Trophectoderm in Control of Murine Embryonal Carcinoma

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ABSTRACT

It has been shown previously that the intact blastocyst of the mouse can regulate tumor formation and colony formation of murine embryonal carcinoma. This effect is consistent with the close histogenetic correspondence between embryonal carcinoma and the inner cell mass of the blastocyst. The ability of inner cell mass, blastocele fluid, and inner and outer surfaces of trophoderm to abrogate colony formation of a variety of malignant tumors has now been tested. Direct contact of the embryonal carcinoma cells with the blastocele surface of trophoderm proved to be necessary for abrogation of colony formation of embryonal carcinoma. This effect was not seen with any of the other tumors tested. Some tumors, which lack a normal cellular counterpart in the blastocyst, grew poorly in the blastocyst unless a fistula was made in the wall of the blastocyst. Colony formation of the embryonal carcinoma was regulated in blastocysts with fistulas, but the other tumors were not regulated under these conditions. It is concluded that colony formation of embryonal carcinoma cells is regulated by direct contact with the trophoderm of its corresponding embryonic field in an unknown but specific manner.

INTRODUCTION

The observation that embryonal carcinoma cells, the stem cells of teratocarcinoma, could spontaneously differentiate into benign cells and tissues (10, 20, 21) and that this naturally occurring differentiation could be modulated in vitro led to the idea that direction of differentiation might serve as an alternative to cytotoxic therapy for cancer (19). The notion took a leap forward with the demonstration of Brinster (2), using Gardner's techniques (6), that embryonal carcinoma cells of one strain of mouse, when injected into blastocysts of another strain, were regulated to the point that they and their progeny behaved as embryonic cells. These cancer-derived cells, together with the normal embryonic cells, formed chimeric mice. This observation was extended in several laboratories, and although lines of embryonal carcinoma vary in their production of tumor-free chimeras, it is clear that blastocysts can regulate some embryonal carcinoma cells to the point that they and their progeny behave as apparently normal embryonic cells (8, 14, 15, 17, 18, 24).

Such results prompted us to undertake an examination of the immediate effect of the blastocyst and its several parts on embryonal carcinoma and other malignant cells. Quantitative assays were required if insight into the mechanism of this regenerative process was to be acquired. To this end, an assay was developed that took advantage of the tumor-forming ability of the cancer cells (22). In this assay, the incidence of tumors produced by cloning embryonal carcinoma cells in either the testis or intraperitoneum was compared to the incidence obtained after single embryonal carcinoma cells were injected into blastocysts, which were subsequently transplanted to the appropriate sites in the animal. Two of 3 embryonal carcinomas tested in this assay were regulated, but a leukemia, sarcoma, and melanoma were not regulated. This suggested a degree of specificity for the reaction (23).

Wells developed a colony assay which took advantage of the ability of malignant cells to form colonies in vitro (27). In this essay, the incidence of colonies produced by cloning embryonal carcinoma cells in microculture plates was compared to that obtained after single cancer cells had been incorporated into blastocysts, which were then cultured individually in similar plates. An embryonal carcinoma (EC 247) which was regulated in the tumor assay was also regulated in the colony assay and, because of its ease of growth in tissue culture, it has been chosen as the prototype tumor for these studies. EC 247 has been demonstrated to have a restriction point in G1 of the cell cycle, at which point the cells respond to the regulatory effect of the blastocyst (27). This is extremely important technically, because if cells are synchronized to a period just prior to the restriction point, short exposures to an inducing environment are effective in regulating colony formation.

Now, we wish to report on the specificity of the blastocyst in regulating colony formation of malignant cells. Under the appropriate conditions, EC 247 was regulated by the blastocyst, but L1210 leukemia, B-16 melanoma, and CHO cells were not regulated. When the components of the blastocyst (blastocele fluid, ICM, and trophoderm) were examined for their ability to regulate colony formation of EC 247 cells, only the blastocele surface of trophoderm proved regulatory through direct contact with the embryonal carcinoma cells.

MATERIALS AND METHODS

The tumors and cells used are listed in Table 1 with their strain of origin, method of propagation, and other characteristics. The methods for acquiring, washing, incubating, and injecting blastocysts and tumor cells have been described previously (22, 23, 27). For cloning of all cell types and for culture of blastocysts, MEM+10 was used to which were added sodium pyruvate, antibiotics, and 10% heat-inactivated fetal calf serum, the latter selected for lack of cytotoxicity (11). Cloning of embryonal carcinoma cells with or without blastocysts used p.o.-controlled pipets with micropipettes. Cells were transferred to the cloning site under direct observation (22, 23, 27). When synchronized cells were required, they were obtained using a modification of the shakeoff techniques developed by Wells (27).

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3 Present address: Department of Pathology, University of Granada, Granada, Spain.
4 The abbreviations used are: EC 247, a particular cell line of embryonal carcinoma; CHO, Chinese hamster ovary; ICM, inner cell mass of blastocyst; MEM+10, modified Eagle's minimal essential medium.
When monolayers of trophoblast were required, single blastocysts were allowed to hatch in a 20-μl drop of MEM+10 under washed paraffin oil. After hatching, the terminally differentiated trophoblast derived from the trophectoderm attached and spread on the substrate (27). The embryonal carcinoma cells were placed on the monolayer of trophoblast at a distance from the inner cell mass. Colony formation was assessed after 6 days.

In the experiments to test the effect of ICMs on embryonal carcinoma, immunosurgery was used to isolate them free of trophectoderm (26). ICMs from incompletely expanded blastocysts (lacking endoderm) and others from blastocysts immediately prior to hatching (with endoderm) were placed singly in 20 μl of MEM+10 under paraffin oil, and embryonal carcinoma cells were placed on them using micropipets attached to a Leitz micromanipulator. These preparations were examined after 16 hr to ensure that the embryonal carcinoma cells remained attached to the ICM. To attach ICMs to embryonal carcinoma, the methods of Fujiwara and Martin (5) for production of aggregate chimeras were adapted. These used phytohemagglutinin (1000 μg/ml, Difco type A).

For the injection of blastocysts with single or as many as 5 cells, the blastocysts and cells were placed in a drop of MEM+10 under a layer of washed paraffin oil. Blastocysts were attached to the holding pipet by suction, and single cells, which were aspirated into injecting pipets, were then injected into the blastocoele using a Leitz micromanipulator. After injection, the blastocyst collapsed and reexpanded within 2 hr of incubation, and only those containing an easily recognizable cancer cell were utilized in the experiment (22, 23, 27). Controls for these experiments sometimes consisted of injecting the embryonal carcinoma cells into the perivitelline space (the space between the zona pellucida and the trophectoderm). These blastocysts were then cultured in vitro to assess colony formation. Because of the close correspondence in the results of this control versus that from cloning cells directly in microtiter plates, in experiments to test the effect of ICMs and the tropheostromatic vesicles from blastocyst was devised based upon those of Gardner et al. (7) and Pa-

A method for the production of trophectodermal vesicles from blastocysts was devised based upon those of Gardner et al. (7) and Papaloianou (16). The blastocysts were kept in tissue culture for 24 hr after removal from the animal to ensure maximum size and turgor. The method is illustrated in Fig. 3a. Blastocysts were attached to a large holding pipet using minimal suction (Fig. 3e). An injecting pipet containing the cancer cell was inserted into the blastocyst avoiding the ICM, and the embryonal carcinoma cell was placed against the trophoblast opposite to the ICM. The injecting pipet was then lowered over the blastocyst, and the inner cell mass was amputated and removed by aspiration. Amputation was performed slowly to keep the embryonal carcinoma cell always in view and to avoid rupture of the trophectoderm. The cut edges of the trophectoderm were forced together and sealed by needle pressure against the plastic floor of the dish. After removal of the needle, the trophectodermal vesicles collapsed, and on reexpanding, those in which a clearly visible cancer cell could be seen with the microscope were used in the experiments (Fig. 3b). These trophectodermal vesicles were then aspirated into p.o.-controlled pipets and placed in tissue culture.

In experiments to test the effect of conduits on the regulatory ability of the blastocoele surface of trophectodermal vesicles on embryonal carcinoma cells, a single cell was injected into the blastocyst and positioned at a point on the trophectoderm opposite to the ICM (Fig. 4, a and b). The tip of the needle was amputated so that it formed a conduit across the trophectoderm; then, the ICM was amputated as in the method for production of trophectodermal vesicles. The appearance of a vesicle with a conduit and containing a cancer cell is illustrated in Fig. 4c.

For experiments in which embryonal carcinoma cells were maintained in the blastocoele in the absence of contact with the cells of the blastocyst, the technique for making fistulas was modified (see Fig. 5). An oil-filled injection pipet bearing the cancer cell in about 10 cell volumes of MEM+10 was inserted into the blastocyst, the cell was retained in the tip of the pipet, and the pipet was amputated through the oil. This resulted in a situation in which the cancer cell was bathed by blastocoele fluid but denied contact with the tissue culture medium or the cells of the blastocyst. These blastocysts were cultured for 24 hr, a period of time sufficient for regulation of colony formation of embryonal carcinoma to occur in intact blastocysts (27). The pipets with the cells were then removed from the blastocysts, and the cells now bathed by tissue culture medium were then free to grow out of the tip of the pipet. Colonies were scored in the usual way.

In experiments to test the effect of blastocoele fluid on embryonal carcinoma cells directly, microcultures were made in the tips of injecting pipets (Fig. 6, a to d). To this end, blastocoele fluid was aspirated into an injecting pipet, followed by a cell, followed by fluid from a second blastocyst. The pipet was plugged by aspirating oil, and its tip was

### Table 1

Tumors and cells used, including strain of origin, method of propagation, and other characteristics

<table>
<thead>
<tr>
<th>Tumor and origin</th>
<th>Host strain</th>
<th>Method of propagation</th>
<th>Cloning site and efficiency</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC 247 (Lehman et al., 1974)</td>
<td>129</td>
<td>Monolayer tissue culture or in vivo</td>
<td>In vitro, 40-70%; in vivo (testis), 10%</td>
<td>Originated OTT 6050 near diploid, maintained as &quot;clean&quot; embryonal carcinoma by rapid passage, multipotent but extremely undifferentiated</td>
</tr>
<tr>
<td>402A7 embryonal carcinoma (Isa and Sanders, 1976)</td>
<td>129</td>
<td>Ascites</td>
<td>Intrapertoneum, 20-40%</td>
<td>Nullipotent</td>
</tr>
<tr>
<td>L1210 leukemia (animal/human tumor bank)</td>
<td>DBA/2</td>
<td>Ascites</td>
<td>Intrapertoneum, 25-35%; in vitro, 30-40%</td>
<td>Clones in vitro only in the presence of feeder layer</td>
</tr>
<tr>
<td>B-16 melanoma</td>
<td>C57BL/6</td>
<td>Monolayer or in vivo</td>
<td>In vitro, 30%</td>
<td>Solid tumor readily produced in vitro monolayer cultures</td>
</tr>
<tr>
<td>CHO cells</td>
<td>NA*</td>
<td>In vitro monolayer</td>
<td>In vitro, 70%</td>
<td>CHO; from Masaru Imada</td>
</tr>
</tbody>
</table>

* NA, not applicable.
amputated. The end result was a glass capillary 1 to 2 cell diameters in width and roughly 1 mm long that was sealed with oil at each end. Such cultures contained 2 blastocyst volumes of blastocele fluid (or growth medium in the case of controls) with one embryonal carcinoma cell in the middle. These were then maintained under oil for the desired period of incubation at 37° (Fig. 7 a to c). To determine rates of exchange in such cultures, dye studies were performed with Fast green (Sigma; 75 mg/ml). There was no significant loss or dilution of dye within the pipet during the brief interval required to create the culture, but diffusion does occur during later incubation. Cells were rescued by covering the fragment of pipet with a drop of growth medium, breaking the pipet with forceps, and transferring the section of capillary bearing the cancer cell to the cloning assay with a p.o.-controlled pipet.

The experiments reported here have been in progress over a 3-year period, and changes in cloning technique have occurred with time. For instance, the data in Tables 2, 5, and 7 were generated in Terasaki plates (Flow Laboratories) utilizing small volumes of medium; those in Tables 3, 4, and 8, a and b, in Linbro plates (Flow Laboratories) with larger volumes of medium. We attribute many of the differences in cloning efficiency of the experiments in the tables to these differences in plating techniques used. Note that the use of differing techniques did not alter the systematic differences between the respective experimental and control groups as confirmed by statistical analysis with the Mantel-Haenzsel extension of $\chi^2$ (13).

RESULTS

Specificity of Regulation of Colony Formation of EC 247 Cells. In studies reported previously, tumor formation of 2 of 3 embryonal carcinomas tested was regulated by the blastocyst, but tumor formation of L1210 leukemia, B-16 melanoma, and Sarcoma 180 cells was not regulated (23). This led to the idea that regulation of tumor formation by an embryonic field had a degree of specificity and that only tumor cells with a normal counterpart in the embryonic field (embryonal carcinoma and inner cell mass of the embryo, for example) would be regulated.

Thus, the first experiments tested the specificity of blastocyst regulation of colony formation of a variety of malignant cells. Single EC 247, L1210 leukemia, B-16 melanoma, and CHO cells were injected into either the blastocele (experimental group) or the perivitelline space (control group), and the number of colonies was compared for each tumor (Table 2). Significantly fewer colonies were obtained in relation to control when the embryonal carcinoma, melanoma, and CHO cells were injected into blastocysts, but the leukemia cells cloned equally well in either situation (Table 2).

Although these observations on colony formation with EC 247

### Table 2

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Inside blastocele</th>
<th>Statistical significance (%)</th>
<th>Perivitelline space</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC 247</td>
<td>14/159 (9)</td>
<td>99.5</td>
<td>68/160 (43)</td>
</tr>
<tr>
<td>L1210 leukemia</td>
<td>38/131 (29)</td>
<td>NS</td>
<td>29/110 (26)</td>
</tr>
<tr>
<td>B-16 melanoma</td>
<td>31/132 (23)</td>
<td>99.0</td>
<td>37/107 (35)</td>
</tr>
<tr>
<td>CHO cells</td>
<td>10/43 (23)</td>
<td>99.5</td>
<td>30/40 (75)</td>
</tr>
</tbody>
</table>

Amounts in parentheses, percentage of colony formation.

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and L1210 leukemia paralleled the experience with control of tumor formation reported previously (23), the B-16 melanoma and CHO cells did not behave as expected. In searching for an explanation for these observations, it was postulated that some cells, capable of cloning at high incidence in tissue culture, might not be able to survive in the blastocele for nutritional reasons. Trophodermal cells, which form the wall of the blastocyst, are connected by tight junctions, which preclude entrance of macromolecules into the blastocele (9). In addition, the longer blastocysts are maintained in culture, the less likely they are to implant when placed in the uterus (1). This combination of the anatomy of the blastocyst and the inadequacy of the in vitro environment might account for the apparent control of B-16 and CHO cells in the colony assay.

This postulate was tested by making a fistula in the wall of the blastocyst, thereby allowing diffusion of fluid across the trophoderm (Fig. 1).

Blastocysts with glass conduits (to keep the fistula open) collapsed, but upon incubation, the blastocyst never completely regained its original volume in the presence of a functional conduit (Fig. 2, a to c). Treated blastocysts achieved a volume of about 2/3 that of control blastocysts. In addition, the trophoderm, which normally becomes cuboidal with collapse of the blastocele, remained cuboidal in shape and failed to flatten as the blastocele was partially reformed. The combination of small size of the blastocyst and the thickness of the trophoderm layer proved to be useful clues as to the patency and function of conduits (Fig. 2a). The data indicate a slow outflow of fluid through the conduit. It is not known if molecules from the tissue culture medium could diffuse against the current into the blastocyst.

If blastocysts were impaled with solid glass spears or with conduits that were plugged with oil (Fig. 2b), they collapsed, and the trophoderm became cuboidal. After incubation in MEM+10 for 1 hr, they became fully reexpanded with flat trophoderm as in the untreated controls. Cuboidal and flat trophodermal cells appeared equally differentiated with the electron microscope, and in addition, each had the usual tight and gap junctions and desmosomes connecting adjacent cells (Fig. 8 and 9). The conclusion was reached that glass per se does not have an adverse effect on the blastocyst.

Blastocysts with functional conduits were somewhat slower in hatching than controls, but after 24 to 48 hr in culture, they often developed a collar of cells where the conduit penetrated the zona pellucida. Indeed, some of the embryos appeared to hatch by trophoderm growing along the pipet and through the zona pellucida. In other cases, hatching occurred in an apparently normal manner. The hatched embryos attached to the substrate and appeared to be relatively normal in appearance with inner cell mass overlying a patch of granular polyplid postmitotic trophoblast (Fig. 2b).

It became immediately apparent, when the effect of the anatomy of the blastocele wall was neutralized with a fistula, that regulation of colony formation by the blastocyst in vitro was as specific as was the regulation of tumor formation by the blastocyst in vivo. The data in Table 3 support this contention. EC 247 produced fewer colonies in blastocysts with fistulas than controls. In contrast, B-16 and CHO cells produced colonies in blastocysts with fistulas at control levels. Comparison of data in Tables 2 and 3 indicates that colony formation of EC 247 was
Role of Blastocoele Fluid in Regulation of Colony Formation of EC 247. Because of short cell survival in closed microculture systems of the required volume (\(10^{-3}\) µl), it was decided to use EC 247 cells synchronized to the restriction point for control of colony formation (27), expose these cells to blastocoele fluid for 4 hr, and then rescue them in 50 µl drops of MEM+10 to determine if there had been an effect on colony formation. This protocol allowed time for reaction of the cells with regulatory factors, but the short exposure would negate nonspecific toxic effects. The details are outlined in “Materials and Methods” (Figs. 6 and 7). If anything, EC 247 cells grew as well in microcultures of blastocoele fluid as in controls, because 23 of 31 (74%) cells microcultured for 4 hr in blastocoele fluid produced colonies after rescue into MEM+10 in relationship to 99 to 130 (76%) that formed colonies after cloning in MEM+10 with no intervening microculture in blastocoele fluid. It was concluded that blastocoele fluid was not regulatory of colony formation of EC 247. In addition, it would appear that EC 247 cells preferred an exposure to blastocoele fluid, because 74% of them formed colonies, whereas 46% formed colonies (12 colonies of 26 cultures) after microcultures in MEM+10 followed by rescue into large volumes of MEM+10.

It was decided to confirm the above observations by contriving a situation in which EC 247 cells would be exposed to blastocoele fluid but would be unable to contact the trophoderm or ICM (Fig. 5). After 24 hr, these experimental cells would be rescued from the blastocoele and tested for their ability to form colonies in MEM+10. It is to be noted that the experimental cells cloned equally well after rescue from the blastocoele (28 colonies from 59 specimens tested or 47%) as they did when cultured directly in growth medium (172 colonies from 360 specimens or 48%). Also, the cells were actively cycling during their exposure to blastocoele fluid, since 27 of the 28 cells destined to form colonies divided during this time.

It is concluded that, under the conditions of the experiment, blastocoele fluid lacked a regulatory effect on colony formation of EC 247 cells. By implication, regulation must be by direct cell-cell contact with either ICM or trophoderm.

Role of ICM and Trophoblast in Regulation of Colony Formation of EC 247. Experiments testing for an effect of ICM and trophoblast on embryonal carcinoma cells required similar technology, so they were performed simultaneously. ICMs were isolated by immunosurgery from early blastocysts, which were incompletely expanded, and from old ones just prior to hatching. The ICMs derived from the former were balls of 12 to 16 cells which lacked endoderm, but those from the latter ones had a cap of newly formed endodermal cells (Fig. 10a). The EC 247 cells were placed on the ICMs, but some floated free and attached to the substratum where some grew successfully. For controls, EC 247 cells were grown on plastic near, but not touching, an ICM. Neither early or late ICMs controlled colony formation of embryonal carcinoma cells under the conditions of the experiments. Nine of 16 (56%) embryonal carcinoma cells placed on ICMs formed colonies in relationship to the controls in which 19 of 48 (40%) cells placed beside ICMs formed colonies. It appeared as though the embryonal carcinoma cells grew better on the ICM than beside it.

In a confirmatory set of experiments, single embryonal carcinoma cells were aggregated with early ICMs in the presence of phytohemagglutinin for 1 hr as described in “Materials and Methods.” During the time, embryonal carcinoma and ICM became a tightly compacted cell mass which would then be transferred to the colony-forming assay without loss of contact of the cancer and embryonic cells. Continued contact was verified at 24 hr. Again, the embryonal carcinoma cells failed to integrate into the developing ICMs and formed colonies in 28 of 35 cases (80%) as compared to 98 of 116 colonies cloned in the control (84%). This is not statistically significant difference. It is to be noted that this experiment was performed 2 years after the previous experiment. The differences in techniques described in “Materials and Methods” plus progression of the tumor account for the differences in the efficiency of cloning between the experiments.

To test if trophoblast was regulatory of colony formation of EC 247 cells, blastocysts were allowed to hatch in vitro, and single EC 247 cells were placed upon the differentiated postmitotic trophoblast cells, which were attached to glass and which were at a distance from the ICM (Fig. 11). Control EC 247 cells were placed beside the attached embryos but not in contact with them. Thirty-one of 48 cells (65%) placed on trophoblast formed colonies in relation to 11 of 21 (52%) placed beside the trophoblast. It was concluded that, under the circumstances of the experiment, no evidence of control of colony formation by trophoblast could be demonstrated. By elimination, it was concluded that the blastocoele surface of trophoderm must regulate colony formation of EC 247 cells by direct cell-cell contact.

Regulation of Colony Formation of EC 247 Cells by Contact with the Blastocoele Surface of Trophoderm. The method for the production of trophodermal vesicles containing EC 247 cells is illustrated in Fig. 3a and described in “Materials and Methods.” Typical vesicles containing cancer cells are illustrated in Fig. 3b.

When trophodermal vesicles lacking cancer cells were cultured in vitro, they attached to the substratum and formed a granular patch of postmitotic well-differentiated trophoblastic giant cells. No evidence of ICM was ever observed in the outgrowth of these vesicles.
fistulas, 2 control lines were used. We knew that CHO and B-16


cell-cell contact. Of Papaioannou (16), who reported fewer than expected colonies


cells were not regulated in blastocysts with fistulas, so we


The results of the experiments in which EC 247 cells, B-16


malignant cell, and CHO cells were cloned in trophectodermal


vesicles. The data are in accord with those of Papaioannou (16), who reported fewer than expected colonies from embryonal carcinoma cells injected into trophectodermal vesicles.

To exclude the possibility that, as in the blastocyst, the anatomy of the trophectoderm forming the wall of the vesicles might impose a deleterious nutritional environment for the cells, vesicles were made that had patent fistulas across the trophectoderm.

Because of the technical difficulty in making vesicles with fistulas, 2 control lines were used. We knew that CHO and B-16 cells were not regulated in blastocysts with fistulas, so we practiced the technique by putting CHO cells in vesicles with fistulas. The same number of colonies was produced as was obtained with cloning of these cells. This indicated the reproducibility of the technique. B-16 or EC 247 cells were placed in vesicles with fistulas, and the data are in Table 5. Note B-16 cells cloned better in trophectodermal vesicles with fistulas than in ones lacking fistulas. Finally, the embryonal carcinoma cloned on plastic at the 66% level, in vesicles at 9%, and in vesicles with fistulas at 15%. It was concluded that the blastocoele surface of trophectoderm regulates colony formation of EC 247 by direct cell-cell contact.

To confirm this conclusion, synchronized EC 247 cells were used. EC 247 cells synchronized to M plus 3 hr are controlled within 4 hr of exposure to the inducing environment, but those synchronized to M plus 8 hr must complete the cell cycle and reach the restriction point to be controlled. Thus, they would not be controlled in the 4-hr period of time. Synchronized cells were injected into trophectodermal vesicles, rescued after 4 hr, and tested for colony formation. The data are given in Table 6. Note that cells at M plus 8 hr cloned in the vesicles at the same rate as did the controls, but the cells at M plus 3 hr cloned at incidences significantly less than either the controls or cells at M plus 8. It was thus concluded that the blastocoele surface of trophectoderm regulates colony formation of EC 247 cells. In addition, the restriction point at which EC 247 cells were responsive to the control by the blastocyst must be about 4 hr after mitosis. The latter idea was confirmed in an experiment in which cells at M plus 3 and 8 hr were injected into blastocysts and rescued after 4 hr. The data, in Table 7, are compatible with what has been shown previously, that cells at M plus 8 hr have passed the restriction point for control (27) and that the restriction point is about 4 hr after mitosis under the current experimental conditions.

DISCUSSION

The results of the experiments in which EC 247 cells, B-16 melanoma cells, and CHO cells were cloned in trophectodermal vesicles are in Table 4. Colony formation of each was abrogated in vesicles injected 4 hr after mitosis under the current experimental conditions. Synchronized HC and B-16 melamine were placed in trophectodermal vesicles with fistulas at the 66% level, in vesicles at 9%, and in vesicles with fistulas at 24%. It was thus concluded that the blastocoele surface of trophectoderm regulates colony formation of EC 247 by direct cell-cell contact.

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The purpose of these experiments was to determine which of the cell types of the mouse blastocyst was responsible for regulation of colony formation of embryonal carcinoma cells as a step in attaining our long-term goal, the development of a noncytotoxic form of therapy for this carcinoma. In addition, it was hoped that, by taking advantage of the neoplastic attributes of embryonal carcinoma cells, which are the neoplastic counterpart of ICM cells, information on regulation of development in the blastocyst might eventually be obtained.

We observed control of colony formation of embryonal carcinoma by trophectodermal vesicles similar to that observed for control of colony formation by the intact blastocyst. In reaching this position, it was necessary to exclude nonspecific effects imposed upon the contents of the blastocoele or trophectodermal vesicles by the microanatomy of the wall of the blastocyst. Tight junctions which interconnect trophectodermal cells preclude passage of macromolecules between these cells (3).

The effect of the anatomy of the blastocyst on the regulation of carcinoma cells was studied by leaving the broken tips of injecting pipets as conduits across the trophectoderm. The colony-forming ability of EC 247 was regulated by blastocysts with conduits, but that of CHO and B-16 cells was not regulated under these conditions. In addition, colony formation of L1210 leukemia was unaffected by the blastocyst. When the anatomy of the blastocyst is taken into account, regulation of colony formation by the blastocyst closely parallels that of tumor formation by the blastocyst reported previously (23). It is concluded that regulation of colony formation of the blastocyst is just as specific for embryonal carcinoma as was that of tumor formation. At this point, we do not know how the conduits produce their effects. Possibly, the conduit corrects a nutritional effect as postulated, or we may be dealing with the outflow of an inhibitor.

Table 4

<table>
<thead>
<tr>
<th>Tumor</th>
<th>No. of colonies/no. of vesicles injected</th>
<th>Clone control (colonies/ cells cloned)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC 247</td>
<td>4/44 (10)*</td>
<td>42/120 (35)</td>
</tr>
<tr>
<td>B-16 melanoma</td>
<td>6/34 (17)</td>
<td>68/200 (34)</td>
</tr>
<tr>
<td>CHO cells</td>
<td>5/21 (24)</td>
<td>61/120 (51)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage of colony formation.

Table 5

<table>
<thead>
<tr>
<th>Tumor</th>
<th>No. of colonies/no. of cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO cells</td>
<td>10/19 (52)*</td>
</tr>
<tr>
<td>B-16 melanoma</td>
<td>8/16 (50)</td>
</tr>
<tr>
<td>EC 247</td>
<td>3/20 (15)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage of colony formation.

Table 6

<table>
<thead>
<tr>
<th>Tumor</th>
<th>No. of cells rescued/no. of vesicles injected</th>
<th>Clone control (colonies/ cells cloned)</th>
<th>Statistical significance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC 247</td>
<td>44/118 (37)*</td>
<td>154/253 (61)</td>
<td>99.5</td>
</tr>
</tbody>
</table>

* M + 3, mitosis plus 3 hr; M + 8, mitosis plus 8 hr.

Table 7

<table>
<thead>
<tr>
<th>Tumor</th>
<th>No. of cells rescued/no. of blastocysts injected</th>
<th>Clone control (colonies/ cells cloned)</th>
<th>Statistical significance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC 247</td>
<td>M + 3*</td>
<td>50/80 (83)</td>
<td>99.5</td>
</tr>
<tr>
<td>M + 8</td>
<td></td>
<td>50/80 (83)</td>
<td>Not significant</td>
</tr>
</tbody>
</table>

* M + 3, mitosis plus 3 hr; M + 8, mitosis plus 8 hr.

* Numbers in parentheses, percentage of total.
Irrespective of how the conduits function, the observation that the blastocele surface of trophectodermal vesicles regulates colony formation was confirmed using another technical approach. In the confirmatory experiments, cells were synchronized to minimize time of exposure to the regulatory environment. Regulation of colony formation by trophectodermal vesicles showed a cell cycle restriction point similar to the intact blastocyst. This is in accord with the data of Scott et al. (25) who have showed a cell cycle restriction point similar to the intact blastocyst surface of trophectodermal vesicles, and therefore in blastocysts, was the result of direct contact of the cancer cells with trophectodermal cells. This conclusion was strengthened by the inability of ICMs to regulate colony formation, as well as the inability of blastocele fluid to regulate it.

It is now accepted that embryonal carcinoma cells are the neoplastic equivalent of ICM cells of the mouse embryo (4, 13). The tumor (22) and colony assays (27) could therefore logically be construed as assays utilizing the neoplastic attributes of embryonal carcinoma cells to determine the mode of regulation of the ICM. Before this attractive notion can be accepted, the fate of the embryonal carcinoma cells that do not form tumors or colonies in the blastocyst must be determined. There is in the chimera experiments irrefutable evidence that some embryonal carcinoma cells are regulated by the blastocyst to the point that they take part in normal development. Thus, we favor the notion that the assays, at least in part, measure developmental effects of the blastocyst on embryonal carcinoma. It is to be reiterated, however, that this has not been proved at this time.

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REFERENCES

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Fig. 1. Steps for making fistulas through the wall of the blastocyst (see "Materials and Methods"). a, placement of the pipet (p) and cell in the blastocyst; b, amputation to create the conduit of the fistula.

Fig. 2. a, blastocyst with a functional fistula. Note the thick layer of trophectoderm lining the zona pellucida and the relatively small size of this blastocyst in relationship to those in Fig. 2, b and c. ×150. b, blastocyst impaled on a solid glass needle. The ICM is on the right. Note how thin the trophectoderm is in relationship to that of Fig. 2a. ×150. c, blastocyst cultured in vitro for the same period of time as those in Fig. 2, e and b. Note the thin trophectoderm hatching through the zona pellucida. ×150. d, blastocyst with a fistula allowed to hatch, attach to the substrate, and grow. It did this in apparently normal fashion. Note the inner cell mass (icm), polyploid trophoblast (t), and the conduit (c). ×150.

Fig. 3. a, method of making trophectodermal vesicles (see "Materials and Methods"). The ICM is dissected from a blastocyst injected previously (p) with a cancer cell. b, 2 trophectodermal vesicles each containing a EC 247 cell (arrows). Note the vesicles are attached to the scar made by the scalpel. ×250.
Fig. 4. Method of making trophectodermal vesicles with conduits. In a, the cancer cell is placed on the trophectoderm away from the ICM, and the injecting pipet is severed as in Fig. 1b. The blastocyst is then positioned as in Fig. 4b, and the ICM is removed. c, the injecting pipet, now forming a conduit across the cuboidal trophectoderm and providing a free communication between the incompletely expanded vesicle (note cancer cell at arrow) and the tissue culture milieu. × 300.

Fig. 5. Technique for placing a cancer cell in the blastocele but denying it contact with ICM, trophectoderm, or the extra blastocyst tissue culture medium. The cancer cell in MEM+10 is left in the tip of the injecting pipet (a), and the pipet is cut off through the oil (b).

Fig. 6. Method of microculturing embryonal carcinoma cells in blastocele fluid. In a, an oil-filled pipet is inserted into a blastocyst; in b, the fluid is aspirated from the blastocyst; in c, a single cell is aspirated into the pipet; d, the pipet is plugged by aspirating oil and cut off through the oil phase.

Fig. 7. a, appearance of a microculture similar to the one described in Fig. 6. In this one, an additional volume of fluid has been aspirated. Note the oil plugs at either end and the cell at the arrow. × 150. b, pipet crushed 24 hr previously to rescue the cells. Note there are 4 embryonal carcinoma cells. × 250. c, the same preparation after 4 additional days. Note the colony of cancer cells. × 250.
Fig. 8. Electron micrograph of a blastocyst that had a fistula to illustrate the character of the cytoplasm and membranes of the mural trophoderm. The zona pellucida is above. Note the desmosomes and tight junctions typical of trophodermal cells. x 21,000.

Fig. 9. Typical gap junction found between trophodermal cells. x 114,700.
Fig. 10. In a, this ICM had been in vitro for 24 hr. Note the cap of endoderm (arrow) and 4 proliferating embryonal carcinoma cells. × 125. In b, same culture, photographed after 6 days, illustrating the growth of the cancer cells. × 125.

Fig. 11. Blastocyst that hatched. The ICM is labeled. A single EC 247 cell was placed on the monolayer of trophoblast 36 hr previously. The cells have proliferated (arrow) and have formed a clump of at least 8 cells. × 170.
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