Heterogeneity of Malignant Cells from a Human Colonic Carcinoma

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ABSTRACT

Three subpopulations of malignant cells were isolated from a primary cell culture of a single human colonic carcinoma. The variant lines were tumorigenic in athymic nude mice given fluent fibroblasts, while colony formation by HCT 116 was showed the highest colony formation in agarose and on confluent layers of mouse fibroblasts. HCT 116a showed the highest colony formation in agarose and on confluent fibroblasts, while colony formation by HCT 116 was higher than that of HCT 116b in both of these systems. All of the variant lines were tumorigenic in athymic nude mice given injections of $10^6$ cells, but the time between inoculation and tumor development (latency period) was approximately 10 times longer for HCT 116b as for HCT 116a and 8 times longer than for HCT 116. HCT 116b was not tumorigenic at an inoculum of $5 \times 10^6$ cells, while both HCT 116 and 116a were tumorigenic at this level. However, HCT 116a was clearly more tumorigenic than was HCT 116 on the basis of the number of animals developing tumors at inocula of both $10 \times 10^6$ and $5 \times 10^6$ cells and on the basis of their differences in latency periods. While all the cell lines had near diploid numbers of chromosomes, each line showed a distinct histological pattern when grown as xenografts in athymic nude mice.

INTRODUCTION

Recent investigations have indicated morphological and functional heterogeneity among the malignant cells of individual colonic carcinomas. Studies from our laboratory demonstrated morphological heterogeneity among the malignant cells purified from individual primary human colonic carcinomas (5). Dexter et al. (8) described the isolation of 2 clones of malignant cells from a single colonic carcinoma. These 2 clones were different with regard to several in vitro properties, including morphology, cloning efficiency in soft agar, and saturation density. However, differences between the in vivo tumorigenic capabilities of the 2 clones were not described. Recently, we described the isolation of 2 subpopulations of malignant cells from an established cell line of human colonic carcinoma (HT29) with different tumorigenic capabilities (14). In this study, we describe the characterization of 3 subpopulations of malignant cells with different tumorigenic capabilities isolated from a primary culture of a human colonic carcinoma.

MATERIALS AND METHODS

Tissue Culture. A primary human colonic carcinoma, designated HCT 116, was obtained from surgery through the auspices of the UAB Tissue Procurement Service. The tumor was prepared for tissue culture as described previously (2, 6). Briefly, the tumor was minced into 1- to 2-cm pieces in McCoy's tissue culture medium containing 20% FBS and antibiotics [gentamicin 4.3 µg/ml], streptomycin (90 µg/ml), and penicillin (90 units/ml), washed extensively in the same medium, and disaggregated with 0.25% trypsin (Grand Island Biological Co., Grand Island, N. Y.) for 8 periods of 20 min as described previously for human colonic carcinoma (4). Cells obtained by this procedure were greater than 90% viable by trypan blue exclusion. Cells (10³) or trypsinized chunks of tissue remaining after the eighth trypsin treatment were plated into 75-cm² flasks in the medium described above and maintained at 37° in a humidified atmosphere of 5% CO₂.

Once they were established, cultures were maintained in the same medium described above except that FBS levels were reduced to 10%. Confluent cultures were subcultured with 0.25% trypsin in Joklik's tissue culture medium containing 0.1% EDTA.

Separation of Subpopulations. Primary cultures of HCT 116 contained 3 morphological variants. These variants (designated HCT 116, HCT 116a, and HCT 116b) were separated and isolated by sedimentation of cells from primary cultures in a linear density gradient of Percoll and tissue culture medium (1.02 to 1.13 g/ml). Procedures similar to those described previously were utilized for centrifugation, collection of fractions containing separated cells, and tissue culture (3, 4, 17). Briefly, separation of $10 \times 10^6$ cells from primary cultures was achieved after centrifugation at 1000 x $g$ for 30 min at 4° in a CRU 5000 centrifuge. The sterile density gradient was collected by displacement with 60% sucrose, and the cells were harvested by centrifugation at 97 x $g$ for 7.5 min after dilution of the fractions with tissue culture medium. Pelleted cells were resuspended in $5 \times 10^5$ of the growth medium containing 10% FBS (described above) and inoculated into 25-cm² tissue culture flasks until sufficient numbers of cells had grown for subculture and subsequent inoculation of 75-cm² flasks.

Determination of Growth Characteristics in Vitro. Saturation density and doubling times were determined from growth curves as we have described previously (6). Cells ($10^6$) were inoculated into 25-cm² dishes and counted every other day after trypsinization until cell numbers were constant for a 6-day period. Cell counts on duplicate cultures were performed with hemocytometer chambers.

Determination of growth in semisolid medium was performed as we have described previously (14, 15). Briefly, $5 \times 10^4$ cells were suspended in the growth medium containing 0.5% agarose and inoculated onto previously formed underlayers of 1% agarose containing growth medium in 9.6-cm² tissue culture dishes. The dishes were equilibrated overnight in 5% CO₂ at 37° and then sealed with Parafilm to prevent drying. After 3 weeks of incubation, colony formation was determined by microscopically counting colonies of 20 or more cells.

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counting, agarose cultures were fixed with glutaraldehyde. Control dishes were fixed with glutaraldehyde immediately after solidification of the 0.5% agarose containing cells.

The ability to grow on confluent layers of C3H10T½ mouse fibroblasts in 25-sq cm flasks was determined by plating 5 x 10⁴ cells and microscopically counting the resultant colonies after 7 days. Cocultures of tumor cells and fibroblasts were given a complete medium change 4 days after seeding and immediately prior to scoring colonies on the seventh day.

Cancer was determined with the commercially available radioimmunoassay as we have described previously (13). CEA assays were performed on cells from confluent cultures and on spent media which had been in contact with confluent cultures of cells for 72 hr. In the case of HCT 116a cells, which will not form completely confluent cultures, assays were performed on cells and spent media from cultures containing the highest cell density obtained with this line.

Modal chromosome numbers were determined as described previously after blocking cultured cells at metaphase by a 24-hr Colcemid treatment (14). Briefly, treated cells were resuspended in water for 10 min at 37°C, harvested by centrifugation, fixed in methanol:acetic acid (3:1, v/v) for 1.5 hr, applied to slides, and stained with 20% Giemsa for 7 min.

Tumorigenicity. Athymic nude mice were given s.c. injections with trypsinized or scraped cells in tissue culture medium without HFBS. Animals were observed daily for the formation of tumors, and the period between injection and tumor development (at least 1 cm in diameter) was referred to as the "latency period" in this study. Tumors were removed from the parent cultures for the remainder of the studies described in "Materials and Methods." Density ranges were determined by refractometry of the individual fractions as described by Pertot and Laurent (17). Fractions were determined to contain a given cell type on the basis of whether colonies of the cell type were observed in cultures derived from the cells recovered from that fraction.

Table 1

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Fractions in which cell type was recovered</th>
<th>Density range for pure cultures of the cell type</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT 116a</td>
<td>1.026–1.068</td>
<td>1.026–1.032</td>
</tr>
<tr>
<td>HCT 116</td>
<td>1.037–1.080</td>
<td>1.050–1.074</td>
</tr>
<tr>
<td>HCT 116b</td>
<td>1.044–1.082</td>
<td>1.086–1.092</td>
</tr>
</tbody>
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The cells isolated from the gradient at a density of 1.026 g/ml contained a mixture of fibroblasts and HCT 116a cells. The number of cells recovered at this density amounted to approximately 6% of the cells recovered from the gradient. After growth in culture, HCT 116a cells were separated from contaminating fibroblasts by a second density gradient centrifugation. No fibroblasts were observed in cultures initiated with cells from a density of 1.092 g/ml (HCT 116b). Cells recovered at this density amounted to less than 3% of the cells recovered from the density gradient. When cells grown from cultures initiated with low-density HCT 116a cells or high-density HCT 116b cells were applied to a second density gradient centrifugation, they exhibited the full range of densities shown by the original cells from the primary cultures.

Since HCT 116 was present in considerably greater concentrations than the other subpopulations, it was isolated by repeated differential trypsinization and reseeding at low cell concentrations in a manner similar to that used by Dexter et al. (9) for the isolation of a subpopulation of cells from a mammary tumor and by Tan et al. (23) for the removal of fibroblasts from cultures of mouse colon carcinoma cells. Our procedure is briefly described below. Cultures of cells obtained from the density range of 1.050 to 1.074 g/ml were allowed to grow to near confluency. These cultures contained HCT 116a cells, HCT 116b cells, and fibroblasts, but crude estimates by phase-contrast microscopy indicated that on the order of 75 to 90% of the surface area of the flask was covered with HCT 116 cells. In order to obtain pure HCT 116 cells, flasks were subjected to our normal subculture procedure involving 0.25% trypsin:0.1% EDTA except that instead of allowing a 10-min exposure, the trypsin:EDTA mixture was removed after 3 min at 37°C. The cells were then harvested by centrifugation and inoculated into a 25-sq cm flask. Fresh trypsin:EDTA was added to the original culture flasks, and the above procedure was repeated for 3 additional periods. Cells harvested after the fourth period of trypsin:EDTA exposure were seeded into flasks at a concentration of 10⁴ cells/flask and allowed to grow to near confluency (approximately 10 days). Examination of the cultures by phase-contrast microscopy indicated that no other cell types except HCT 116 were present. However, the cultures were again subjected to the same differential trypsinization procedure described above. Cultures of HCT 116 cells obtained from the fourth trypsin:EDTA treatment were utilized as parent cultures for the remainder of the studies described below. The characterizations described below were performed at passage 10 or above for each of the "purified" cell types.
**In Vitro Growth Characterization.** The growth characteristics of the 3 variant populations are presented in Table 2. Doubling times for the 3 variants were not significantly different. The saturation densities of HCT 116 and HCT 116a were approximately twice that of HCT 116b. It should be noted, however, that HCT 116a would never grow to confluency (i.e., completely cover the surface area of the flask). All of the variants formed colonies when grown in semisolid medium or on confluent layers of mouse fibroblasts. HCT 116a cells formed the highest number of colonies in soft agarose culture and on mouse fibroblasts, while HCT 116 cells showed higher levels of colony formation than did HCT 116b under those conditions. It is interesting to note that the efficiency of colony formation was generally higher on confluent fibroblasts than in semisolid medium for all 3 variants. HCT 116a cells had very high levels of CEA relative to HCT 116 cells, while HCT 116b cells contained negligible levels of this antigen.

**Tumorigenicity.** The 3 subpopulations showed variation with respect to their tumorigenic abilities when injected s.c. into athymic nude mice (Table 3). The relative tumorigenic abilities of the variants were consistent with their in vitro abilities for growth in semisolid medium and on confluent fibroblasts. HCT 116a cells formed tumors at both high and low inocula of mice and had the shortest latency periods relative to the other variant subpopulations (5 and 9 days for inocula of 10 × 10^5 and 5 × 10^6 cells, respectively). HCT 116 cells showed intermediate tumorigenic ability as approximately 50% of the animals given injections at the lower inocula developed tumors with an average latency period of 16 days. Injection of 10 × 10^6 HCT 116b cells resulted in xenograft formation in 50% of the recipient animals; however, latency periods were much higher than for HCT 116a or HCT 116. Inoculation of 5 × 10^6 HCT 116b cells failed to produce tumors in 6 mice observed for periods ranging from 2 to 4 months (depending upon the survival of the individual mouse). In order to determine the stability of the variant lines, tumorigenicity experiments were conducted at 9 and 13 months after the establishment of pure cultures of the subpopulations. No differences in their tumorigenicity were observed at these times.

Histological examination of the tumors grown in nude mice indicated that all 3 types of cells gave rise to epithelioid tumors. Tumors from HCT 116a cells are less differentiated than are tumors resulting from the injection of HCT 116 or HCT 116b cells (Figs. 5 to 7). Tumors from HCT 116b cells show a consistently different histological pattern from those obtained from HCT 116 cells. All 3 variants showed a modal chromosome number of 46 (counting at least 50 mitoses). Less than 10% of the mitoses contained 45 chromosomes in each of the variants, and when 45 chromosomes were present, the missing chromosome appeared to be a random loss.

**DISCUSSION**

Heterogeneity of the malignant cells comprising individual tumors has been described for a number of cellular and functional properties. Among others, these include morphology and state of differentiation (18, 19), metastatic and invasive ability (9-11, 16), karyotype (1), and pharmacological response to drugs (7, 12). More complete summaries of the identification and isolation of heterogeneous subpopulations of malignant cells have recently been published (7, 20). The studies described in this report demonstrate the potential complexity of human colon cancer with regard to the presence of diverse cell types with variable functional capabilities.

Several investigators (7-9, 20) have summarized some implications of tumor heterogeneity in relation to therapeutic approaches. The existence of discrete subpopulations raises several questions regarding both the biological expression of cancer and its treatment. From the standpoint of the chemotherapy of cancer, heterogeneous subpopulations of malignant cells suggest that some subpopulations in an individual tumor may respond well to particular therapeutic regimens while others may not respond at all. Current evidence would seem to indicate that this is indeed the case in some systems (12). It follows then that the identification and characterization of the subpopulations of cells comprising an individual tumor might be of paramount importance in its treatment. In vitro techniques, such as the stem cell soft agar assay (22), for the prediction of drug sensitivity may be quite lacking for many tumors in that subpopulations comprising a small percentage of the cells could be insensitive to the particular drug tested and yet go undetected because of their small contribution to the total numbers of colonies observed in untreated controls (14). The currently available evidence does suggest that stem cell assays are more effective for predicting which drugs will not be effective for treating a patient's tumor, rather than predicting drugs to which a particular tumor would be sensitive (24).

Morphological heterogeneity of purified cells from colonic cancer has been described (3, 5, 21). Dexter et al. (8) described the isolation of 2 clones of cells derived from a single human colonic cancer. These clones showed some variation in their in vitro growth properties, but differences in their tumorigenic capability in athymic nude mice were not described. We have described the isolation of a subpopulation of cells from...
an established human colon carcinoma (HT29) which forms metastasis in nude mice, whereas the parental cells will form tumors only at the primary site of injection (14). However, this subpopulation of cells could have developed as a result of progression during the long period in which the cells have been in culture or as a response to the treatment of the cells with 5-fluorouracil. In this study, we have characterized variant cell subpopulations from a single primary colon carcinoma which have different degrees of tumorigenic potential as judged by their abilities to form tumors in nude mice. An important question arising from the identification of heterogeneous subpopulations concerns the nature of their origin. Are these subpopulations the result of different stages of differentiation, or do they represent different levels of progressive mutation? We cannot answer this question at present, but it would be relevant to point out that most of the cells of all the subpopulations described in this study contain normal chromosome numbers. This is in contrast to the subpopulations isolated from human colon carcinomas which have been described in previous reports (8, 9). While chromosome data and the state of differentiation observed on histological sections of nude mouse tumors arising from the injection of the subpopulations are certainly not proof that these subpopulations are related by a common path of differentiation, the evidence presently available suggests that they might be a good model for studies of differentiation in human colon carcinoma.

The utility of in vitro markers for the prediction of tumorigenic potential has been of considerable interest in recent years. There are, however, relatively few systems for which cells of differing tumorigenic and aggressive abilities derived from the same tumor are available for comparison and evaluation of markers. HCT 116 and its variants were characterized with respect to their abilities to grow on confluent fibroblasts and in semisolid medium. It is interesting to note that, in this system, increasing tumorigenic potential of the individual subpopulations is associated with high colony formation on fibroblasts and in agarose. On the other hand, the characterization of metastatic and nonmetastatic cells from the established cell line HT29 indicated that the more aggressive metastatic cells showed lower colony formation in semisolid medium than did their nonmetastatic counterparts (14). Studies performed with subpopulations of cells isolated from a murine mammary tumor also indicated that the more aggressive cell types in vivo did not necessarily show a high degree of tumorigenic potential by the criteria of in vitro markers of tumorigenicity (9). Thus, it does not appear to be a general rule that in vitro markers reflect the in vivo tumorigenic potential of cell lines.

While the variant cell lines established in this study have retained the morphologies seen in the primary explant of the original colon tumor, they have been in culture for an extended period of time. As with all long-term cultures, one must be aware of the possibility of changes occurring in the cell lines. Thus, the properties of the cells carried in vitro may not at present be reflective of properties of the cells comprising the original tumor.
Fig. 1. Phase-contrast microscopy of HCT 116 cells in culture. × 100.
Fig. 2. Phase-contrast microscopy of HCT 116a cells in culture. × 100.
Fig. 3. Phase-contrast microscopy of HCT 116b cells in culture. × 100.
Fig. 4. Phase-contrast microscopy of HCT 116 colony on confluent mouse fibroblasts. × 100.
Fig. 5. Section of a xenograft from a nude mouse given an injection of HCT 116a cells. H & E, x 100.
Fig. 6. Section of a xenograft from a nude mouse given an injection of HCT 116 cells. H & E, x 100.
Fig. 7. Section of a xenograft from a nude mouse given an injection of HCT 116b cells. H & E, x 100.
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