Establishment and Biological Properties of a Guinea Pig Colonic Adenocarcinoma Cell Line Induced by N-Methyl-N-nitrosourea

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ABSTRACT

A colonic adenocarcinoma cell line, designated GPC-16, was established in culture from tissue removed from an outbred guinea pig which received 56 mg of N-methyl-N-nitrosourea intrarectally over a 28-week period. The GPC-16 has been maintained for more than 30 in vitro passages and was tumorigenic in both the autochthonous host and nude mice. The cells were aneuploid with a wide range in chromosome number (54 to 172) in a bimodal distribution. The cells grew in vitro as a tight monolayer and demonstrated numerous desmosomes by electron microscopy. In vitro-passaged cells had a doubling time of 36 hr and a 5.1% plating efficiency in agar.

INTRODUCTION

The intrarectal administration of MNU has been reported recently to induce colonic neoplasms in guinea pigs in a high incidence (9 of 10 animals; Ref. 14). The lesions were infiltrative or constrictive carcinomas and resembled large bowel tumors in humans. In order to assess the value of this tumor as a model of colon cancers, the immunobiology of MNU-induced colon tumors in guinea pigs was studied. Our intention in this portion of the study was to cultivate MNU-induced colon cancer cells for use in immunological studies and to characterize the cells for comparison to colon cell lines of human origin.

MATERIALS AND METHODS

Guinea Pigs. A total of 240 (120 male and 120 female) guinea pigs (Cavia porcellus) was used. The guinea pigs were outbred and supplied from NIH.

Tumor Tissue. The primary tumor was induced in the distal colon of a female guinea pig by the biweekly intrarectal instillation of 1 mg MNU as described previously (10). The guinea pig received this carcinogen for 28 consecutive weeks (total dose of MNU, 56 mg). A midline laparotomy was performed 10 days after the last dose of MNU, and the colon was found to be firm and fibrotic from 2.5 to 7.5 cm from the anorectal junction. The colon was transected 4 cm from the anorectal junction (as low as possible through the midline approach), and the proximal 11 cm were resected with formation of a ventral-end colostomy (1). The mucosal surface of the caudal end of the colon was irregularly roughened. Portions of the excised segment was fixed in 10% buffered formalin for examination by light microscopy or transferred into GM for primary culture.

Culture Media. The GM used for primary culture and first passage was McCoy's modified Medium 5A (Grand Island Biological Co., Grand Island, N. Y.) supplemented with gentamicin (0.1 mg/ml) and mycostatin (75 units/ml) for prophylaxis against bacterial and fungal contaminants, insulin (0.05 units/ml) for enhancement of cell growth, and 10% fetal bovine serum (Grand Island Biological Co.). Following initial in vitro adaption to this medium, mycostatin was deleted, and the concentration of gentamicin was reduced to 0.05 mg/ml for the second to 17th passages. Thereafter, insulin was removed, and the cells were passaged in a less expensive maintenance medium (Eagle's minimal essential medium; Grand Island Biological Co.) with better short-term buffering capacity (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 10 mM N-tris(hydroxymethyl)methylglycine) and with gentamicin (0.05 mg/ml) and 10% fetal bovine serum.

Primary Culture. A portion of the excised segment of colon with roughened mucosa was minced with scalpel blades. The fragments were transferred equally to the 2.1-sq cm wells of a plastic 24-well cluster dish (Falcon Plastics, Oxnard, Calif.), fed with 0.5 ml of GM the following day, and then left undisturbed for 4 days. The GM (1 ml/well) was changed 2 times a week. After 3 weeks, 5 of 24 wells had 1 to 2 colonies of epithelial-like cells. Fibroblasts were removed by washing the cultures 2 times with PBS without Ca2+ or Mg2+ (Grand Island Biological Co.) and then treating the cultures with 0.125% trypsin-0.05% EDTA (Grand Island Biological Co.) in PBS. After 1 min, the trypsin solution was pipetted off, leaving a layer of solution in contact with the cultured cells. The majority of fibroblasts detached within 2 to 3 min at 37°. One ml of GM was added to each well to stop the trypsinization process and to resuspend the unattached cells. Each of the wells was pooled into a 25-sq cm flask. Since only fibroblasts grew in the 25-sq cm flask, it was discarded.

Subculture and Establishment of an Epithelial-like Cell Line. The attached cells were trypsinized again 2 weeks later, but this time, the dish was incubated at 37°C for 10 min. One ml of GM was added to each well and vigorously pipetted to help detach and disperse the epithelial cells. The cell suspensions of the 5 wells were pooled in a 25-sq cm flask. The selective process to remove fibroblasts was repeated after the third subculture. The remaining cells with a fibroblastic morphology were lost during successive subcultures.

The antibiotic-free spent medium from an actively growing culture of 32nd passage GPC-16 was tested microbiologically for Mycoplasma contamination by standard protocols (6). No contamination was found in either broth or agar cultures.

Different passages of GPC-16 cells were aliquoted in GM with 5% dimethyl sulfoxide, frozen in the vapor phase of liquid nitrogen, and stored in the liquid phase. The cells were suc-
cessfully replated as needed throughout this study.

**Morphological Observations.** GPC-16 cells were observed with an inverted phase-contrast microscope. Cells in culture at different passages were rinsed in PBS and stained with either Wright-Giemsa, Giemsa, or hematoxylin and eosin after methanol fixation. Cytocentrifuge preparations of trypsinized cells were stained with PAS and mucicarmine after methanol fixation.

A pellet obtained from the harvest of a confluent fourth passage monolayer was fixed in 2% glutaraldehyde at pH 7.4 for 1 hr and postfixed in 2% osmium tetroxide for 45 min. Cells were embedded in Epon, and ultrathin sections were stained with 2% uranyl acetate and Reynold’s lead citrate and examined by a transmission electron microscope (Philips 301).

**Growth Characteristics.** Each of thirty 25-sq cm plastic flasks was inoculated with $1 \times 10^5$ thirtieth passage GPC-16 cells in 5 ml of GM. At various times, 3 randomly chosen flasks were harvested by trypsinization, and the number of cells in each flask was determined by hemocytometer counts. The average of triplicate cell counts was plotted against time. The flasks were fed after 6 and 10 days. The doubling time was estimated from the early logarithmic growth phase (11), and the saturation density was estimated from plateau phase. Plating efficiency was calculated by counting macroscopic colonies (methanol fixed, stained with 0.1% crystal violet) formed 9 to 10 days after inoculation of 35-mm Petri dishes (Falcon Plastics) with a defined number (100 and 500, 5 dishes/dilution) of 12th passage GPC-16 cells in 3 ml of GM.

**Growth in Semisolid Media.** GPC-16 cells (34th passage, 2 x $10^5$ cells) were suspended into a final concentration of 0.3% agar (Difco Laboratories, Detroit, Mich.). One ml of this suspension was layered over a base layer of 0.5% agar in 35-mm Petri dishes. The cultures were incubated in a humid atmosphere at 37°C with 5% CO$_2$ in air. The cultures were terminated after 13 days in culture, and the number of colonies was counted by microscopic analysis. A colony consisted of 20 or more cells. Colony size aggregates were counted in control plates at the initial time of plating, and their number was deducted from the final colony count (8, 9, 12).

**Cytogenetic Analysis.** Ignition-dried and air-dried slide preparations of 31st passage GPC-16 metaphase chromosomes were prepared by standard methodology (4). The slides were stained with Giemsa, and metaphases were analyzed for exact chromosome counts.

**Tumorigenicity.** GPC-16 cells were tested for their tumorigenic potential at the 18th passage in the autochthonous host and at the 26th and 31st passages in 3 nude mice (BALB/c, nu/nu, supplied by Dr. R. Jacobson, Cornell University) (5). Animals were inoculated s.c. or i.p. with $1 \times 10^7$ viable cells, and the animals were killed 21 or 51 days later. All resulting tumors were examined grossly and histologically.

**RESULTS**

**Morphology.** The primary tumor at 4 cm from the anorectal junction consisted of anaplastic cells with occasional duct formation consistent with an adenocarcinoma of the colon (Fig. 1). Following plating of the primary tumor and selective trypsinization of the fibroblast-like cells, discrete epithelial colonies remained and grew slowly during the early passages (Fig. 2). The cells formed confluent monolayers which demonstrated density-dependent inhibition of growth (Fig. 3). Cytocentrifuge preparations of cells from confluent monolayer and tissue sections of the primary tumor showed minimal reactivity with either PAS or mucicarmine. This was similar to the surface epithelium of normal adjacent colonic mucosa; normal goblet cells within Lieberkühn’s crypts reacted intensely with both mucicarmine and PAS. Ultrastructural studies of cultured GPC-16 cells demonstrated typical epithelial characteristics (Fig. 4).

**Growth Characteristics.** Chart 1 illustrates the growth curve of GPC-16 cells. The cells had their shortest doubling time of 36 hr between Days 3 and 4. The saturation density, determined 14 days after initiation, was $2.8 \times 10^6$ cells/sq cm. Plating efficiency was 51.6%.

**Growth in Semisolid Media.** GPC-16 cells had a 5.1% plating efficiency in agar. Colony size rarely exceeded 40 cells/colony.

**Cytogenetic Analysis.** Chart 2 illustrates the range and distribution of chromosomal counts for GPC-16 metaphases. The cells exhibited a wide range in chromosome number (54 to 172) including both a hypodiploid and hypertriploid mode. Common markers to all of the metaphase spreads were an increased number of submetacentric chromosomes above that expected for a normal female guinea pig and the presence of metacentrics (Fig. 5).

**Tumorigenicity Studies.** GPC-16 cells were inoculated i.p. into the autochthonous host. When killed for examination 3 weeks later, there were numerous cysts (1 to 2 mm in diameter) on the serosal surface of the distal colon (proximal to the stoma) and uterus. Histopathologically, these cysts were lined by, and the adjacent tissue invaded by, neoplastic cells similar to those seen in the primary tumor (Fig. 1). Tumor cells persisted when they were heterotransplanted into nude mice. When tumor cells were inoculated i.p., multiple small (less than 1 mm in diameter) cysts, lined by neoplastic cells, formed on the parietal peritoneal surfaces but caused no grossly apparent abdominal enlargements. Inclusions (s.c.) produced grossly visible nodules; the nodule from a s.c. inoculation of 31st passage GPC-16 cells grew to a final and persistent size of 4 x 4 x 3 mm when the mouse was killed 51 days post inoculation (Fig. 6).
women (17). Furthermore, despite advances in surgical techniques, colon carcinogenesis necessitated the development of neoplasms which develop from nonadenomatous epithelium similar to those of normal guinea pig cells (7). Furthermore, the cells grew in their autochthonous host and in nude mice, and the resulting tumors were morphologically similar to those seen in the original tumor.

GPC-16 represented one of 11 attempts to culture histologically confirmed adenocarcinomas of the guinea pig colon. The main problem in the majority of the attempts was overgrowth of the small epithelial colonies by fibroblasts. Differences in trypsin sensitivity allowed the fibroblasts to be selectively removed from GPC-16 cells. GPC-16 grew as tight clusters of epithelial-like cells. These clusters eventually coalesced into confluent monolayers. Evidence in support of their epithelial nature was the presence of desmosomes and the uniform apposition of plasma membranes.

The growth characteristics of GPC-16 were similar to the ranges seen for human colon carcinoma cell lines (3, 8, 13, 16). The growth rate (doubling time, 36 hr) was slower than most human cell lines but was the same as the one reported for LoVo (3). The plating efficiency (51.6%) was similar to that reported for WiDr (51%; Ref. 16), and the growth in semisolid media (5.1%) resembled that of HT 29 (4.7%; Ref. 13). An important exception to these similarities was that GPC-16 reached a much lower saturation density (2.8 x 10^5 cells/sq cm) than the human colon carcinoma cell lines. This was probably due to contact inhibition of growth of GPC-16 cells seen morphologically by the failure of the cells to pile atop each other. This observation has usually been correlated with a lack of tumorigenic potential. However, this was not borne out by the transplantation studies of GPC-16 cells. Kimball and Brattain (8) reported recently that a subpopulation of HT 29 cells showed similar contact inhibition of growth yet increased metastatic potential over their noncontact-inhibited counterparts. Thus, GPC-16 provides further support to the discrepancies between in vitro and in vivo tests for defining the malignant state (2, 8, 13).

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REFERENCES


Fig. 1. Histological appearance of primary adenocarcinoma of the colon. Neoplastic cells form irregular nests or ducts lined by plump or flattened cells embedded in a collagenous stroma. H & E, x 300.

Fig. 2. Typical colony of epithelial-like GPC-16 cells at the second passage. Tumor cells are polygonal with a single large nucleus, multiple nucleoli, and abundant cytoplasm. Phase-contrast, x 150.

Fig. 3. Appearance of GPC-16 cells at the 28th passage, demonstrating maximum growth density. Giemsa, x 150.

Fig. 4. Ultrastructure of GPC-16 cells at the fourth passage. in a, cytoplasm contains a small amount of rough endoplasmic reticulum and large numbers of free polysomes. Plasma membranes of adjacent cells show desmosomes (arrows) and indigisting microvilli (M), x 22,500. in b, cytoplasm contains dilated cisternae (C) of rough endoplasmic reticulum. Plasma membranes of adjoining cells are in uniform apposition and demonstrate numerous desmosomes (arrows) and attached tonofilaments. x 30,250.
Fig. 5. Typical metaphase preparation of GPC-16 cells at the 31st passage. Giemsa. × 750.

Fig. 6. Microscopic appearance of nodule produced 51 days after the s.c. inoculation of \(1 \times 10^7\) GPC-16 cells into a nude mouse. In a, the nodule is mostly well delineated from the adjacent normal s.c. tissue and has been transected at its ventral border in the figure. The box delineated by white lines is shown at higher magnification in b. H & E. × 30. In b, neoplastic cells form ducts (right) with cellular debris in their lumena or are embedded in a dense collagenous matrix (left). H & E. × 300.
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