CONCISE REPORT

Human Placental Conditioned Medium Reverses Apparent Commitment to Differentiation of Human Promyelocytic Leukemia Cells (HL60)

By Harald von Melchner and Klaus Höffken

Using a system of sequential daughter cell transfers in semisolid medium, we have analyzed self-renewal and differentiation of human promyelocytic leukemia cells (HL60) in presence of all-trans retinoic acid and human placental conditioned medium (HPCM). We find that retinoic acid induces coordinated losses of self-renewal potential which are followed by phenotypic differentiation. The latter occurs as an all-or-none event and is reversible in the presence of HPCM. Thus, HL60 cells that apparently had terminally differentiated (as estimated by the ability to reduce nitroblue tetrazolium [NBT]) can lose their differentiation marker and reenter the proliferative pool.

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increased proliferation. However, this includes cells that apparently had terminally differentiated. Evidence is shown that HL60 cells exhibiting a generally accepted marker for terminal differentiation, i.e., the ability to reduce nitroblue-tetrazolium (NBT), can lose this marker and reenter the proliferative pool.

**MATERIALS AND METHODS**

*Cell cultures.* Promyelocytic leukemia cells (HL60) were continuously passaged in RPMI 1640 medium (Gibco Europe Inc, UK) supplemented with 10% (v/v) preselected and heat inactivated fetal calf serum (Biochrom, West Berlin). The cells were grown at a concentration of $2.5 \times 10^5$ cells/mL at 37°C in a humid atmosphere of 10% CO$_2$ and were fed two to three times per week. Similar cultures were prepared with medium containing either $1 \times 10^{-6}$ mol/L all-trans retinoic acid (Sigma Chemical Co, St Louis) or 10% (v/v) human placental conditioned medium (HPCM), or both $1 \times 10^{-6}$ mol/L retinoic acid and 10% HPCM. The HPCM used was partially purified (stage II) and was kindly provided by Dr Nicos A. Nicola from The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia. The concentration of HPCM used for all experiments was that which stimulated growth and differentiation of 150 to 200 granulocyte-macrophage colony forming cells per $1 \times 10^6$ human bone marrow cells.

*Agar cultures.* Cells from liquid cultures were harvested during the exponential growth phase and were grown at a concentration of 100 cells/mL in 35 mm plastic Petri dishes containing 2 mL of agar/medium as previously described.

*Sequential recloning of paired daughter cells.* Sequential recloning of paired daughter cells was performed as previously described. The detailed procedure used for the present experiments is outlined in Fig 1.

*Replating of daughter cell colonies.* Single colonies developing during an incubation time of 14 days were harvested and replated at a concentration of 100 cells/mL as previously described (Fig 1). Remaining cells from single colonies were assayed for their ability to reduce nitroblue-tetrazolium (NBT).

*Assays for differentiation.* Cells derived from colonies or liquid cultures were diluted in RPMI medium to give a final concentration of maximum $2.5 \times 10^5$ cells/mL. The cell suspensions were mixed with a phorbol myristate acetate (PMA) (Sigma)-NBT (Sigma) solution and after incubating for 20 minutes at 37°C in a water bath, cell differentiation was estimated as previously described.

**RESULTS AND DISCUSSION**

We first investigated whether HPCM has any influence on growth and differentiation of HL60 cells. For this purpose, cells were grown in liquid cultures that contained either HPCM or retinoic acid, or both HPCM and retinoic acid. At daily intervals, proliferation was estimated by viable cell counting and differentiation was assessed by estimating the fraction of cells already had differentiated (Fig 2, right).

There are three mechanisms that may explain this observation. First, HPCM may have increased replication of those cells which failed to differentiate in retinoic acid. Second, growth factor dependent granulocyte-progenitor cells may have arisen in presence of retinoic acid, which when exposed to CSA, produce a progeny with granulocytic features. Third, HPCM may have stimulated differentiated cells to resume division and thus to reenter the proliferative pool.
Differences in the proliferation of daughter cells were observed. In cultures containing HPCM, the frequency of dividing cells was significantly increased (Fig 1B). Differentiation of HL60 cells in presence or absence of retinoic acid was studied. After an additional incubation time of 72 hours, the frequency of dividing cells in cultures with HPCM was higher than in cultures without HPCM. As shown in Table 1, the percentage of dividing cells is significantly higher in presence of HPCM, indicating that division is resumed by cells which had been rendered nondividing. A constant excess of dividing cells above the seeded number of undifferentiated (NBT-negative) cells (Table 1), indicates that NBT-positive cells must have regained proliferative potential. Because all colonies arising from these cultures fail to exhibit the NBT differentiation marker (data not shown), it is feasible to conclude that differentiated cells which regain proliferative potential concomitantly lose their differentiation marker.

To establish the time interval during which differentiated HL60 cells respond to HPCM, cells which had been induced to differentiate by retinoic acid were seeded in soft agar as described above. At various intervals following plating,
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Table 1. Frequency of Differentiated HL60 Cells Resuming Division in Presence of HPCM

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Day of Liquid Culture</th>
<th>% NBT Positive</th>
<th>% Dividing Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>HPCM</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>50</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>67</td>
<td>26</td>
</tr>
</tbody>
</table>

Cells from liquid cultures containing $1 \times 10^{-8}$ mol/L all-trans retinoic acid were washed and plated in soft agar either in presence or absence of 10% (v/v) HPCM. After 72 hours of incubation the frequency of dividing cells among 10,000 seeded cells was counted using an inverted microscope.

Table 2. Frequency of Differentiated HL60 Cells Resuming Division After Delayed Addition of HPCM

<table>
<thead>
<tr>
<th>Day of Liquid Culture</th>
<th>% NBT Positive</th>
<th>% Dividing Cells After Addition of HPCM at</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HPCM 0h 24h 48h 72h 96h</td>
</tr>
<tr>
<td>7</td>
<td>67</td>
<td>6 71 33 20 8</td>
</tr>
<tr>
<td>2</td>
<td>82</td>
<td>4 38 26 26 16</td>
</tr>
</tbody>
</table>

Cells from liquid cultures containing $1 \times 10^{-8}$ mol/L all-trans retinoic acid were plated in soft agar. At various intervals following plating 10% (v/v) HPCM was added to duplicate culture dishes. After 72 hours of incubation following the addition of HPCM, the frequency of dividing cells among 10,000 seeded cells was counted using an inverted microscope.

HPCM was added to the cultures and 72 hours thereafter, the frequency of dividing cells was estimated as described above. As shown in Table 2, the proportion of dividing HL60 cells is highest in cultures receiving HPCM immediately following plating. This proportion, however, decreases progressively with time elapsing between plating and HPCM addition, and no further stimulation is noticed at intervals longer than 72 hours. This indicates that the ability of nondividing (differentiated) HL60 cells to resume division in the presence of HPCM is restricted in time.

In conclusion, the present experiments have shown that in the presence of CSA (HPCM), a significant proportion of differentiated HL60 cells loses its differentiation marker and regains proliferative potential. This suggests that a phenomenon generally accepted as terminal differentiation is reversible. It appears that in HL60 cells, the expression of a differentiation program consists of a series of unidirectional but notably reversible events eventually leading to terminal differentiation. Hence, there is apparently no detectable "commitment" along the differentiation pathway of HL60 cells until the late granulocyte stage. It is only at this stage that cells undergo a single and irreversible event which yields them nondividing and nonresponsive to CSA.

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REFERENCES


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