 3T3 cells than in the transformed cell lines [4]. This result indicates different physical states of the plasma-membrane of these cell lines and might well be due to different surface concentrations of cholesterol. Because of lack of some experimental details of this interesting work, a more detailed discussion of it is not possible at present.

It is of considerable interest, whether the above findings of higher cholesterol contents of transformed derivatives of 3T3 cells compared to normal cells are true also for other pairs of normal and transformed cell lines.

Cell surface areas of WI 38 and WI 38 VA 13 A cells unfortunately are not known to us. However, the cholesterol contents of 6.60 pg/cell and 8.47 pg/cell for WI 38 and WI 38 VA 13 A, respectively [15], appear to parallel our findings of a higher cholesterol surface concentration in transformed cells. Similarly it has been found that cholesterol content of plasma-membrane preparations of several hepatoma from mouse and rat are significantly larger than that of the corresponding normal mouse or rat liver cells [27].

These results may point to a general phenomenon. However, the dependence of the effects mentioned on growth conditions, such as lipid content and composition of growth medium etc., as well as the lipid composition of many more comparable cell lines have to be studied before a more detailed conclusion may be drawn. These studies, as well as those on the lipid composition of isolated plasma membrane preparations are in progress in our laboratory at present.

If indeed the above generalization can be verified experimentally, interesting clues as to the nature of the deviations from normal of the regulation mechanism of cell division in transformed cells appear obvious. Experimental evidence has been obtained for a fairly narrow temperature region of phase separation in 3T3 cells, which is shifted to about growth temperature upon stimulation of cell division by serum or trypsin treatment [6]. This shift of the range of phase separation was interpreted as a basic triggering process for secondary membrane functions leading to initiation of DNA synthesis [6]. Such a regulation mechanism might only function properly with a steep characteristic, as evidenced by the narrow temperature transition present in normal cells. Upon broadening of the transition region due to increased cholesterol in the membrane of transformed cells, the regulation characteristic might be disturbed grossly and growth control be disabled. In this context, anomalies in feed-back regulation of cholesterol synthesis observed in hepatoma [28] and to a lesser degree in WI 38 VA 13 A cells [29] gain considerable interest, as they may be intimately connected with, if not the cause of, the deviations of cholesterol surface concentrations in transformed cells discussed above.

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