Membrane Changes and Adenosine Triphosphate Content in Normal and Malignant Transformed Cells
(concanavalin A/simian virus 40/golden hamster embryo)

ISRAEL VLODAVSKY, MICHAEL INBAR, AND LEO SACHS

Department of Genetics, Weizmann Institute of Science, Rehovot, Israel
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ABSTRACT. Transformed fibroblasts had a low content of ATP when grown at a high cell density and a high content of ATP when grown at a low cell density. Concanavalin A agglutinated transformed cells with a low, but not those with a high, ATP content. Transformed cells with a high ATP content gained agglutinability after ATP depletion by inhibitors of the energy-generating systems, and those with a low ATP content lost their agglutinability after restoration of a high ATP content by glucose. Fixation of the surface membrane by formaldehyde, glutaraldehyde, or LaCl3, inhibited agglutination of cells with an ATP content that allows agglutination. Normal fibroblasts grown at a high or a low cell density were not agglutinated by concanavalin A. Depletion of the cellular ATP content of normal cells induced agglutination only in cells grown at a high, but not at a low, cell density. A similar number of concanavalin A molecules was bound to the surface membrane of agglutinating and nonagglutinating fibroblasts. It is suggested that a high content of ATP inhibits the movement of concanavalin A binding sites, and that a low content of ATP allows, in transformed cells, a new distribution of binding sites to form the clusters required for cell agglutination. Agglutinability of transformed cells is determined by ATP content, and in normal cells changes in the content of ATP are by themselves not sufficient to induce agglutination. Transformed cells, therefore, do not have a control, presumably for membrane stability, that exists in normal cells.

Concanavalin A (Con A) (1–3) can bind specifically to glucose- or mannose-like sites on the surface membrane of normal and malignant transformed cells. Interaction of this protein with specific sites can be used as a probe to study structure and function of the cell-surface membrane. With this probe, differences between normal and transformed cells have been shown with respect to agglutinability by Con A (4–8), the number and distribution of Con A binding sites (9–12), the location of aminoacid and carbohydrate transport sites (13), and Con A-induced cell toxicity (14–16). We have suggested that Con A sites on the surface membrane are associated with two activities, one that binds Con A molecules and another that determines agglutination (6, 7). The activity that determines agglutination is associated with induction of a new distribution of binding sites to form clusters (17–19). The present studies were undertaken to determine the role of energy-generating systems in the control of membrane changes required for agglutination by Con A and the difference between the control in normal and transformed cells.

MATERIALS AND METHODS

Cell Cultures. Transformed cells were a line of fibroblasts from golden hamster embryo cells transformed in vitro after infection with polyoma virus and a line derived from a simian virus (SV) 40-induced hamster tumor (Flow Laboratories, Bethesda, Md.). The normal cells were fibroblasts from secondary cultures of golden hamster embryos. Cells were cultured in plastic petri dishes in Eagle's medium with a 4-fold concentration of amino acids and vitamins (H-21, Grand Island Biological Co., N.Y.) and 10% fetal-calf serum (4); they were subcultured every fourth day and 10⁴ cells were seeded per 100-mm petri dish. There was no detectable mycoplasma contamination when the cultures were tested on mycoplasma agar (20). For the experiments, cells were dissociated by incubation with 0.02% EDTA for 15–30 min at 37°, washed three times with phosphate-buffered saline (PBS; 8.0 g of NaCl, 0.2 g of KCl, 1.15 g of Na2HPO4, 0.2 g of KH2PO4 per 1 liter of water, pH 7.2) and suspended in PBS at a density of 1 to 2 × 10⁶ cells per ml. The cells were then incubated for 30 min at 37° with or without metabolic inhibitors in the presence or absence of glucose, before testing for agglutinability and ATP content.

Assay for Agglutination. Con A was obtained from Miles-Yeda. To test for agglutination, 0.5 ml of the lectin at different concentrations in PBS was mixed with 0.5 ml of the cell suspension to give a density of 10⁴ cells per ml in a 35-mm petri dish. The density and size of aggregates was scored in a scale from – to +++++ after 30-min incubation at 24° (4).

Assay for Con A Binding. Con A was labeled with [3H]acetic anhydride (21) to give a specific activity of 3 × 10⁶ cpm/mg. For binding of [3H]Con A to cells, 0.5 ml of labeled Con A diluted to different concentrations in either PBS or PBS containing 0.1 M α-methyl-D-mannopyranoside (α-MeM), was mixed with 0.5 ml of cell suspension in a centrifuge tube and incubated for 30 min at 24°. The cells were then washed three times with 5 ml of PBS, the pellet was dissolved in 0.1 M NaOH, and the radioactivity was counted in Triton scintillation fluid (Koch–Light Labs, England). To calculate the amount of Con A bound specifically, the amount bound in the presence of α-MeM was subtracted from the amount bound in the absence of α-MeM (9, 10). The results on Con A binding

Abbreviations: Con A, concanavalin A; PBS, phosphate-buffered saline (pH 7.2); α-MeM, α-methyl-D-mannopyranoside; SV40, simian virus 40; DNP, 2,4-dinitrophenol; CCCP, carbonyl cyanide m-chlorophenylhydrazone.
are given as specific binding in cpm/1000 µg of cell protein. Total cell protein was measured by the method of Lowry et al. (22).

**Determination of Cellular ATP Content.** Cellular ATP content was assayed by the firefly luciferin–luciferase method (23). Buffered extract of desiccated firefly lantern (Sigma Chemical Co.) was reconstituted and incubated for 24 hr at 4° to degrade any endogenous ATP. After centrifugation, the supernatant was used for 3–4 days with no loss of activity. Cellular ATP was extracted by boiling a sample of cells for 5 min in distilled water, followed by immediate cooling and freezing in liquid air. The precipitate was removed by centrifugation and the ATP was measured in a sample of 10–50 µl of the supernatant, by addition of a sample of 50 µl of the firefly extract in a total volume of 3 ml of buffer. The light emission was counted immediately for 0.1 min in a liquid scintillation counter. The intensity of the emitted light increased linearly from 10^-4 to 10^-6 M ATP.

**RESULTS**

**Agglutinability of transformed cells and the cellular ATP content.**

Previous results on the agglutination of transformed cells by Con A have indicated, that transformed cells were usually not agglutinated at 1 day but that they were agglutinated at 4 days after subculture (6, 10). Transformed cells grown under conditions that enhanced cell replication were agglutinated even at 1 day after subculture (10). In the present experiments, when the transformed cells were seeded at a density of 10^6 cell per 100-mm petri dish, the cells were not agglutinated at 1 day after seeding, they gained agglutination on the second day, and reached maximum agglutinability on the fourth day (Fig. 1). However, when the transformed cells were seeded at a density of 5 × 10^4 or 10^5 cells per 100-mm petri dish, they gained maximum agglutinability 2 days after seeding (Fig. 2). Measurements of the cellular ATP content indicated in both experiments that the gain of agglutinability correlated with a high density of cells and was also associated with a decrease in the content of cellular ATP (Figs. 1 and 2).

Incubation of agglutinating transformed cells at 2 days after seeding with 1 mM glucose for 30 min, resulted in restoration of a high ATP content and completely abolished agglutination (Fig. 3). The agglutination of cells at 4 days after seeding was partially abolished by the same concentration of glucose. The possibility that agglutination was inhibited by 1 mM glucose as a result of its interaction with the Con A carbohydrate binding sites was ruled out, since 1 mM α-methyl-D-glucopyranoside, which is 10–20 times more efficient than glucose as a hapten inhibitor of Con A, did not affect either the ATP content or cell agglutination (Fig. 4). In all cases, the cultures contained not more than about 5% of cells in mitosis.
and used that with hamster cells. Cells depletion and tested was content agglutinability and further inhibition of the glycolytic pathway (iodoacetate and N'-ethylmaleimide) or when oxidative phosphorylation was inhibited and sodium fluoride (NaF) was present in the reaction mixture (Fig. 4). NaF by itself had no effect on ATP content or cell agglutination (Table 1). Addition of 1 mM glucose for the last 5 min of a 30-min incubation with the metabolic inhibitors, was sufficient for both restoration of cellular ATP content and inhibition of the agglutination of nonagglutinating transformed cells induced by the inhibitors.

Protein synthesis is inhibited when the cellular ATP content is decreased (24). Our experiments have indicated that 95% inhibition of protein synthesis of nonagglutinating transformed cells by puromycin or cyclohexamide, which had no effect on ATP content, did not affect cell agglutination. The low content of ATP that induced agglutination with Con A did not induce a higher degree of agglutinability than in cells with a high ATP content with the lectins from wheat germ (25) and soybean (26).

The effect of metabolic inhibitors on agglutination of normal cells is summarized in Table 2. The results indicate that when normal cells were seeded at a low density, depletion of more than 90% of the cellular ATP content did not induce agglutination. Treatment of these cells with DNP or oligo-

**Control of agglutination by depletion and restoration of the cellular ATP content**

In order to further determine the relationship between cellular ATP content and cell agglutination, the effect of metabolic inhibitors that inhibit cellular energy generating systems was tested with nonagglutinating transformed cells. The results summarized in Table 1 indicate that nonagglutinating transformed cells gained agglutinability after depletion of the cellular ATP content and that restoration of a high ATP content by addition of glucose abolished this agglutination. ATP generated by glycolysis or by oxidative phosphorylation was effective as an energy source for inhibition of cell agglutination. The presence of glucose prevented both ATP depletion and the gain of agglutination induced by inhibitors of respiratory electron transfer [sodium azide (NaN₃) and sodium cyanide (NaCN)] or oxidative phosphorylation [2,4-di-

**Fig. 4.** Effect of metabolic inhibitors and glucose on the agglutinability and ATP content of SV40-transformed hamster cells. Cells were seeded at a density of 10⁶ cells per 100-mm petri dish and used 48 hr after seeding. Similar results were obtained with hamster cells transformed by polyoma virus. \( \alpha \)-MG = \( \alpha \)-methyl-D-glucopyranoside.

**Fig. 5.** Specific binding of radioactively labeled Con A molecules to SV40-transformed hamster cells in the presence or absence of metabolic inhibitors and glucose. Similar results were obtained with normal cells. —O—O, control or control + 1 mM glucose; •—•, 10 mM NaN₃; △—△, 1 \( \mu \)M CCCP; ▲—▲, 1 \( \mu \)M CCCP + 1 mM glucose.

**Fig. 6.** Inhibition of agglutination by fixation of aldehydes and LaCl₃. A, agglutinating SV40-transformed hamster cells seeded at a density of 10⁶ cells per 100-mm petri dish and used 4 days after seeding. B, nonagglutinating SV40-transformed cells 48 hr after seeding. Cells were fixed with 2.5% glutaraldehyde for 60 min, with 10% formaldehyde for 4 hr, and with 1 mM LaCl₃ for 15 min. After aldehyde fixation, cells were incubated with PBS containing 0.2 M glucose for 10 min and then washed twice with PBS (17, 19). Fixation with LaCl₃ was in 30 mM Tris buffer with 0.14 M NaCl (pH 7) and reversed by two washings with this buffer.
mycin in the presence of sodium fluoride had a small effect (+). However, depletion of the ATP content of normal cells seeded at a high density induced cell agglutination, but to a lower extent than in the case of nonagglutinating transformed cells. There was no, or only a small, effect of glucose on both restoration of the ATP content and the inhibition of agglutination in normal cells. Binding experiments with radioactively labeled Con A have shown that a similar number of Con A molecules were bound to the membrane of agglutinating and nonagglutinating transformed (Fig. 5) and normal cells. Treatment of nonagglutinating transformed and normal cells with 1 or 10 μg of purified trypsin per ml for 10 min induced agglutinability without decreasing the ATP content.

Inhibition of cell agglutination by fixation of the surface membrane

Fixation of agglutinating transformed cells with 10% formaldehyde, 2.5% glutaraldehyde, or 1 mM LaCl₃ inhibits agglutination (17, 19). In the present experiments, the high degree of agglutination (+ + + +) with 250 μg of Con A per ml in the unfixed cells was reduced by fixation to +. Cells incubated with LaCl₃ (27) and then washed twice reached the same degree of agglutination as unfixed cells. The gain of agglutination in nonagglutinating transformed cells induced by the metabolic inhibitors was also inhibited by fixation with formaldehyde and glutaraldehyde and reversibly inhibited by fixation with LaCl₃ (Fig. 6) and incubation at 4°C.

**Table 1. ATP content and agglutinability of transformed cells after treatment with metabolic inhibitors**

<table>
<thead>
<tr>
<th>Metabolic inhibitor</th>
<th>Site of action</th>
<th>Inhibitor</th>
<th>ATP content (μg of Con A per ml)</th>
<th>PBS</th>
<th>PBS containing 1 mM glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>PBS None</td>
<td></td>
<td>100*</td>
<td>±</td>
<td>163</td>
</tr>
<tr>
<td>Electron</td>
<td>NaNO₂, 5 mM</td>
<td></td>
<td>17</td>
<td>++++</td>
<td>123</td>
</tr>
<tr>
<td>transfer</td>
<td>NaCN, 1 mM</td>
<td></td>
<td>7</td>
<td>++++</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>DNP, 0.2 mM</td>
<td></td>
<td>12</td>
<td>++++</td>
<td>82</td>
</tr>
<tr>
<td>Uncouplers</td>
<td>CCCP, 0.5 μM</td>
<td></td>
<td>14</td>
<td>++++</td>
<td>90</td>
</tr>
<tr>
<td>Energy</td>
<td>Oligomycin 0.5</td>
<td>μM</td>
<td>13</td>
<td>++++</td>
<td>102</td>
</tr>
<tr>
<td>Glycolytic</td>
<td>Iodoacetate 2 mM</td>
<td></td>
<td>8</td>
<td>++++</td>
<td>6</td>
</tr>
<tr>
<td>pathway</td>
<td>NEM 1 mM</td>
<td></td>
<td>1</td>
<td>++++</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>NaF 20 mM</td>
<td></td>
<td>107</td>
<td>104</td>
<td>-</td>
</tr>
</tbody>
</table>

The experiments were performed with simian virus(SV)40-transformed hamster cells. The cells were seeded at a density of 10⁶ cells per 100-mm petri dish and used in the experiments 48 hr after seeding. Oligomycin and CCCP were added to the cells as solutions in ethanol and methanol, respectively, to give a final concentration of alcohol of less than 0.1%. *NEM = N-ethylmaleimide.*

* 100% ATP = 2 × 10⁻¹⁸ mol per cell = 9.8 nmol/mg of cell protein.

**Table 2. ATP content and agglutinability of normal cells after treatment with metabolic inhibitors**

<table>
<thead>
<tr>
<th>Metabolic inhibitor</th>
<th>Low density</th>
<th>High density</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Agglutination</td>
<td>ATP Agglutination</td>
</tr>
<tr>
<td></td>
<td>content (250 μg of Con A per ml)</td>
<td>content (250 μg of Con A per ml)</td>
</tr>
<tr>
<td>Control</td>
<td>100*</td>
<td>94</td>
</tr>
<tr>
<td>NaNO₂, 5 mM</td>
<td>9</td>
<td>13</td>
</tr>
<tr>
<td>NaNO₂, 5 mM + NaF, 25 mM</td>
<td>6</td>
<td>± 3</td>
</tr>
<tr>
<td>DNP, 0.5 mM</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>DNP, 0.5 mM + NaF, 25 mM</td>
<td>8</td>
<td>+ 4</td>
</tr>
<tr>
<td>Oligomycin 1 μM</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td>Oligomycin, 1 μM + NaF, 25 mM</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

The experiments were performed with normal hamster cells. The cells were seeded at a density of 10⁴ and 5 × 10⁴ cells per 100-mm petri dish for the low and high density, respectively, and used in the experiments 48 hr after seeding. Cells 4 days after seeding at the low seeding level gave similar results to cells 2 days after seeding at the high seeding level.

* 100% ATP = 2.6 × 10⁻¹⁸ mol per cell = 15.3 nmol/mg of cell protein.

**DISCUSSION**

Our results have shown that malignant transformed fibroblasts grown at a low cell density had a high content of cellular ATP and that transformed cells grown at a high cell density had a low content of ATP. The experiments on cell agglutination by Con A indicate that Con A agglutinated transformed cells with a low but not with a high ATP content. Nonagglutinating transformed cells with a high ATP content gained agglutinability after ATP depletion by inhibitors of the cellular energy-generating systems, like sodium azide and DNP. Agglutinating transformed cells with a low ATP content lost their agglutinability after restoration of their ATP content by an energy source like glucose. Restoration of the cellular ATP content and inhibition of cell agglutination by glucose was inhibited by sodium fluoride, which inhibits the glycolytic pathway.

Normal fibroblasts grown at a low or a high cell density were not agglutinated by Con A. Depletion of the ATP content of normal cells induced agglutination only in cells grown at a high, but not at low, cell density. In normal cells, glucose had no, or only a small, effect on restoration of the ATP content and inhibition of cell agglutination.

The results indicate that in transformed cells agglutination is determined by a low ATP content. However, in normal cells a low ATP content is required but not sufficient for the gain of agglutination. Agglutination of transformed cells by the lectins from wheat germ and soybean was not associated with a low content of cellular ATP. This again indicates (6) a difference in the mechanism of agglutination by Con A and these two lectins. In some experiments, in contrast to results with Con A, addition of glucose enhanced the agglutinability, whereas ATP depletion decreased the agglutinability of transformed cells by the wheat-germ lectin (to be published).

We have previously shown that formation of clusters of Con A-binding sites on the surface membrane is inhibited...
by fixation of the fluid state of the membrane. Fixation also inhibited cell agglutination, although the fixed and unfixed cells bound a similar number of Con A molecules. Movement of Con A sites on the membrane to form clusters is therefore required for cell agglutination (17, 19). We assume that agglutinating fibroblasts have a higher degree of membrane fluidity, which allows cluster formation by Con A, than non-agglutinating fibroblasts. The present results indicate that fixation inhibited agglutination of cells with an ATP content that allows transformed cells to agglutinate. We suggest that the high content of ATP in nonagglutinating transformed cells stabilized the membrane (28) and inhibited the formation of clusters of Con A-binding sites that are required for cell agglutination, and that the Con A-binding sites in agglutinating transformed cells that have a low ATP content are more mobile and form clusters more readily. In normal fibroblasts a low ATP content is required, but is by itself not sufficient for agglutination. The transformed cells, therefore, do not have a control, presumably for membrane stability, that exists in normal cells.

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