Cell Lines from Human Colon Carcinoma with Unusual Cell Products, Double Minutes, and Homogeneously Staining Regions

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INTRODUCTION

Cultured malignant cell lines provide a valuable resource material for cancer research. Large numbers of cell lines from different tumor types are required to reflect the diversity of cell type and cell response of tumors observed by a clinician. Sequential biochemical, physiological, and immunological analyses made possible by the accessibility of the cultured cells can lead to improved and new methods for accurate diagnosis, define new subclassifications of tumors, aid in assessment of prognosis, and suggest new approaches for development of anticancer agents.

Cell lines derived from human colon carcinomas have been reported (9, 20, 26, 38, 42, 43, 46). Ultrastructural studies reveal brush borders and numerous cell junctions (9, 20, 38, 46). Synthesis of CEA is the most frequently reported biochemical marker of malignant colonic cell lines. In this report, we describe 2 colon carcinoma cell lines, derived from the same tumor specimen, with characteristics that differ significantly from previously reported colon carcinoma lines. The possible origin of these unique cell lines, unusual cell products, and cytogenetic aberrations, DM and HSR's are the reasons for this report.

CASE REPORT

A 55-year-old Caucasian female was diagnosed as having a carcinoma of the sigmoid colon after a brief interval of pain and dysuria. Preoperative levels of electrolytes, minerals, and enzymes were normal. The CEA level of 15 mg/ml in a nonsmoker was consistent with an extensive tumor. On May 11, 1977, an invasive carcinoma of the colon was resected in continuity with the dome of the urinary bladder. Surgical specimens were obtained for pathology studies and tissue culture.

A majority of the histology sections revealed a moderately undifferentiated adenocarcinoma of the colon with atypical pseudo-gland formation in the muscle and serosal layers (Fig. 1). The cytological details of the invasive glands and clusters of cells were not unusual, except for one area in which the tissue spaces between the malignant glands were completely filled with a second kind of epithelial-like cells that were suggestive of a poorly differentiated carcinoid. No rosette, cord, or duct-like structures were observed. The cells were large; immaturity was emphasized by the large globular nucleus and large nucleolus. Mitotic figures were infrequent.

Clinical episodes that might be related to our subsequent findings included a severe hyponatremia in the postoperative period, labile blood pressure, hypercalcemia (15.4 mg-100 ml, and worsening liver function tests. Chemotherapy was initiated postoperatively, but the patient expired 4 months later.

MATERIALS AND METHODS

Fragments of the colon carcinoma were minced, placed in glass flasks, covered with a thin layer of GEM 1717 (developed at Denver General Hospital) or Roswell Park Memorial Institute Medium 1640 supplemented with 10% heat-inactivated FBS and incubated at 37°C. After 24 hr, the cultures were tightly capped and placed in a dry incubator at 37°C. Every 2 to 4 days, 1 to 5 ml of fresh medium were added to the cell cultures. At no time was the medium completely replaced with fresh medium. Subcultures were made by shaking the flask and pouring a portion of the medium and suspended cells into a new culture flask. The original culture flasks were retained. When successful subcultures solely comprised of tumor cells were obtained, the cultures were considered established. Cell cultures were periodically tested for mycoplasmas by the bisbenzamide fluorescent-staining method of Chen (7).

Heterologous transplantation of the cultured COLO 320 cells were performed, as previously described (38).

Cells grown on coverslips were used for phase and other light microscopy studies. HCl-toluidine blue and silver stains were performed according to the method of Grimmelius (13). Samples of cells were prepared for electron microscopy as described by Mallory (25).

Cell products were assayed in 7-day-old spent-culture medium. Parallel assays were performed on fresh medium supple...
were determined by Associated Laboratories (Wichita, Kan.) and ACTH were performed by Nichols Institute (San Pedro, Calif.). Analyses of catecholamines were performed by Laboratory Procedures (Woodland Hills, Calif.). Serotonin synthesis was assayed by Bioscience Laboratories (Van Nuys, Calif.). PTH, a-fetoprotein, and alkaline phosphatase levels were determined with FBS. Assays for ectopic production of calcitonin and ACTH were performed by Nichols Institute (San Pedro, Calif.). PTH, a-fetoprotein, and alkaline phosphatase levels were determined by Associated Laboratories (Wichita, Kansas). Analyses of catecholamines were performed by Laboratory Procedures (Woodland Hills, Calif.). Serotonin synthesis was assayed by Bioscience Laboratories (Van Nuys, Calif.).

The cell lines were tested for CEA production by a radioimmunoassay kit (Roche Diagnostics, Nutley, N. J.) in April 1978 and January 1979. Seven-day-old spent medium supplemented with 10% FBS was used to examine glucose 6-phosphate isozymes by cellulose-acetate electrophoresis, as described previously (38).

Isozyme phenotypes were analyzed in the laboratory of Dr. J. Fogh (Sloan-Kettering Institute). Starch-gel electrophoresis (14) of tumor cell cytosols was performed for the following isozymes: "red cell" acid phosphatase, adenosine deaminase, first and third loci of phosphoglucomutase, ESD, and mitochondrial glutamate-oxaloacetate. The cytosol from 10⁷ tumor cells lysed by freeze-thaw in 0.5 ml 0.9% NaCl solution was used to examine glucose 6-phosphate isozymes by cellulose-acetate electrophoresis, as described previously (38).

Standard cytogenetic methods were used to obtain chromosome preparations. G-, Q-, and C-banding were obtained by the methods of Lavappa (19), Lin et al. (23), and McKenzie and Lubs (27). COLO 320 was examined at 1.5, 3.0, 11.0, 16.0, and 20.0 months after initiation of the culture. Some of the cytogenetic preparations at 3, 11, and 16 months were made from the same subculture. COLO 321 was examined 1.5, 11, and 20 months postinitiation. Twenty to 75 metaphases were examined, and a minimum of 10 karyotypes were made from each chromosome harvest. The Hoechst-bromodeoxyuridine methods of Latt (18) and Goto et al. (10) were used to study the replicative pattern of the chromosomes and DM.

RESULTS

The primary cultures began to rapidly metabolize the culture medium within the first 24 hr postinitiation and were subcultured by pouring half of the supernatant into new flasks. The cultures initiated with Roswell Park Memorial Institute Medium 1640 supplemented with 10 and 15% FBS continued to proliferate. It was possible to split the cultures 1:2 every 2 or 3 days. Fibroblasts were not apparent in the cultures. Forty-five days postinitiation, 2 of the original primary cultures and the subcultures derived from them were designated COLO 320 and COLO 321. Mycoplasmas were observed in some subcultures after approximately 1 year.

Heterologous Transplants. Tumor nodules were apparent 8 days postinjection of 8 x 10⁶ cells (COLO 320) in all 3 of the irradiated C3H/HeJ mice used as heterologous hosts. Examination of histology sections of the tumor nodules revealed poorly differentiated tumors with large areas of necrosis.

Morphology. The cells of COLO 320 and COLO 321 proliferated both attached to the culture flask and in suspension. Phase microscopy revealed small flat clumps and isolated small round cells (Fig. 2A). Occasionally, elongated or multinucleated cells were observed.

Coverslip preparations were stained with acidified toluidine blue or Grimelius' silver nitrate. These stains were used to ascertain whether secretory granules, often present in endocrine and paracrine cells, could be observed. Neither stain revealed the presence of secretory granules.

The original tumor specimen was not examined by electron microscopy at the time of surgical excision. A tissue block was later deparaffinized, refixed, and stained for electron microscopy. The overall quality of the preparation was poor. Few organelles were observed in this preparation. One cell appeared to have 10 to 15 secretory granules. The nuclear material had numerous crystalloid structures that probably resulted from the preparation of the specimen for electron microscopy.

Electron microscopic studies of COLO 320 and COLO 321 were similar. The cells were spheroid. Microvilli were absent. Occasional desmosomes were observed. The presence of multiple convoluted Golgi apparatuses reflected a possible secretory activity. The dilated endoplasmic reticulum was moderately rough. Single ribosomes and polysomes were numerous. Cytoplasmic fibrils were observed in the perinuclear region near vesicles and near the cytoplasmic membrane. Large lipid vacuoles, a few clear vacuoles, and rare dense osmiophilic vacuoles, similar in size to secretory granules observed in endocrine cells, were present. The dense osmiophilic vacuoles were more frequent in COLO 321 than in COLO 320. A representative micrograph of COLO 321 is depicted in Fig. 2B.

Cell Products. Levels of hormones and polypeptides tested in spent medium from COLO 320 are summarized in Table 1. PTH, ACTH, serotonin, norepinephrine, and epinephrine were present in quantities above control levels. Alkaline phosphatase, calcitonin, and dopamine were not detectable. a-Fetoprotein and CEA also were not present in detectable quantities.

Isozymes. Results of the isozyme analysis for COLO 320 were as follows: red cell acid phosphatase type B, adenosine deaminase type 2, first locus of phosphoglucomutase type 1, third locus of phosphoglucomutase type 2, ESD type 1, mitochondrial glutamate-oxaloacetate transaminase type 1, and glucose-6-phosphate dehydrogenase type B. It should be noted that the ESD isozyme was present in much greater quantity than were the other isozymes in COLO 320 and 4 other cell lines tested at the same time.
Cytogenetics. The earliest cytogenetic preparations from COLO 320 and COLO 321 revealed numerous DM in nearly 100% of the metaphases. The DM were darkly stained with Giemsa (Fig. 2C), lightly stained with trypsin G-banding (Fig. 2D), and pale with quinacrine dihydrochloride stain. Centromeric heterochromatin, as revealed by C-banding was absent in DM (Fig. 2E). Hoechst-bromodioxurydine differential staining confirmed that the DM replicated in vitro (Fig. 2F). The number of DM per metaphase varied from 2 to several hundred in both COLO 320 and COLO 321 (Chart 1).

The chromosome mode of COLO 320 was 51. Chromosome banding was performed on COLO 320; a complete metaphase and markers from 4 additional metaphases are represented in Fig. 3. Our analysis of the chromosome markers was as follows:

M1: del(1)(qter→p31;)
M2: t(1;?) (1qter→1p36::?)
M3: t(1;5) (1pter→1p23 or 1p13::5p13 or 5p15→5qter)
M4: B-like marker with dark bands near the centromere and lower one-third of the long arm.
M5: t(22;11;14) (14qter→cen→2?q37::11q13→11qter)
M6: t(22;7;14) (14qter→14q17::?pi?→2q37::?)
M7: t(?;?) (2qter→2p2::?)
M8: t(4;7?) (4pter→4q37::?q71?→7qter)
M9: t(?;?) (5qter→5p1::?)
M10: del(3)(pter→3q132; or q133;)
M11: t(3;9) (3qter→3q21::9p2?→9qter)
M12: t(9;15) (9qter→cen→15qter)
M13: t(13;7?) (13qter→13q11::?q21→1?p21;)
M14: dic(13;13;13) (13qter→13p1::13p1?→13qter). One centromeric constriction was more pronounced than the other.
M15: Small metacentric; possible 11p to 11q14 with some additional light-staining material.
M16: E-size submetacentric; possible a deleted 12q.
M17: t(19;?) (19;?):
M18: t(22;?) (22pter→22q13::?)
M19: del(22)(pter→q11;)
M20: Small isochromosome DM

Variable sizes of small ring chromosomes were also observed in 10 to 15% of the metaphases. When ring chromosomes were present, the number of rings per metaphase varied from

* Nomenclature for chromosomes is according to Refs. 31 and 32.
were seldom observed. The chromosome mode shifted upward by 2, but we were unable to detect the cause of the altered mode. One complete karyotype and examples of markers from 3 additional metaphases are depicted in Fig. 5. Our analysis of the markers was as follows:

M1: Same as M1 of COLO 320-DM
M2: Same as M2 of COLO 320-DM
M3: Same as M3 of COLO 320-DM
M4: t(2;?) (2qter→2p1?:?)
M5: Same as M10 of COLO 320-DM
M6: t(4;?) (4qter→4p1?:?)
M7: Same as M11 of COLO 320-DM
M8: t(9;12?) (9qter→9p2?:12?q13→12qter)
M9: Same as M12 of COLO 320-DM
M10: t(11;?) (11qter→11p1?:?)
M11: t(13;20) (13qter→13q11::20q17→20pter or dic(13;20) (13qter→13p1?:20q17→20pter)
M12: Same as M13 of COLO 320-DM
M13: Same as M14 of COLO 320-DM
M14: t(14;?) (14qter→cen→?)
M15: t(15;?) (15qter→cen→?)
M16: Same as M17 of COLO 320-DM
M17: Same as M16 of COLO 320-DM
M18: Small metacentric
M19: Very small unidentifiable piece of chromatin
M20: (X;HSR:?) (Xpter→Xq21?:;HSR:?)
M21: (HSR:;X;HSR:?) (HSR→?:Xp22?:Xq21?:;HSR:?)

After the HSR marker was observed in one subculture, other subcultures of COLO 320 were examined. One subculture continued to have DM and marker complements very similar to the earliest cytogenetic preparation. Another subculture had HSR markers in 65% of the metaphases. The remaining cells continued to have the same percentage of metaphases with markers; cultures that had DM in the majority of the metaphases as did the subcultures analyzed at 16 months, i.e., cultures appeared to have approximately the same cytogenetic patterns.

DISCUSSION

APUD cells were initially characterized by a capacity to produce catecholamines or indolamines after uptake and decarboxylation of amine precursors (34, 35). Cell products such as cholinesterases, nonspecific esterases, and α-glycerophosphate dehydrogenase as well as secretion of a variety of amines and polypeptides are now considered more consistent properties of an APUD cell (41). Pearse theorized that the APUD cells were probably of neurogenic origin (33).

Biochemical studies of COLO 320 suggest that the line is of neuroendocrine origin rather than of colonic adenoepesthepidial origin. Endocrine cells between adenoepesthepidial cells have been reported (5, 6, 8). Malignant transformations are rare. Histological and ultrastructural studies reveal numerous secretory granules in the cytoplasm of colonic endocrine cells. The number, size, and staining characteristics of the granules are variable (5, 8). Information on the hormones released by the colon endocrine cells is minimal (44). However, serotonin, enteroglucagon, Substance P, vasoactive intestinal peptide, somatostatin, and/or a number of less defined amines or peptides have been detected (5, 8, 11, 34, 35).

The pathology of the tumor specimen indicated that 2 cell types were present. One cell type was typical of an adenocarcinoma. The second cell type was very undifferentiated and resembled a carcinoid. Unfortunately, we were unable to obtain good quality ultrastructural studies on the original tumor specimen, which may have clarified the patient’s diagnosis. The only clinical symptom suggestive of an apudoma was hypercalcemia. Possibly, the patient had 2 types of cancer, or malignant cells of an original clone mutated and resulted in the development of new clonal types.
COLO 320 and COLO 321 differ considerably from previously reported colon carcinoma cell lines (9, 20, 26, 38, 43, 46). In most cases, colon carcinoma cell lines grow slowly, adhere tightly to the culture flask, form dense cell colonies, have cell membranes with interdigitating brush borders, and produce variable quantities of CEA. In contrast, COLO 320 and COLO 321 grow very rapidly, high-density epithelial colonies are not apparent, and the cells are easily loosened from the culture flask. Ultrastructure studies revealed relatively uniform, round cells with few microvilli or desmosomes. Repeated assays of COLO 320 and COLO 321 were negative for CEA.

Assays for endocrine-like products indicated that COLO 320 secreted serotonin, PTH, ACTH, catecholamines, and elevated levels of ESD isozyme, which are characteristics common to APUD cells. None of the previously reported colon carcinoma cell lines are known to produce these cell products. However, the question remains as to whether all such endocrine-secreting cancers arise from precursor APUD cells, or whether transformed intestinal cells may become so undifferentiated that they develop the capacity to produce polypeptides with endocrine function, just as a variety of tumor types produce CEA.

Cytogenetic analyses of COLO 320 and COLO 321 revealed a number of marker chromosomes derived from structural rearrangements of normal chromosomes. There have been very few banded cytogenetic characterizations of colon carcinomas (16, 38, 39, 42). Therefore, substantive conclusions concerning whether there are nonrandom patterns of chromosome involvement in markers are not possible at this time. Furthermore, these unusual cell lines may not have the same chromosome aberrations that might be found in adenocarcinomas of the colon.

The most striking cytogenetic feature of the cell lines was the presence of numerous DM. Tumors of neurogenic origin are most frequently reported to have DM (22, 24). It is tempting to suggest that the presence of DM in COLO 320 and COLO 321 is further evidence of the neuroendocrine origin of the cells. However, DM have been observed in a number of other human tumor types (3, 12, 15, 18, 29, 36, 40, 45). In a survey of 35 of our malignant tumor cell lines, at least one metaphase of 75 metaphases examined had DM. The number and frequency of DM per cell varied considerably among the different cell lines. Only one additional cell line, a carcinoma of the rectum with atypical morphology, had large numbers of DM similar to COLO 320 and COLO 321. The significance of DM in malignant cells has not yet been elucidated.

Although we have no direct evidence, DM were probably present in the original tumor. DM have been observed in fresh tumor specimens and cultured tumor lines of a variety of tumors. Studies by Levan et al. (22), Barker and Hsu (3), and ourselves have demonstrated that DM replicate synchronously with other chromosomes and, therefore, have the potential to persist in vitro. Our studies also indicate that in some subcultures, the DM have persisted in nearly the same frequency for approximately 1.5 years.

Recently, there has been speculation that DM are related to the de novo appearance of unusual chromosomes or markers with long HSR’s (2, 21). Levan et al. (21) described a murine sarcoma line, SEWA1R, that had DM when passaged in vivo. The DM were lost after 3.5 months of in vitro cultivation, and new marker chromosomes appeared with the same staining properties as those of DM. The in vitro line was again passaged in animals, the DM reappeared in the same frequency as in the original tumor. Balaban-Malenbaum and Gilbert (2) described a human neuroblastoma cell line that had 2 cell populations. In one cell population, DM were observed. In the second cell population, no DM were evident, but marker chromosomes with HSR’s were present. Since there were marker chromosomes common to both cell populations, it was probable that both cell populations were derived from a common precursor.

We observed a similar phenomenon in some subcultures of COLO 320 and COLO 321. In both cases, DM were observed in metaphases from the earliest cytogenetic analyses. A number of subcultures lost DM and gained HSR markers after 1 to 1.5 years. Although occasional metaphases were observed that had a few DM in addition to the HSR markers, the majority of the metaphases had either DM or HSR markers. COLO 321 had a few DM that were observed in addition to the HSR markers. Whether these DM are different from the numerous DM observed in early harvests cannot be determined at this time. In any case, the number of DM was drastically reduced in 99% of the cells with HSR markers.

The basis for the sudden appearance of a new marker with a HSR and concurrent disappearance of DM is not clear. Alterations of in vitro conditions, such as addition of antibiotics and variations in percentages of FBS supplement, have not resulted in the formation of HSR markers in cultures with DM or gain of DM in cultures that had cell populations with HSR markers. It is conceivable that tumor cells with HSR markers and without DM were present in a very small percentage of cells of the original tumor. Kovacs (17) reported HSR’s in direct preparations in 5 of 14 malignant specimens.

Levan et al. (21) suggested that cells with HSR’s may have a selective advantage in vitro over cells that have DM. Certainly, one subculture of COLO 320 very rapidly became dominated by cells with a HSR marker. However, other subcultures derived from the flask that later had HSR markers in nearly all of the cells and parallel subcultures have had concomitant cell populations with either HSR markers or DM for approximately 6 months.

Analysis of the markers present in the subculture dominated by cells with a HSR revealed less variety in the complements of markers than was found in the early preparations. The markers were not sufficiently uniform to conclude that all cells with a HSR were derived from a single cell that overgrew the other cells with DM. In vitro instability, selection processes, or chromosome harvest technique-induced random losses of chromosomes from metaphases could account for some of the variability in marker complements.

The most recent theory for the origin of DM and HSR’s suggests that gene amplification is responsible for the development of these aberrations. Biedler and Spengler (4) correlated the appearance of HSR’s in methotrexate-resistant cells with manifold increases in the levels of DHFR. Others have demonstrated that induced methotrexate resistance in mouse sarcoma cells results from increased numbers of copies of the DHFR genes (1, 37). In situ hybridization studies have confirmed that DHFR genes are present in the HSR’s of methotrexate-resistant cells (30). No specific cell products have yet been associated with HSR’s or DM occurring in human tumors or human tumor lines. If gene amplification is the origin of both DM and HSR’s, these abnormalities may have biological sig-
significance for tumor cell development and survival against the defenses of the host and cancer therapies.

COLO 320 and COLO 321 should provide an accessible source of cells for studies of the biochemical properties of unusual colon carcinoma and the cytogenetic phenomenon of DM and HSR’s.

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REFERENCES


Fig. 2. A, phase photomicrograph of COLO 320. x 200; B, Electron micrograph of COLO 321. Note the absence of a brush border and presence of a few osmiophilic granules. x 6600; C, Giemsa-stained metaphase of COLO 320 with DM of variable sizes; D, trypsin-treated metaphase of COLO 320. DM stain lightly; E, C-banded metaphase of COLO 320. Note absence of C-bands in DM; F, Hoechst-bromodeoxyuridine differential staining of COLO 320 that indicates DM and chromosomes have replicated.
Fig. 3. A, complete karyotype of COLO 320 11.0 months postinitiation; B to E, additional examples of COLO 320 marker chromosomes from 4 different metaphases. All metaphases had numerous DM.
Fig. 4. A, complete karyotype of COLO 320 16 months postinitiation. Note loss of DM and gain of a marker with a HSR (M20); B to D, additional examples of markers from 3 different metaphases of COLO 320. Note variations in the markers with HSR’s (M20 and M21).
Fig. 5. A, complete karyotype of COLO 321 16 months postinitiation; B to E, additional examples of markers from 4 different metaphases. Note HSR markers (M15).
Fig. 6. A, metaphase from COLO 320 with HSR marker; B, metaphase from COLO 321 with HSR markers. Arrows, markers with a HSR.
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