An in Vitro Assay for Growth Regulation of Embryonal Carcinoma by the Blastocyst

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ABSTRACT

An in vitro assay has been developed in order to examine the mechanism whereby the mouse blastocyst regulates embryonal carcinoma (EC). The assay measures the suppression of EC cell colony formation caused by exposure to the cavity of the blastocyst (i.e., blastocele), and the results are comparable to the previous results with blastocyst-mediated suppression of tumor formation for this same cell line.

The assay has been used to determine the time necessary for the blastocyst to regulate the EC cell. In these experiments, immnosurgery is done to disrupt the interaction between the EC cell and the blastocyst, and the EC cell is then recovered and identified by its ability to form a colony. As compared to control cells, 84% of the EC cells are recovered after 2 hr of exposure to the blastocyst, 57% are recovered after 14 hr of exposure, and 27% are recovered after 24 hr of exposure. This long time course is similar to the prolonged doubling time of labeled EC cells within the blastocele suggesting that the response of the EC cell may be related to the cell cycle within the blastocyst. Data presented show that synchronization of the EC cell cycle (using a mitotic selection procedure) produces synchronization of the response to the blastocyst. The response appears to be closely linked to the GI phase of the EC cell cycle.

INTRODUCTION

EC cells are the malignant stem cells of mouse teratocarcinomas (6, 16). At least some of these malignant cells can respond to the environment of the mouse embryo as if they were normal stem cells in the ICM of the blastocyst. This was first shown by Brinster (1) who injected EC cells into blastocysts and then transferred the injected blastocysts to the uteri of pseudopregnant hosts. Of the offspring produced, one was demonstrably chimeric, having mosaic coat coloring which indicated a contribution by the EC cell. This result has been extended to show that the EC cells contribute to the formation of many tissues in chimeric mice (11, 15) and that only in unusual circumstances do the chimeric mice have tumors (14). Clearly, some EC cells are reprogrammed by the blastocyst to behave as normal embryonic cells incapable of forming a tumor. However, before the mechanism of this reprogramming could be studied, a better assay for the regulatory effect was needed.

An assay was developed which compared the tumorigenicity of EC cells which had been injected into a blastocyst (see Fig. 1a) with the tumorigenicity of control EC cells which had not been exposed to the blastocyst. An EC cell placed inside the blastocele (the cavity of the blastocyst) was much less tumorigenic than EC cells cloned in the absence of a blastocyst; similar results were also obtained by Papaioannou et al. (13). Further, EC cells placed in the perivitelline space (between zona pellucida and trophectoderm) showed no impairment of the ability to form a tumor (17). This served as an important positive control. In contrast, the tumorigenicity of leukemia, melanoma, and sarcoma cells was not affected by injection into the blastocele (17, 18). Thus, regulation of cancer cells by the embryo required close correspondence between tumor cells and the embryonic field and appeared specific. However, the assay was expensive and laborious, and it did not provide the means to examine by direct visualization how the blastocyst functioned or how the EC cell responded.

These problems were resolved by in vitro studies. From the in vivo experiments, one line of EC was identified which was particularly sensitive to growth regulation by the blastocyst and which cloned efficiently in vitro (18). As reported here, this cell line, 247 EC, can be used in a precise clonal assay which measures the suppression of colony formation by EC cells inside the blastocyst as compared to that of EC cells cultured either in the perivitelline space or independently of the blastocyst. In agreement with the in vivo work, each blastocyst can regulate at least 5 247 EC cells. The in vitro assay has served to probe the interaction between the cancer cell and the embryo, and the time course of the interaction and the role of the cell cycle of the EC cell have been examined.

MATERIALS AND METHODS

Cells and Culture Methods. The 247 line of EC cells used in these experiments was kindly provided by John Lehman (Denver, Colo.); the origin and methods of propagation of these cells have been described previously (7). Modified Eagle's medium supplemented with 1 mM sodium pyruvate and 10% fetal calf serum was used as growth medium in all experiments. Drops of growth medium with volumes of 20 µl or greater were used; this drop size has been shown to support clonal growth of EC cells with or without mineral oil. For experiments with nonsynchronized cells, rapidly growing cultures of EC cells were suspended by brief trypsinization (0.05% trypsin and 0.02% EDTA; Flow Laboratories, Inc., Englewood, Calif.), pelleted by slow-speed centrifugation, and resuspended in growth media. Cells were then either injected into blastocysts or placed in the cloning assay to serve as controls for viability.

Infection into Blastocysts. Blastocysts were flushed from the uteri of 3.5-day pregnant Swiss-Webster mice, washed 3 times, and placed in a drop of growth medium under mineral oil. EC cells were added to the drop, and then the appropriate number of EC cells was injected into the blastoceles or into the perivitelline space by methods described previously (17).
Cloning Assay to Determine Colony-forming Ability. Blastocysts containing EC cells (or EC cells alone) were individually cultured in the wells of microculture plates (Falcon) with 20 μl of growth medium. In certain experiments, 1.5 × 10^5 W138 human fibroblasts were included in each culture to serve as a feeder layer control. The cloning technique used micropipetting of individual specimens under direct visual control as described previously (17). After 7 days in culture, each well was scored for the presence of a colony of EC cells (defined as 25 or more EC cells). The results of each experiment were expressed as colony formation (number of wells positive for colony formation/total number of wells used), and for each series of experiments, the results were analyzed using the Mantel-Haenszel extension of χ^2 (10). The results were deemed significant if the null hypothesis was rejected at a significance level of 0.05 or less.

Recovery of EC Cells from the Blastocyst. The injected blastocysts were incubated for the desired period of exposure, and then immunosurgery was performed according to the method of Solter and Knowles (23). In this technique, the blastocysts were placed in pronase [0.2% in phosphate-buffered saline (0.1 M Na₂HPO₄-NaH₂PO₄:0.144 M NaCl, pH 7.2)] for 10 min to digest the zona pellucida, washed 3 times, placed in rabbit anti-mouse antibody (raised against mouse spleen cells and diluted 1:50 in phosphate-buffered saline) for 30 min, washed 3 times to remove excess antibody, and placed in guinea pig complement (diluted 1:8 in growth medium) for 30 min. The trophectoderm underwent complement-mediated immunocytolysis, but the blastocysts maintained sufficient structural integrity to be washed gently by 3 changes of medium and plated in the cloning assay.

Prelabeling of Cells by Phagocytosis of Fluorescent Microspheres. EC cells were cocultured with 0.7-μm fluorescent carboxylated microspheres (Polysciences, Inc., Warrington, Pa.) for a period of 16 hr at 10^3 microspheres/cell. The excess microspheres were removed by 6 changes of medium, and the cells were used in experiments as described. The average cell phagocytosed more than 20 microspheres, and virtually all the cells were labeled. This labeling method did not alter the cloning efficiency of the cells, the length of their cell cycles, or their response to the blastocyst in the assay for colony formation (24). The labeled cells could be readily identified inside the blastocyst over a few cell generations (18).

Monitoring of Cell Division Inside the Blastocyst. Single EC cells prelabeled with fluorescent microspheres were injected into the blastocoele (experimental) or the perivitelline space (control). After 24 hr of incubation, the blastocysts were examined by a combination of phase and fluorescent microscopy at ×200 magnification to determine the number of labeled cells which each culture contained. Observations were made independently by 2 observers, and the blastocysts were rolled to permit visualization from the optimum angle.

Selection of Synchronous EC Cells by the Paired-Daughter Cell Method. Synchronous EC cells were selected by a variation of the mitotic shake-off technique. Rapidly growing cultures of EC cells were gently agitated to release mitotic cells from the substrate, and these were collected in a micropipet and transferred to a drop of growth medium under mineral oil. After a 30-min incubation, the majority of the cells underwent cytokinesis and yielded pairs of daughter cells. These newly produced daughter cells were pooled in another drop and agitated to release mitotic cells from the substrate, and these cells were examined at each time point, and the number of grains per nucleus was counted. The amount of isotope used did not alter the total cycle length.

RESULTS

Development of the Assay. Blastocysts were individually in microtiter wells each containing 20 μl of medium. The cultures were observed daily, and the embryos were developed as follows. They hatched from the zona pellucida (Days 1 to 2), implanted on the plastic substrate (Day 3), and appeared by Days 4 to 7 as a monolayer of polyloid trophoblast with a clump of cells derived from the ICM on the surface. These observations, also described by others (5, 21), provided a basis for comparing the development of blastocysts containing cancer cells (see Fig. 1, a and b). If an EC cell was injected into the perivitelline space and the embryo was cultured as described, then by Days 6 to 7 the colony of tumor cells had usually outgrown the embryo as illustrated in Fig. 1b. In contrast, if the EC cell had been injected into the blastocoele cavity, then usually no colony of EC cells developed. The fate of EC cells failing to form a colony was not apparent against the background of the embryo as will be discussed later. However, the decrease in the rate of colony formation was an easily quantitated end point.

The results (Table 1) indicated a 5-fold suppression of colony formation of 247 EC cells by the blastocyst; this difference is statistically significant at the 0.005 level. The 9% of colonies which were observed is considered within the limit of error for the microinjection technique (6). Further, the control EC cells (in the perivitelline space) showed a cloning efficiency similar to that for EC cells cloned without blastocysts (i.e., no statistically significant difference). It seemed possible that these results were due to separation of the EC from the plastic substrate by a monolayer of cells (created by implantation of the embryo). However, the inclusion of a confluent feeder layer of fibroblasts did not change the results significantly as shown in Table 1. In vitro assay for blastocyst regulation of single EC cells

<table>
<thead>
<tr>
<th>Colony formation (no. of colonies/no. tested)</th>
<th>EC cell inside blastocoele</th>
<th>EC cell inside perivitelline space</th>
<th>EC cell alone</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/10</td>
<td>2/3</td>
<td>21/40</td>
<td></td>
</tr>
<tr>
<td>0/5</td>
<td>2/3</td>
<td>15/40</td>
<td></td>
</tr>
<tr>
<td>1/17</td>
<td>8/19</td>
<td>19/40</td>
<td></td>
</tr>
<tr>
<td>1/13</td>
<td>7/13</td>
<td>18/40</td>
<td></td>
</tr>
<tr>
<td>2/14</td>
<td>9/18</td>
<td>20/40</td>
<td></td>
</tr>
<tr>
<td>2/15</td>
<td>5/12</td>
<td>23/40</td>
<td></td>
</tr>
<tr>
<td>3/13</td>
<td>13/14</td>
<td>23/40</td>
<td></td>
</tr>
<tr>
<td>10/87 (11%)</td>
<td>44/79 (56%)</td>
<td>116/240 (48%)</td>
<td></td>
</tr>
</tbody>
</table>

* Assay done on a confluent feeder layer of W138 fibroblasts.
colonies did so in close proximity to the ICM; apparently such exposure formed colonies at 57% of the level for control; and cells rescued after 14 hr of exposure to blastocysts formed colonies at 84% compared to that for control EC cells (Table 2). Cells rescued until they had grown out of the remnants of trophectoderm. The blastocele cavity collapsed within 1 hr after transfer, and the trophectoderm retained sufficient structural integrity so that the treated cells could be unequivocally identified inside the blastocoele or in the perivitelline space.

**Time Course for the EC Cell Response.** The assay was used to determine the time necessary for the blastocyst to regulate cells of line 247 EC. Single EC cells were injected into the blastocyst, rescued by immunosurgery (23) at various times, and tested for their ability to form colonies. During immunosurgery, the outer layer of the blastocyst (trophectoderm) was destroyed with antibody and complement, but the inner cells (ICM) and the injected cancer cell were protected from the macromolecules by the tight junctions which connect the trophectoderm cells. Thus, inner cells were not exposed to the antibody and survived the technique. The dead trophectoderm was destroyed with antibody and complement, but the ICM portion of the blastocyst, rescued by immunosurgery (23) at various time points, it was shown that the blastocyst was able to control 5 247 EC cells/blastoecyst; colonies formed from cells in the blastocele in only 6% of cases (one of 18) as compared to 86% of cases (31 of 36) from cells in the perivitelline space (statistically significant at the 0.005 level). The upper limit to the number of EC cells which can be regulated by a single blastocyst is not known; experiments with larger numbers of cells have not been done due to the technical difficulties involved. It was concluded that the assay measures a growth-regulatory effect of the blastocyst, and the effect appears to be irreversible since refeeding or passing of the negative cultures has not rescued any EC cells (0 of 20 such negative cultures).

**Table 2**

<table>
<thead>
<tr>
<th>Time of rescue from blastocyst (hr)</th>
<th>Colony formation (no. of colonies/no. tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC cell inside blastocele</td>
</tr>
<tr>
<td>2</td>
<td>7/16</td>
</tr>
<tr>
<td>2</td>
<td>4/14</td>
</tr>
<tr>
<td>2</td>
<td>10/15</td>
</tr>
<tr>
<td>2</td>
<td>8/15</td>
</tr>
<tr>
<td>2</td>
<td>5/8</td>
</tr>
<tr>
<td>2</td>
<td>34/66 (50%)</td>
</tr>
<tr>
<td>14</td>
<td>7/17</td>
</tr>
<tr>
<td>14</td>
<td>4/10</td>
</tr>
<tr>
<td>14</td>
<td>11/27 (41%)</td>
</tr>
<tr>
<td>24</td>
<td>2/14</td>
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<tr>
<td>24</td>
<td>2/14</td>
</tr>
<tr>
<td>24</td>
<td>3/16</td>
</tr>
<tr>
<td>24</td>
<td>3/15</td>
</tr>
<tr>
<td>24</td>
<td>10/59 (17%)</td>
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</tbody>
</table>

*The results are fitted by a least-square line with a correlation coefficient of -0.97. The line extrapolates to show absolute regulation (i.e., no colony formation) after approximately 35 hr of exposure to the blastocyst.

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**Proliferation of EC Cells inside the Blastocyst.** From the time course experiments, it was clear that some EC cells responded rapidly to the blastocyst whereas others required more than 24 hr to respond. The question arose whether or not these cells divided within the blastocoele. To answer the question, the frequency of cell division inside the blastocoele was compared to that in the perivitelline space over a 24-hr period. The results showed a 50% decrease in the rate of division of EC cells within the blastocoele (13 of 40 EC cells divided inside versus 17 of 27 outside). In these experiments, the EC cells were prelabeled with fluorescent microspheres so that they could be unequivocally identified inside the blastocoele or in the perivitelline space.

The suppression of proliferation of EC cells inside the blastocoele could be due to removal of some cells from the cell cycle before mitosis or to slowing of all cells in their traverse of the cell cycle. If the latter explanation were true, the cell cycle of EC cells inside the blastocyst would be roughly twice that of EC cells outside the blastocyst. As will be seen, this agrees with the long time course. It seemed possible that the response of the EC cells was linked to its cell cycle.

**Synchronization of EC Cells to Determine the Role of the Cell Cycle.** The strategy of these experiments was to select EC cells synchronized at a predetermined point in the cell cycle, inject these cells into blastocysts, recover the injected cells after a known period of exposure, and determine whether this exposure had caused the EC cells to lose their ability to form colonies. Synchronous EC cells were obtained by selecting individual cells in the M phase of the cycle and then further selecting those that underwent cytokinesis within 30 min of one another (see Fig. 3). The key to this method was that, after cytokinesis, the newly created daughter cells were tightly cohesive for at least 30 min and could be easily recognized and manipulated as a pair of cells. Later, they could be separated into single cells for use in the experiment. To determine the degree of synchrony in the subsequent cell cycle, samples of paired daughter cells were studied as to their conformity in total cycle length and onset of DNA synthesis. More than 100 daughter cells were examined in 3 separate experiments to determine cycle length. In each experiment, mitotic activity began after 10 hr of incubation and proceeded at a constant rate until after 20 hr of incubation at least 90% of the cells had divided. To determine onset of DNA synthesis, the daughter cells were cultured in [methyl-3H]thymidine, and samples totaling at least 50 cells were prepared at 1-hr intervals for autoradiography. DNA synthesis was first detected in some cells at 3 hr after mitosis, and the number of such cells increased at a constant rate until, by 8 hr after mitosis, 90% of cells had entered S phase. It was concluded that, for 90% of the paired daughter cells, the G, phase varied from 3 to 8 hr and the total cycle length varied from 10 to 20 hr.

**Response of Synchronized Cells after 16 Hr of Exposure to the Blastocyst.** Having established a time schedule for the cell cycle of paired daughter cells (i.e., synchronized EC cells), purified populations of early-G,-phase (mitosis plus 1 to 2 hr)
cells, late-G₁- or early-S-phase (mitosis plus 3 to 4 hr) cells, and S- or G₂-phase (mitosis plus 7 to 8 hr) cells were prepared and tested for their responsiveness to the blastocyst, as shown in Table 3. The cloning efficiency of control cells at the same point in the cell cycle was determined in each experiment.

In the initial experiments, synchronous EC cells were all exposed to the blastocyst for 16 hr, recovered by immunosurgery, and tested for their ability to form colonies. This period of exposure was chosen because in the time course experiments approximately 50% of EC cells randomly distributed in the cell cycle had responded to the blastocyst after 16 hr (i.e., 50% reduction of colony formation). For the synchronous EC cells (Table 2), the response of the EC cells was maximal for exposure during the early G₁ phase; there was a 3-fold reduction in colony formation which was statistically significant at the 0.005 level and which approached the 5-fold reduction seen in Table 1. The response was minimal for exposure during the late G₁ or early S phase; colony formation was not significantly different from that of the control cells. Cells in the late S or G₂ phase were between these 2 extremes (a 40% reduction in colony formation).

It seemed that the EC cells were responding only during the early G₁ phase of the cell cycle. This would explain the lack of response of late-G₁- and early-S-phase cells. To account for the intermediate response of late-S- and G₂-phase cells, it was postulated that these cells were able to cycle and reach the G₁ phase after 16 hr inside the blastocyst. If this were true, then early-G₁-phase cells should respond much more quickly than 16 hr, and late-G₁- and early-S-phase cells should eventually respond after more than 16 hr. This hypothesis was tested.

**The Course for Response of Early-G₁-Phase Cells versus Late-G₁- and Early-S-Phase Cells.** The most sensitive EC cells and the most resistant populations were exposed to the blastocyst for varying times to define further their responsiveness to the blastocyst. To ensure the reliability of the results, each experiment was performed at least 3 times, and a method of statistical analysis was used which takes into account the variability between experiments. As shown in Table 3, early-G₁-phase cells did not respond to a significant degree after 2 hr of exposure to the blastocyst but responded to the same degree after 4 hr of exposure as they had with 16 hr of exposure (3-fold decrease in colony formation statistically significant at the 0.005 level). Late-G₁- and early-S-phase cells did not respond significantly with 4 or 10 hr of exposure (as they had not responded with 16 hr of exposure), but with 24 hr of exposure there was a significant response (3-fold decrease in colony formation statistically significant at the 0.005 level).

### DISCUSSION

Results have been presented which demonstrate growth regulation of EC cells by the blastocyst in vitro. A precise clonal assay has been used which is based on colony formation, and the results agree with those from previous in vivo studies in which the blastocyst was able to abrogate the tumorigenicity of the same EC cell line (18). They also agree with the observations of Papaioannou et al. (13) who injected clumps of EC cells into blastocysts and observed a reduction in colony formation.

Taken together, the data concerning time course, rate of cell division, and response of late-G₁- or early-S-phase cells indicate that the first EC cell cycle inside the blastocyst is approximately 28 to 35 hr long. During this first cycle, it appears that only EC cells in the G₁ phase of the cell cycle respond promptly to the blastocyst. The results also suggest that trophectoderm may play an active role in the mechanism. Immunosurgical destruction of trophectoderm done at early time points blocks regulation of the EC cell even though some degree of proximity to viable ICM is maintained. It may be that a more intimate interaction is necessary between the EC cell and ICM, but it is also possible that trophectoderm is required to trigger the G₁-phase mechanism. The precise relationship of the G₁-phase mechanism to subsequent growth potential and to cellular differentiation is not yet known. Clearly, after the G₁-phase response, there is a lack of growth potential because the cell fails to outgrow the embryo and form a colony under the conditions of the assay.

Does growth regulation occur because the cell becomes committed to normal differentiation and shares the abortive developmental role of the embryo in tissue culture, because it enters a state of growth arrest, or because it dies? The ability to synchronize the response of the EC cell to the blastocyst should be a convenient tool with which to answer these questions. Previous studies have been hampered by the possibility of nonspecific effects and have sought to control this factor through the use of unrelated cancer cells which survive exposure to blastocysts (17, 18). However, when an unrelated cancer cell does not survive, this may merely reflect the idiosyncratic growth requirements of that particular cell and, hence, may not be relevant to EC cells. Using synchronized late-G₁- or early-S-phase cells (selected at 4 hr after mitosis) provides a more meaningful control. One knows that these EC cells survive, do not respond, and can be quantitatively recovered from the blastocyst for at least 16 hr. This group can then be compared to an experimental group of early-G₁-phase EC cells (selected at 1 hr after mitosis) which fully respond after 4 hr of exposure. This comparison opens the way to study the EC cell response in a well-controlled manner.

To date, the results are consistent with the hypothesis that the EC cell responds to a mechanism which is aimed at regulating early development. For instance, the slowed rate of cell division for the EC cell in the blastocyst corresponds to that...
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reported for the ICM cells in the mature blastocyst (2). Such a mechanism might be involved in the process whereby the stem cells of the embryo become committed to differentiation pathways. If the hypothesis is true, our results suggest that the commitment occurs in the G₁ phase of the ICM cell cycle. This suggestion has support from other systems. The commitment to differentiation of several cell types, including 3T3 preadipocytes, myoblasts, and Friend erythroleukemia cells, has been closely linked to events in the G₁ phase of the cycle (8, 9, 12, 19, 20), and the commitment step may be coupled to a state of growth arrest (12, 20). In this regard, it is interesting to note that the ICM becomes arrested in the G₁ phase of the cell cycle during delayed implantation (22).

ACKNOWLEDGMENTS

The author thanks J. Caldwell and A. Lewellyn for technical assistance, G. B. Pierce for his encouragement, and John Berg for assistance with statistical analysis.

REFERENCES

Fig. 1. Colony-forming assay with a single cell of the 247 EC line. In a, the EC cell is placed either in the blastocoele as in this case or in the perivitelline space (PV). The trophectoderm (T), ICM, and zona pellucida (Z) are also identified. × 400. In b, after 7 days in culture, an EC cell from the perivitelline space has formed a colony adjacent to the embryo. × 260.

Fig. 2. Method of rescue of EC cells from the blastocyst. Immediately after immunosurgery, a blastocyst shows immunocystolysis of trophectoderm. An intact ICM can still be seen inside the blastocoele which has not yet collapsed. The cancer cell is not seen. × 510.

Fig. 3. Synchronization of EC cells by the paired-daughter cell method. Cells were selected in the M phase of the cycle, and after 30 min of incubation, cytokinesis has occurred. The cohesive pairs of daughter cells are evident. These are harvested and used at the appropriate time (i.e., mitosis plus the desired length of time). × 700.
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