

Dependence of Cellular Surface Area on Growth Density of 3T3 and SV40-3T3 Cells

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Cell Micrographs, Planimetry, Electric Cell Sizing

The cell surface areas of 3T3 and SV40-3T3 cells both for suspended and for flat cells growing on the culture substrate have been determined in dependence on growth density. The surface areas of flat 3T3 and SV40-3T3 cells turn out to be independent of growth density over a wide range. For 3T3 cells the surface area of flat cells compares well with that determined by Collard and Temmink for suspended 3T3 cells taking the surface area of their coverlayer of microvilli into account. This indicates that upon suspension of flat 3T3 cells the surplus surface area is disposed of in the form of microvilli. For SV40-3T3 cells we obtained a surface area of flat cells which differs by a factor of about two from that of suspended cells as determined by Collard and Temmink including the surface area of microvilli.

Microscopical diameters and electrical ("Coulter") volumina of suspended 3T3 and SV40-3T3 cells depend only little on cell density in the range investigated.

Introduction

Numerous studies have established the prominent role of the plasma membrane in processes of density-dependent regulation of cell proliferation as studied by in vitro cell cultures. It has been shown that physiological properties of the plasma membrane, such as phosphate transport [1–4], sugar transport [4–5], activity of Na/K-ATPase [6–8], passive cation permeability [9, to be published] as well as its physical properties, such as microviscosity [10], agglutinability [11], volume of suspended cells [12] depend strongly on cellular density of the growing cell sheet. A property of the plasma membrane most important not only in its own right but also as a reference quantity for derivation of any specific membrane parameter is its surface area. Membrane surface areas for normal and transformed 3T3 cells have been determined for suspended spherical cells by several authors using optical [13, 14] and scanning electron microscopy [15] or electric measurements of volume by the Coulter principle [14]. However, as these experiments do not pertain to the physiologically relevant state of cells growing flat on their culture substrate, we have determined the surface areas by planimetry of micrographs of flat cells at different population densities on the plate and compared these figures with those obtained for spherical suspended cells

by calibrated microscopy or by electric measurements of cellular volume with a Coulter Counter.

Materials and Methods

Cell lines. Stocks of Swiss 3T3 and SV40-3T3 (line SV101) have been kindly supplied by Dr. M. M. Burger, Basel. The cells were maintained antibiotic-free at 37 °C on 94 mm plastic petri plates (Greiner, Nürtingen, BRD) in Dulbecco's modification of Eagle medium supplemented with 5% or 10% heat-inactivated newborn calf serum in a moistened atmosphere of air with 10% CO₂. Media and supplements were obtained from Flow Laboratories, Bonn, BRD.

Cells were seeded at a density of $1 - 3 \times 10^3 \text{ cm}^{-2}$ for 3T3 cells and SV40-3T3 cells. Growth medium was changed three times weekly. Saturation densities were $2 - 3 \times 10^5 \text{ cm}^{-2}$ for SV40-3T3 cells and $2 - 3 \times 10^4 \text{ cm}^{-2}$ for 3T3 cells. Higher densities for 3T3 cells were achieved by stimulating growth from the resting monolayer with medium containing 10 to 30% newborn serum.

Harvesting of cells. For measurements on suspended cells or for cell counting, the cells were relaxed from the plates by incubation at 37 °C for 3–15 min with 4 ml Tris-buffered modified Earle's solution (116 mM NaCl, 5.4 mM KCl, 0.9 mM NaH₂PO₄·H₂O, 5.1 mM glucose, 0.14 mM phenol red, 20 mM Tris-HCl, pH 7.4) containing 60 µg/ml trypsin (Boehringer, Mannheim, BRD). Cell sus-

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pensions were diluted with the modified Earle solution as required. For some experiments, cells were harvested as described earlier [16]. Cell counts for evaluation of cell density were taken with the Neubauer hemocytometer or the Coulter TF-System, both methods coinciding within 10%.

Microscopy and planimetry. Micrographs of cells growing on the petri plate under complete medium were taken at different cell densities using a Zeiss microscope Universal with phase-contrast optics and water-immersion objective ($40\times$) at a total magnification of $800\times$. Outline areas of the cells were determined with a mechanical planimeter and calibrated using micrographs of the grid of a Neubauer hemocytometer. The surface areas of 10–40 micrographs of different cells were evaluated for each independent preparation (petri plate). For 3T3 cells 43 independent preparations, for SV40-3T3 cells 9 independent preparations were used covering different culture passages of the cells over several months.

Cell heights were determined observing cells with objective Zeiss-planapochromate 63/1.4 Oel Ph under differential interference contrast (Nomarski optics) and using calibrated fine focus adjustment.

Measurements on suspended cells. Under microscopic observation suspended cells exhibit spherical shape. From cell diameters thus determined using a calibrated ocular micrometer, cell surface areas have been evaluated assuming smooth spherical shape. For 3T3 cells more than 20 independent preparations at cell densities below $1.5 \times 10^4 \text{ cm}^{-2}$ have been evaluated measuring 20–50 cells each. For SV40-3T3 cells at cell densities between 1.5×10^3 and $3 \times 10^5 \text{ cm}^{-2}$ more than 50 independent preparations have been evaluated in the same way. Cellular volumes have been measured also by a method based on the Coulter Counter. We have used the Coulter TF-System (Coulter Electronics, Krefeld, BRD), which employs hydrodynamical focusing of the measured cell suspension and thus avoids distortion of the volume distribution by non-axial transit of cells through the aperture. For every independent cell preparation 2×10^3 to 5×10^4 cells were sized, using an aperture current of 0.3 mA at a capillary width of $100 \mu\text{m}$. Cellular volumes were calibrated with latex particles of 12.9, 14.0 and $18.0 \mu\text{m}$ diameter. The number of independent preparations characterized by this method was

39 for 3T3 cells (cell densities 1.5×10^3 to $1.5 \times 10^4 \text{ cm}^{-2}$) and 28 for SV40-3T3 cells (cell densities 1.5×10^3 to $3 \times 10^5 \text{ cm}^{-2}$). The measurements on suspended cells covered many culture passages over 2–3 years.

Results

Outline areas of cells growing on the petri plate were determined by planimetry as described above. Twice the outline area (for upper and lower surface area) is plotted versus cellular density of 3T3 and SV40-3T3 cells in Fig. 1. These experiments did not differentiate between mitotic and flat cells. As the former are represented only by a fraction of at most a few percent, the averages given in Fig. 1 in a very good approximation pertain to flat cells. Within experimental error, we do not observe any dependence of cell surface area of flat 3T3 cells on cell density below $4.4 \times 10^4 \text{ cm}^{-2}$. At all cell densities, 3T3 cells grow in a cellular monolayer without appreciable overlap of cell borders. With this growth habit of 3T3 cells is consistent the conspicuous decrease of cell surface area of 3T3 cells above a density of $4.4 \times 10^4 \text{ cm}^{-2}$, which corresponds to 100% coverage of the plate with cells of an average surface area of $4.5 \times 10^{-5} \text{ cm}^2$ (outline area $2.25 \times 10^{-5} \text{ cm}^2$). In Fig. 1 is shown the theoretical expectation (dashed curve) corresponding to outline areas of 3T3 cells at high cell densities as being strictly reciprocal to cell density. This theoretical expectation neglects cellular surface area arising

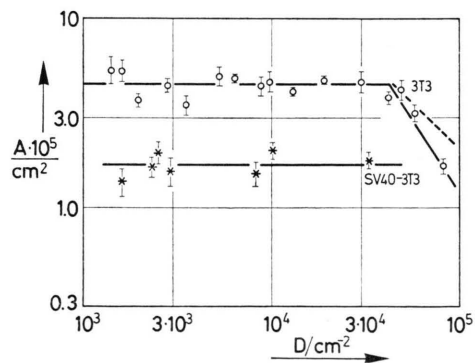


Fig. 1. Cell surface area A in 10^{-5} cm^2 of 3T3 cells ($\bigcirc-\bigcirc$) and SV40-3T3 cells ($*-*$) growing on their culture substrate in dependence on cell growth density D in cells per cm^2 . Each point is mean and standard error of the mean from measurements on 20–30 micrographs of cells. Dashed line: theoretical expectation for cell surface area being strictly reciprocal to cell density.

from three-dimensional shape of the cells. In order to check the validity of approximating cell surface as twice a plane sheet, we have made use of the small depth of focus in observing cells under differential interference contrast (Nomarski optics) and scanned the height of cells above surface of the petri dish using calibrated fine focus adjustment. Maximum height of 3T3 and SV40-3T3 cells at cell densities below 3×10^4 cells/cm² is between 3 and 6 μ m near the cell nucleus and falls to less than 1 μ m approaching the cell border. When 3T3 cells are stimulated to cell densities above 4×10^4 cm⁻² by repeated treatment with elevated serum concentration, cell morphology changes from flat polygonal ("cobble-stone") to spindle-shaped without appearance of appreciable overlap of cell borders. Spindle-shaped 3T3 cells at high cell densities exhibit maximum cell heights of ≤ 9 μ m. Using different geometrical models for approximating three-dimensional shape of cells, one can estimate that at densities of 3T3 cells below 3×10^4 cm⁻² cell surface area is underestimated by 5–10% at the maximum, if cells are approximated as plane double-sheets. At cell densities above 4×10^4 cm⁻², the correction may amount to about 20 to 30%. This contribution seems to account largely for the difference between experimental data and theoretical curve for surface areas of 3T3 cells above 4×10^4 cm⁻² (Fig. 1).

The shape of SV40-3T3 cells below 3×10^4 cells/cm² is flat polygonal to spindle-shaped. Estimates using cell heights reported above in geometrical models of cell shape (*e. g.* segments of cones for spindle-shaped cells) indicate that cell surface area is underrated by about 10–15% at the maximum, if the assumption of a plane double-sheet is used (for scanning electron micrographs of SV40-3T3 cells see [17]). As this correction is largely

independent of cell density below 3×10^4 cm⁻² and, furthermore, is not larger than variations of cell surface area between different cell preparations, we shall neglect it in the following. Cell surface area of SV40-3T3 cells within the range amenable to direct measurement ($< 3 \times 10^4$ cm⁻²) is found independent of cell density (Fig. 1). Above a cell density of 3×10^4 cm⁻², cellular overlap of SV40-3T3 cells is so extensive that a statistically meaningful evaluation of cellular outline areas becomes impossible. However, SV40-3T3 cells from full plates reseeded to lower cell density, after spreading (*i. e.* measured 8–12 h after reseeding) exhibit surface areas indistinguishable from those shown in Fig. 1. This might indicate that cell surface area of SV40-3T3 cells does not change upon increase of cell density beyond 3×10^4 cm⁻². Average cell surface areas of flat 3T3 and SV40-3T3 cells computed from the data corresponding to the horizontal curves in Fig. 1 are given in Table I.

Cell surface areas of suspended SV40-3T3 cells as evaluated from microscopic cell diameters or electrical ("Coulter") volumes assuming smooth spherical shape of cells, exhibit a slight decrease with cell density above 5×10^4 cm⁻². Since this small change of average cell surface area is almost obscured by the variations between different preparations, we may neglect in a first approximation the dependence on cell density. The averages of cell surface areas of SV40-3T3 cells determined over a wide range of cell densities are given in Table I and show excellent agreement between optical and electrical measurements. These experimental results on suspended SV40-3T3 cells were obtained on different series of passages over several years and turn out to be remarkably insensitive to the particular conditions of culturing, harvesting and measuring of the cells.

Table I. Average cell surface areas \pm standard error of the mean from different independent preparations of 3T3 and SV40-3T3 cells (number of independent preparations in parentheses) as measured by different methods described in the text.

Cell type	3T3			SV40-3T3		
Range of cell densities per cm ²	$1.5 \times 10^3 - 4.5 \times 10^4$		$1.5 \times 10^3 - 1.5 \times 10^4$	$1.5 \times 10^3 - 1.5 \times 10^4$		$1.5 \times 10^3 - 3 \times 10^5$
Preparation	flat cells		suspended cells	flat cells		suspended cells
Method	optical		optical electrical	optical		optical electrical
Surface area \pm SEM in 10^{-5} cm ²	4.53 ± 0.14 (43)		0.9–1.3 (38)	1.68 ± 0.08 (9)		0.831 ± 0.008 (51) 0.844 ± 0.012 (28)

For 3T3 cells in suspension from a particular sequence of passages under the same conditions of growth and measurement, cell surface areas do not show variations which exceed those of SV40-3T3 cells, and within this variation are independent of cell density between 1.5×10^3 and $1.5 \times 10^4 \text{ cm}^{-2}$. However, the results for suspended 3T3 cells differ for different growth conditions (concentration and type of serum) and for different procedures of harvesting (*e.g.* type of protease used for release from plate) and of suspension (type of buffer used). Thus, in the case of 3T3 cells it does not appear possible to uniquely characterize the size of suspended cells by a representative average of cell surface areas; in this case we have given in Table I only the range of experimental figures obtained.

Discussion

Cell surface area of flat 3T3 cells evaluated by planimetry of micrographs was found to be $(4.53 \pm 0.14) \times 10^{-5} \text{ cm}^2$ (Table I). The additional surface area contributed by microvilli or blebs on the surface of flat cells may be estimated as less than 20% from scanning electron micrographs [17]. The surface area of flat 3T3 cells thus is larger by a factor of 3.5 to 5 than that obtained for suspended cells assuming smooth spherical shape, *i.e.* neglecting presence of microvilli on the cell surface. The number and size of microvilli on the surface of suspended 3T3 cells has been evaluated, however, by scanning and transmission electron microscopy [15], yielding a total cell surface of $5.2 \times 10^{-5} \text{ cm}^2$, which compares well with our figure for flat 3T3 cells. This agreement supports the notion that upon release (and subsequent rounding) of 3T3 cells from the culture substrate, the entire surplus membrane surface area is disposed of in the form of microvilli.

A similar situation is met in the case of SV40-3T3 cells, although here, according to Table I, the difference of surface areas of flat cells ($1.68 \times 10^{-5} \text{ cm}^2$) and of suspended cells ($0.84 \times 10^{-5} \text{ cm}^2$) is smaller, indicating less membrane surface area to be stored in the form of a coverlayer of microvilli on the surface of suspended SV40-3T3 cells in comparison to 3T3 cells. Qualitatively, this expectation is confirmed by electron microscopic study [15] yielding smaller average length and average density of microvilli on the surface of SV40-3T3 cells com-

pared to 3T3 cells. Similarly, the coincidence of cellular surface areas of suspended SV40-3T3 cells (Table I) evaluated microscopically or electrically, indicates that in the case of SV40-3T3 cells the coverlayer of microvilli is thin and does not lead to an overestimation in the microscopic determination of the diameter of rounded cells. In the case of 3T3 cells, however, a consistent overestimate of microscopical diameters compared to electrically evaluated diameters is observed (Table I and [14]). This discrepancy might be traced to a thicker coverlayer of microvilli in the case of 3T3 cells. In quantitative detail, however, the situation is not as clear: total cell surface of suspended SV40-3T3 cells (including microvilli) $0.82 \times 10^{-5} \text{ cm}^2$ [15] was found about half as large as for flat SV40-3T3 cells $1.68 \times 10^{-5} \text{ cm}^2$ (Table I). In descending order of likelihood the reason for this difference might be sought in another mode of membrane disposal of SV40-3T3 cells upon rounding, methodical details of the two studies, or different cell stocks used, but is not resolved at present.

Different properties of the plasma membrane of 3T3 and SV40-3T3 cells pertinent to the process of rounding of the cells are indicated by the remarkable insensitivity of cell diameter of suspended SV40-3T3 cells to different growth and suspending procedures in contrast to 3T3 cells ([14] and Table I).

Thus, the cell surface areas of flat 3T3 and SV40-3T3 cells as derived in the present study might contribute to an understanding of the membrane dynamics in the process of release from the plate and rounding up. Further work in our laboratory is directed to determination of cell volumes of flat cells and rounded (*e.g.* mitotic) cells in comparison to the corresponding cell surface areas.

In addition, the cell surface areas of flat cells, as derived in the present study, provide for the necessary reference quantity for studies, where specific membrane properties such as in transport studies are to be evaluated for cells growing on their culture substrate.

As an example, we may refer to our earlier work on the cholesterol content of normal and transformed cells which was related to cell surface area of suspended cells considered as smooth spheres [16]. If we relate the cholesterol content per cell of the earlier study [16] to the surface area of flat cells as derived above, we arrive at 280 pmol

cm⁻² for 3T3 cells and 723 pmol cm⁻² for SV40-3T3 cells. This 2.6-fold excess of cholesterol per cm² surface area of SV40-3T3 cells compared to 3T3 cells is of considerable interest with regard to different microviscosities [10, 18] and to different temperature dependence of the surface properties [19, 20] of these cells.

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