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Differentiation of Clonal Lines of Teratocarcinoma Cells: Formation of Embryoid Bodies *In Vitro*

(mouse tumors/tissue culture/pluripotent cells/cell determination/endoderm)

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ABSTRACT **The differentiation *in vitro* of clonal pluripotent teratocarcinoma cells is reported. The first stage of this process is the formation of simple embryoid bodies which are identical to those found in animals bearing intraperitoneal teratocarcinomas. They consist of an inner core of embryonal carcinoma cells surrounded by a layer of endodermal cells which produce Reichert's membrane. The endodermal cells become apparent shortly after the embryonal carcinoma cells have formed aggregates which are loosely attached to the substratum. One clonal teratocarcinoma line was found to produce complex cystic embryoid bodies *in vitro*. Following formation of the endodermal cells, extensive differentiation to a wide variety of cell types occurs. There are similarities between the process of embryoid body formation and the early events of differentiation of the mouse embryo.**

Mouse teratocarcinomas are a useful alternative to embryos for the study of mammalian cell determination (the process by which multipotential cells become committed to a particular developmental pathway), as well as for the study of subsequent terminal differentiation. The stem cells of these tumors, known as embryonal carcinoma cells, are pluripotent: like the cells of the early embryo, they can differentiate to form derivatives of all three primary germ layers. Kleinsmith and Pierce (1) first demonstrated this by showing that a single embryonal carcinoma cell injected intraperitoneally could give rise to a teratocarcinoma containing a wide variety of differentiated tissues. Embryonal carcinoma cells also have ultrastructural (2, 3), biochemical (4, 5), and antigenic (6) properties in common with early embryos. Unlike the cells of the early embryo, however, embryonal carcinoma cells are relatively easy to obtain in large numbers and to culture *in vitro*.

Several clonal lines of embryonal carcinoma cells have been isolated *in vitro* (7–11). When these cells are injected into mice they give rise to teratocarcinomas containing a wide variety of both mature and immature tissues. Since subclones of these lines also give rise to teratocarcinomas on reinjection, it is evident that pluripotent cells can be maintained *in vitro*.

We describe here the differentiation of clonal teratocarcinoma cells under defined conditions *in vitro*. The importance of differentiation of pluripotent cells *in vitro* is the opportunity it affords to study cell determination under defined conditions. We have, therefore, focussed our attention on the earliest stages of differentiation *in vitro*. The results described below indicate that it is an orderly process which mirrors the development of the early embryo.

MATERIALS AND METHODS

Cell Cultures. The pluripotent SIKR teratocarcinoma line from which subclones were isolated has previously been described (9, 12). Clonal lines were also isolated from cultures derived from tumor LS402C-1684 kindly provided by Dr. L. Stevens. This tumor is composed only of embryonal carcinoma that has lost the ability to differentiate (13). All cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and were found to be free of mycoplasma infection as determined by the [^{3}H]thymidine-autoradiography method (14).

Subclones were prepared using a modification of the technique described by Macpherson (15): Mass cultures or single colonies of embryonal carcinoma cells were trypsinized and single cells were isolated in a finely drawn pasteur pipette and seeded in a microdrop of medium in a 17 mm tissue culture dish (Linbro multiwell tray). The drop was carefully scanned under an inverted phase contrast microscope with a 10× objective to confirm that only one cell was present. To each well, medium containing 4×10^5 feeder cells was added and the cultures were incubated at 37°. Within 10 days, colonies of embryonal carcinoma cells became apparent in up to 40% of the wells seeded with single cells. The colonies were then passaged to a 50 mm tissue culture dish containing a feeder layer.

Stock cultures of such embryonal carcinoma cells were passaged every 3–4 days by plating 5×10^6 cells with 3×10^6 feeder cells per 90 mm dish. Approximately 5×10^7 cells were harvested from each dish at passage. Embryonal carcinoma cells which were subsequently passaged without feeder cells were seeded at 10^7 cells per 90 mm dish and were morphologically homogeneous with few feeder cells detectable.

Cells of the STO line, which are thioguanine-resistant and ouabain-resistant were used as feeders. This cell line was derived from a continuous line of SIM mouse fibroblasts (16) and was kindly supplied by Dr. A. Bernstein. The cell line does not appear to release any C-type viruses as determined by the XC plaque assay and measurement of reverse transcriptase in the culture medium (A. Bernstein personal communication).

Feeder layers of STO cells, incapable of division, were prepared by incubation with a freshly prepared solution of mitomycin C (Sigma) at 10 μg/ml for a minimum of 2 hr, and subsequent replating at approximately 10^6 cells per 50 mm dish or at 3×10^6 cells per 90 mm dish. Cells that had escaped the killing treatment could easily be detected by their sensitivity to medium containing hypoxanthine, aminopterin, and thymidine (17), their inability to incorporate [^{3}H]hypoxanthine, and their resistance to ouabain. Under our conditions, however, there was never any growth of the mitomycin-C-treated cells.

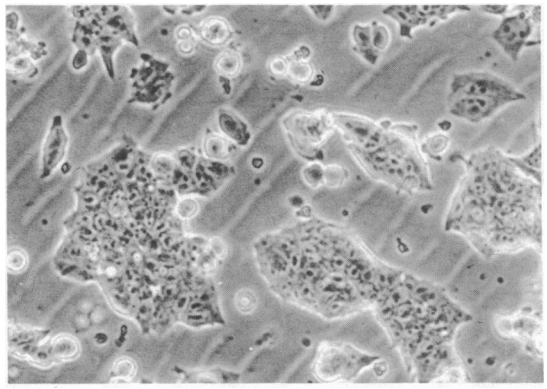

FIG. 1. Homogeneous population of pluripotent embryonal carcinoma cells derived from an isolated single cell, twenty-four hours after plating a single cell suspension without feeder cells. Phase contrast ×230.

Light and Electron Microscopy. Cell aggregates formed during growth *in vitro* tended to detach from the substratum; those which were not already floating could be detached by gently pipetting the medium over them. The aggregates were allowed to settle in a conical centrifuge tube, resuspended in phosphate-buffered saline, and pelleted by centrifugation. For light microscopy the pellet was fixed in 10% formalin/saline, embedded in wax, and sectioned using standard procedures.

For electron microscopy, the pellet was fixed with 3% glutaraldehyde in phosphate-buffered saline for a minimum of 1 hr, and post-fixed with 1% OsO_4 for 45 min. After dehydration, the pellet was stained with uranyl acetate, embedded in Spurr resin, and sectioned. The sections were post-stained with lead citrate.

Alkaline Phosphatase Histochemistry. Aggregates formed *in vitro* were washed in Tris-buffered saline and fixed in acetone at 4° for 16 hr. They were then dehydrated in ice-cold absolute acetone, cleared in ice-cold xylene, and embedded in wax at 45°. Sections were cut and mounted at 4° and stained with naphthol ASMX phosphate and fast blue BB salt (Sigma) at room temperature (18).

RESULTS

Embryonal Carcinoma Cultures from Isolated Single Cells. Clonal cultures of embryonal carcinoma cells [previously referred to as C cells (12)] were obtained from isolated single cells. The four clones studied in detail all have similar properties. Growth of these cells is dependent on the presence of feeder cells even when they are passaged at high density. The cultures are homogeneous and the cells have the distinctive morphology of embryonal carcinoma cells; they are approximately 15 μm in diameter with a large nucleus, containing a single large basophilic inclusion, and with relatively little cytoplasm (Fig. 1). Subcutaneous injections of the cells into syngeneic 129 mice gives rise to teratocarcinomas containing embryonal carcinoma cells as well as a variety of differentiated tissues including nerve, cartilage, keratinizing epithelium, and epithelial cysts.

Stock cultures of embryonal carcinoma cells grow well in the first passage without added feeders. As described below, under these conditions the cells form aggregates and differentiated cells became apparent. However, after two to three passages without added feeders they begin to die. The cells survive for longer periods when passaged on gelatin-coated dishes. Both the feeder layers and the gelatin coating of the substratum appear to increase the spreading and attachment of the cells to the substratum; however, the feeder cells must have some additional effect, since the cells do not survive indefinitely when passaged on gelatin-coated dishes.

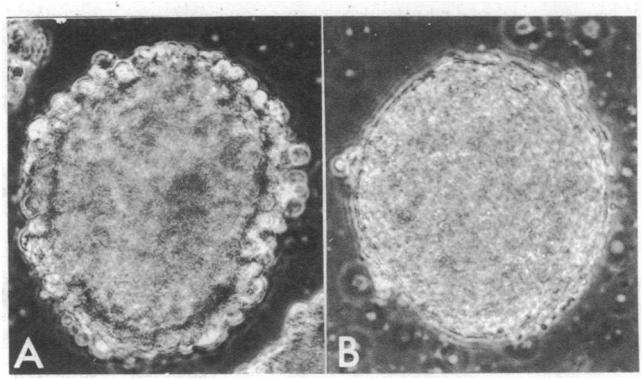

FIG. 2. Aggregates of embryonal carcinoma cells 5 days after plating a single cell suspension. Phase contrast ×200 (approx.). (A) Pluripotent cells. The endodermal cell layer is apparent. (B) Nullipotent cells. No endodermal layer has formed.

In experiments using the colony isolation method of cloning (12) similar results were obtained; again, homogeneous populations of embryonal carcinoma cells could be obtained on feeder layers. Previously (9, 12), only heterogeneous cultures (mixtures containing both embryonal carcinoma cells and a fibroblast-like cell type called E cells) were obtained by colony isolation from the SIKR line of teratocarcinoma cells. In those experiments, the cells were passaged in the absence of feeder layers. The results described here suggest that the fibroblast-like E cells acted as feeder cells during the growth of embryonal carcinoma cells and provided strong selection for those colonies that originated from two cells, or in which differentiation to E cells established heterogeneity at an early stage.

Early Stages of Differentiation: The Formation of Embryoid Bodies In Vitro. When embryonal carcinoma cells are cultured in the absence of added feeders, they multiply rapidly for several days and form tight rounded aggregates which are initially well attached to the surface of the dish. If the medium is changed daily and the cultures are maintained for several weeks, differentiation to a wide variety of cell types is observed, as described below.

The first morphologically detectable change in the cells occurs as the clumps become tighter, rounder and less well attached to the substratum. At this time a distinctive outer layer of cells surrounding the clumps becomes apparent (Fig. 2A). These structures have a striking resemblance to the embryoid bodies found in ascites fluid of animals bearing intraperitoneal teratocarcinomas (19). Such simple embryoid bodies formed *in vivo*, which resemble the 5- to 6-day-old mouse embryo, have an inner core of embryonal carcinoma cells surrounded by a single layer of endodermal cells (20, 21) which produces the mucopolysaccharide layer known as Reichert's membrane (22). The results described below confirm that the clumps formed *in vitro* are embryoid bodies.

The clumps formed *in vitro* after plating a single cell suspension have an outer layer of cells which is separated from

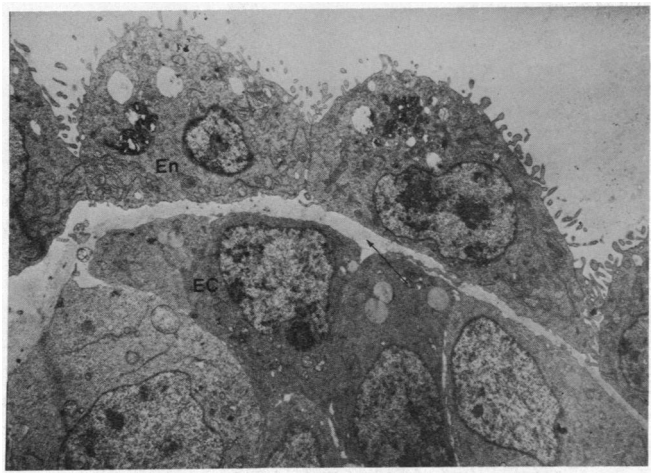

FIG. 3. Electron micrograph showing part of an aggregate formed by pluripotent cells. EC: Embryonal carcinoma cells in the core of the aggregate. En: Endodermal cells of the outer layer, with plentiful swollen endoplasmic reticulum and prominent microvilli on their outermost surface. Note contrast with cytoplasm of embryonal carcinoma cells, which has few organelles. Arrow points to Reichert's membrane found between the inner and outer cells. ×3,220

the core of embryonal carcinoma cells by an eosinophilic extracellular material. Electron microscopy further confirmed the similarity seen between these aggregates and embryoid bodies formed *in vivo* (Fig. 3). The inner cells have the distinctive structure of embryonal carcinoma cells; that is, a large nucleus, relatively little cytoplasm which is almost devoid of organelles except for mitochondria, and a large number of free ribosomes. In contrast, the cells of the outer layer have the characteristics of the endodermal cells of embryoid bodies (19). They contain plentiful swollen endoplasmic reticulum and have prominent microvilli on their outermost surface. The hypertrophied basement membrane material between the outer and inner cells has the characteristic fibrillar structure seen in Reichert's membrane (22).

Biochemical differences between the cells of the inner mass and those of the outer layer were demonstrated by histochemical staining. High levels of alkaline phosphatase are found in embryonal carcinoma cells (4). Bernstine *et al.* (23) have shown that this enzyme is undetectable in the endodermal layer of embryoid bodies formed *in vivo*. When embryoid bodies formed *in vitro* were stained for alkaline phosphatase it was found that the enzyme is present at high levels in the cells of the inner core and at very low levels in the outer layer (Fig. 4). In addition, periodic acid Schiff (PAS) (24) staining showed that the eosinophilic membrane found between the outer layer and the central core of cells contains mucopolysaccharide, as does Reichert's membrane (22). The cells of the outer layer themselves stain with PAS, as would be expected if they were synthesizing this basement membrane in large quantities. Thus, by all criteria available, these embryoid bodies formed *in vitro* are identical to simple embryoid bodies formed *in vivo*.

The clonal lines described above were isolated from cultures of the SIKR teratocarcinoma line which had been passaged *in vitro* for many months. A homogeneous embryonal carcinoma line was also obtained by subcloning from a considerably earlier stock of SIKR. The embryoid bodies formed *in*

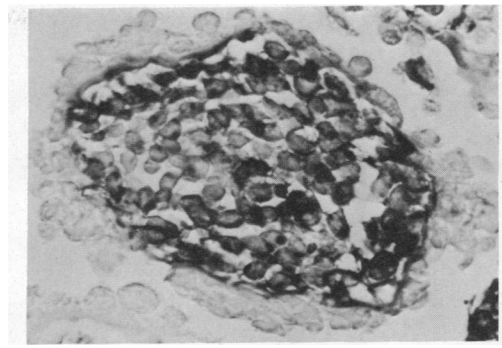

FIG. 4. Localization of alkaline phosphatase activity in aggregates formed by pluripotent cells. Wax-embedded aggregates were sectioned and stained for the enzyme. Embryonal carcinoma cells in the central core of the aggregate show high enzyme activity. Endodermal cells of the outer layer show no activity. ×187.5

vitro by these cells, however, are not all of the simple type described above. Many of them were of a larger, more complex variety with a fluid-filled cavity, and contain a variety of differentiated cell types (Fig. 5). They are similar to the cystic embryoid bodies produced *in vivo* by some teratocarcinoma lines adapted to intraperitoneal growth (20, 21). It is not known why this subline of SIKR cells produces cystic embryoid bodies *in vitro*, while others produce only the simple form. This difference between subclones may be related to the number of generations that the cells have been grown *in vitro*.

Clonal cultures of embryonal carcinoma cells derived from a tumor in which differentiation does not occur also form aggregates when plated on tissue culture dishes. In contrast to those which are formed by pluripotent embryonal carcinoma cells, however, these aggregates of "nullipotent" (23) cells have a smooth outer surface (Fig. 2B) and no endodermal cell layer is detectable in cross-sections. No differentiation of these cells is observed *in vitro*.

Subsequent Differentiation from Embryoid Bodies. Following embryoid body formation *in vitro*, extensive differentiation is observed when the bodies attach to a substratum. Embryoid bodies floating in the medium can be reattached by plating in fresh medium, and 6–10 days later endodermal cells migrate out and form a halo around the colonies (Fig. 6A). Migration and cell multiplication continue over the next few weeks and

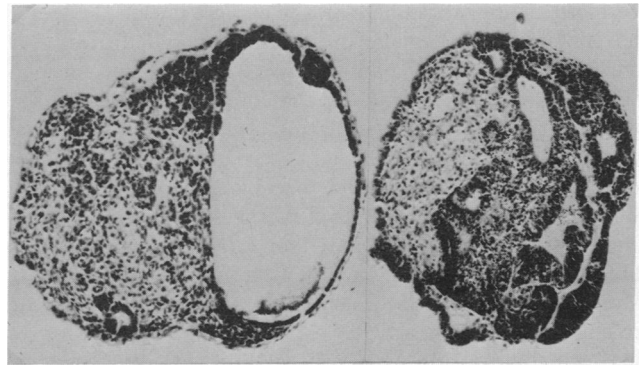

FIG. 5. Cystic embryoid bodies formed in vitro. Aggregates found floating in the medium approximately 2 weeks after plating a single cell suspension. ×80 (approx.).

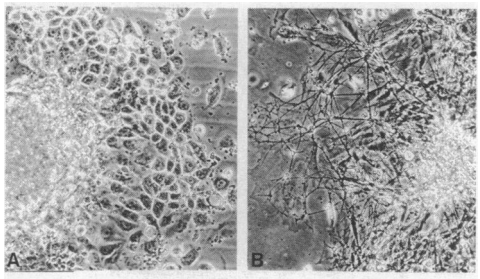

FIG. 6. Subsequent differentiation from embryoid bodies formed *in vitro*. (A) Pluripotent cells were plated as a single-cell suspension. Four days later embryoid bodies were found floating in the medium. These were reattached to the substratum by plating in fresh medium. Seven days later cells had migrated out to form a halo around the embryoid body. Phase contrast ×91.2. (B) Neural differentiation at the periphery of a halo of differentiated cells surrounding an embryoid body, twenty days after plating a single cell suspension. Phase contrast ×52.8.

the cultures rapidly become confluent, dense, and multilayered. It should be noted that there is sometimes a considerable amount of cell necrosis and lysis during the initial period of embryoid body formation and subsequently in the dense centers of the differentiating colonies.

A variety of cell types are apparent after several weeks, among which may be identified: keratinizing epithelium, endodermal cysts, fibroblasts, cartilage, adipose tissue, beating muscles, pigmented cells, and neural cells (Fig. 6B). A detailed description of the differentiation from embryoid bodies formed *in vitro* will be given later.

DISCUSSION

We have shown here that isolated single cells from a pluripotent teratocarcinoma line can give rise to morphologically homogenous populations of embryonal carcinoma cells. Under suitable conditions *in vitro* these cells differentiate to form a wide variety of cell types. These observations on the behavior of embryonal carcinoma cells *in vitro* parallel those of Kleinsmith and Pierce (1), that a single embryonal carcinoma cell can give rise to a teratocarcinoma *in vivo*. The advantage of studying differentiation of these cells *in vitro* is that it becomes possible to study the events of cell determination and differentiation on large populations of cells under defined conditions.

The differentiation of various cell types in these cultures is a complex and to some extent disorganized process. We have, therefore, concentrated here on the earliest visible changes in the population undergoing differentiation. In cultures of all five of the pluripotent clonal lines examined, the first morphologically detectable change is the formation of simple embryoid bodies. Shortly after plating of a single cell suspension of embryonal carcinoma cells on tissue culture dishes, the cells form small aggregates, which become progressively larger, rounder, and less well attached to the substratum. Some of these aggregates detach and are found floating in the medium. This phenomenon closely parallels the events described by Martin and Rubin (25) when chick embryo fibroblasts are grown on bacteriological dishes. Their observations suggest that cell clumps form when cell–cell adhesion is greater than that of the cells to the substratum. Consistent with this interpretation is the fact that in the presence of feeder cells, or when the substratum is coated with gelatin, the embryonal carcinoma cells show greater attachment and spreading on the substratum and fail to form three-dimensional aggregates. Under these conditions the population remains homogeneous.

The endodermal layer of the embryoid bodies appears as the cell aggregates become less well attached to the substratum. Although it is possible that the appearance of the endoderm is due to reassortment of an already heterogeneous population, it seems more likely that the embryonal carcinoma cells on the outside of the clumps differentiate to endoderm and the inner cells remain unchanged. This mechanism is similar to that proposed for the early events of embryogenesis in the mouse (26). The first determination that occurs in the mouse embryo is the formation of the trophectoderm. There is now good evidence (27) that the cells that become committed to form trophectoderm do so as a result of their position on the outer surface of the morula. In the development of the mouse embryo, the second cell type to appear after the establishment of the blastocyst is the endoderm, which forms only on the free surface of the inner cell mass (adjacent to the blastocoel). The stimulus for the formation of endoderm may also be a positional one, as it has been found that inner cell masses isolated from the embryo form endoderm over the whole of their outer surfaces (28, 29).

Thus, the first event of differentiation of pluripotent teratocarcinoma cells *in vitro* is similar to that of the inner cell mass of the mouse embryo. One difference, however, is that the proximal endoderm of the embryo, which covers the surface of the inner cell mass, does not produce Reichert's membrane, which is produced by the endodermal cells of embryoid bodies. In the embryo, Reichert's membrane is produced by endodermal cells that have migrated away from the inner cell mass and have come to lie on the inner surface of the mural trophoblast (22). However, the proximal endoderm of embryos developed *in vitro* does produce Reichert's membrane-like material (36).

In studies on the formation of the primary germ layers in Amphibia, it has been demonstrated that the mesoderm is formed by the influence of the endoderm on the ectoderm (30). The endoderm is also implicated in mesoderm formation in the chick embryo (31). It is possible that in our cultures too the endoderm may play an inductive role in the formation of mesoderm from the pluripotent embryonal carcinoma cells. In this regard, it is noteworthy that those clonal embryonal carcinoma cells that are incapable of differentiation *in vivo* or *in vitro* do not form an endodermal layer *in vitro*.

The observations described here are consistent with what is already known about the differentiation of teratocarcinoma cell lines *in vitro*. Rosenthal *et al.* (7) reported that limited differentiation occurred in aggregates from clonal cultures grown in bacteriological dishes. In those experiments embryoid body formation apparently occurred *in vitro*, but it was not recognized as the primary event since subsequent differentiation was poor. Jami and Ritz (11) also briefly noted the formation of embryoid bodies *in vitro* by a clonal line of embryonal carcinoma cells, but no further differentiation was observed. In both these cases, this was probably because the embryoid bodies were not allowed to attach; extensive differentiation of cells in embryoid bodies occurs only after attachment to a substratum (32, 33). Recently Lehman *et al.* (34) have reported differentiation in long-term cultures of a non-clonal teratocarcinoma line and Jakob *et al.* (10) have noted that differentiation of a clonal embryonal carcinoma

line occurs *in vitro*. It would be interesting to know if the first stage in the differentiation of these cells is also the formation of endodermal cells.

The observations described here indicate that the early differentiation of teratocarcinoma cells *in vitro* is not a disorganized process, but that it parallels the early development of the mouse embryo. Since the formation of endodermal cells occurs under controlled conditions in large populations of cells, it should be possible to use biochemical and immunological techniques to study this particular cell determination and the possible role of the endoderm as an inducer of subsequent differentiation. It will also be of interest to establish whether the endodermal cells which arise *in vitro* are nonmalignant, as are the differentiated derivatives of embryonal carcinoma cells *in vivo* (35). If so, it should be possible to study the processes that lead to the loss of malignancy during embryonal carcinoma differentiation.

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