Multipotentiality of Single Embryonal Carcinoma Cells*

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SUMMARY

In order to test the hypothesis that embryonal carcinoma cells are multipotential stem cells of a teratocarcinoma, an in vivo cloning technic was designed. Small embryoid bodies containing mostly embryonal carcinoma were obtained from ascitic conversion of a murine teratocarcinoma and were dissociated with trypsin to form a suspension of single cells; the single cells were picked up in small capillary tubes and transplanted directly into mice.

From over 1700 single cell grafts, 44 clonal lines were obtained; 43 of these lines were teratocarcinomas composed of as many as fourteen well differentiated somatic tissues in addition to embryonal carcinoma. These 43 lines varied in their degree of differentiation, capacity to produce embryoid bodies, and in growth rate. The remaining clonal line showed limited potential for differentiation, producing only yolk sac, trophoblast, and embryonal carcinoma.

The results demonstrated the multipotentiality of single embryonal carcinoma cells, as well as the heterogeneity of the embryonal carcinoma of a teratocarcinoma. The capacity of single embryonal carcinoma cells to differentiate into benign tissues supports neither the dogma of the irreversibility of the malignant transformation nor the somatic cell mutation theory of cancer. These findings were interpreted as giving strong support to the stem cell theory of cancer.

A teratocarcinoma is a bizarre neoplasm composed of foci of undifferentiated malignant cells interspersed with a chaotic array of somatic tissues; these somatic tissues, representing each of the three embryonic germinal layers, are found in various stages of differentiation. In some of the earlier descriptions of teratocarcinomas their obvious neoplastic characteristics were overlooked, and the tumors were interpreted as maldeveloped parasitic twins. Present evidence, however, supports the theory that the somatic tissues of teratocarcinomas arise by morphogenesis from undifferentiated malignant stem cells.

This theory was first proposed by Askanazy (1) in 1907 as the result of detailed histologic studies on benign cystic ovarian teratomas. He concluded that the well differentiated somatic tissues of the teratoma developed by embryonic differentiation from either a single multipotential type of cell or from a group of cells composed of representatives of each of the embryonic germinal layers. Support for Askanazy's concept was provided by Jackson and Brues (11), who observed in a murine ovarian teratocarcinoma that more mitoses occurred in the undifferentiated cells than in the adult-appearing somatic tissues.

They postulated that some of the rapidly growing cells matured under a delicate control mechanism that kept cell division and maturation relatively constant, thus allowing both differentiated and undifferentiated cells to persist. More recently, morphologic studies on teratocarcinomas in mice by Fawcett (3) and Fekete and Ferrigno (4) both led to the same conclusion that teratocarcinomas arise by morphogenesis from undifferentiated stem cells. Extensive studies by Stevens et al. (26, 28) on the early development of spontaneous teratocarcinomas in newborn mice lent further support to this concept.

The first experimental proof for this theory was obtained by Pierce and co-workers (18, 19, 21), who approached the problem by selecting reproducible aggregates of embryonic cells from a murine teratocarcinoma and testing their capacity to differentiate. These aggregates were produced by ascitic conversion in the following manner: When a suspension of teratocarcinoma was injected intraperitoneally into mice, free-floating cystic aggregates of embryonic cells developed in the hemorrhagic ascitic fluid of the host. Although the teratocarcinoma was composed of as many as fifteen differentiated cell types, the aggregates consisted of only two or three cell types. These cystic aggregates were composed of a layer of visceral yolk sac overlying a loose mesenchyme; foci of embryonal carcinoma were sometimes, but not always, found embedded in the mesenchyme. Since these aggregates showed an organization similar to that of early mouse embryos, they were referred to as embryoid bodies.
When embryoid bodies were grafted subcutaneously in mice, fully differentiated teratocarcinomas or benign cystic teratomas resulted. Since the percentage of grafts resulting in teratocarcinomas correlated with the percentage of embryoid bodies containing embryonal carcinoma, it was concluded that embryonal carcinoma was the multipotential tissue of this tumor. To obtain further support for this contention, embryoid bodies which had been grafted subcutaneously were serially sectioned at stepwise intervals of time following implantation. From microscopic examination of these grafts it was apparent that the teratocarcinomas were originating in the embryonal carcinoma of the embryoid bodies. The multipotentiality of embryoid bodies was confirmed by Stevens (27), who transplanted these structures into the anterior chamber of eyes of mice and observed their development into differentiated teratocarcinomas.

Although the multipotentiality of embryonal carcinoma has been demonstrated, the question arises whether this multipotentiality resides within single embryonal carcinoma cells or whether it is only a property of a population of these cells. Although embryonal carcinoma cells appear morphologically alike, it is conceivable that functionally they are a heterogeneous population with various potentialities and that individually few or none of the embryonal carcinoma cells are totipotent. Some evidence for the heterogeneity of the population of embryonal carcinoma cells was obtained by Pierce et al. (20), who showed that sublines of teratocarcinomas with limited potential for differentiation could be developed by ascitic conversion. The definitive experiments, however, required the cloning of cell populations from single embryonal carcinoma cells.

Although Braun (2) cloned single cells of teratomas of plants and demonstrated their multipotentiality, there are no published reports of successful cloning of teratocarcinomas of animals. Cloning experiments, to be successful, require cell lines capable of growing well in tissue culture (12, 22-24); embryonal carcinoma does not grow well as a monolayer. The alternative to cloning in vitro is the direct transplantation of single cells into animals. This has been accomplished by several investigators, starting with preparations of free leukemic or ascites cells and isolating single cells in micropipettes (5, 7-10, 14). However, the murine teratocarcinoma, as previously mentioned, does not grow intraperitoneally as a suspension of single cells but rather grows as cellular aggregates (embryoid bodies). This paper demonstrates the multipotentiality of single embryonal carcinoma cells and presents the technic which was successful in cloning embryonal carcinoma by enzymatic dissociation of cells from fresh tissue, followed by in vivo transplantation of single cells.

**MATERIALS AND METHODS**

**Tumor.**—The tumor employed was strain 402 AIII, a subline of a transplanted teratocarcinoma isolated from the testis of strain 129 mice, originally described by and acquired from L. C. Stevens (25) of the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. The stock tumor was maintained intraperitoneally in strain 129 mice and transplanted at about 4-week intervals. Solid peritoneal implants of the stock tumor contained embryonal carcinoma plus as many as fourteen differentiated somatic tissues. The ascitic fluid contained small "embryoid bodies" less well developed than those described previously; they contained only two cell types: a central core of embryonal carcinoma, invested with a layer of visceral yolk sac (Fig. 1). These embryoid bodies were used as the source of single cells in these experiments because they contained the greatest concentration of embryonal carcinoma cells of any tissue available.

**Attempts at in vitro cloning.**—In preliminary experiments embryoid bodies were dissociated with trypsin and cultured as a monolayer (15). Various culture media were tried: Eagle's concentrates or NCTC-109 (Microbiological Associates) were combined with 2, 10, 25, or 50 per cent serum; horse, calf, human, and mouse serum were all tried. Because of the poor growth of these cells irrespective of the media employed, it was not surprising that attempts to clone with Sanford's capillary tube technic (23, 24), Puck's plating technic (22), or Lwoff's microdrop technic (12) all failed. It was decided, therefore, to devise a direct in vivo technic for cloning embryonal carcinoma.

**In vivo cloning technic.**—Owing to fragility of embryonal carcinoma cells the cloning technic was designed to minimize handling and damage to the cells and to return them immediately to an animal. The donor animals were killed when their abdomens were distended, and the hemorrhagic fluid containing the embryoid bodies was aspirated aseptically into a 5-ml. syringe containing 0.5 ml. of 3 per cent sodium citrate solution to prevent clotting. The fluid was centrifuged for 1 minute at a very low speed, and the supernatant bloody fluid was discarded. The precipitated embryoid bodies were mixed twice with 10 ml. of Hanks balanced salt solution (BSS); each time the embryoid bodies were allowed to settle out before decanting the wash fluid.

The embryoid bodies were dissociated into single cells by being suspended in 15 ml. of 0.25 per cent trypsin in magnesium- and calcium-free BSS containing 0.1 per cent sodium citrate. The suspension was allowed to stand at room temperature for 10 minutes, with periodic gentle shaking. The solution was triturated gently with a syringe and diluted twentyfold in conditioned culture medium. The medium was composed of 2 parts fetal calf serum and 8 parts BSS in Eagle's concentrates to which had been added phenol red, 5 µg/ml; penicillin, 100 units/ml; and streptomycin, 25 µg/ml. The medium was conditioned by allowing the teratocarcinoma to grow in it for 24 hours, after which it was slowly frozen and thawed several times and filtered through a Millipore filter to remove any bacteria and cell debris.

The technic of Sanford et al. (24) was modified for these experiments. Sterile ¼-inch segments of capillary tubing (Aloe Scientific #48302, 1 mm.) were allowed to fill by capillary action from the diluted cell suspension and were examined microscopically for the presence of single cells (Fig. 2). The concentration of the cell suspension was manipulated so that most of the tubes contained no cells or a single cell.
In the first experiment four capillary tubes, each containing a single cell, were grafted subcutaneously into anesthetized strain 129 mice. Poor results were obtained from this technic. Since Sanford et al. (24) have pointed out that even the best culture medium is a grossly imperfect environment for a single cell, it was decided to return the single cells to their natural physiologic environment as soon as possible.

Capillary tubes containing single cells were attached to an orally controlled 12-inch length of Intramedic polyethylene tubing (PE-190/S12, I.D. = .047 inch). Intraportal or subcutaneous incisions were made in anesthetized mice and the contents of the capillary tubes deposited. The capillary tubes were discarded. Before being used again the end of the polyethylene tubing was flamed to insure that no viable cells accidentally contaminated it.

The contents of 100 capillary tubes examined microscopically and known to contain a single cell were deposited directly on glass slides to check the efficiency of the method. When the drops were examined microscopically, 83 contained a single cell, whereas seventeen of them contained no cell. It was concluded that these seventeen represented cells which stuck to the inside of the capillary tubing, and thus not every animal received a single cell. More important, these results also served as a check on the accuracy of the microscopic examination of the capillary tubes and suggested that no animal received more than a single cell.

Animals which produced tumors were killed, and the clonal line was maintained for at least five generations by the intraperitoneal transplantation of 0.1 ml. of the ascitic fluid. Two pieces of the solid peritoneal implants from each animal were fixed in Bouin's fluid, embedded in paraffin, sectioned, and stained routinely with hematoxylin and eosin. Animals which produced no tumor within 6 months were killed, and the graft sites were examined.

RESULTS

Table 1 summarizes the number of clones obtained in these experiments. As can be seen, the cloning efficiency was much higher when cells were grafted intraperitoneally (11 per cent) than subcutaneously (0.2 per cent). Of the 44 clones obtained, 42 were obtained intraperitoneally. Since the embryoid bodies used as the source of single cells were maintained intraperitoneally, it was not too surprising to find that these cells grow better intraperitoneally than subcutaneously.

### TABLE 1
RESULTS OF CLONING TECHNIC

<table>
<thead>
<tr>
<th>Method</th>
<th>Graft site</th>
<th>Total cells implanted</th>
<th>No. of clones</th>
<th>Success (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube implanted with single cell</td>
<td>Subcutaneous</td>
<td>840</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>Single cell deposited without tube</td>
<td>Subcutaneous</td>
<td>578</td>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td>Single cell deposited without tube</td>
<td>Intraperitoneal</td>
<td>372</td>
<td>42</td>
<td>11.0</td>
</tr>
</tbody>
</table>

The clonal lines were analyzed for the variety and number of tissue types produced, growth rate, and type of embryoid bodies produced. Of the 44 clones, 43 were true teratocarcinomas composed of differentiated tissues from each of the three embryonic germinal layers (Figs. 3–6).

One clone, NRS-Cl.44, was unusual in that it contained only embryonal carcinoma, parietal and visceral yolk sac, and some giant cells closely resembling trophoblastic giant cells (Fig. 7). This clone produced no embryoid bodies, but the hemorrhagic ascitic fluid contained small cellular aggregates, some consisting of about a dozen parietal or visceral yolk sac cells around a clump of eosinophilic hyaline material, whereas others consisted of clumps of trophoblastic giant cells.

Although the remaining 43 clones were all teratocarcinomas, there were consistent differences in the degree of differentiation; some of the tumors were well differentiated, whereas others were poorly differentiated. The degree of differentiation of most fell somewhere between these extremes, as is shown graphically by the normal distribution found when the average degree of differentiation for all clonal lines is plotted in a bar graft (Chart 1). Several examples are given in Table 2 of clonal lines which differed greatly in their ability to produce somatic tissues.

Not only was there variation in the degree of differentiation of the clonal lines, but there was also a difference within the clonal lines in the capacity to produce a particular type of tissue. For example, stratified squamous epithelium, produced by 79 per cent of a series of stock tumors, was never produced by ten of the clonal lines, whereas six of the clonal lines produced it in more than 50 per cent of the tumors examined. Other tissue types showed similar variability in appearance in the clonal lines (Table 2). Notochord appeared in one clone al-
TABLE 2
INCIDENCE (PER CENT) OF DIFFERENTIATED TISSUES, GROWTH RATE, AND EMBRYOID BODY PRODUCTION OF SEVERAL CLONES

<table>
<thead>
<tr>
<th>TISSUES PRESENT</th>
<th>STOCK TERATOCARCINOMA</th>
<th>CLONES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NRS-C19</td>
<td>NRS-C18</td>
</tr>
<tr>
<td>Number of tumors:*</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>Embryonal carcinoma</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Astrocytes</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Ependyma</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Simple glands</td>
<td>76</td>
<td>40</td>
</tr>
<tr>
<td>Trophoblast</td>
<td>39</td>
<td>20</td>
</tr>
<tr>
<td>Squamous epithelium</td>
<td>79</td>
<td>10</td>
</tr>
<tr>
<td>Mesenchyme</td>
<td>71</td>
<td>30</td>
</tr>
<tr>
<td>Cartilage</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>Bone</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Smooth muscle</td>
<td>57</td>
<td>0</td>
</tr>
<tr>
<td>Striated muscle</td>
<td>43</td>
<td>0</td>
</tr>
<tr>
<td>Notochord</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ciliated epithelium</td>
<td>71</td>
<td>30</td>
</tr>
<tr>
<td>Visceral yolk sac</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Parietal yolk sac</td>
<td>79</td>
<td>10</td>
</tr>
<tr>
<td>Growth rate (days)</td>
<td>28</td>
<td>35</td>
</tr>
<tr>
<td>Embryoid bodies</td>
<td>2-layered</td>
<td>2-layered</td>
</tr>
</tbody>
</table>

* At least five generations.

though it never appeared in the stock series of tumors; however, this probably reflects the rarity of this type of tissue in our stock lines of teratocarcinoma rather than the emergence of a new tissue potentiality.

In general, there was little difference in the growth rates of the teratocarcinomas. Furthermore, calculations of growth rates were only approximations, since cell counts were not done at the time of inoculation and not all animals could be killed at exactly the same stage of tumor growth. A few clonal lines, however, showed about one-half the growth rate of the other clones; this difference could not be due to experimental error alone and must reflect an inherent difference between the clones.

The clones were analyzed for differences in the production of embryoid bodies (Table 2). Most of the clonal lines produced small embryoid bodies composed of embryonal carcinoma invested by visceral yolk sac just like those produced by the parent teratocarcinoma; however, several clonal lines produced larger cystic embryoid bodies composed of three cell types: embryonal carcinoma, visceral yolk sac, and mesenchyme (Fig. 8). Although this type of embryoid body has been described in other lines of teratocarcinoma, it is not produced by the parent line used in these experiments. Furthermore, some of the clonal lines produced no embryoid bodies, but they did produce free-floating single embryonal carcinoma cells or parietal yolk sac carcinoma cells in the ascitic fluid.

DISCUSSION

Cloning technics which require cells to be maintained in tissue culture for a substantial period of time have the distinct disadvantage of subjecting the cells to prolonged selective and inductive influences of the artificial environment. Many cell lines lose specific functions when grown in vitro. Therefore, problems requiring homogeneous populations of cells may be better approached by cloning in vivo where extraneous selective and inductive influences are minimized. Prior to the present experiments, only free-floating ascites or leukemic cells were used in in vivo cloning experiments. The present findings show that, with gentle treatment, single cells can be enzymatically dissociated from larger cellular aggregates and directly transplanted into animals to give clonal lines. The general applicability of this method was demonstrated in another experiment from this laboratory in which the properties of clonal lines of melanoma were investigated (6).

In regard to teratocarcinomas, several conclusions are warranted. Single embryonal carcinoma cells are multipotential and capable of producing all the somatic tissues of a well differentiated teratocarcinoma. Furthermore, the results also indicate that all embryonal carcinoma cells are not alike but that they vary in their capacity for differentiation, growth, and production of embryoid bodies. An important point to note in this regard is that we started with a very homogeneous population of embryonal carcinoma cells—namely, those contained in immature embryoid bodies; thus, it is possible that the experiment was weighted against showing the true heterogeneity of embryonal carcinoma. If single embryonal carcinoma cells from a well differentiated teratocarcinoma had been transplanted, perhaps a much greater variation in clones would have resulted.

The demonstration of the heterogeneity of embryonal...
carcinoma in teratocarcinomas gives strong support for
the stem cell theory of cancer (13). Similar results on
the heterogeneity of malignant cell populations were
found in the cloning experiments of Hauschka and Levan
(7) and Makino and Kano (14), where chromosomal
variations were used as markers. It has been postulated
that perhaps all neoplasms consist of a heterogeneous
population of cells, a small number of which are undiffer-
entiated, highly malignant stem cells; the rest are less
malignant and more differentiated. The type of differ-
entiation is determined by the cell of origin of the
tumor. Thus, in teratocarcinomas, where the stem cell is of
germinal cell origin, multipotential differentiation is
possible. On the other hand, in a neoplasm of glandular
origin, the differentiation of the stem cells would be limited
to simple glands.

Although experiments upon the pathogenesis of terato-
carcinoma (17) lend strong support to the stem cell theory
of cancer, they do not support the theory of somatic cell
mutation or the dogma regarding the irreversibility of the
malignant change. The results show conclusively that
embryonal carcinoma cells have the capacity to differ-
entiate into somatic, adult-appearing tissues; since these
tissues have been shown to be benign (17, 21), it is obvious
that the malignant stem cells are constantly differentiating
spontaneously into benign, normal-appearing cells, a
phenomenon difficult to reconcile with a somatic mutation
or an irreversible type of change.

If cancer cells are not irreversibly changed and do
spontaneously undergo differentiation, which in some
instances results in benign cells, it would seem appropriate
that cancer therapy, rather than attempting to kill or
extirpate all the cells of a tumor, might attempt to direct
the spontaneously occurring differentiation toward the
production of benign tissues as we have postulated pre-
viously (17). In this regard, the recent work of Niu (16),
using ribonucleic acids to slow growth by directing differ-
entiation in mouse ascites tumors, is of great interest.

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FIG. 1.—Small embryoid body consisting of a core of embryonal
   carcinoma invested with a layer of visceral yolk sac. X 300.

FIG. 2.—Single cell (arrow) isolated in a capillary tube. X 100.

FIG. 3.—Low-power view of an area from one of the well differ-
   entiated clones, showing cartilage, ciliated glands, mesenchyme,
   primitive neuroepithelium, and embryonal carcinoma. X 120.

FIG. 4.—Higher-power view of an area from Fig. 3, showing the
   embryonal carcinoma with primitive neuroepithelium (arrow)
   developing in it. X 300.
FIG. 5.—Embryonal carcinoma, mesenchyme, ciliated glands, and keratinized stratified squamous epithelium from one of the clones. X 300.

FIG. 6.—Bone, mesenchyme, cartilage, and cuboidal epithelium developing in one of the cloned teratocarcinomas. X 300.

FIG. 7.—Low-power view of the undifferentiated clone which was not a teratocarcinoma. This tumor contained embryonal carcinoma, parietal yolk sac cells, and trophoblastic giant cells. X 120.

FIG. 8.—Large cystic embryoid body containing embryonal carcinoma and mesenchyme invested with a layer of visceral yolk sac. X 300.
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