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Tumorigenicity of embryonal carcinoma as an assay to study control of malignancy by the murine blastocyst

(differentiation/melanoma)

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ABSTRACT **A bioassay, based on tumorigenicity, has been developed to determine the mechanism whereby the blastocyst of the mouse controls malignant expression of embryonal carcinoma. The assay is based upon the incidence of tumors obtained when known numbers of cells of the 402AX strain of embryonal carcinoma are injected into strain 129 mice, compared to the incidence obtained when the same number of embryonal carcinoma cells are incorporated into Swiss–Webster blastocysts that are then injected in strain 129 animals. The results indicate that the blastocyst can regulate one embryonal carcinoma cell consistently; it may have a slight effect on three, but it cannot regulate four or five of them. The position of the embryonal carcinoma cell in the blastocyst is important. Regulation occurs if the embryonal carcinoma cell is placed in the blastocoele cavity, but enhancement of tumorigenicity is obtained if it is placed between the zona pellucida and the trophectoderm. By contrast, the blastocyst is unable to regulate a single B-16 melanoma cell placed in the blastocoele cavity, indicating a degree of specificity for the regulatory process.**

During experiments to determine the validity of the classification of testicular tumors, it was demonstrated by direct means that embryonal carcinoma cells were the stem cells of mouse teratocarcinomas capable of differentiating into the chaotically arranged somatic tissues characteristic of these malignancies (1). The differentiated tissues derived from embryonal carcinoma proved to be benign and no menace to the host (2). Then it was established that this phenomenon is not limited to teratocarcinomas. Direct experiments with a squamous cell carcinoma (3), and adenocarcinomas of the breast and colon (4), as well as indirect evidence from electron microscopy (5), indicated that the stem cells of other carcinomas also can differentiate into populations of benign cells. Although little has been done with sarcomas, the spleen-colonizing assay of Till and McCulloch (6) provided a means of demonstrating that stem cells of leukemias may also differentiate. *In toto*, these observations have led to the postulate that malignancies are caricatures of the normal process of tissue renewal (7), and that direction or enhancement of differentiation in neoplastic cells might serve as either an adjuvant or an alternative to cytotoxic therapy of cancer.

Efforts to direct the differentiation of embryonal carcinoma by using tissue extracts, hormones, and nucleic acids were unsuccessful (unpublished data). However, tissue extracts modulate growth and differentiation of certain leukemias (8), and some chemicals modulate the differentiation and growth of melanoma (9) and Friend erythroleukemia (10).

In 1974, Brinster (11) reasoned that because embryonal carcinoma appeared to be the equivalent of embryonic epithelium of the mouse blastula (4½ days of gestation), possibly the environment of the early embryo might be able to regulate the differentiation of embryonal carcinoma cells. This proved to be the case because when embryonal carcinoma cells were injected into mouse blastocysts and the injected blastocysts were put into the uteri of pseudopregnant mice, chimeric mice were born, as evidenced by the coat colors of the animals (11).

In 1975, Mintz *et al.* (12) confirmed Brinster's observations, and, in addition, using isoenzyme markers, determined the proportions of cells in various tissues that were derived from the cancer cell and from the normal embryonic cells. One of their chimeric animals proved to be fertile, and sperm derived from the embryonal carcinoma cells conjugated with normal ova and produced apparently normal offspring. In addition, Papaioannou *et al.* (13) showed that when 20 embryonal carcinoma cells were placed in the blastocyst (it is composed of about 80 cells), a chimeric mouse was born with tumors. Thus, there was a limit to the number of cancer cells that could be controlled by the blastocyst.

The mechanism whereby the blastocyst regulates growth and differentiation of embryonal carcinoma cells is unknown. Little quantitative data have been published on the production of chimeric mice with tumor cells, so it can be assumed that the procedure is too complex to serve as an assay.

We postulated that a comparison of the incidence of tumors from small numbers of embryonal carcinoma cells injected into animals alone or within blastocysts might be made sufficiently quantitative to serve as the required assay, provided a line of embryonal carcinoma could be found that would produce tumors from a few cells, and blastocysts could be found that were not carcinogenic in their own right.

This is the report of the development of a negative assay to determine the mechanism whereby the blastocyst regulates embryonal carcinoma cells. The assay is negative in the sense that if the blastocyst successfully regulates an embryonal carcinoma cell, tumors will not develop.

MATERIALS AND METHODS

The embryonal carcinomas employed were: OT6050, originally obtained from L. C. Stevens and maintained in this laboratory *in vitro* (14); F-9, a nullipotent line of embryonal carcinoma isolated by Bernstine *et al.* (15) and obtained from G. Sato; 402AX, a teratocarcinoma developed by L. C. Stevens, was obtained from M. Edidin; one line of 402AX had been carried *in vitro* for many years (16). A subline of tumor 402AX was acquired from A. Isa, who had obtained it from Edidin and had carried it for many years in the ascites form (17). The ascites was diluted appropriately with 5% fetal calf serum in minimal essential media (18), and, prior to use, the cells were tested for viability by trypan blue exclusion. An amelanotic subline of the B-16 melanoma was obtained from S. Gordon and maintained *in vitro*. Cells from solid tumors were dissociated in 0.025% trypsin.

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Abbreviation: i.p., intraperitoneally.

Table 1. Growth of single embryonal carcinoma cells

Exp.	Alone		In blastocysts	
	Animals	Tumors	Animals	Tumors
1	19	7	—	—
2	6	3	5	0
3	8	4	8	0
4	3	2	3	0
5	15	5	16	0
6	10	2	9	2
7	7	5	4	1
Totals	68	28	45	3
	41% tumors		7% tumors	

Expanded blastocysts were flushed with balanced salt solution, at the appropriate time after fertilization, from the oviducts and uteri of Swiss–Webster mice that had been induced to superovulate according to Runner's method (19). Blastocysts and a few tumor cells were placed in a small drop of 3 mg of bovine serum albumin per ml of balanced salt solution under a layer of paraffin oil that had been selected for lack of toxicity and equilibrated with balanced salt solution and 5% CO_2 and 95% air. Blastocysts were injected with tumor cells, using a Leitz micromanipulator and a technique learned from Markert (20). Injected blastocysts were washed free of any adherent embryonal carcinoma cells and were injected intraperitoneally (i.p.) in strain 129 animals. Embryonal carcinoma cells from the same drop were then aspirated into microcapillary needles and injected i.p. in strain 129 animals to serve as controls.

DEVELOPMENT OF THE ASSAY

The success of the assay depended upon finding a strain of teratocarcinoma cells that would produce tumors in significant numbers upon injection of small numbers of tumor cells. OT6050, F-9, and the *in vitro* strain of 402AX cells did not produce tumors when small numbers of cells were injected i.p. or subcutaneously in strain 129 animals. The ascites line of strain 402AX did not produce tumors when injected subcutaneously; however, about 40% of single cells, when injected i.p. in strain 129 mice, gave rise to typical embryonal carcinomas with minimal evidence of endodermal differentiation.

Because blastocysts may be teratocarcinogenic when transplanted into the testes of adults (21), it was necessary to select ones of low tumorigenicity. Swiss–Webster blastocysts, when placed in the testes of strain 129 hosts, developed into benign teratomas in only 10% of instances. When transplanted i.p. in strain 129 animals, they never gave rise to teratomas. The i.p. implantation sites were congested, measured about a millimeter in diameter, and were necrotic and surrounded by a vascular and round cell response, suggesting homograft rejection. As a result, it was decided to use the ascites form of 402AX cells injected i.p. directly or within Swiss–Webster blastocysts to determine if tumorigenicity could serve as an assay to investigate the mechanism whereby blastocysts direct differentiation of embryonal carcinoma cells.

Table 2. Growth of single embryonal carcinoma cells in collapsed blastocysts

Exp.	Alone		In collapsed blastocysts	
	Animals	Tumors	Animals	Tumors
8	19	4	18	11
9	10	3	7	5
Totals	29	7	25	16
	24% tumors		64% tumors	

Table 3. Growth of various numbers of embryonal carcinoma cells

Cells	In controls			In blastocysts		
		Tumors			Tumors	
	Animals	No.	%	Animals	No.	%
3	92	56	61	61	27	44
4	16	13	81	12	9	75
5	8	7	88	12	10	83

RESULTS

The results of seven experiments in which single embryonal carcinoma cells, alone or within blastocysts, were injected i.p. in strain 129 animals, are listed in Table 1. Forty-one percent of the single cells developed into tumors; the earliest, 25 days after injection, the latest by 40 days. In contrast, only 7% of single cells incorporated in blastocysts produced tumors when injected i.p. Histologically, these were typical 402AX embryonal carcinomas with some endodermal differentiation.

Little variation in the data obtained from individual experiments was observed, except in experiment 6, in which 20% of single cells without blastocysts gave rise to tumors, and 22% of those in blastocysts gave rise to tumors. This is the only discordant result. In seeking an explanation for it, we noted that for experiment 6, we had lengthened the bevel on the injecting pipettes to facilitate penetration of trophectoderm. It was our impression that the long bevel on occasion entered and collapsed the blastocoele cavity prior to injection of the cell. Thus, a few of the tumor cells might have escaped to lie between the zona pellucida and the trophectoderm. This possibility was tested in experiments in which blastocysts were purposely collapsed and a single tumor cell was placed between the zona and the trophectoderm. Tumorigenicity of such cells was compared to that of single cells injected without blastocysts (Table 2). Note that whereas tumorigenicity was blocked when single cells were placed in the blastocoele cavity (Table 1), it was enhanced when the cells were placed between the trophectoderm and the zona pellucida (Table 2). Some unexplained variation occurred in the results of cloning (Tables 1 and 2), but the data were highly significant within each experiment.

Because injection of 20 cells into a blastocyst resulted in mice with tumors (13), it was decided to determine the precise number of embryonal carcinoma cells that could be controlled by a blastocyst. Table 3 compares tumorigenicity when three, four, or five cells were injected into animals alone or within blastocysts. It is clear that blastocysts cannot regulate four or five embryonal carcinoma cells and probably cannot regulate three of them with consistency.

Because the assay is negative, an additional positive control was required. To this end, B-16 melanoma cells were employed (Table 4). Single B-16 melanoma cells, when injected into the testes of C57BL/6J mice, produced tumors in 60% of cases. Similarly, 51% of single B-16 melanoma cells incorporated into Swiss–Webster blastocysts produced tumors when injected into

Table 4. Growth of B-16 melanoma

Exp.	In controls		In blastocysts	
	Animals	Tumors	Animals	Tumors
1	2	1	2	1
2	7	5	8	4
3	6	3	5	1
4	20	12	20	12
Totals	35	21	35	18
	60% tumors		51% tumors	

the testes of C57BL/6J mice. These data indicate that tumor cells are not killed nonspecifically when injected into blastocysts and, from a standpoint of understanding the mechanism of direction of differentiation of carcinoma cells, indicate a degree of specificity for the reaction.

DISCUSSION

The assumption that tumorigenicity could serve as an assay to determine the manner in which the blastocyst regulates embryonal carcinoma has been borne out by the experiments cited. The data indicate that the blastocyst can control a single embryonal carcinoma cell, provided that cell is placed within the blastocoele cavity. If the cell is placed between the zona pellucida and the trophectoderm, the malignant phenotype is not controlled, and there even appears to be an enhancement of tumorigenicity. This experiment was a direct positive control, ruling out lack of growth due to killing of the cells as a result of the manipulations. In addition, it suggested that trophectoderm, or at least its surface, normally resting on the zona pellucida, is incapable of regulating embryonal carcinoma cells. The data do not exclude the possibility that regulation might occur if the cancer cell were on the blastocoele surface of the trophectoderm.

An additional positive control was obtained when the blastocyst was shown to be incapable of regulating the malignant phenotype of single B-16 melanoma cells injected into the blastocoele cavity. Tumors were produced in about equal incidence from melanoma cells transplanted to the testes alone or within blastocysts. Although there was a slightly lower incidence of tumors produced from cells in blastocysts than from cells alone, we feel that the difference is a reflection of the technical manipulations rather than any specific effect of the blastocysts.

There are interesting implications from the observation that the blastocyst can regulate one type of malignant cell and not another. Our explanation for this effect is that embryonal carcinoma cells and inner cell mass cells are similar morphologically (1), functionally (2), and biochemically (15). Apparently, the environment provided by the blastocyst for regulation of inner cell mass also regulates closely corresponding malignant cells. This reaction has some developmental specificity because melanocytes (from which melanoma cells are derived) develop later in embryogenesis and are not regulated by the blastocyst.

Ruben (22) postulated that persistent embryonic fields might be able to regulate cancer cells, and grafted a lymphosarcoma into the regenerating limb of an amphibian, but regulation of malignancy did not occur. Among possible explanations for this result is the lack of correspondence between the tumor and embryonic field employed. On the basis of our experience, it can be postulated that a few malignant tumor cells will be regulated by the closely corresponding embryonic field. In this sense, parietal yolk sac carcinomas might be regulated by the embryo at the time of outgrowth of proximal and distal endoderm, the Wilms' tumor of the kidney might be regulated by the environment at the time of nephrogenesis, and a chondrosarcoma by prechondrogenic portions of a somite. It is anticipated that regulation will occur irrespective of the causative agent of the tumor because differentiation of malignant into benign cells has been demonstrated in chemically (3) and virally (4) induced tumors and in spontaneous ones (2), and the present experiments indicate the required degree of histogenetic specificity.

Whether or not the mechanism of regulation of malignant cells by normal embryonic fields can be refined for clinical use in the treatment of the patient with metastasis remains to be seen, but the use of malignant cells as probes to study the normal mechanisms of differentiation may be as important to the study of developmental biology as the use of mutation has been to the study of genetics or myelomas to the study of immunoglobulins.

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1. Kleinsmith, L. J. & Pierce, G. B. (1964) *Cancer Res.* **24,** 1544–1551.
2. Pierce, G. B., Dixon, F. J. & Verney, E. L. (1960) *Lab. Invest.* **9,** 583–602.
3. Pierce, G. B. & Wallace, C. (1971) *Cancer Res.* **31,** 127–134.
4. Pierce, G. B., Nakane, P. K., Martinez-Hernandez, A. & Ward, J. M. (1977) *J. Natl. Cancer Inst.* **58,** 1329–1345.
5. Pierce, G. B. (1966) *Cancer* **19,** 1963–1983.
6. Till, J. E. & McCulloch, E. A. (1961) *Radiat. Res.* **14,** 213–222.
7. Pierce, G. B., Shikes, R. & Fink, L. M. (1978) in *Cancer: A Problem of Developmental Biology,* ed. Markert, C. L. (Prentice-Hall, Englewood Cliffs, NJ), pp. 27–48.
8. Metcalf, D. & Moore, M. A. S. (1970) *J. Natl. Cancer Inst.* **44,** 801–808.
9. Silagi, S. (1967) *Cancer Res.* **27,** 1953–1960.
10. Friend, C., Preisler, H. D. & Scher, W. (1974) in *Current Topics in Developmental Biology,* eds. Monroy A. & Moscona, A. A. (Academic, New York), Vol. 8, pp. 81–101.
11. Brinster, R. L. (1974) *J. Exp. Med.* **140,** 1049–1056.
12. Mintz, B., Illmensee, K. & Gearhart, J. D. (1975) in *Teratomas and Differentiation,* eds. Sherman, M. I. & Solter, D. (Academic, New York), pp. 59–82.
13. Papaioannou, V. E., McBurney, M. W. & Gardner, R. L. (1975) *Nature (London)* **258,** 70–73.
14. Lehman, J. M., Speers, W. C., Swartzendruber, D. E. & Pierce, G. B. (1974) *J. Cell Physiol.* **84,** 13–28.
15. Bernstine, E. G., Hooper, M. L., Granchamp, S. & Ephrussi, B. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 3899–3903.
16. Edidin, M. & Gooding, L. R. (1975) in *Teratomas and Differentiation,* eds. Sherman, M. I. & Solter, D. (Academic, New York), pp. 109–121.
17. Isa, A. M. & Sanders, B. R. (1976) *Transplantation* **21,** 195–198.
18. Hoppe, P. C. & Pitts, S. (1973) *Biol. Reprod.* **8,** 420–426.
19. Runner, M. N. & Palm, J. (1953) *J. Exp. Zool.* **124,** 303–316.
20. Markert, C. L. & Petters, R. M. (1977) *J. Exp. Zool.* **201,** 295–302.
21. Stevens, L. C. (1968) *J. Embryol. Exp. Morphol.* **20,** 329–341.
22. Ruben, L. N. (1956) *J. Morphol.* **98,** 389–403.