Isolation of a Cellular Subpopulation from a Human Colonic Carcinoma Cell Line

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ABSTRACT

A population of cells with increased resistance to 5-fluorouracil was isolated from cultures of the human colonic carcinoma cell line HT29. The resistant cells (HTFU) showed an altered morphology by light and electron microscopy and demonstrated contact inhibition in vitro. DNA assays and chromosome counts showed that HT29 cultures exhibit both hyper- and hypoaneuploidy, while HTFU cultures appear exclusively hypoaneuploid. One year after the isolation of HTFU, both cell lines showed equal sensitivity to 5-fluorodeoxyuridine while HTFU cells retained comparative insensitivity to 5-fluorouracil. Carcinoembryonic antigen production was not demonstrated in pre- or postconfluent cultures of HTFU, although carcinoembryonic antigen was present in both cell lines and media of HT29 cultures. Growth in semisolid medium was demonstrated for both cultures; however, HTFU showed a lower plating efficiency than did HT29. Tumors were observed in all of the nude mice given injections of HT29 or HTFU cells. Tumors formed from HTFU cells were smaller, and frequently the primary site receded after 6 to 8 weeks. Although in vitro tests suggested a reduced tumorigenic potential for HTFU cells, metastasis was observed only in mice given injections of cells from the HTFU line.

INTRODUCTION

A major problem in the study of tumor biochemistry is the cellular heterogeneity of the tumor (2-4, 6, 25). The cell variants within a single tumor may show significant differences in functional capability, such as in the ability to metastasize (8, 20, 27). Many investigators have taken advantage of functional heterogeneity to select distinct cell subpopulations such as in the isolation of lectin-resistant cells (24, 26). Of particular interest in the study of human cancer has been the implication that some cell populations in a tumor may be resistant to the drug. These subpopulations may exhibit variable degrees of drug resistance and may represent a minority of the cells present in the original tumor mass. Using the human colonic carcinoma cell line HT29 (9), we have isolated and characterized a cell population which is highly insensitive to FUra.

MATERIALS AND METHODS

Cells and Culture Conditions. The human colonic carcinoma cell line HT29 was provided by Dr. Jorgen Fogh (Sloan Kettering Institute, New York, N. Y.). Cells were cultured in plastic flasks (Corning Glass Works, Corning, N. Y.) at 37° in 5% CO2 in a air in McCoy's enriched medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 20% fetal calf serum (Grand Island Biological Co.) and antibiotics (6.4 µg of gentamicin per ml, 90 µg of penicillin per ml, and 90 µg of streptomycin per ml).

Cultures of the HT29 cell line were grown in the presence of FUra (Sigma Chemical Co., St. Louis, Mo.) in concentrations ranging from 10⁻² to 10⁻⁸ M. Cells were exposed to the drug for 2 weeks, after which FUra was omitted from the media. FUra-resistant cells were those which survived the highest concentration of the drug and could be successfully maintained in culture when the drug was removed.

Growth Curve. Cultures of the cell line HT29 and the drug-resistant cells (HTFU) were grown as described above with an initial inoculum of 3 x 10⁵ cells/25-sq cm flask. At 2-day intervals, duplicate cultures were harvested with 0.25% trypsin and 0.2% EDTA in tissue culture medium, and cell counts were performed with hemocytometer chambers. Approximately 1 x 10⁷ cells from each subcultured flask were prepared subsequently for DNA assay (15).

Morphology. Cell cultures were stained in situ by fixing the cells in methanol and staining with Giemsa (Fisher Scientific, Fair Lawn, N. J.). Cells were prepared for EM were grown to confluence and then subcultured as described above. The cell pellet was fixed in 2% glutaraldehyde and postfixed in 1% osmium tetroxide. The fixed pellet was dehydrated with ethanol and acetone and embedded in plastic. Sections were stained with uranyl acetate and lead citrate.

Determination of Chromosome Number. Cell cultures were blocked at metaphase by exposure to Colcemid for 24 hr. The cells were resuspended in water and incubated at 37° for 10 min. After centrifugation, the pellet was fixed in methanol:acetic acid (3:1, v/v) for 1.5 hr. The suspension was applied to slides and treated with trypsin (1:250; Difco Laboratories, Inc., Detroit, Mich.) for 7 sec and then 20% Giemsa for 7 min.

CEA Assay. CEA content was determined for both cells growing in culture and in ambient media. Cells from each line were plated at an inoculum of 1 x 10⁶ cells. Cultures were fed daily. The volume of exhausted media for each culture was determined, and then a 1-ml aliquot was removed, filtered

1 Supported by Grant CA 21520 from NIH through the National Large Bowel Project and Grant PDT-109 from the American Cancer Society.
2 To whom requests for reprints should be addressed.
3 The abbreviations used are: FUra, 5-fluorouracil; EM, electron microscopy; CEA, carcinoembryonic antigen; FdUrd, 5-fluorodeoxyuridine.

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through a 0.22-μm filter, and stored at −20° until the time of assay. Cell cultures were terminated after 5 and 17 days. Cells were counted, diluted to a concentration of 1 × 10^6 cells/ml, and frozen and thawed twice. CEA content was measured by the indirect procedure outlined in the Hoffman-LaRoche manual (Hoffman-LaRoche Inc., Nutley, N. J.) (3, 11). The indirect method, which uses perchorlic acid extraction, is used to measure titers ranging between 0.5 and 25 ng/ml.

**Growth in Semisolid Media.** Cells (2 to 8 × 10^4) were suspended into a final concentration of 0.27% agarose (Pharmacia Fine Chemicals, Piscataway, N. J.). One ml of this suspension was layered over a base layer of 0.5% agarose in 35-mm Petri dishes (Corning Glass Works). Agarose cultures were incubated in a humid atmosphere at 37° with 5% CO_2 in air. After 2 days in culture, 1 ml of tissue culture media was applied to each agarose plate to provide sustenance for the cells and prevent drying of the agarose. The feeding solution was replenished every 2 to 3 days. All cultures were terminated after 24 days, and the number of colonies was counted by microscopic examination. A colony consisted of 20 or more cells. Appropriate controls were counted at the initial time of plating to determine the number, if any, of colony-size aggregates. This number of aggregates was deducted from the final colony count (12).

**Tumorigenicity.** Cells to be used for injection were grown to confluence in 35-mm Petri dishes (Corning Glass Works). Cells were scraped from the Petri dishes without use of proteolytic enzymes or EDTA and resuspended in tissue culture medium at a final concentration of approximately 10 × 10^6 cells/0.1 ml. A 0.1-ml volume was injected s.c. into the flank of genetically athymic nude mice. Tumors were removed after 6 to 8 weeks and histological sections were prepared and stained with hematoxylin and eosin.

**RESULTS**

**Isolation of Resistant Cells.** Cultures of the HT29 cell line were exposed to various concentrations of FUra for a 2-week period. Cells in cultures exposed to high concentrations of FUra (10^-2 and 10^-3 M) died within the 2-week exposure period. With the exception of cultures exposed to 10^-4 M FUra, massive cell death occurred in the remaining cultures during the 2- to 3-week period following drug removal. Similar latent effects have been reported for patients treated with FUra (22). During the first week of exposure to the drug, a flattened cell type was observed in cultures exposed to 10^-4 to 10^-7 M FUra. This cell type became more prevalent due to the death of the majority of cells of HT29. Four weeks post drug removal, the flat cells (HTFU) were the only cells observed in the cultures listed above. The growth rate of the newly isolated HTFU cells was initially slow but increased to its current rate approximately 3 months post drug removal.

**Growth Curves.** The rate of growth was determined for HT29 and HTFU cell lines over an 18-day period. HT29 cells adhered to the flask within 24 hr. As shown in Chart 1A, proliferation began within 2 days and continued throughout the duration of the experiment. The HT29 cells became confluent on Day 9 and then proceeded to display extensive cell piling. Assay of DNA content indicated a direct relationship between cell number and quantity of DNA. Expressed relative to cell number, the HT29 cells contained an average of 25.2 ± 1.1 (S.D.) μg DNA per 10^6 cells. Chromosome counts indicated a bimodal distribution of 44 to 46 and 60 chromosomes with the majority of cells showing the higher average.

At the same inoculum, HTFU cells showed complete adherence to the flask within 2 to 3 hr. As seen in Chart 1B, proliferation was immediate. Confluency was microscopically observed on Day 5. Although cell numbers increased until Day 9, cell piling was not observed until Days 7 and 8. Cell concentration did not appear to significantly increase beyond 6 × 10^6 cells/25-sq cm flask. This saturation density of 35-mm Petri dishes was reached after 8 to 9 days. Repetition of the growth curve after HTFU was in continuous culture for 1 year indicated no significant differences in saturation density. DNA content appeared to vary directly with cell number; however, the DNA concentration before saturation density was 19.2 ± 0.63 μg DNA per 10^6 cells, while afterwards the concentration was 16 ± 0.65 μg DNA per 10^6 cells. Chromosome analysis indicated a modal count of 44 to 46 chromosomes.

**Morphology.** HT29 cells, as seen in Fig. 1, grew in vitro as discrete semispheroid clusters, with the cells maintaining a rounded appearance. HTFU cells assumed a flattened morphology, as shown in Fig. 2.

Cultures of HT29 and HTFU were grown to confluency and prepared for EM. As seen in Figs. 3 and 4, HTFU contained a greater number of mitochondria and a more elaborate system of endoplasmic reticulum (smooth and rough), while HT29 contained a higher proportion of lysosomes and Golgi apparatus. The plasmalemma of HTFU cells frequently appeared fuzzy and microvilli occurred as sporadic clusters. The plasmalemma of HT29 cells seemed more defined, and microvilli occurred individually and at more regular intervals.

**Sensitivity to FUra and FdUrd.** The sensitivities of the 2 cell lines to FUra and FdUrd were determined after both cell lines were replenished every 2 to 3 days. All cultures were terminated after 24 days, and the number of colonies was counted by microscopic examination. A colony consisted of 20 or more cells. Appropriate controls were counted at the initial time of plating to determine the number, if any, of colony-size aggregates. This number of aggregates was deducted from the final colony count (12).

**Use of Drug Resistance to Separate Cell Subpopulations**

Chart 1. Growth curves were performed as described in “Materials and Methods.” Growth is indicated by cell number (○) and DNA content (□). A, HT29 cells; B, HTFU cells. Analysis of the rate of cell proliferation during the initial period of exponential growth indicated mean doubling times of 1.2 and 2.8 days for HT29 and HTFU cells, respectively.
had been grown in a drug-free environment for 1 year. HT29 and HTFU cultures were exposed to various concentrations of FUra and FdUrd for 2 weeks. After the exposure period, cultures were terminated and cell counts were performed. Drug sensitivity was defined as the maximum drug concentration in which the cells could double in number during the exposure period. The sensitivities of HT29 and HTFU to the drugs are indicated in Table 1. A flattened cell, similar to HTFU cells, was isolated from HT29 cells exposed to 10^{-4} through 10^{-6} M FUra. We observed a maximum of 140 flattened cells per million HT29 cells, which is a frequency of approximately 0.01%. The flattened cell was not observed in HT29 cells exposed to FdUrd.

**CEA.** Preconfluent HT29 and HTFU cultures were terminated after 5 days in culture. The number of cells and volume of media from each culture were determined and assayed for CEA. No evidence of CEA was found in HTFU cells or media. Assay of CEA in HT29 cultures indicated concentrations of 2.7 and 2.2 ng CEA per 10^6 cells from the monolayer and media, respectively. Repetition of this experiment on confluent cultures similarly indicated an absence of CEA in HTFU cells and media. Concentrations of 1.9 and 3.7 ng CEA per 10^6 cells from the monolayer and the collected media, respectively, were observed in confluent cultures of HT29.

**Growth in Agarose.** Growth in agarose was observed in both HT29 and HTFU cultures. HTFU cultures demonstrated an 8% plating efficiency. Colony size rarely exceeded 100 cells/colony. A plating efficiency of approximately 60% was observed in HT29 cultures, and colony size varied from 20 to 500 cells/colony. Agarose culture, using the higher inoculum of HT29 or HTFU, yielded a lower plating efficiency and smaller colonies.

**Tumorigenicity.** Palpable tumors formed in 3 of 3 nude mice that were given injections of HT29 cells within 14 days. Tumor size increased with time, and no gross signs of metastasis were observed. The tumors appeared moderately differentiated. Tumor formation was observed in 3 of 3 nude mice given injections of HTFU cells on the tenth day after injection. Tumor size increased slightly during the next 2 weeks, after which all tumors receded. The tumors were poorly differentiated. Six weeks postinjection, metastases under both forelegs were observed in 2 mice given injections of HTFU. Kyriazis et al. (13) have similarly reported the occurrence of metastases to the regional lymph nodes following s.c. injection of malignant cells. Using sterile procedures, the foreleg metastases were recovered and injected s.c. into the flank of 2 nude mice. Two months postinjection, tumors had formed at the injection sites. Subsequently, metastases were found under the forelegs and in the lungs of both mice. Chromosome analysis performed on the metastatic cells yielded a modal chromosome number similar to that observed with HTFU cells.

**DISCUSSION**

The difficulty in identifying and separating cellular subpopulations within a tumor persists as a significant obstacle in the study of tumor biochemistry. We attempted to separate cell populations from a colonic carcinoma cell line by utilizing the different functional capabilities of the populations. HTFU cells, isolated from HT29 cultures following exposure to FUra, demonstrated an altered morphology and contact inhibition in vitro. As summarized in Table 1, growing cultures of HT29 and HTFU differed appreciably in DNA content. The hyper- and hypoaneuploidy of the HT29 cell line has been reported (9). The exclusive hypoaneuploidy of the HTFU cells suggested that we had isolated a distinct cell subpopulation.

EM was performed on HT29 and HTFU cultures in an attempt to visualize cellular variations. The cell surface of HTFU cells differed qualitatively from that of HT29 cells. The changes in microvillar arrangement may be related to an alteration in cell-to-cell interactions. Schirmacher et al. (24) have reported similar changes in the microvilli of cells with different metastatic capabilities.

Although FUra is one of the most effective chemotherapeutic agents used against colonic carcinoma, its mechanism(s) of antitumor activity remains enigmatic due to the complexity of the metabolism of the drug (1, 14, 21, 22). Knowledge of the mode of action of a drug becomes increasingly important in light of evidence indicating the presence of drug-resistant subpopulations within certain tumors (6, 10, 17, 25, 30). It has been suggested that FUra and its derivatives can affect the following areas of cell metabolism: DNA synthesis; RNA synthesis; ribosomal maturation and function; and mitochondrial DNA synthesis (1, 14, 19, 21, 22, 28, 29). Our experiments were not initially designed to test the mechanism by which HTFU cells maintain a relative insensitivity to FUra; however, due to the similarity with work performed on other drug-resistant mammalian cells, a general hypothesis may be forwarded. The action of FUra on DNA appears mediated by the conversion of the drug to the active metabolite 5-fluorodeoxyuridine 5'-monophosphate which inhibits dTMP synthesis and eventually results in death due to thymine deprivation (14, 17, 19, 22). It has been reported that in some cells FUra resistance is caused by a decreased thymidine kinase activity, the enzyme which converts FdUrd to 5-fluorodeoxyuridine 5'-monophosphate (28, 29). Other drug-resistant cells which vary significantly in their sensitivity to FUra and FdUrd do not show a decreased thymidine kinase activity (17). Laskin et al. (14) have shown that drug uptake may be more rapid in FUra sensitive cells when compared to cells with less sensitivity. Our studies suggest that the metabolism of FUra and FdUrd is equivalent in HTFU cells and is similar to the metabolism of FdUrd in HT29.

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Table 1: Summary of characteristics of HT29 and HTFU cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>DNA content (pg/10^6 cells)</th>
<th>Modal chromosome (x 10^{-4} M) to</th>
<th>Sensitivity (%)</th>
<th>CEA content (ng/10^6 cells)</th>
<th>Plating efficiency in agarose</th>
<th>Tumor formation in nude mice</th>
<th>Evidence of metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT29</td>
<td>25.2</td>
<td>44-46, 60</td>
<td>0.1</td>
<td>1.9</td>
<td>60</td>
<td>3/3</td>
<td>No</td>
</tr>
<tr>
<td>HTFU</td>
<td>19.2</td>
<td>44-46</td>
<td>1.6</td>
<td>0.0</td>
<td>8</td>
<td>3/3</td>
<td>Yes</td>
</tr>
</tbody>
</table>

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4 CEA content assays were performed on confluent cultures.
cells. The higher sensitivity of HT29 cells to FURA could be caused by (a) an altered transport mechanism for drug uptake or (b) the use of a metabolic path affecting some aspect of cell metabolism other than DNA synthesis.

The isolation of a drug-resistant subpopulation demonstrates certain problems which may be encountered in in vivo and in vitro drug-screening programs. The low frequency of HTFU cells in the HT29 population could allow the subline to go undetected in either drug-screening systems. The reduced tumor-forming ability of HTFU in nude mice could present serious problems in an in vivo test system. Similarly, the differences in cell cycling time and plating efficiency between HTFU and HT29 cells could lead to inaccurate results in in vitro test systems, such as in procedures which measure the colony-forming ability of drug-treated cells cultured in semisolid media (23). As discussed by other workers, any method used to predict effective cancer chemotherapy may be seriously impaired by the presence of preexisting drug-resistant subpopulations within a tumor (10, 25).

The FURA-resistant line was further characterized by these criteria: CEA production; growth in semisolid media; and tumor formation in nude mice. Because the glycoprotein CEA is frequently associated with the cell surface, we assayed the CEA in the cells and the media in which they were grown. CEA was detected in the cells and media of HT29 cells, although at lower concentrations than have been reported for more recently established colon carcinoma cell lines (7). The low CEA content of HT29 cells may be caused by any of the following conditions: (a) an inherent difference in CEA content between cell lines; (b) an overall decrease in CEA content with continued propagation of a cell line; (c) differences in cell culture conditions; and (d) differences in the methodology of CEA assay. We have shown previously that extraction with perchloric acid, as is used in the commercially available indirect assay, reduces CEA content (11). Therefore, although CEA was not detected in HTFU cells or media, we can not state with absolute assurance that HTFU cells are totally devoid of CEA. We can state that HTFU cells contain a lower concentration of this glycoprotein than is found in an equal number of HT29 cells.

While there are many criteria used to characterize tumor cells (including abnormal karyotypes, elevated saturation density in vitro, histology, and presence of specific antigens), 2 criteria are most frequently used to operationally define a cell line as being malignant: growth in semisolid media and tumor formation in animals (3, 4, 6, 12, 18, 27). HTFU cells satisfied these criteria, although they demonstrated a lower plating efficiency and a reduced tumor-forming ability than was observed with HT29 cells. Although the results of in vitro tests suggested a reduced tumorigenic potential, HTFU cells were capable of forming metastases in nude mice. The malignant nature of the metastatic cells was confirmed by their ability to form a tumor and metastasize when injected into a nude mouse. Metastasis was not observed following injection of HT29 cells. The apparent discrepancy between the results of in vitro and in vivo tests has been noted by others, and our data merely confirm that the criteria which define cancer are inadequate as an index of the degree of the malignant state (5, 18). Because our studies do not exclude the possibility that HT29 cells could metastasize under certain conditions, we can state only that HTFU cells appear to show a greater potential towards the formation of metastasis.

In summary, we have used drug resistance to isolate a cell subpopulation from the human colon carcinoma cell line HT29. The drug-resistant cells, which do not appear highly differentiated, demonstrate 2 important specializations in addition to increased FURA insensitivity, contact inhibition in vitro and increased metastatic capability in vivo. We are presently investigating both phenomena.

ACKNOWLEDGMENTS

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Fig. 1. In situ stain of HT29 cells. Giemsa, ×100.
Fig. 2. EM micrograph of HT29 cell from a confluent culture. The following structures are indicated with arrows: microvilli (MV), mitochondria (Mi), and endoplasmic reticulum (ER), ×13,500.
Fig. 3. EM micrograph of HT29 cell from a confluent culture. Microvilli (MV) and Golgi apparatus (Go) are indicated with arrows, ×10,200.

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