Title
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Publication Date
1976-07-01

Peer reviewed
Cell Shape and Hexose Transport in Normal and Virus-Transformed Cells in Culture

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Key words: sugar transport, cell shape, transformed chick cells, methyl cellulose, scanning electron microscopy.

Abbreviations used: ZDG, 2-deoxy-D-glucose; 3MG, 3-O-methyl-D-glucose; Meth C, methyl cellulose; CB. cytochalsin B.

LBNL/DOE funding & contract number: DE-AC02-05CH11231

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The rate of hexose transport was compared in normal and virus-transformed cells on a monolayer and in suspension. It was shown that: 1) Both trypsin-removed cells and those suspended for an additional day in methyl cellulose had decreased rates of transport and lower available water space when compared with cells on a monolayer. Thus, cell shape affects the overall rate of hexose transport, especially at higher sugar concentrations. 2) Even in suspension, the initial transport rates remained higher in transformed cells with reference to normal cells. Scanning electron micrographs of normal and transformed chick cells revealed morphological differences only in the flat state. This indicates that the increased rate of hexose transport after transformation is not due to a difference in the shape of these cells on a monolayer.

The relation between the geometry of cells, transport rates, and growth regulation is undoubtedly very complex, and our knowledge of these relationships is still very elementary. In a recent review on the influence of geometry on control of cell growth, Folkman and Greenspan (1) pointed out that the permeability of cells in a flat versus a spherical state may indeed be very different. The growth properties of cells on a surface and in suspension have been compared often (1 -5). However, with one exception, little is known about the changes in transport properties when cell shape is changed. Foster and Pardee (6) demonstrated that the active transport of a-aminoisobutyric acid was reduced 2.5 times in suspension cultures of Chinese hamster cells with respect to the cells grown on a coverslip. They attributed this to the smaller surface area of suspended cells. While it is not clear why active transport should be dependent on the surface area available, it is possible that once the cells assume a spherical configuration, the carrier proteins are redistributed in such a way as to make them less accessible to the substrate. What happens to facilitated and nonmediated diffusion when cells are placed in suspension has not been determined.

The transport of hexoses into animal cells in culture has been shown to occur by facilitated diffusion (7). In chick embryo fibroblasts in culture, there is more than one component to this transport system: a saturable carrier-mediated transport with a Km for 2-deoxy-D-glucose (2DG) of about 1-5 mM (8-11) and a nonsaturable component which may include a low affinity transport site and/or nonmediated diffusion (8, 10, 11). It was shown previously (11) that growth rate, cell density, glucose deprivation, and virus transformation may alter not only the overall transport rate but also the rate of transport of one mode relative to the other. The rate of the nonmediated uptake, at least, is dependent upon the surface area available, and in addition total level of transport is dependent on the available water space (which in turn is roughly related to cell volume). Since it is estimated that the surface area may change as much as tenfold when cells go from a flat configuration to a spherical one (I), it is apparent that the cell shape could play a role in overall transport characteristics of cultured cells.

Using glucose analogues, 2DG and 3-0-methylglucose (3MG), we compared the transport properties of normal and virus-transformed cells in suspension and on monolayers. It was found that both the rates as well as the total levels of transport were decreased after the cells were placed in suspension, with the nonsaturable component being most affected. Nevertheless, a difference in the initial rates of transport between normal and transformed cells remained, indicating that the difference is independent of cell shape.
Materials and Methods

Primary cultures, free of resistance-inducing factor, were prepared from the body walls of 10-day-old C/O or C/B type SPF embryos (H and N Inc., Redmond, Washington). Embryos were decapitated, minced, washed with Tris-saline buffer, and digested with 0.25% trypsin (Gibco). After 15 min the suspended cells were poured into a “stop bath” containing 2 parts cold medium 199 (Gibco) and 1 part calf serum (Microbiological Associates Inc.). This process was repeated twice. The single cells were plated in 100-mm culture dishes (Falcon) at 3 X 10^6 cells/plate in medium 199 supplemented with 2% tryptose phosphate broth (Gibco) and 1% each of chick and calf serum. Half the plates were infected with 0.2 ml of Schmidt-Ruppin, subgroup A of Rous sarcoma virus (5 X 10^6 focus-forming units/ml) 0-4 hr after primary seedings. Since fungicides were shown previously (1 2) to alter the transport characteristics of chick cells in culture, none were added to the culture medium. Secondary cultures were prepared at 5 X 10^5 cells per 35-mm plate. Calf serum and medium glucose concentrations were doubled at this time.

Suspension Cultures

Methyl cellulose. Twenty-four hours after secondary plating, cells were removed with trypsin (0.5%), washed with Hanks’ buffer and placed in 1.8% methyl cellulose (Meth C; Dow Chemical) suspension (3). The suspension was prepared as follows: In 100 ml of boiling water 2.4 grams Meth C was dispersed. The mixture was then placed in an ice bath to allow the Meth C to form a “solution”. After autoclaving, 300 ml of this solution was mixed with 100 ml concentrated medium 199 (4 times the solid ingredients) to yield a final medium concentration of 1.8% Meth C, 2% each tryptose phosphate and calf serum, and 1% chicken serum. The cell suspension, 10-20 ml, was then placed in 50-ml plastic tubes (Falcon) which contained 2 ml of 0.9% agar in medium 199 and centrifuged for 20 min at 3,000 rpm. They were then washed 3 more times in 10 ml of Hanks’ buffer without glucose. The recovery of cells was about 60%, but the clumping that occurred at lower Meth C concentrations was prevented. In addition, both normal and transformed cells were healthy as judged by trypan blue exclusion, and the plating efficiencies were comparable to cells removed by trypsin.

Trypsin. The time of trypsin treatment was found to be very critical and had to be regulated precisely. The plates were rinsed with Hanks’ buffer and 1 ml of 0.05% trypsin was added. The cells were left exactly 15 min at 37°C and then removed gently by a wide mouth pipette and combined in one tube. The trypsin was removed by centrifugation (3 min at 2,000 rpm) and the cells were washed 3 times in Hanks’ buffer without glucose.

EDTA. Cells were removed from plates according to the procedure described by Burger and Martin (1 3). EDTA was removed by centrifugation as above, and cells were examined for viability with trypan blue and for replating efficiency (see Results).
Measurements of Sugar Uptake

All transport studies were performed at 25°C except for long-term (10 min and longer) uptake experiments with 3MG which were done at both 25°C and 37°C in a CO2 incubator. [3H]-2DG, [3H]-3MG, and [3H]-L-glucose (2-6 µCi/ml; New England Nuclear) were made up in various concentrations in Hank’s buffer or medium 199 without glucose. The use of medium 199 was especially important for long-term 3MG uptake studies since the metabolic characteristics of cells changed drastically after 30 min in Hanks’ or Krebs-Ringer bicarbonate buffers (M. J. Bissell, unpublished).

Monolayer cultures. Plates were rinsed 3 times with 25% Hank’s buffer without glucose, and 1 nil of the appropriate sugar concentration was added to duplicate plates at 25°C. Incubation was terminated by aspirating the medium and then rinsing the monolayer with cold Hanks’ plus glucose. In the case of 2DG uptake, the sugar is phosphorylated and thereby trapped in the cell. Inhibitors of sugar uptake such as cytochalasin B (CB; 2.1 X 10^-5M; Aldrich Chemical Company) and phloretin (1 mM; K and K Laboratories) were added to the wash medium in the 3MG uptake studies to prevent efflux (17). Under these conditions, the [3H]-3MG counts associated with the cells were shown to remain essentially unchanged after 5 washes. To ascertain that the pool had not been diminished due to initial washings, 5 sec incubation with [3H]-L-glucose was performed on sister cultures at room temperature. The uptake of L-glucose is extremely slow under these conditions and the amount of sugar remaining after each wash can be used to correct for contamination due to extracellular adherence (D. Fick, personal communication). The values obtained after 5 washes or after correction for L-glucose were comparable. Uptake studies in the presence of CB were performed as above except that 2.1 X 10^-6M CB was present during incorporation (11).

Suspension cultures. Both trypsin and Meth C cells obtained as described above were suspended in 1 ml of appropriately labeled sugar and placed on a gyratory shaker (200 rotations per min; New Brunswick), at room temperature. Experiments performed at 37°C in CO2 incubators and without shaking gave similar patterns, with higher rates, although the results were less reproducible. The incorporation was interrupted by addition of 10 ml ice-cold Hanks’ buffer plus glucose. The cells were centrifuged at 200 rpm for 2 min, and the washing was repeated 2 more times in the presence of 10 µg/ml CB. Correction for L-glucose was performed as described above.

Scanning Electron Microscopy of Monolayer and Suspension Cultures

Monolayer cultures. Secondary normal and virus-transformed fibroblasts were seeded at 5 X 10^5 cells per 35-mm plate containing two sterile coverslips (15-mm diameter, no. 2 thickness: ERIE Scientific, Sybron Corporation, N.Y.). The medium was aspirated and the monolayer was washed 3 times with Hanks’ buffer containing glucose. The cells were fixed by adding 4 in1 of 1% glutaraldehyde (Polysciences) in Hanks’ buffer (pH 7.4) and left at room temperature for 24 hr. The fixed cells were then washed 3 times in 0.1 M Sorensen’s buffer at room temperature. The coverslips were postfixfixed with 1% OsO4 in 0.1 M Sorensen’s buffer, pH 7.4, for 30 min at room temperature. They were then rinsed thoroughly
with distilled water and stained with 2% uranyl acetate (aqueous) for 1 h at room temperature. After again rinsing with distilled water, the coverslips were placed in a metal spring and subsequently dehydrated in a series of graded ethanols, transferred in graded series of Freon 113 (Dupont), and critical point dried in Freon 13. The coverslips were then coated with platinum-carbon on a rotating-tilting stage on a vacuum evaporator (Mikros VE-10).

The specimens were viewed in a field emission scanning electron microscope (Coates and Welter, SV Special), with an accelerating voltage of 20 KV and a 45° angle. Photographs were taken at 1,300 X magnification with an Ilford FP₄ film.

**Cells in suspension.** Poly-L-lysine coated glass coverslips were used as a substrate for processing suspended cells. The technique was a slight modification of that reported by Mazia et al. (15) and Sanders et al. (16). Cells recovered from Meth C or after trypsin removal were fixed in suspension by addition of 10 nil of 1% glutaraldehyde in Hanks’ buffer. After 24 hr at room temperature, the fixed cells were washed 3 times in 10 ml of 0.1 M Sorensen’s buffer (centrifuged 2 min at 2,000 rpm) and the final cell concentration was adjusted to 2.0 X 10⁶ cells per ml. Clean glass disks (the same type used for monolayer cells, above) were covered with 100 1.11 drops of 1 mg/ml poly-L-lysine hydrobromine (mol. wt. 350,000, Sigma) in 0.1 M Sorensen’s buffer. pH 7.35. After 1 hr at room temperature, the coated coverslips were washed in distilled water and then were partially blotted with filter paper and placed in 35-mm petri dishes in a moist chamber. Immediately a 100 µl drop of the cell suspension was placed on each coverslip. The chamber was stored at 4° for approximately 14 hr. The cells were then fixed with 1% glutaraldehyde for the second time for 15 min at room temperature and treated as described for the monolayer cultures above.

**Other Techniques**

Growth rates were monitored by a 1 hr pulse of [³H] -thymidine (2 µCi/ml, New England Nuclear) and subsequent analysis of the acid-precipitable counts. Cell number was determined using a Coulter particle counter. To determine DPM per mg protein, 1% SDS in 0.01N NaOH was added directly to monolayer plates or to cell pellets. An aliquot was taken for the measurement of radioactivity and counted in a Packard Model 3375 scintillation counter after addition of 15 ml of Aquasol (New England Nuclear). Cell protein was determined from another aliquot by the method of Lowry et al. (17). Flow microfluorometry was performed according to Bartholomew et al. (18).

**Results**

Most of the techniques available for removing cells from a monolayer disrupt the integrity of the plasma membrane in one form or another. Cell removal by rubber policemen yielded a largely broken cell population. EDTA could be used for removing chick cells from the monolayer only if very stringent controls were observed (13). However, even with stringent controls, the cell yield was too low for uptake studies. In addition, cell removal by EDTA under our culture conditions yielded a population of which only 20% were judged viable by trypsin blue exclusion test and which subsequently had a low plating efficiency. We therefore decided to examine uptake characteristics of cells in suspension, after careful removal by trypsin. Since trypsin easily
removes some surface components, we decided also to keep the cells for 24 hr in suspension under viable conditions to allow the “repair” of any damages done by trypsin. A procedure was developed to permit recovery of cells from Meth C suspension (see Methods). The clumping was minimal under these conditions, and trypan blue exclusion test and plating efficiency were comparable to trypsin-removed cells. The percentage of cells (both normal and transformed) which picked up the dye was 6-10% for trypsin-treated cells and 8-12% for Meth C grown cells.

Scanning electron micrographs indicated that asynchronous populations of normal and Rous-transformed chick cells appear to differ significantly in morphology only in the flat state (Figs. 1-3). A comparison of 2DG transport of these cells indicated that the transport rate is decreased in suspension cultures (Fig. 4). The decrease in overall transport rate of trypsin-treated cells could have been the result of general damage to the membrane. However, the initial difference in 2DG uptake between normal and transformed cells was still apparent in suspension cultures. In addition, cells grown in Meth C for an additional 24 hr had transport characteristics similar to trypsin-removed cells in suspension (Fig. 4). It is also apparent from Fig. 4 that at higher glucose concentrations the magnitude of difference between normal and transformed cells is lower in suspension as opposed to monolayer cultures.

The contribution of nonmediated uptake (and/or low affinity mediated uptake) to overall transport rates becomes significant at higher sugar concentrations. Thus this component of the transport system was drastically reduced in suspension cultures. We had previously proposed (11) that there is a CB-sensitive (high affinity carrier-mediated transport) and a CB-insensitive component to the glucose uptake system in chick cells in culture. To determine which component was reduced in suspension, we measured 2DG uptake with and without CB. It is clear from Fig. 5 that 2DG transport in suspension is mainly by the CB-sensitive component.

To ascertain that the transport itself and not phosphorylation was affected in suspension, we measured the rate of 3MG transport. The results were similar to that detailed above (Fig. 6).

Finally we measured the total “water space” available to hexoses in normal and virus-transformed cells by measuring the saturation level of 3MG uptake. In doing so, we took advantage of the procedure described by Kletzien et al. (16) except that phloretin was replaced by CB (10 &ml) since the latter was even more effective in retaining 3MG within the chick cells during the washing procedure. The requirements for using 3MG to calculate the available water space were fulfilled as follows: 1) The uptake of sugar was shown to reach equilibrium rapidly with time in both monolayer and suspension cultures. 2) The intracellular sugar concentration was shown not to be irreversibly sequestered by the cells since the plateau was strictly concentration-dependent and since rapid efflux and accelerative exchange diffusion occurred. 3) Both CB and phloretin were shown to trap the sugar within the cell (data not shown). The water space was calculated (14) from the plateau for 3MG uptake, assuming that at equilibrium intracellular and extracellular concentrations were similar (8,9) and is shown in Table I.

Discussion

The rate of hexose transport in cultured cells varies with population density, growth rate, position in the cell cycle, and viral transformation. Similarly, cell shape and “topology” of cells in culture
vary with these same factors. The possible influence of cell shape on various transport processes in chick cells has not been determined previously. A comparison of Fig. 1 and Figs. 2 and 3 reveals that gross morphological changes after transformation are observed only in monolayer cultures. Normal and transformed cells kept in suspension after trypsin or grown in Meth C have heterogeneous surface morphology probably reflecting the cell cycle stage as described for CHO cells (19). No obvious morphological differences exist in suspension between normal and transformed cells. Pictures taken of suspended cells at higher magnifications show that while each of these cell types display an array of morphological variations, no obvious differences are detected between normal and virus-transformed fibroblasts.

The data presented here indicate that once the normal and transformed cells are removed from the monolayer and placed in a suspension, they transport glucose analogues at a slower rate, especially at high sugar concentrations. The differential between normal and virus-transformed cells, however, is retained. Two conclusions may be drawn from the fact that the magnitude of the difference is very similar to that shown by the cells on a monolayer at low 2DG concentrations: 1) The difference in the rate of 2DG transport between normal and transformed cells is not due to cell shape, topology, or stage of cell cycle (our unpublished flow microfluorometric data). 2) The high affinity hexose carrier protein (Km for 2DG 1-5 mM) does not appear to be extremely trypsin-sensitive. It therefore may not be located in an exposed site on the membrane. Much lower concentrations of trypsin than those used here have been shown to remove other proteins such as LETS (20) from the cell surface.

It may be recalled from the introduction that the contribution of nonmediated diffusion (and/or low affinity carrier diffusion) to transport is considerable at high sugar concentrations and is also CB-insensitive [at CB concentration of 1 µg/ml (1 l)]. The fact that the uptake was reduced more drastically at higher sugar concentration when placed in suspension and that it was very CB-sensitive may indicate that the second component of transport is a protein sensitive to trypsin and thus absent in suspension. However, since no change was observed in Meth C grown cells, it is more likely that the additional decrease in high sugar concentration is simply a reflection of the decrease in surface areas when cells are placed in suspension. Our results would thus support the contention that the uptake of sugar in cultured cells is by a single carrier-mediated transport (7) and that the “second” component of sugar uptake (8) is indeed nonmediated diffusion, and, as such, its rate is directly related to available surface area. The fact that the level of 3MG uptake at saturation is still lower in cells in suspension can be explained by the smaller available 3MG space of suspended cells as shown in Table I. Foster and Pardee also observed a 25% decrease in intracellular fluid space when hamster cells were grown in suspension cultures (6). They used two separate techniques for estimation of the intracellular fluid space for monolayer and suspended cultures, which makes the comparison difficult. Our data indicate a larger reduction in available 3MG space when cells are placed in suspension, especially for virus-transformed cells.

It is unlikely that uptake properties of a cell can explain its growth behavior. Nevertheless, there is no question that increased nutrient uptake - by whatever mechanism - gives tumor cells a selective advantage for growth (21). The fact that normal cells in suspension do not grow and their uptake rate is very low, and the fact tumor cells have increased uptake capacity even in the rounded state and do grow may have implications for the avascular phase of tumor survival and metastasis in vivo.
Acknowledgments

We wish to thank Dr. Nichola Neff for critical reading of the manuscript and Daniel Fick for helpful discussions. This work was supported, in part, by the U.S. Energy Research and Development Administration and, in part, by National Cancer Institute Grant NO. CA-I 4828-03.

References

Figures and Tables

FIGURE 1

Scanning electron micrograph of normal (top) and Rous-transformed (bottom) chick cells. Electron microscopy was performed as described in Methods; X 1,300 (reduced in printing to X 1,170).
FIGURE 2

Scanning electron micrograph of normal (top) and transformed (bottom) chick cells after trypsin removal (see Methods); X 1,300 (reduced in printing to X 1,170).
Scanning electron micrograph of normal (top) and transformed (bottom) chick cells after 24 hr in Meth C suspension (see Methods); X 1,300 (reduced in printing to X 1,170).
[\textsuperscript{3}H]-2 DG uptake versus 2 DG concentration (left panel) and versus time (right panel) in normal (open symbols) and transformed (closed symbols) cells. Transport studies were conducted on monolayer (Plate; ○, ●), trypsin-removed cells (Tryp; Δ, ▲), or methyl cellulose grown cells (Meth C; □, ■) as described in Methods. The concentration of 2 DG was 1 mM in the time study. Each point represents the average of duplicate samples.
[\textsuperscript{3}H]-2 DG uptake in trypsin-removed cells in presence (Δ, ▲) and absence (○, ●) of CB in normal (open symbols) and transformed (closed symbols) cells.
$[^3]H$-3MG uptake in normal (open symbols) and transformed (closed symbols) cells. Transport studies were conducted on monolayer (○, ●) and Meth C removed cells (□, ■) at 1.0 mM 3MG for the indicated times as described in Methods. 3MG transport in trypsin-removed cells was similar to these results.
TABLE I. Available Water Space of Chick Fibroblasts \( \mu l/\text{mg Protein} \)

<table>
<thead>
<tr>
<th></th>
<th>Monolayer</th>
<th>Trypsin-removed</th>
<th>Meth C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>4.8 ± 0.8</td>
<td>3.3 ± 0.5</td>
<td>3.2 ± 0.5</td>
</tr>
<tr>
<td>Transformed</td>
<td>5.6 ± 1.2</td>
<td>3.5 ± 0.5</td>
<td>3.2 ± 0.6</td>
</tr>
</tbody>
</table>

The water space was calculated as described (16) from the plateau of 3MG uptake. 3MG uptake in the presence of high levels of CB, or 10-second pulse with \[^{3}H\]L-glucose were used to correct for extracellular adherence.

The values for normal cells represent mean and standard deviation of 6 experiments. The values for transformed cells represent 3 experiments.