Malignant Hemangioendotheliomas Produced by Subcutaneous Inoculation of Balb/3T3 Cells Attached to Glass Beads

Abstract. *The Balb/3T3 mouse embryo cell line has been frequently used in cancer research as representative of nontumorigenic cells with the characteristic in vitro properties of postconfluence inhibition of cell division, low saturation density, and anchorage dependence. On the reasoning that anchorage dependence might also apply in vivo, each of nine mice were subcutaneously inoculated with an average of 15,400 Balb/3T3 cells attached to two glass beads 3 millimeters in diameter. After 8 weeks, all the mice had developed large bloody tumors that microscopically proved to be hemangioendotheliomas. The inoculation of Balb/3T3 cells alone or beads alone produced no tumors. Transplants of each tumor into normal mice grew to kill the animal within 6 weeks. Tumor cells from collagenase-disaggregated tumor tissue had a plating efficiency of 21.2 percent compared to that of normal adult subcutaneous fibroblasts of less than 0.1 percent. The tumor cells in vitro closely resembled Balb/3T3 cells in appearance and were tumorigenic at a dose of 10^1 cells. A second, repeat experiment produced the same type of tumors grossly and microscopically in 17 of 25 mice between 99 and 211 days after inoculation of the Balb/3T3 cells attached to glass beads. These findings require a reassessment of the postulate that low saturation density, postconfluence of cell division, and anchorage dependence are characteristic in vitro properties only of nonneoplastic cells.*

The Balb/3T3 mouse embryo cell line (*1*) exhibits the properties of low saturation density (4×10^4 to 5×10^4 cells per square centimeter) and postconfluence inhibition of cell division (*2, 3*) as the result of being carried in continuous exponential growth at low cell density with minimal cell-cell contact. These properties have been correlated with the fact that the line is nontumorigenic when inoculated subcutaneously into syngeneic mice (*4*). An earlier 3T3 line produced from random-bred Swiss mouse embryo (*5*) possesses the same in vitro properties as the Balb/3T3 line but cannot be tested for tumorigenicity because of the histocompatibility barrier. If Balb/3T3 cells are not carried in continuous exponential growth, they can become spontaneously neoplastically transformed (*6*). For this reason, authentic clone A31 Balb/3T3 cells were obtained from the American Type Culture Collection, Rockville, Maryland, for use in these studies.

Balb/3T3 cells have been quite frequently used in cancer research as representative of nontumorigenic cells and have been compared with neoplastically transformed derivative lines, especially those transformed by SV40 virus, with regard to morphology (*7*), postconfluence inhibition of cell division (*2, 3*) and of locomotion (*8*), response to factors affecting growth (*9*) and locomotion (*10*), agglutinability by lectins (*11*), cyclic adenosine monophosphate metabolism (*12*), membrane transport of glucose, amino acids, and nucleosides (*13*), ganglioside and glycosyl transferase content (*14*), and alterations in surface concentration of H2 antigens (*15*). Because its low saturation density makes foci of piled up, morphologically transformed cells easy to see, the Balb/3T3 line has also been used in the study of neoplastic transformation in vitro by viruses (*16, 17*) and chemicals (*18*).

Balb/3T3 cells also have the property of anchorage dependence, or the inability to divide in vitro unless attached to a solid substrate (*19*). I reasoned that anchorage dependence might also apply in vivo, and that Balb/3T3 cells might grow and produce tumors if they were inoculated subcutaneously attached to a solid substrate. The experiments to be described showed that this was indeed the case. Glass beads, 3 mm in diameter (Kimax, No. 5663-F28, Arthur H. Thomas Company, Philadelphia), were placed in a 60-mm plastic petri dish, and 5 ml of tissue culture medium (Dulbecco-Vogt modified Eagle's minimal essential medium with 10 percent fetal bovine serum) containing approximately 3×10^6 singly suspended, trypsin-detached Balb/3T3 cells (clone A31, passage 83, No. CCL 163, obtained from the American Type Culture Collection) were added. The next day, two beads with attached cells were inoculated subcutaneously under ether anesthesia into each of nine Balb/c mice by making a 1-cm incision through the skin with scissors, opening the subcutaneous space over one side of the back by blunt dissection, dropping the beads into the space, and closing the incision with skin clips. The number of 3T3 cells attached per glass bead under these conditions averaged 7700, determined by counting in a hemocytometer the number of cells detached with trypsin from 500 beads. The calculated maximum possible number of cells per bead (surface area $\times$ saturation density) was 14,000 cells. Each of another group of 20 mice was inoculated subcutaneously with two glass beads that had been incubated in tissue culture medium without attached Balb/3T3 cells. A third group of animals consisted of four subgroups of ten mice each that were inoculated subcutaneously with Balb/3T3 cells suspended in tissue culture medium at doses of 10^3, 10^4, 10^5, and 10^6 cells per mouse. By the end of 8 weeks after inoculation, all nine mice inoculated with Balb/3T3 cells attached to glass beads had developed tumors approximately 2 cm in diameter, whereas the groups inoculated with glass beads alone or Balb/3T3 cells alone had no tumors (nor did they develop tumors during 4 months of subsequent observation). When the skin was reflected, all of the tumors were similar in gross and microscopic appearance. They were of a dark reddish-brown color, covered with a thin fibrous capsule, and were fluctuant to touch. They could be separated from the fascia overlying the muscles of the back by blunt dissection but were firmly attached to the skin. Cut sections revealed a large central area of friable reddish-brown tissue mixed with similarly colored fluid indicative of old hemorrhage. The two glass beads were found loosely enmeshed in this tissue. The peripheral area of the cut section was 1 to 4 mm thick and consisted of glistening, pale pink translucent tissue. Microscopic sections showed that this peripheral area was composed of tightly packed, highly undifferentiated, large, polygonal tumor cells that in some areas were interspersed between anastomosing small blood channels that were frequently seen to be lined by tumor cells (Fig. 1). It appeared that these blood channels had ruptured to produce the degenerating tumor tissue and old hemorrhage seen in the central portion of the tumor. Groups of tumor cells at the border of the tumor were invading the surrounding muscular and fibroareolar tissue. The microscopic diagnosis was malignant hemangioendothelioma (*20*).

Small portions of each of the nine tumors were trocar-transplanted subcutaneously into ten mice. The transplants all grew to form large tumors that killed the animals within 6 weeks. The appearance of the transplanted tumors closely resembled the original tumors in gross and microscopic appearance, indicating that the central areas of hemorrhage in the original tumors were not secondary to the presence of the glass beads.

Taking advantage of the known high plating efficiency of Balb/3T3 cells [30 to 50 percent (*1*)], I proceeded to recover the tumor cells in vitro free of normal stromal cells by planting the cells from collagenase-disaggregated tumor tissue at 100 cells per petri dish. The mean number of colonies appearing in 12 dishes 2 weeks later was 21.2, standard error 0.87 (plating efficiency 21.2 percent). For comparison, I determined that the mean number of colonies in ten petri dishes 2 weeks after planting 10^4 cells from collagenase-disaggregated connective tissue, scraped from the dorsal skin of adult Balb/c mice, was 5.0, standard error 1.33 (plating efficiency 0.05 percent). I also determined the plating efficiency of collagenase-disaggregated fibroblasts that had proliferated and grown into an approximately 1-cm wad of nylon wool implanted subcutaneously 3 weeks previously. In this case the mean number of colonies in nine petri dishes planted at 10^4 cells per dish was 8.7, standard error 0.7 (plating efficiency 0.087 percent). Thus, the very low plating efficiency of normal connective tissue cells (less than 0.1 percent) assured me that the colonies derived from plating tumor tissue cells at 100 cells per dish were practically all tumor cells. The tumor cell colonies were all similar in appearance, making it unlikely that the tumor tissue was made up of a mixture of host-derived and Balb/3T3-derived tumor cells.

In addition to their high plating efficiency, the tumor cells also resembled Balb/3T3 cells in their morphology, being polygonal and tending to form a monolayer with a tile mosiac-like pattern. The appearance of clonal colonies of the tumor cells was distinctive. The peripheral half of their radial extent consisted of a monolayer of cells closely resembling Balb/3T3 cells, while in their central portion a layer of rounded, refractile cells rested on top of the monolayer. In mass culture the tumor cells formed a monolayer of polygonal cells that continued to divide slowly to form rounded, refractile cells that adhered to the monolayer; this behavior was very different from that of adult connective tissue cells, which formed overlapping strata of parallel-oriented spindle-shaped cells, or that of a line of spontaneously transformed adult connective tissue cells, which formed a multilayered feltwork of crisscrossing spindle cells.

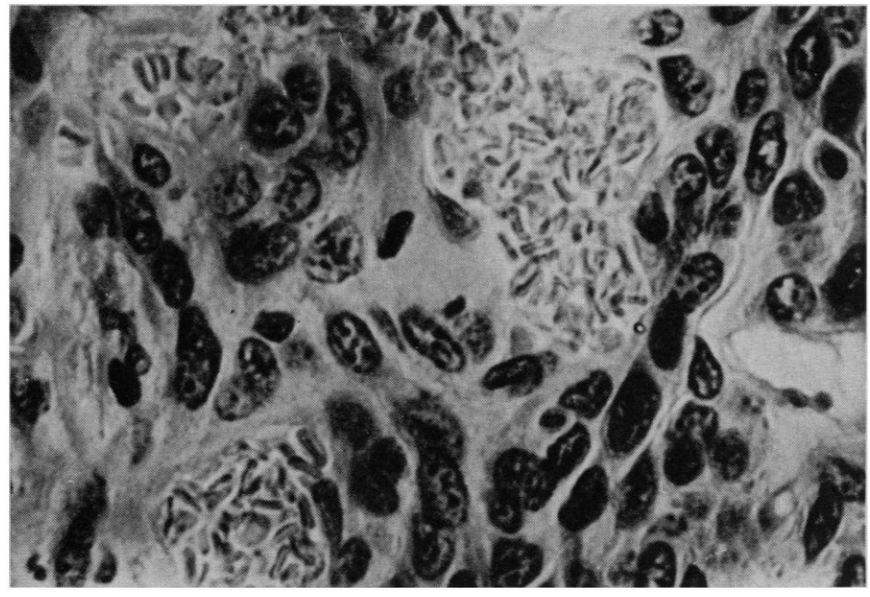

Fig. 1. Histologic section of tumor developing from Balb/3T3 cells attached to glass beads. The tumor cells have formed vascular channels containing red blood cells. One of the tumor cells bordering the lumen of a vascular channel is in the anaphase stage of mitosis. The microscopic diagnosis is malignant hemangioendothelioma.

The tumorigenicity of the cultured tumor cells, determined by inoculating graded doses of cells subcutaneously into groups of 10 mice at each dose, was 2/10 at 10^4 cells per dose, 7/10 at 10^5 cells, 7/10 at 10^6 cells, and 10/10 at 10^7 cells per dose. The tumors all reached a size of 1.5 cm in average diameter within 3 weeks. They were very similar in both gross and microscopic appearance to the original tumors produced by inoculating the Balb/3T3 cells attached to glass beads.

A complete repeat experiment was performed in which two glass beads with attached Balb/3T3 cells were inoculated subcutaneously as before into each of 25 mice. Two humors appeared by the 99th day after inoculating, 11 more between the 152nd and 167th days, and four more by the 211th day, when the experiment was terminated. All of these tumors were similar in gross and microscopic appearance to those described in the first experiment.

In view of the fact that malignant hemangioendotheliomas appeared at the site of subcutaneous inoculation of Balb/3T3 cells attached to 3-mm glass beads, whereas inoculation of cells or glass beads alone produced no tumors, and adhering to the conventional definition of a neoplastic cell population as one that produces tumors when inoculated subcutaneously, Balb/3T3 cells must be considered neoplastic. This will require a reassessment of the current concept that postconfluence inhibtion of cell division, low saturation density, and anchorage dependence are characteristic in vitro properties only of nonneoplastic cells.

Since the saturation density of Balb/3T3 cells increases with increase in the serum concentration of the culture medium (*21*), the existence of a many-fold higher serum concentration in vivo seems a plausible, although of course unproved, explanation for some of the continued proliferation of the Balb/3T3 cells on the glass beads to the point where they finally no longer required attachment to a solid substrate in order to divide. This loss of anchorage dependence by the Balb/3T3 cells can be identified as an example of tumor progression (*22*).

The possibility that the tumors were derived from host cells rather than the glass-attached Balb/3T3 cells appears unlikely for the reasons that the Balb/3T3 cells alone or beads alone pro-

duced no tumors, the high plating efficiency of the tumor cells was like that of the Balb/3T3 cells, and the morphology of the tumor cells in culture was similar to that of Balb/3T3 cells.

There are now two pieces of evidence that the Balb/3T3 cell is a vascular endothelial cell: It looks like an endothelial cell by scanning electron microscopy (*7*), and it forms tumors that resemble those derived from vascular endothelial cells. The evidence is further supported by the resemblance in morphology and behavior between endothelial cells in vivo and Balb/3T3 cells in vitro. Endothelial cells forming the walls of capillaries appear as a tightly adherent monolayer of nondividing polygonal cells that are activated to grow by wounding and that do not grow as a multilayer, since this would tend to occlude the lumen of the capillary.

It appears that the in vitro properties of the Balb/3T3 cloned line of probable endothelial cells should not be used as the standard for nontumorigenic mouse cells, not only because the cells represent just one of dozens of embryonic cell types, but also because they are neoplastic when inoculated subcutaneously attached to a solid substrate.

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References and Notes

S. A. Aaronson and G. J. Todaro, *J. Cell. Physiol.* **72**, 141 (1968).

E. Martz and M. S. Steinberg, *ibid.* **79**, 189 (1972).

3. Other terms for this phenomenon have been contact inhibition of cell division (*1*), density dependent inhibition of growt [M. G. Stoker and H. Rubin, *Nature (Lond.)* **215**, 171 (1967)], and topoinhibiti [R. Dulbecco, *ibid.* **227**, 802 (1970)].

4. S. A. Aaronson and G. J. Todaro, *Science* **162**, 1024 (1968).

5. G. J. Todaro and H. Green, *J. Cell Biol.* **17**, 299 (1963).

6. C. W. Boone, E. L. Lundberg, T. Orme, R. Gillette, *J. Natl. Cancer Inst.* **51**, 1731 (1973).

7. K. R. Porter, G. J. Todaro, V. Fonte, *J. Cell Biol.* **59**, 633 (1973).

M. H. Gail and C. W. Boone, *Exp. Cell Res.* **64**, 156 (1971).

A. Lipton, D. Paul, M. Henehan, I. Klinger, R. W. Holley, *ibid.* **74**, 466 (197 P. S. Rudand, W. Eckhart, D. Gospodarowicz, W. Seifert, *Nature (Lond.)* **250**, 337 (1974).

10. A. Lipton, I. Klinger, D. Paul, R. W. Holley, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2799 (1971); R. R. Burk, *ibid.* **70**, 369 (1973).

C. H. O'Neill and M. E. Burnett, *Exp. Cell Res.* **83**, 247 (1974); W. Eckhart, R. Dulbecco, M. M. Burger, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 283 (1971); G. L. Nicolson, *Nat. New Biol.* **233**, 244 (1971).

S. Bannai and J R. Sheppard, *Nature (Lond.)* **250**, 62 (19 L. J. deAsua, E. Rozengurt, R. Dulbecco, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 96 (1974).

W. E. C. Bradley and L. A. Culp, *Exp. Cell Res.* **84**, 335 (1974); K. J. Isselbacher, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 585 (1972); R. Kram, P. Mamont, G. M. Tomkins, *ibid.* **70**, 1432 (1973).

14. R. O. Brady and P. T. Mora, *Biochim. Biophys. Acta* **218**, 308 (1970); L. M. Patt and W. J. Grimes, *J. Biol. Chem.* **249**, 4157 (1974); S. Roth and D. White, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 485 (1972).

15. E. Tsakraklides, C. Smith, J. H. Kersey, R. A. Good, *J. Natl. Cancer Inst.* **52**, 1499 (1974).

J. L. Jainchill and G. J. Todaro, *Exp. Cell Res.* **59**, 137 (1970).

17. S. A. Aaronson, J. L. Jainchill, G. J. Todaro, *Proc. Natl. Acad. Sci. U.S.A.* **66**, 1236 (1970).

18. J. A. DiPaolo, K. Takano. N. C. Popescu, *Cancer Res.* **32**, 2686 (19 M. A. Basombrio and R. T. Prehn, *Int. J. Cancer* **10**, 1 (1972).

19. M. Stoker, C. O'Neill, S. Berryman, V. Waxman, *Cancer* **3**, 683 (1968).

20. The consultative advice and opinion of Dr. Harold L. Stewart, Division of Cancer Cause and Prevention, National Cancer Institute, in making the diagnosis of malignant hemangioendothelioma is acknowledged.

21. R. W. Holley and J. A. Kiernan, *Proc. Natl. Acad. Sci. U.S.A.* **60**, 300 (1968).

22. L. Foulds, *Neoplastic Development* (Academic Press, New York, 1969).

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Anomeric Specificity of 3-O-Methyl-D-glucopyranose against Alloxan Diabetes

Abstract. *The individual α and β anomers of the nonmetabolized glucose analog 3-O-methyl-D-glucopyranose (3MG) were studied as protective agents against the alloxan toxicity to pancreatic beta cells in an in vivo rat model. The α 3MG provides greater protection than either the β or the equilibrated compound, as indicated by plasma glucose concentrations 24 hours after the experiment. This specificity suggests that the beta cell membrane is extremely stereospecific, and that glucose or 3MG provide protection against alloxan injury directly by an interaction with the cell membrane and not by subsequent metabolism of the protecting compound.*

It has been reported (*1*) that the α anomer of D-glucose provides better protection than the β anomer does against the toxic effects of alloxan on pancreatic beta cells in fasted rats. These data suggested a highly stereospecific protection site, but did not exclude an effect that might be secondary to a selective transport or metabolism of the α anomer as compared to the β anomer. 3-*O*-Methyl-D-glucopyranose (3MG) is not metabolized in rats (*2*) and several laboratories have shown that it is able to provide protection against alloxan toxicity (*3*).

Commercial crystalline 3-*O*-methyl-D-glucose (Sigma) was analyzed and found to be in the α-D-pyranose form (98 percent α- and 2 percent β-D-pyranose) (*4*). In water solution, it underwent a mutarotation similar to that of α-D-glucose to give an equilibrium mixture of 32 percent of α and 68 percent of β anomer. Removal of the α anomer from the equilibrium mixture (*5*) gave the pure β anomer (96 percent β- and 4 percent α-D-pyranose form) (*6*). Thus, the biological activity of the α and β anomers and of the equilibrium mixture could be compared.

As shown in Table 1, at a dose of 0.28 mmole, α-3MG exerted protection against alloxan toxicity, near normal plasma glucose concentrations being observed 24 hours after alloxan administration, but β-3MG was less effective. These studies confirm that the protective site involves a highly stereospecific conformation of the carbohydrate molecule and does not result from a metabolite.

Table 1. Effect of α and β anomers of 3-*O*-methyl-D-glucopyranose (3MG) against alloxan diabetes. Male rats weighing approximately 200 g were fasted for 24 hours. The crystalline α or β anomers (0.28 mmole) rapidly dissolved in 0.5 ml of saline solution, or a solution of the equilibrium mixture (68 percent of β and 32 percent of α, total 0.28 mmole, in 0.5 ml of saline), were infused over 60 seconds, followed in 4 seconds by a rapid infusion (0.5 ml) of alloxan (40 mg/kg). The animals were fed freely and plasma glucose was determined 24 hours later to express the degree of alloxan diabetes. Control animals receiving neither alloxan nor 3MG had a mean plasma glucose concentration of 164 ± 2 mg/100 ml (N = 21). S.E.M., standard error of the mean; N, number of animals.

Substances injected	Plasma glucose (mg/100 ml)	
	Mean ± S.E.M.	N
Alloxan alone	431 ± 17	6
α-3MG and alloxan	195 ± 26*	6
β-3MG and alloxan	359 ± 25	6
Equilibrium mixture and alloxan	305 ± 47	6

* $P < .005$; Student's t-test comparing α versus β.