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Establishment of Mouse Colonic Carcinoma Cell Lines with Different Metastatic Properties[1]

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ABSTRACT

Tumorigenic cell lines were established in culture from three transplantable mouse colonic carcinomas designated CT 26, CT36, and CT 51. The cultured lines were characterized for the retention of the biological characteristics of the parental lines. All three cultured lines retained the ability to form tumors *in vivo*. Serially transplanted parental lines CT 26 and CT 51 grew at a faster rate than did CT 36 and showed a greater propensity for the formation of lung metastases. Similar characteristics were exhibited by the tumors formed from the injection of cultured cells. The cultured cell lines were also evaluated with respect to a number of *in vitro* markers for cancer. Cultured CT 26 and CT 51 cells formed tumors at lower inocula than did CT 36. CT 26 and CT 51 showed anchorage-independent growth and lack of contact inhibition, while CT 36 grew as a strict monolayer and did not form colonies in 0.27% agarose. CT 26 had the highest saturation density of the cell lines when grown in media supplemented with either 10 or 2.5% fetal bovine serum, while CT 51 had the lowest saturation density under these conditions. The varying degrees of malignancy exhibited by the three cell lines and the overall retention of the biological characteristics of the parental lines by the cultured lines suggest that the cultured cells (without the contaminating stromal elements present in the serially transplanted lines) will provide suitable material for the investigation of the molecular bases of these malignant characteristics.

INTRODUCTION

Recent reports have described the development of a series of chemically induced transplantable murine tumors of colonic origin carried in BALB/c mice (7–9). Of particular interest in this series of tumors are their varying biological and pharmacological properties which are considered representative of the spectrum of properties observed in human colonic carcinoma. In serial transplantation, these murine colon tumors (designated CT26, CT 51, and CT 36) show variability with respect to rates of growth, latency periods between transplantation and the development of tumors and death of the recipient animal, responses to experimental therapeutic regimens, and abilities for metastatic spread. CT 26 is the most aggressive of the 3 tumors by the criteria of growth rate and metastatic spread, while CT 36 is the least aggressive. CT 51 is intermediately aggressive but resembles CT 26 more closely than it resembles CT 36.

The availability of large numbers of malignant cells in tissue culture without the other types of cells found in the stroma of tumors (*e.g.*, blood cells and fibroblasts) would facilitate studies of the underlying molecular bases for the various properties exhibited by these tumors *in vivo* (3, 4). Long-term cultures of all 3 tumors have been established (6, 17), but the cell lines have not been extensively characterized. It is essential for molecular studies that established cell lines be shown to retain the characteristics of the serially transplanted parent tumors. The objects of this study were 2-fold: to establish tumorigenic cell lines from the 3 transplantable tumors which, when injected back into BALB/c mice, would have similar growth characteristics to those of the parental lines, and to determine the degree of correlation between the *in vivo* characteristics of tumors from these cell lines and several commonly utilized *in vitro* markers for cancer (15).

MATERIALS AND METHODS

Establishment of Cell Lines. Transplantable Tumors CT 26, CT 36, and CT 51 were obtained in BALB/c mice from Dr. T. H. Corbett of Southern Research Institute, Birmingham, Ala. The characterisitcs of these tumors in serial transplantation have been described in detail (7–9). With sterile technique, tumors (1 to 2 cm in diameter) were excised and minced into 1- to 2-cu mm pieces in McCoy's tissue culture medium supplemented with 20% HFBS[4] and antibiotics (4.3 μg of gentamicin per ml, 90 units of streptomycin per ml, and 90 μg of penicillin per ml). Tissue pieces were washed 3 times for 10-min periods with stirring, followed by decantation of the medium. Following the third wash period, the pieces of tissue were disaggregated with 0.25% trypsin (Microbiological Associates, Bethesda, Md.) for 8 periods of 20 min as described previously for human colonic carcinoma (3). Cells obtained from all 3 tumors by this procedure were greater than 90% viable by trypan blue exclusion. Cells from all 8 periods of trypsin digestion were combined, sedimented at 97 × *g* for 7.5 min, resuspended in medium, filtered through Nitex (TETKO, Inc., Elmsford, N. Y.) with a pore diameter of 48 μm, and inoculated into 75-sq cm tissue culture flasks. Cultures were maintained at 37° in a humidified atmosphere with 5% CO_2. At an initial inocula of 2 × 10^6 cells, CT 26 and CT 51 cultures became confluent after 10 to 14 days, while CT 36 cultures required approximately 3 weeks to reach confluency. Cultures received complete media changes every 2 to 3 days. Primary cultures were subcultured with 0.25% trypsin as described previously (2) and, for maintenance, were replated at 1 × 10^6 cells in 75-

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[4] The abbreviation used is: HFBS, heat-inactivated fetal bovine serum.

sq cm flasks. Routinely, cultures were passaged weekly and maintained in medium supplemented with 10% from HFBS. All of the studies in this report were performed with cells from passages 10 to 30. The absence of *Mycoplasma* contamination was indicated by a commercially available *Mycoplasma* test kit (Flow Laboratories, Rockville, Md.).

Determination of *in Vitro* growth parameters. Growth parameters were determined from cultures obtained by seeding 10^3 to 5×10^3 cells in 9.6-sq cm Falcon tissue culture dishes. Saturation densities were determined by counting cells every other day until the numbers of cells in 9.6-sq cm dishes were approximately constant for 8 days. All counts were performed with a Model ZF Coulter Counter on duplicate cultures dispersed by 0.1% trypsin (subsequently neutralized with 1% HFBS in 0.9% NaCl solution). Mitotic indices were determined by counting cells stained with a mixture of crystal violet and methylcellulose, as described by Glinos and Werrlein (11). Generation time was determined as described by Paul (16). Determination of the ability to grow on fibroblasts was performed by seeding 10^4 cells from the tumor lines onto confluent C3H/10T½ mouse fibroblasts in 25-sq cm flasks. Growth in 0.27% agarose on underlayers of 0.5% agarose was determined for inocula ranging from 10^2 to 10^4 cells of each line, as described previously (13).

Tumorigenicity. Cells were dispersed as described above and injected s.c. into animals in concentrations ranging from 10^5 to 4×10^6 cells. Animals were observed daily for up to 4 months for the development of tumors, and the period between injection and the appearance of a tumor 1 to 2 cm in diameter was recorded for each animal (referred to as latency period in this study). Cultures of metastatic cells were initiated after removal and treatment of the entire lung by the disaggregation procedure described above. The tumorigenicity of metastatic cells was determined by injecting 10^6 cells from the third passage cultures. In other experiments, lungs with grossly evident metastases were minced into small pieces (1 to 2 cu mm) and transplanted back into BALB/c mice.

RESULTS

Morphology of Cultured Cells. While CT 36 appeared homogeneous by phase-contrast microscopy, CT 26 and CT 51 were heterogeneous with respect to cellular morphology (Figs. 1 to 3). We attempted to resolve the heterogeneity of these cultures by cloning. Clones of CT 26 and CT 51 (derived from single cells plated into individual wells of Linbro microtiter plates or from colonies grown in 0.27% agarose) were cultured as described in "Materials and Methods." Over 20 cultures of different clones were examined, and each was found to be heterogeneous with respect to cellular morphology. It is not known whether the observed heterogeneity is due to the state of differentiation of individual cells or is possibly associated with the phase of the cell cycle of particular cells. Both CT 26 and CT 51 show a lack of contact inhibition, while CT 36 grows as a strict monolayer without any evidence of cellular piling.

Growth Properties of Cultured Cells. Growth parameters of the 3 colon tumors *in vitro* are listed in Table 1. CT 26 and CT 51 had similar generation times and grew at a considerably faster rate than did CT 36. Interestingly, the saturation density of CT 51 was only 67% that of CT 36. CT 26 had the largest mitotic index during proliferation, while that of CT 51 was slightly greater than that of CT 36. The relative rates of growth of the 3 cell lines *in vitro* approximated the reported relative *in vivo* growth rates of the parental transplantable lines carried in BALB/c mice (9).

Each of the cell lines was characterized for a number of *in vitro* growth properties associated with cancer, including reduced requirements for serum supplementation of growth media, growth on confluent fibroblasts, and anchorage-independent growth in semisolid medium. Reduction of HFBS from 10 to 2.5% did not affect the generation time or saturation density of CT 26 cultures. On the other hand, when CT 51 and CT 36 were grown in 2.5% HFBS, generation times were increased and saturation densities were reduced to approximately 20% of those for cultures grown in 10% HFBS (Table 1). While all 3 lines were capable of growth on confluent fibroblasts, only CT 36 was not capable of forming colonies in 0.27% agarose (Table 2). CT 26 and CT 51 showed large, concentration-dependent colony formation in 0.27% agarose, except for the highest inoculum tested (Table 2).

Tumorigenicity and the Formation of Metastases. CT 26 and CT 51 were highly tumorigenic at doses ranging from 10^5 to 4×10^6 cells, but inocula of less than 4×10^6 CT 36 cells failed to produce tumors in the 4-month observation period for animals given injections (Table 3). As the size of the inoculum was increased, latency periods for the appearance of palpable tumors (1 to 2 cm in diameter) were decreased for both CT 26 and CT 51. While the period of time between injection and death appeared to be dose dependent for CT 51 cells, the size of the inoculum did not appear to have a significant effect on animals given injections of CT 26. At inocula of 4×10^6 CT 36 cells, the latency periods for the appearance of tumors were 2 to 3 times as long as those for similar inocula of CT 27 and CT 51. The period between the injection of equal doses of cells and death was approximately twice as long for CT 36 as for the other lines (Table 3). The relative growth behavior of tumors resulting from the 3 cultured cell lines was quite similar to that

Table 1

Growth characteristics of murine colonic carcinoma lines in vitro

Cell line	Generation time (hr)	Saturation density (10^4 cells/sq cm)	Saturation density in 2.5% HFBS (10^4 cells/sq cm)	Mitotic index (no. of mitoses/100 cells)
CT 26	20.5	35	35.7	2.9
CT 51	24	10	2.7	2.2
CT 36	34	15	4.2	1.9

Table 2

Soft agarose culture of murine colonic carcinoma lines

Cell line	Inoculum (cells/9.6 sq cm dish)	Plating efficiency (%)
CT 26	10^2	22
	10^3	41
	10^4	30
	10^5	4
CT 51	10^2	46
	10^3	25
	10^4	28
	10^5	6
CT 36[a]		

[a] No colony formation at any inoculum.

Table 3
Tumorigenicity of cultured murine colonic carcinoma lines

Cell line	No. of cells in inoculum	No. of animals inoculated/no. of tumors	Average no. of days for tumor to attain diameter of 1 to 2 cm	Average no. of days for death to occur after inoculation
CT 26	1×10^5	8/10	15	38
	1×10^6	10/10	12.5	33
	4×10^6	9/10	9.5	33
CT 51	1×10^5	3/5	17	64
	1×10^6	9/12	10.5	48
	4×10^6	9/10	8	31
CT 36	1×10^5	0/7		
	1×10^6	0/8		
	4×10^6	4/5	20	66

observed by Corbett *et al.* (9) for the parental transplantable tumors.

Metastatic ability was evaluated in separate experiments from the tumorigenicity studies. In initial experiments, lungs were removed 3 weeks after the injection of 4×10^6 cultured CT 26 cells, minced as described in "Materials and Methods," and subsequently transplanted to new recipients. Approximately 50% of the animals implanted with the same lung tissue from any single mouse given injections of cultured CT 26 cells developed tumors. Consequently, in an effort to improve tumor takes, cells from lungs of injected mice were cultured prior to the inoculation of new recipients.

Cultures of metastatic cells from minced-lung tissue were initiated, as described in "Materials and Methods," 3 weeks after the injection of cultured CT 26 or CT 51 cells. Metastatic nodules were apparent on the surface of 10 of 10 animals given injections of CT 26 and 7 of 8 animals given injections of CT 51. Separate cultures were initiated from the lungs of each mouse with metastatic nodules. The resulting culture from the mouse given injections of CT 51, but without grossly evident lung metastases, failed to survive 2 passages. After 3 passages, 10^6 cells from each of 5 of of the cultures of metastatic cells of both CT 26 and CT 51 were injected into 3 BALB/c mice, and all animals subsequently developed tumors. Grossly evident lung metastases were apparent at autopsy of the recipient animals. The morphological appearances of the cultures derived from metastatic cells were heterogeneous and identical to those of the parental CT 26 and CT 51 lines. After 40 days postinjection of cultured CT 36 cells, mice with tumors had no apparent metastases. Cultures derived from these lungs appeared fibroblastic in nature and, after 3 passages, failed to give rise to tumors at doses as high as 6×10^6 cells.

DISCUSSION

We have established cultured cell lines from 3 chemically induced transplantable murine colon tumors. Both *in vitro* and *in vivo* characterizations of the 3 cell lines indicate that they closely resemble cells of the serially transplanted tumors from which they were derived. The biological properties of the parental tumors in serial transplantation have been described in detail (7–9). Serially transplanted Tumors 26 and 51 were found to be very similar with regard to growth properties and metastatic propensity, with Tumor 26 being slightly more aggressive *in vivo* than Tumor 51. Serially transplanted Tumor 36 had a slower rate of growth and rarely metastasized. Tumors that resulted from injecting 4×10^6 cultured cells from each line closely approximated the reported results with serially transplanted tumors (arising from 20 mg trocar implantations) with regard to the length of time before tumor appearance, death of the recipient animal, and metastatic ability (7–9, 12).

The relative malignancy and aggressiveness of the parental lines and cultured cell lines *in vivo* was also reflected in a number of *in vitro* characterizations for cancer. CT 26 cells consistently displayed malignant attributes by all of the *in vitro* markers utilized: growth and cell density were unaffected by decreasing concentrations of HFBS in culture medium; high plating efficiencies were obtained in semisolid medium; culture morphology showed a lack of contact inhibition; and cells grew on confluent cultures of mouse fibroblasts. Although cultured CT 36 cells are tumorigenic at high doses, this line does not grow in 0.27% agarose, nor does it show a lack of contact inhibition. In addition, low concentrations of HFBS result in reduced growth rates and lower saturation densities. CT 51, which appears to be of intermediate malignancy with respect to the serially transplanted parental tumors (1, 7–9), is also intermediate to the other cultured lines with respect to *in vitro* markers for cancer. CT 51 plating efficiencies in semisolid medium are similar to those of CT 26; cultures show a lack of contact inhibition, and CT 51 cells grow on confluent fibroblasts. However, reduced concentrations of HFBS lower the growth rate and saturation density of this line.

These results suggest that the cultured cell lines of these colonic tumors provide suitable models for studies directed at elucidating the molecular bases for the various biological properties listed above. Of particular importance in this regard is the retention of the ability for metastasis by cultured CT 26 and CT 51 cells. The availability of cultured cell lines offers investigators greater precision in designing experiments to study the metastatic process (10). Moreover, the absence of stromal elements in the cultured lines will facilitate studies aimed at the molecular bases of the metastatic process. Whereas models for the study of metastasis or selective implantation of tumor cells have been developed for a murine lymphosarcoma (5), sarcoma, and melanoma (10, 14), there are currently no metastatic models of colonic cancer carried in cell culture. One drawback of this model with regard to human disease is, of course, the lack of metastasis to the liver (a major site of metastases from human colonic carcinoma). However, studies of these cultured cell lines should provide more valuable information for the metastatic process of a carcinoma than do the types of tumors previously available for these studies.

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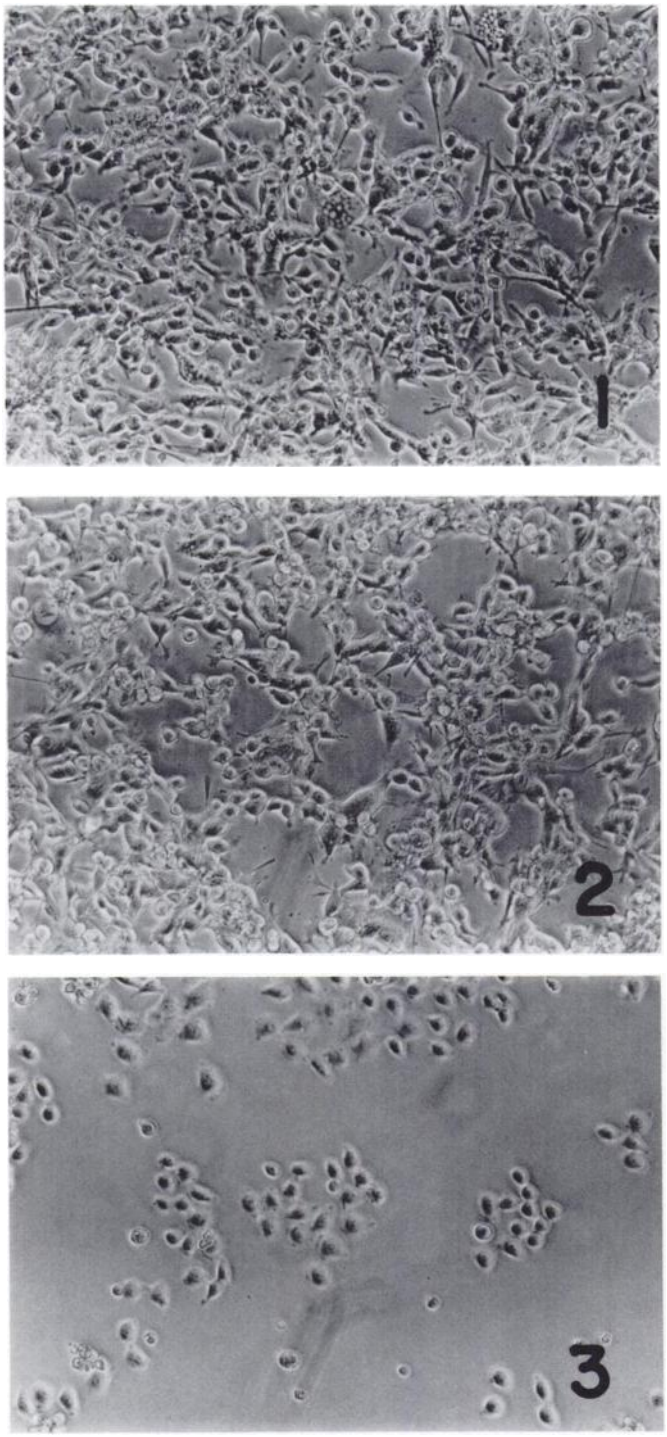

Fig. 1. Phase-contrast microscopy of CT 26 cells in culture. × 100.
Fig. 2. Phase-contrast microscopy of CT 51 cells in culture. × 100.
Fig. 3. Phase-contrast microscopy of CT 36 cells in culture. × 100.