Establishment of a Human Carcinoembryonic Antigen-producing Colon Adenocarcinoma Cell Line


Department of Clinical Chemistry and Laboratory Medicine [B. D., L. Y. Y., M. J. A., J. M. T.] and Department of Surgery [M. M. R.], The University of Texas System Cancer Center M. D. Anderson Hospital and Tumor Institute, Houston, Texas 77025

SUMMARY

A human carcinoembryonic antigen-producing colon carcinoma cell line has been established. The cells form acinar structures and signet ring cells. The lumen of the acini presents microvilli and a glycocalyx. Neighboring cells show desmosomes and terminal bars. The cells present an aneuploid karyotype with a modal number of 49. No marker chromosomes are found, although a significant proportion of cells show an altered A2 chromosome and an extra B. Exponentially growing cultures produce 54 ng of carcinoembryonic antigen/10⁶ cells. Kinetic parameters are as follows: doubling time, 37 hr; mitotic index, 0.8%; labeling index, 31%; generation time, 30 hr; G₁ phase, 7 hr; S phase, 18 hr; G₂ phase, 5 hr; growth fraction 90%. This cell line, designated line LoVo, represents an in vitro model for human colon carcinoma.

INTRODUCTION

Tissue culture is a research tool that permits adequate experimentation of cellular processes under precise environmental conditions. In most instances, explantation in vitro disrupts physiological controls that operate in vivo, and the transplanted cells may lose the identifying characteristics that permit precise classification. However, these cells may retain some morphological or functional markers that allow identification of the tissue source (i.e., immunoglobulin or CEA production, melanosomes, etc.) (1). Recently, great interest has been devoted to obtaining permanent cell cultures of human large bowel origin in order to pursue research in tumor immunology and cell kinetics. Since 1970, one of us (M. M. R.) has engaged in a systematic trial of establishing cell lines of human colon carcinoma. The present report describes the establishment and biological characteristics of 1 such cell line (line LoVo) that retained morphological and physiological properties of the in vivo counterpart, including the formation of acinar structures and the production of CEA.

MATERIALS AND METHODS

Tissue Culture. A fragment of the tumor, obtained from a metastatic nodule in the left supraclavicular region of a 56-year-old patient with histologically proven diagnosis of adenocarcinoma of the colon, was minced under sterile conditions within 1 hr after excision. Explants smaller than 1 cm² were placed in T-flasks containing Ham's F-12 medium, supplemented by 20% fetal calf serum, glutamine, vitamins, and antibiotics. Primary cultures were fed regularly by partially changing the growth medium. After peripheral growth from the explants was initiated, cells were removed and cultures were allowed to progress to confluent monolayers. Subculturing was subsequently carried out every 2 weeks by decanting the medium, washing with N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid Tricine buffers (Grand Island Biological Co. Laboratories, Santa Clara, Calif.), and incubating with 0.25% trypsin for 20 min at room temperature. The cells were dislodged in clumps from the monolayer by brisk agitation, centrifuged, washed with buffer, and transferred to 12-oz Owens bottles. After 2 years in continuous culture, a variety of harvesting procedures designed to provide single cell suspensions with the least decrease in cell viability was explored. These techniques utilized different enzymes, temperature conditions, and incubation intervals. The methods are summarized in Table 1. Presently, stock subculturing is effected weekly using Method 15, which uses hyaluronidase and trypsin. However, for experimental purposes, Method 16 (similar to Method 15, but with 10 times the concentration of trypsin) is used because it yields a higher percentage of viable single cells. Cultures are examined periodically for evidence of Mycoplasma contamination using morphological (including electron microscopy) and biochemical methods.

Morphological Studies. Cells from stock cultures were seeded in 150-mm Petri dishes containing sterile slides and were allowed to reach exponential growth. Slides were rinsed in 0.9% NaCl solution and either air dried or fixed in pure methanol or neutral buffered formalin. Cells were stained with Giemsa, Wright's, hematoxylin and eosin, PAS, diastase digestion of PAS, Meyer's, Alcian blue, colloidal iron, Sudan black, oil red, phosphotungstic acid-hematoxylin, and methyl green-pyronin.

Electron Microscope Studies. A pellet obtained from the harvest of a single cell culture was fixed in 2.5% glutaraldehyde at pH 7.2 for 45 min and postfixed in 1% osmium tetroxide for 2 hr before dehydration, embedment, and sectioning for electron microscopy.
Table 1
Harvesting methods

<table>
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<tr>
<th>Method</th>
<th>Hyaluronidase, 5 min at 37°</th>
<th>Neuraminidase, (500 units/ml) at -37°</th>
<th>Versene, 0.02%, room temperature (sec)</th>
<th>Viookase, 2.5%, at 37° (min)</th>
<th>Trypsine, 0.25%, at 37° (min)</th>
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Cytogenetic Studies. Actively growing cultures were incubated with Colcemid, 0.1 μg/ml (Ciba Laboratories, Summit, N. J.), for 6 hr. Cells were harvested and “squash” preparations were made according to standard techniques. Preparations were observed under a phase microscope, suitable metaphases were analyzed, and 18 cells were photographed for routine karyotype analysis.

Growth Kinetic Studies. For determination of the growth kinetics curve of line LoVo, stock cultures were harvested as a single cell suspension and aliquots of 2 or 4 × 10⁶ cells were seeded into 60-mm Petri dishes containing 5 ml of nutrient medium. Two cell counts in each of 2 replicates/time point were made every 3 hr for 63 hr and every 24 hr thereafter for an additional 160 hr with a Model ZBI electronic particle counter (Coulter Electronics, Inc., Hialeah, Fla.). The values obtained were analyzed by linear regression techniques with the assistance of a Cyber 73 digital computer (Control Data Corp., Minneapolis, Minn.).

The MI was defined simultaneously on floating and on monolayer cells at various phases of growth utilizing cytacentrifuge preparations stained with aceto-orcein. In addition, cell viability on replicate aliquots was monitored by trypan blue exclusion.

The LI of cells in various growth phases was defined by pulse labeling with [³H]Tdr (1 μCi/ml; specific activity, 3.0 Ci/mmol) for 30 min at 37°. The cells were harvested and cytacentrifuge preparations were processed for radioautography.

Cell cycle analysis was performed by seeding aliquots of 5
Human Colon Carcinoma in Culture

× 10⁴ cells in 60-mm Petri dishes and allowing them to reach exponential growth. All cells were pulse labeled with [³H]Tdr (1 μCi/ml; specific activity, 3.0 Ci/m mole) for 30 min at 37°C. The cells were washed twice with medium and were reincubated with fresh medium. Samples were harvested every 2 hr for 120 hr. Cytocentrifuge preparations were processed for radioautography.

Continuous labeling was carried out by dispensing [³H]Tdr (2 μCi/ml; specific activity 3.0 Ci/m mole) to replicate dishes. Samples were collected every 3 hr for 50 hr and processed for radioautography.

In all radioautography procedures, a 50% solution of Ilford K5 emulsion (Polysciences, Inc., Warrington, Pa.) in distilled water was used. The radiolabeled cells were exposed for 1 to 2 weeks and developed in Kodak D19 developer (Eastman Kodak Co., Rochester, N. Y.). Labeled cells (or metaphases) were those that had more than 5 grains overlaying the nucleus. All experiments were performed at least twice with 2 replicate cultures per time point.

**CEA Synthesis.** The method used to assay CEA produced by LoVo cells was the double-antibody triple-radioimmunoassay of Egan et al. (2). Purified antigen and antibody for these studies were obtained from Dr. C. W. Todd, Duarte, Calif., through the courtesy of Dr. H. Fritsche, Chief, Section of Chemistry, Department of Clinical Chemistry and Laboratory Medicine, M. D. Anderson Hospital. Five million cells were subcultured in 12-oz Owens bottles containing 35 ml of fresh medium. The supernatant was collected on Day 5, the boundary between exponential and stationary phase of growth. The total volume was determined, the cells were harvested and counted, and CEA activity was measured directly. Reproducibility and sensitivity were assessed by utilizing various dilutions of supernatants from replicate cultures. Other studies compared results obtained by the Egan et al. technique to those of the Hoffmann-LaRoche kit method (Hoffmann-LaRoche, Inc., Nutley, N. J.).

**RESULTS**

**Tissue Culture.** Primary cultures showed slow but progressive peripheral growth issuing from the single explants. The cells were polygonal and cuboid, with large vesicular nuclei. Although basically growing as a firmly attached monolayer, a number of cells were found floating in the supernatant medium, usually in small clumps. The number and size of these clumps increased as the incubation interval was prolonged. It was very difficult to break up these clumps by either mechanical means or by enzyme treatment. Many cells detached from the monolayer by subculturing procedures would also come off as clumps. It was subsequently learned (see "Ultrastructural Studies") that line LoVo presented large numbers of junctional complexes (desmosomes) between neighboring cells. These complexes became more prominent as the culture aged and are considered the source of our difficulty in obtaining single cell suspensions. For this reason, it became necessary to investigate various means to obtain single cell suspensions while retaining cellular viability, in order to utilize LoVo cells for subsequent experiments. Different methods explored are summarized in Table 1. Although none yielded a pure single cell suspension, Method 16 was considered adequate for further work and was used in investigations detailed below.

**Morphological Studies.** Line LoVo cells are cuboidal and columnar and rarely polygonal. They present a large vesicular nucleus usually located at 1 end. Most cells are mononuclear, although occasional multinucleated giant cells are noted. As the cultures age, signet ring cells appear that contain amorphous eosinophilic material (Fig. 1). This material stains positive with PAS and is not removed by previous diastase, neuraminidase, or hyaluronidase treatment. However, no fat or mucin could be demonstrated by appropriate cytochemistry. In older cultures, groups of cells arrange in true acinar patterns (Fig. 2). The lumen of these acini contains material with properties identical to those described for signet ring cells. The free border of the cells lining the lumen appear fuzzy, suggesting the presence of cilia or a glycocalyx. These morphological characteristics have remained stable over the period of continuous propagation, and acinar structures are observed even after subculturing single cell suspensions.

**Ultrastructural Studies.** Cellular morphology was similar to that observed at the light microscope level. Cells were cuboidal and columnar and frequently arranged in a glandular pattern. Junctional complexes between neighboring cells were represented by desmosomes (Fig. 3) and by large terminal bars at the free border of cells lining the lumen of the gland (Fig. 4). The free border displayed numerous, rather uniform microvilli and, frequently, a fuzzy coat (glycocalyx) (Fig. 5). Very few intracytoplasmic vacuoles and rare ergastoplasmic profiles were noted. However, there was a profusion of microfibril bundles arranged in haphazard fashion and a multitude of free ribosomes. The nuclei were large and invariably displayed a single nucleolus. The nucleoli were strikingly associated with the nuclear membrane and revealed a clear nucleolonema and discrete areas of pars amorpho (Fig. 6). The Golgi apparatus was not prominent, and lysosome-like structures were rare.

**Cytogenetic Studies.** Karyotype analysis revealed an aneuploid human male karyotype with numerical and structural alterations and a modal chromosome number of 49 (range, 48 to 51). The extra chromosomes resembled those of the B or C group (Denver-Chicago classification) (Fig. 7). Most of the chromosomal changes varied from one metaphase to another, even though the majority of metaphases had 49 chromosomes. However, in many of the analyzed cells, 2 distinct abnormalities were usually noted: an altered, possibly A2 chromosome and an extra element morphologically similar to a B chromosome.

**Growth Kinetic Studies.** Stock cultures of LoVo cells plated from a single cell suspension in Petri dishes showed a lag in resuming exponential growth of about 36 hr (Chart 1). There was no density-dependent difference in the doubling time of exponentially growing cells (Chart 2). The average doubling time (T_d) is 36.9 hr with a 95% confidence interval of 34.3 to 39.9 hr. Under the conditions of our experiments, the cells grow exponentially for 5 days, after
which they enter stationary growth phase as defined by no
net change in cell counts.

The MI, the LI, and the cell viability (trypan blue exclu-
sion) were defined on monolayer cultures at each growth
phase. Cells in lag phase (24 hr) showed no mitotic cells,
the LI was 43%, and cell viability was about 80%. A MI of
0.8%, a LI of 31%, and 92% viable cells were recorded for
exponentially growing monolayer cells (3 days of culture).
Cells in stationary phase showed a MI of 0.1%, a LI of 1%,
and 80% viable cells. Cells floating in the supernatant of
exponentially growing cultures had a MI of 1.7% but only
68% viable cells. No mitotic cells were noted floating on
the supernatant of stationary phase cultures, where only
25% of the cells excluded trypan blue.

Cell cycle analysis performed by the PLM technique is
shown on Chart 3. The length of each phase of the cell cycle
assessed at the level of 50% labeled metaphases was de-
formed as follows: generation time ($T_g$, 36 hr; G1 phase, 7 hr; S
phase, 18 hr; and G2 phase, 5 hr.

The percentage of labeled nuclei of LoVo cells in con-
tinuous incubation increased slowly with a rate of 3.0/hr,
reaching a plateau of about 90% after 20 to 24 hr (Chart 4). Thus,
up to 3 months. There was essential agreement between CEA levels detected by both assay methods used. Thus, supernatants of 5-day-old cultures (exponential growth) contained 52 ng of CEA/10⁶ cells measured by the Hoffman-LaRoche method and 53.8 ng of CEA/10⁶ cells assayed by the method of Egan et al. (2). This method proved to be very sensitive (0.2 ng) and precise (coefficient of variation, <8%) and showed results comparatively similar to those obtained by the CEA assay kit of Hoffmann-LaRoche. Results were expressed in terms of cell number rather than in terms of milliliters of supernatant medium because such treatment reflects more accurately the magnitude of CEA synthesis of a given cell population. Thus, our results cannot be compared to the levels of CEA synthesis by other cell lines reported in the literature (3, 5, 7). Nevertheless, if expressed in such arbitrary terms, the level of 22.8 ng of CEA/ml measured on the 5th day of culture of the 87th passage of line LoVo relates favorably to the 17 ng of CEA/ml measured on the 7th day of culture of an early passage of H129 cells (3) and to the 32 and 34 ng of CEA/ml recorded, respectively, on the 8th day of culture of Passage 3 of line HRT-18 and Passage 5 of line HCT-8 (7).

The cytogenetic analysis revealed a human aneuploid karyotype with a modal chromosome number of 49. The fact that some of the chromosomal changes varied from one metaphase to another suggests some degree of karyotype instability. On the other hand, the presence of similar abnormalities (altered A2 chromosome and an extra B chromosome) in a significant proportion of these cells is highly suggestive of a monoclonal origin. Two previously reported human colon carcinoma cell lines had a modal chromosome number of 48 (7). The chromosomal alterations exhibited by these 2 lines are not similar to those observed in the LoVo line. More precise identification of the chromosomal changes seen in human colon carcinoma cell lines will require cytogenetic analyses using modern banding techniques.

The growth rate of LoVo cells (Td = 36 hr) is somewhat slower than that reported for other colon carcinoma cell lines. However, as judged from the continuous labeling experiments, most of the cells (90%) are in the proliferative pool. The LI of stationary phase cells is extremely low, indicating a low proportion of S-phase cells. When such cells are replated in fresh medium, the LI increases to values higher than those observed with exponentially growing cells. This suggests that stationary cultures may be blocked in G₁ phase, probably by depletion of an essential metabolite. These cells would start to proliferate as a wave of partially synchronized cells once they are replated in complete nutrient growth.

A discrepancy is noted between the summation of T₈ and T₉ intervals obtained by the continuous labeling method and by the PLM techniques (24 hr and 12 hr, respectively). This discrepancy is attributed to the fact that values determined by continuous labeling estimate cell cycle parameters for the entire proliferating population, while the average values obtained by the PLM technique reflect the transit of the most rapidly proliferating cells.

From the above considerations, it is evident that LoVo cells represent an invaluable cell line displaying markers expected from malignant elements of intestinal origin. These markers include the synthesis of CEA and the production in vitro of true acinar structures, similar to those observed in histological sections of malignant tissues of the alimentary tract. Thus, we expect to utilize these cells for a
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variety of studies concerning tumor immunology, cellular pharmacology, and biochemical mechanisms involved in the synthesis of CEA.

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REFERENCES


Fig. 1. Monolayer culture showing a signet ring cell. PAS, × 480.
Fig. 2. Acinar structure containing amorphous material. PAS, × 480.
Fig. 3. Numerous desmosomes (arrows) are illustrated in this electron micrograph on the lateral surfaces of adjacent LoVo cells. Uranyl acetate-lead citrate, × 7650. Inset, detail of the desmosomes’ dense plaques on opposing cell surfaces with their converging bundles of cytoplasmic filaments. Uranyl acetate-lead citrate, × 49,875.
Fig. 4. An electron micrograph of 4 LoVo cells arranged characteristically around a central lumen showing the distinct areas of the zonula occludens and continuous zonula adherens (arrows) in the terminal junctions of these cells. The free borders demonstrate numerous microvilli. Uranyl acetate-lead citrate, × 7200.
Fig. 5. Free border of 2 adjacent LoVo cells displaying the fuzzy coat of the glycocalyx (arrows). Uranyl acetate-lead citrate, × 9000.
Fig. 6. Typical association of a large nucleolus (arrow) with nuclear membrane. Uranyl acetate-lead citrate, × 7875.
Fig. 7. Aneuploid karyotype of a colon adenocarcinoma cell line obtained from the 8th subculture. Modal chromosome number, 49. Brackets and arrows, numerical and structural chromosomal changes.

Human Colon Carcinoma in Culture

Figure 1

Figure 2
Establishment of a Human Carcinoembryonic Antigen-producing Colon Adenocarcinoma Cell Line
