“Flat” Morphology Induced in Cultured Transformed Cells by Polyphloroglucin Phosphate

Hans Peter Schnebli and Marianne Burger
Friedrich Miescher-Institute, P. O. Box 273, CH-4002 Basel, Switzerland

SUMMARY

Polyphloroglucin phosphate and polyphloretin phosphate (known as inhibitors of hyaluronidase), at concentrations of 0.1 to 2 µg/ml, induced “flat” morphology within 24 to 48 hr and very low saturation density in cultured transformed mouse fibroblasts. The effect was reversible. Cells cultured in the presence of polyphloroglucin phosphate for 70 days resumed their “transformed” morphology 48 to 72 hr after removal of the drug.

INTRODUCTION

Low saturation density and “flat” morphology of cells in tissue culture is often regarded as a sign of “normal” behavior. Transformed variants or revertants selected for their flat morphology (10, 11) exhibit many characteristics of untransformed cells. In attempts to understand the basis for contact inhibition of mitosis, a number of agents have been used to induce flat morphology in cultured transformed cells. These include the addition of cyclic adenosine 3’,5’-monophosphate analogs (7, 15), histones (1), dimethyl sulfoxide (8), monovalent concanavalin A (3), or protease inhibitors (6, 12) to the culture medium or adsorption of polycations and histones to the growth substratum (9). Treated cells often attain an elongated, more parallel-oriented shape (6-8).

Earlier, after proteases were reported to release cells from contact inhibition (2, 14), we were able to inhibit selectively the growth of transformed cells with protease inhibitors (13) and to induce flat morphology in these cells (12).

The present experiments were prompted by the report of Vasiliev et al. (17) that hyaluronidase stimulates the growth of stationary-phase mouse embryo fibroblasts. Although we were unable to reproduce Vasiliev’s observation, we report here the restoration of flat morphology to transformed cells by inhibitors of hyaluronidase (4).

MATERIALS AND METHODS

Chemicals. PPP and PGP were gifts of AB Leo, Helsingborg, Sweden. Hyaluronidase from bovine testes (14,000 units/mg) was purchased from Sigma Chemical Co., St. Louis, Mo.; insulin was from Terapeutisk Laboratorium Novo, Copenhagen, Denmark.

Cells. 3T3 mouse fibroblasts and their simian virus 40-transformed derivative line (SV3T3) were a gift from Dr. H. Green, Massachusetts Institute of Technology, Cambridge, Mass. The cells were grown in Dulbecco’s modified Eagle’s medium (Gibco No. H-21) containing 10% fetal calf serum (Colorado Serum Co., Denver, Colo.). The cells were checked for Mycoplasma (5) regularly and were free of contamination throughout the study.

A subline of SV3T3 designated SV3T3-P was grown in the presence of PGP for 10 weeks. It was passaged every 3rd or 4th day as follows. Cells were trypsinized and plated in normal medium at a density of approximately 5,000 cells/sq cm; 8 to 24 hr later, when the cells were firmly attached, PGP at a final concentration of 0.25 to 1 µg/ml was added. PGP could not be added at the time of passage as it interfered with the attachment of the cells to the plate. Addition of PPP and PGP to the growth media did not change the pH (7.4).

RESULTS AND DISCUSSION

Failure of Hyaluronidase to Cause “Overgrowth” in 3T3 Cells. An attempt at initiating “overgrowth” in contact-inhibited cells by treatment with hyaluronidase is illustrated in Table 1. As can be seen, hyaluronidase had no effect on 3T3 cells when added to the growth medium (up to 30 µg/ml); however, the cells were responsive to insulin stimulation as well as to increased serum. The morphology of the 3T3 monolayer was not visibly altered by the hyaluronidase treatment.

Vaheri et al. (16) have shown that hyaluronidase (up to 100 µg/ml) does not release chick embryo fibroblasts from density-dependent inhibition, although their cells responded to a large number of stimuli including neuraminidase. In contrast, Vasiliev et al. (17) reported that hyaluronidase could stimulate growth in stationary mouse embryo fibroblasts. It appears likely that this was due to an impurity in his enzyme preparation (300 units/mg protein compared to 14,000 units/mg used here and in Vaheri’s experiments).

Short-Term Effects of PPP and PGP. Addition of PGP (0.5 to 2 µg/ml) or PPP (0.1 to 1 µg/ml) to SV3T3 cells profoundly altered their morphology. The cells became very flat within 24 to 48 hr and avoided all overlapping. This resulted in a monolayer of low cell density and “cobblestone” appearance as is illustrated in Fig. 1B. Py3T3 cells were affected similarly but became somewhat less flat.
Table 1

Overgrowth effect in 3T3 cells

3T3 cells were plated in 3.5 cm Falcon Petri dishes (2 to 3 x 10³ cells) and reached a stable density of 58,000 cells/sq cm within 2 to 3 days. At that point, hyaluronidase or insulin (dissolved in 50 μl medium) or 200 μl serum (to increase the concentration from 10 to approximately 20%) was added to the cultures. Cells were counted at intervals by use of a Coulter counter.

<table>
<thead>
<tr>
<th>Treatment of cells</th>
<th>Cell density (cells/sq cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>59,000</td>
</tr>
<tr>
<td>24 hr</td>
<td>58,000</td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td></td>
</tr>
<tr>
<td>10 μg/ml, 72 hr</td>
<td>58,000</td>
</tr>
<tr>
<td>30 μg/ml, 72 hr</td>
<td>59,400</td>
</tr>
<tr>
<td>Insulin</td>
<td></td>
</tr>
<tr>
<td>10 μg/ml, 48 hr</td>
<td>76,000</td>
</tr>
<tr>
<td>Serum, 20%</td>
<td></td>
</tr>
<tr>
<td>24 hr</td>
<td>82,000</td>
</tr>
<tr>
<td>72 hr</td>
<td>98,000</td>
</tr>
</tbody>
</table>

Treatment of cultures with high cell densities led to detachment of overlapping cells, while cells attached to the Petri dish flattened and formed a continuous monolayer. It appears paradoxical that, although cells treated with PGP adhered more tenaciously to the Petri dish than untreated cells, unattached or suspended cells (e.g., during subculture) failed to attach in PGP-containing medium (in as little as 0.25 μg/ml).

The flattening effect was also reflected in the growth curves of treated cells. As can be seen in Chart 1, the saturation density of SV3T3 was reduced in a dose-dependent fashion by PGP. However, in contrast to the action of a number of protease inhibitors (13) this inhibitory effect is not limited to transformed cells. 3T3 cells were also inhibited in their growth and became extremely flat.

The mechanism by which PGP induces flat morphology is not clear. PGP, which is highly negatively charged, could bind to and alter the plastic surface of the Petri dishes. This can severely change the morphology and growth behavior of cells, as was demonstrated elegantly by Macieira-Coelho and Avrameas (9). Alternatively, PGP could interact with a serum component or with the cells directly. Its effect on the cellular synthesis and turnover of mucopolysaccharides, which may be responsible for the "stickiness" of cells, is currently under study. However, in view of the failure of hyaluronidase to stimulate overgrowth in quiescent 3T3 cultures, it appears unlikely that the effects of PGP and PPP are (solely) due to the inhibition of hyaluronidase.

Long-Term Effects of PGP. It seemed possible that continuing culture of transformed cells in PGP-containing medium would select for cells with decreased ability to overgrow a monolayer, i.e., with higher sensitivity to contact inhibition. A subline, SV3T3-P, was grown for 10 weeks in PGP and passed 18 times during this period, exposing it to PGP, 0.25 to 1 μg/ml, for 2 to 3 days between each passage (see "Materials and Methods"). When allowed to grow in the absence of PGP, the SV3T3-P cells maintained a flat shape for only 1 to 2 days and then gradually resumed the spindle shape typical for SV3T3 cells (Fig. 1C). The SV3T3-P cells grew to a saturation density similar to that of SV3T3. Furthermore, the SV3T3-P was as sensitive to the "flattening" effect of PGP as was the SV3T3 line (Fig. 1D). The 10-week treatment with PGP thus selected neither for a PGP-resistant cell nor for a cell with altered growth properties.

REFERENCES

Hans Peter Schnebli and Marianne Burger


Fig. 1. Effect of PGP on the morphology of SV3T3 cells. A, untreated SV3T3 cells; B, SV3T3 cells grown in the presence of PGP, 1 μg/ml, for 48 hr; C, SV3T3-P cells (grown for 10 weeks in PGP; see “Materials and Methods”) not treated with PGP during the last 48 hr; D, SV3T3-P in the presence of PGP, 1 μg/ml. Phase contrast microscopy. All photographs at same magnification. Bar, 100 μm.
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