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Cytoplasmic Suppression of Tumorigenicity in Reconstructed Mouse Cells[1]

Jerry W. Shay[2] and Harold Werbin

Department of Cell Biology, The University of Texas Southwestern Medical Center, Dallas, Texas 75235 [J. W. S.], and Programs in Biology, The University of Texas at Dallas, Richardson, Texas 75083-0688 [H.W.]

ABSTRACT

Previous cybrid studies aimed at demonstrating cytoplasmic suppression of tumorigenicity have been generally inconclusive because of (*a*) the use of mutagens or carcinogens to introduce nuclear-coded and cytoplasmic-coded genetic markers and (*b*) dilution of putative cytoplasmic suppressors with tumorigenic cytoplasm of whole cells used in the cybrid construction. We have circumvented these potential problems by examining tumorigenicity in reconstructed cells made from tumorigenic karyoplasts and nontumorigenic cytoplasts and by using a ricin-antiricin selection to obtain the reconstructed cells.

Karyoplasts from tumorigenic NIH/3T3 cells that were derived from a clone that had survived incubation with benzo(*a*)pyrene-*trans*-7,8-dihydrodiol-9,10-epoxy (anti) and been passaged 17 times were fused to NIH/3T3 cytoplasts derived from nontumorigenic cells. The cytoplasts were loaded with antiricin antibody prior to fusion. Ten clones which survived ricin selection were not tumorigenic in nude mice. These findings offer support for the presence of cytoplasmic factors in nontumorigenic mouse cells that suppress benzo(*a*)pyrene epoxide-induced tumorigenicity.

INTRODUCTION

A wide variety of animal cells either in tissue culture or *in vivo* become tumorigenic after exposure to diverse chemical carcinogens. While activated nuclear oncogenes are found in many carcinogen-induced tumors (1), there is considerable uncertainty whether the tumorigenic state can be attributed solely to oncogene activation (2, 3). A mitochondrial role in chemical carcinogenesis may be proposed because many diverse lipophilic carcinogens modify mitochondrial DNA more effectively than nuclear DNA in the same cell (4, 5) and induce mutations (6–8) in the mitochondrial genome. Because there does not appear to be repair of mitochondrial DNA damage (9, 10), these and other findings prompted several groups to explore by means of hybrid and cybrid experiments a cytoplasmic involvement in tumorigenesis. The results of these studies have been reviewed recently by Sager (11) and by Shay (12). Some studies have shown that tumorigenic cytoplasms do not induce tumorigenicity following their fusion to whole normal cells, and it has been generally inferred that the cytoplasm of tumor cells does not contribute to the tumorigenic state (13, 14). Yet, both Sager (11) and Shay (12) cite in their reviews converse studies in which the expression of tumorigenicity is blocked from 20 to 90% when normal cytoplasts are fused to tumorigenic cells or to tumorigenic karyoplasts (nuclei surrounded by a rim of cytoplasm). In the latter instance such cells are referred to as recons or reconstructed cells. These observations are the basis for the recent concept of "cytoplasmic suppressors of tumorigenicity" (11) which should be distinguished from another recent concept "nuclear encoded tumorigenic suppressors" (11, 15, 16).

In the present study, we have sought more definitive evidence for cytoplasmic suppression by (*a*) using reconstructed cells in which dilution of cytoplasmic suppressors is minimized and (*b*) not using mutagens or carcinogens to introduce genetic markers into the nucleus or cytoplasm. Our experimental protocol was influenced by two recent findings with respect to BPDE[3]- and benzo(*a*)pyrene-induced carcinogenesis. (*a*) Exposure of embryo and fibroblast cells BPDE does not immediately convert them to tumorigenicity, exemplified in this and another study (17). The cells require extensive passaging before this occurs. An important interpretation of these data is that a single mutation acting dominantly by itself does not immediately lead to full expression of tumorigenicity. (*b*) In the case of two-stage mouse skin carcinogenesis induced by exposure to benzo(*a*)pyrene followed by application of phorbol esters, the DNA isolated from skin tumors does not contain Ha-*ras* oncogenes activated by mutation nor does it transfect NIH/3T3 cells (18). In contrast the DNA extracted from 7,12-dimethylbenz(*a*)anthracene- and dibenz[*c*,*h*]acridine-induced skin tumors is active in transfection and contains mutated *ras* oncogenes. These findings indicate that either this oncogene is not acting in a dominant manner or factors besides oncogenes are involved in tumor induction and that diverse chemical carcinogens may promote cancer by the interplay of alternate mechanisms. Such considerations prompted us to explore suppression of tumorigenicity in recons.

Mouse NIH/3T3 cells were exposed to BPDE, and then one clone was passaged until it became tumorigenic when assayed in the nude mouse (NIH/3T3 BPDE 17). Karyoplasts from these cells were fused to cytoplasts obtained from nontumorigenic NIH/3T3 cells. A ricin-antiricin selection procedure permitted isolation of recons without the need to resort to mutagenesis to introduce nuclear- and mitochondrial-encoded gene markers. Because the karyoplasts and cytoplasts are from the same cell type, we refer to these recons as homologous ones. The data obtained lend credence to the concept that there are suppressors of tumorigenicity in the cytoplasm of mouse cells.

MATERIALS AND METHODS

Carcinogen. The BPDE was obtained from the Midwest Research Institute and dissolved in dimethyl sulfoxide that had been stored several months over the drying agent Aquacide I.

Animal Cells. Immortal NIH/3T3 mouse cells were obtained from Dr. Robert Weinberg. These cells were derived from Swiss mouse embryo cells and became immortal spontaneously rather than by mutagenesis (19). The cells were cultivated in Dulbecco's modified Eagle's medium:Ham's F-12 (1:1, v:v) containing 10% fetal calf serum, 1.98 mM L-glutamine, and 2.8 mM glucose. The cells were never allowed to become confluent, since this leads to the appearance of spontaneous transformants. A population of NIH/3T3 cells was exposed twice in serum-free medium to 1 μg/ml of BPDE for 1 h, 24 h apart, in a 48-h period. A few clones became tumorigenic after only 17 passages (approximately 35 population doublings), and one of these, NIH/3T3 BPDE 17, was used to make the homologous recons (Table 1). At the time of injection, recons and controls were passaged approximately 65 doublings beyond the initial BPDE treatment.

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[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: BPDE, benzo(*a*)pyrene-*trans*-7,8-dihydrodiol-9,10-epoxy (anti); NIH/3T3 BPDE 17, a clone of NIH/3T3 that became tumorigenic after exposure to BPDE and 17 passages; tum$^-$ or tum$^+$, testing negatively or positively for tumorigenicity in nude mice.

Table 1 *Progression to tumorigenicity as a function of passage number of BPDE-treated NIH/3T3 cells*

	No. of cells injected	No. of tumors/no. of mice given injections
NIH/3T3 mixed population (untreated)[a]	2×10^7	0/2
NIH/3T3 (10 subclones after passage 17) (untreated)	10^7	0/10
NIH/3T3 BPDE 8[b]	10^7	0/2
NIH/3T3 BPDE 10	10^7	0/2
NIH/3T3 BPDE 12	10^7	0/2
NIH/3T3 BPDE 15	10^7	1/2
NIH/3T3 BPDE 17	10^7	2/2
NIH/3T3 BPDE 17 x NIH/3T3 BPDE 17 hybrids	10^7	4/4

[a] Cells are never allowed to become confluent.
[b] The numeral following BPDE indicates the passage number.

Homologous Reconstructs. NIH/3T3 BPDE 17 (tum$^+$) cells were enucleated in Falcon T-25 flasks in 5 μg/ml of cytochalasin B with 85% efficiency (7500 rpm, 37°C, 30 min). The karyoplasts and a few whole cells that detached during centrifugation were subjected to a differential adhesion for 1.5 h to remove whole cells and karyoplasts containing large amounts of cytoplasm. Based on previous experiments (20) we estimate that ~5% of the cytoplasm is associated with these karyoplasts. During the differential adhesion, nontumorigenic NIH/3T3 cells were enucleated in 10 μg/ml of cytochalasin B (8000 rpm, 25 min). Cytoplasts were obtained with 99% enucleation efficiency. The cytoplasts were incubated with 50 μg/ml of mitomycin C for 2 h in order to kill any unenucleated cells (21). The cytoplasts were washed, trypsinized, counted, pelleted, and incubated for 10 min with 100 μl of a monoclonal antiricin antibody (provided by W. E. Wright) in a hypertonic solution of 0.5 M sucrose and 10% polyethylene glycol according to Wright (22). This procedure allows the antibody to be taken up by the cytoplasts in pinosomes (23). Then prior to degradation of the antibody, the cytoplasts were subjected to hypotonic shock in serum-free medium:distilled water (6:4, v:v). This causes rupture of the pinosomes with ensuing distribution of the antiricin throughout the cytoplast (23). One million cytoplasts were washed 3 times and mixed with the karyoplasts obtained from 5×10^5 BPDE 17 tumorigenic cells. Following fusion with polyethylene glycol, the cells were incubated for 48 h in medium with 10 ng/ml of ricin (provided by E. Vitteta). Fig. 1 depicts a schematic flow sheet for the preparation of homologous recons.

Chromosomal Analysis. Using log-phase cells, the chromosome compositions of the parental cells and recons were analyzed on the air-dried chromosome preparations as described previously (24). Twenty metaphase spreads were counted for each clone analyzed. NIH/3T3 cells are aneuploid containing approximately 58 chromosomes per cell. In all instances the chromosome content of reconstructed cells was consistent with their origin.

Tumorigenicity Testing. Congenitally athymic nude BALB/c mice, homozygous for the *nu/nu* allele, that were 4 to 6 wk old were used for testing the tumorigenicity of the parental cells and recons. Cells were prepared in medium without serum at a concentration of 2×10^6 per 0.1 ml and injected s.c. in the neck or thigh area of lightly anesthetized mice. If tumors (1 to 2 cm in diameter) have not arisen by the end of 4 mo, the cells are considered nontumorigenic.

RESULTS

Homologous Recons. NIH/3T3 cells exposed twice over a 48-h period to BPDE are not tumorigenic when clones are tested at passages 8, 10, and 12 but do become so at passage 15 (one of two mice tested positively) and at passage 17 where both mice tested were positive (Table 1). A population of NIH/3T3 cells not incubated with carcinogen was nontumorigenic as were ten subclones of untreated NIH/3T3 cells that were passaged along with the BPDE-treated ones and that were tested at passage 17 and beyond in nude mice (Table 1). During the passaging of both the BPDE-treated and untreated cells, they were carefully monitored and never allowed to become confluent. Fig. 1 depicts a flow sheet for the preparation of homologous recons using karyoplasts from NIH/3T3 BPDE 17 (tum$^+$) cells rescued by NIH/3T3 (tum$^-$) cytoplasts containing ricin antibody (explained in "Materials and Methods"). None of the controls (NIH/3T3 cytoplasts, NIH/3T3 BPDE 17 karyoplasts, cytoplasts mixed with karyoplasts, and cytoplasts fused to cytoplasts and mixed with karyoplasts fused to karyoplasts and NIH/3T3 BPDE 17 tum$^+$ cells) survived selection in 10 ng/ml of ricin when 10^5 cell parts or 10^5 whole cells were plated. When NIH/3T3 cytoplasts were fused to NIH/3T3 BPDE 17 karyoplasts 165 colonies surviving ricin selection were obtained and 10 were cloned. None was tumorigenic (Table 2). Such findings are significant only when examined in relation to the results obtained on several other controls that were run simultaneously. Recons from normal cell parts (10 of 10 clones) were negative in contrast to those prepared from tumor cell parts at passage 17 which were positive (4 of 4 clones; Table 2). NIH/3T3 BPDE 17 cells were fused to themselves, and four clones that were tested for tumorigenicity were all positive (Table 1). If any cybrids had been formed in the fusion of NIH/3T3 BPDE 17 karyoplasts and NIH/3T3 cytoplasts, they would have survived the selection also. While it is unlikely that all ten clones were cybrids, our data are still significant because whether the clones were recons or cybrids they were all suppressed (Table 2).

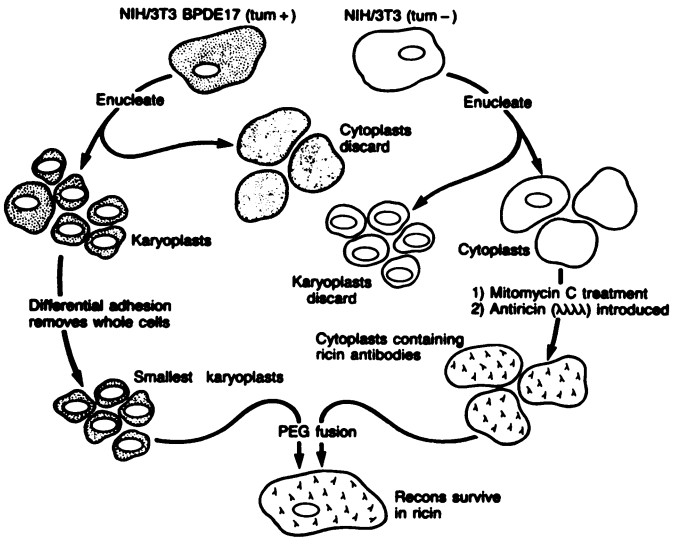

Fig. 1. Flow sheet for construction of homologous recons between NIH/3T3 BPDE 17 tum$^+$ karyoplasts and NIH/3T3 tum$^-$ cytoplasts. *PEG*, polyethylene glycol.

Table 2 *Suppression of tumorigenicity in homologous recons of NIH/3T3 cells*
The number of population doublings of the recons and controls at the time of injection was approximately 30.

Recon cells injected[a]	No. of clones tested	No. of cells injected	No of tumors/no. of mice given injections[b]
NIH/3T3(C) x	10	5×10^6	0/10
NIH/3T3(K)		10^7	0/10
NIH/3T3 BPDE 17(C) x	4	5×10^6	3/4
NIH/3T3 BPDE 17(K)		10^7	4/4
NIH/3T3(C) x	10	5×10^6	0/10
NIH/3T3 BPDE 17(K)		10^7	0/10

[a] C, cytoplast; K, karyoplast.
[b] Each mouse was given an injection of a different clone of reconstructed cells.

DISCUSSION

NIH/3T3 cells require extensive passaging after exposure to BPDE before they become tumorigenic (Table 1). Similar observations have been made with other carcinogens, benzo(*a*)pyrene (17, 25), 3-methylcholanthrene (26), *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (26), dimethylsulfate (17), and *N*-methylnitrosourea (17), and other cells, Syrian hamster embryo cells (17, 26) and fetal guinea pig cells (26). This period after carcinogen exposure and prior to the onset of tumorigenicity, which has not been defined molecularly, is referred to as progression.

The karyoplasts that were used to construct recons were derived from a clone that had survived exposure to 1 μg/ml of BPDE twice in a 48-h period and that had progressed to tumorigenicity after 17 passages. This clone was tumorigenic by passage 15 as well as at passage 17 (Table 1). The suppression observed in the reconstructed cells (10 of 10 clones) was of karyoplasts obtained from these tumorigenic NIH/3T3 cells at passage 17. Even though these cells may consist of both tum^+ and tum^- cells, it is unlikely that all recons would be made only from tum^- cells, because in one control experiment NIH/3T3 BPDE 17 cells were fused to themselves, and the 4 clones tested were tum^+ (Table 1). In addition, recons made from karyoplasts fused to cytoplasts, both isolated from NIH/3T3 BPDE 17, were tum^+ (Table 2).

The total suppression of tumorigenicity observed in our recon experiments agrees with the recent data of Israel and Schaeffer (27). They rescued karyoplasts of spontaneously transformed rat epithelial cells with nontumorigenic cytoplasts of the same cell type. In these homologous recons there was virtually total suppression of tumorigenicity. Of sixty-eight animals given injections only one developed a tumor after 1 yr. Data from Sager's laboratory (11) are not as conclusive: homologous recons prepared from karyoplasts of tumorigenic Chinese hamster embryo fibroblasts and nontumorigenic cytoplasts were in some cases suppressed while in others tumorigenic.

In earlier studies (13, 14, 28) attempts were made to suppress the tumorigenicity of whole cells by fusing normal cytoplasts to them, *i.e.*, by cybrid formation. The data obtained were not as decisive as are the recon data reported here and those of Israel and Schaeffer (27). In the cybrid data reviewed by Shay (12) suppression varied from 20 to 90% in various cybrids made from Chinese hamster embryo fibroblasts, rat thyroid cells, mouse myogenic cells, and rat hepatocytes. Similarly cybrids made by Israel and Schaeffer (27) with rat epithelial cells varied in suppression from 14 to 100%.

There are several factors that may influence the success or failure of cybrid and recon experiments designed to uncover cytoplasmic suppressors of tumorigenicity: (*a*) the relative amounts of tumorigenic and nontumorigenic cytoplasm in cybrids; (*b*) the time interval that cybrids or recons are passaged prior to testing their tumorigenicity; (*c*) whether mutagenesis with carcinogens was used to introduce genetic markers on the cells; and (*d*) the specific cell combinations used. Hence it is not surprising that investigators have had varying results in such experiments. Thus cybrids obtained from the fusion of whole human HeLa cells with normal human diploid primary skin fibroblast cytoplasts are tumorigenic after prolonged cultivation, even though at this time the mitochondrial DNA is almost exclusively fibroblast (29). Also when normal cytoplasts from rat embryo cells were fused to whole rat glioma cells, the cybrid clones were tumorigenic as were recons prepared from karyoplasts of tumorigenic Y-1 cells and nontumorigenic AMT cytoplasts (30). The lack of repression in these instances may reflect other unknown factors not enumerated.

Israel and Schaeffer (27) point out several difficulties in interpretation that arise when examining suppression in heterologous cybrids. This led them to use homologous cybrids and recons. Because most mutagens are also carcinogens, the cells they used were spontaneous mutants that arose during growth of the cells in 6-thioguanine or in tevenel, an analogue of chloramphenicol. The use of ricin-antiricin selection in the present studies precluded the necessity of mutagenesis of NIH/3T3 cells to introduce nuclear- and cytoplasmic-coded markers. In the experiments of Israel and Schaeffer and those presently described the results are decisive. These findings suggest that cytoplasmic suppression of tumorigenicity may be more easily evinced by the use of recons made from cell parts of unmutagenized cells. Of course the use of recons also minimizes the possible dilution effects of the tumorigenic cytoplasm on the suppressors.

The work of Stanbridge (15), Klinger and Kaelbling (31), and Sager (11) with human cells has revealed that tumorigenicity is suppressed in hybrids of tumorigenic and nontumorigenic cells. Rare revertant cells that have lost specific chromosomes from the nontumorigenic parent become tumorigenic. This finding and others have led to the concept of nuclear suppressors of tumorigenicity. To ascertain whether the factor(s) responsible for cytoplasmic suppression are different from those that mediate nuclear suppression will require the isolation and identification of both kinds of suppressors.

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