A New Colon Carcinoma Cell Line (LIM1863) That Grows as Organoids with Spontaneous Differentiation into Crypt-like Structures in Vitro

Robert H. Whitehead, Jennifer K. Jones, Anastasia Gabriel, and Robyn E. Lukies

ABSTRACT

A new colon carcinoma cell line (LIM1863) has been characterized. This cell line is unique in that the culture consists of organoids which are morphologically and functionally organized. Histological studies of the organoids show that the cells are arranged around a central lumen and the nuclei are polarized to the periphery. Two major morphological types are present: a columnar cell with a polarized, structurally normal brush border and goblet cells. The cells are also functionally mature and express the brush border enzymes amidopeptidase N, dipeptidyl peptidase IV, alkaline phosphatase, and sucrase-isomaltase. These enzymes are localized to the luminal membrane and the apical cytoplasm (of some cells). The goblet cells contain mucus and this mucus is secreted into the lumen. This functional differentiation suggests that the organoids contain precursor cells capable of differentiating along both the columnar and goblet cell pathways. At present no endocrine cells have been detected by morphological or histochemical analysis. The organoids have been in continuous culture with regular passaging for 21 months and also grow and differentiate normally in serum-free medium.

INTRODUCTION

The failure of current therapeutic regimens to improve the survival of colon cancer patients suggests that a better knowledge of the biology of this tumor is required before improvements in survival are seen. Such biological studies require the availability of cell lines derived from colon carcinomas which have retained features of the tissue of origin, the colonic mucosa.

Although there are now a large number of cell lines derived from colon carcinomas (1-15) only a few of these have retained any of the properties of the colonic mucosa. Some lines form polarized monolayers with a relatively normal brush border (8, 9, 14, 15), and some are functionally active and demonstrate vectorial fluid transport (16, 17). In addition many of the cell lines can be induced to differentiate either morphologically (14, 18-20) or functionally in that expression of various brush border enzymes can be induced by treatment with differentiation agents (21-24).

Recently cell lines have been described that are morphologically heterogenous with cells of different characteristics being present even in cloned cultures (11, 14, 15). In addition McBain et al. (13) have recently described a cell line that shows some of the properties associated with normal colonie mucosa. We wish to describe a new colon carcinoma cell line that exhibits many of the properties associated with normal colonic mucosa. The cells grow as organoids and are organized around a central lumen. The cells express a brush border on the luminal surface and are polarized. Both morphologically mature columnar and goblet cells are present in these organoids.

MATERIALS AND METHODS

Clinical Specimen. A representative portion of a carcinoma of the ileocecal valve was received following a right hemicolectomy performed on a 74-year-old Caucasian female. Histologically, the tumor was classified as a poorly differentiated ulcerated carcinoma which extended through the full thickness of the muscle wall.

Culture Conditions. The tissue was cultured using an explant culture method (25). In brief, the specimen was washed well with PBS2 (pH 7.2) and then soaked in 0.01% sodium hypochlorite for 20 min at room temperature. The tissue was rinsed again in PBS and cut into approximately 1-mm3 pieces. These small tissue pieces were aliquoted into 25-cm2 flasks in a small volume of growth medium (1.5 ml). The growth medium was RPMI 1640 plus 10% fetal calf serum (Commonwealth Serum Laboratories, Melbourne, Australia), 0.6 μg/ml insulin (Commonwealth Serum Laboratories), 1 μg/ml hydrocortisone (The Upjohn Co., Sydney, Australia), 10-4 M α-thioglycerol (Sigma Chemical Co., St. Louis, MO), 50 μg/ml penicillin, and 50 μg/ml streptomycin (25). After 4 days of culture at 37°C, the volume of the medium was increased to 4 ml and was then changed weekly.

Histological Staining. Pellets of LIM1863 cells were embedded in a small volume of 1% agar and fixed in 10% formalin. The pellet was embedded and sectioned using standard histological techniques.

Electron Microscopy. The cell organoids were fixed in Karnovsky's fixative (pH 7.30) overnight. The organoids were washed in PBS, postfixed in 2% osmium tetroxide, and block stained with uranyl acetate. The organoids were then dehydrated through a graded series of acetone solutions, embedded in Epon-Araldite, thin sections cut on a Reichart ultramicrotome, and stained with lead citrate. Sections were examined on a Siemens 101 electron microscope.

Attachment Studies. Surfaces were prepared by preincubation with a solution of laminin, collagen, or fibronectin for 1 h at 37°C. The attachment factor was then removed, the flask were rinsed, and the organoids were added at a final concentration of 300 organoids/flask. After 2 and 5 days the flask were examined, and the number of organoids attached to the surface and the number that showed spreading of cells onto the surface were counted. The attachment factors tested were collagen I (rat tail collagen) (26), collagen IV (Sigma), laminin (Bethesda Research Laboratories, Gaithersburg, MD), and fibronectin (Collaborative Research, Inc., Lexington, MA).

Proliferation Studies. The percentage of cells synthesizing DNA has been determined using a BUdR uptake method (27). In brief, the cells were incubated in growth medium containing 10-5 M BUdR (Sigma), and organoids were harvested at 2, 4, 6, and 32 h. The cells were pelleted and embedded in a drop of 1% agar and fixed in absolute ethanol. The pellets were sectioned and stained by immunoperoxidase using an α-BuDR monoclonal antibody (28) and standard immunoperoxidase techniques. This method has recently been shown to yield results equivalent to standard [3H]thymidine methods (29).

Immunohistochemistry and Histochemistry. The organoids were pelleted and either embedded in a small volume of 1% agar and frozen in OCT (Miles Laboratories, Inc., Naperville, IL) or placed directly in OCT and frozen. Frozen sections were cut, air-dried, fixed in cold acetone, and stained by immunoperoxidase. Antiserum used were directed against human sucrase-isomaltase, maltase-glucosamylase, dipeptidyl peptidase IV, aminopeptidase N (all kindly supplied as monoclonal antibodies by Dr. H-P. Hauri, Department of Pharmacology, University of Basel.

The abbreviations used are: PBS, phosphate buffered saline; BuDR, bromodeoxyuridine; CE, carcinoembryonic antigen; HITES, RPMI 1640 based medium supplemented with hydrocortisone, insulin, transferrin, estradiol, and selenium (33); ITS, 5 μg/ml insulin, 5 μg/ml transferrin, and 5 μg/ml streptomycin.

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1 To whom requests for reprints should be addressed.

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Biocenter, Basel, Switzerland) (30), large and small intestinal mucus antigens, (supplied by Dr. Jeng Ma, Department of Biochemistry, Monash University, Melbourne, Australia) (31), and CEA (the rabbit α-CEA antibody was obtained from Dakopatts a/s, Copenhagen, Denmark).

The periodic acid-Schiff-diastase and alkaline phosphatase staining was done using standard histochemical methods.

Cells were stained for the presence of endocrine granules using antisera to cholecystokinin, neuropeptide Y, bombesin (all obtained from Cambridge Research Biochemicals, Ltd., Cambridge, England) glucagon, substance P, vasoactive intestinal peptide, somatostatin (all obtained from Amersham International, England), and chromogranin (32) (Hybritech, Inc., San Diego, CA).

Other Culture Media. Two serum-free media, HITES and RPMI 1640 supplemented with ITS (Collaborative Research, Inc.) were tested.

Karyotype Studies. Cultures of organoids were cultured in growth medium containing 0.8 μg/ml Colcemid (Sigma) for 30 min. The organoids were rinsed in PBS and disaggregated using a solution of 0.1% trypsin, 0.02% EDTA in PBS. The spread of the chromosomes was improved by the addition of 4 μg/ml Colcemid to the hypotonic treatment. The chromosomes were spread and stained for G banding using standard techniques.

Xenograft Studies. The packed organoids were injected s.c. into 6 BALB/c nu/nu mice. Each mouse received at least 10⁷ cells. The mice were observed weekly for tumor growth. The tumors were removed using sterile techniques and divided into 2 parts, one part being used for histological studies and the other for culture studies.

RESULTS

Establishment of Cell Line. After 4 weeks in culture, floating clumps of cells (Fig. 1) were noted and harvested from the growth medium by centrifugation and returned to the culture flasks. There was no evidence of attachment of epithelial cells to the culture flasks even though fibroblasts grew out from the explants and formed a monolayer. When the floating clumps of cells increased in number sufficiently, the cultures were split and could be expanded in this way. The cells were stored in liquid N₂ in RPMI 1640 plus 20% fetal calf serum and 10% dimethyl sulfoxide by disaggregating the clumps as much as possible by vigorous pipetting and then allowing the cells to incubate in the freezing mixture for 10 min at 4°C before commencing the freezing process. The cell line has now been in continuous culture for 21 months and has been split 1:2 weekly during that period. It has been proven impossible to obtain a single cell suspension from the cell clumps despite the

Fig. 1. LIM1863 cell clumps in culture. Individual organoids can be seen. Phase contrast, × 200.

Fig. 2. Section through LIM1863 organoids embedded in agar. The organized nature of the structure of the organoids can be clearly seen. Many organoids have a lumen and the cell nuclei are polarized to the periphery. →, incomplete fission between 2 organoids. × 125.

Fig. 3. Section through an organoid embedded in agar showing morphological organization and differentiation. Both columnar and goblet cells can be seen. × 1000.
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Fig. 4. Electron micrograph showing a well formed typical goblet cell. The cell has few microvilli. × 4500.

Fig. 5. Electron micrograph showing well formed brush border. The microvilli have both cores and a terminal web. The cells are columnar and the nuclei (N) are polarized to the base of the cells. × 4500.

Table 1 Percentage of LIM1863 organoids attaching to surfaces treated with attachment factors

<table>
<thead>
<tr>
<th>Surface treatment</th>
<th>% attachment 2 day</th>
<th>% attachment 5 day</th>
<th>% spread 2 day</th>
<th>% spread 5 day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Type I collagen</td>
<td>35</td>
<td>30</td>
<td>23</td>
<td>3</td>
</tr>
<tr>
<td>Type IV collagen</td>
<td>26</td>
<td>23</td>
<td>21</td>
<td>16</td>
</tr>
<tr>
<td>Laminin</td>
<td>8</td>
<td>13</td>
<td>4</td>
<td>13</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Attachment was assessed microscopically after washing. A cell clump was considered to have spread if flattened cells were seen at the periphery of the attached cell clump.

use of a range of proteolytic enzymes. Thus, it has not been possible to clone these cells.

Histology. Sections through the agar-embedded aggregates revealed a very organized structure. The aggregates comprised a series of small organoids; many of the organoids consisted of a ring of cells arranged around a central lumen. Many of the cells were columnar and the nuclei were polarized to the outer rim (Fig. 2). The lumen often contained dead cells or cellular material indicating that a process of differentiation, maturation, and cell shedding was occurring. In very large cell clumps, areas of central necrosis were seen. Ultrastructural studies confirm this structure (Fig. 3) and also have shown the presence of morphologically mature goblet (Fig. 4) and columnar cells (Fig. 5). The columnar cells had a well formed brush border with the microvilli being polarized to the luminal membrane. The cells were linked at the luminal margin by tight junctions and junctional complexes (Fig. 5).

Attachment Studies. The floating clumps of cells will not attach and spread spontaneously. Prolonged trypsination will disaggregate the cell clumps to some degree; however, the resultant cell suspension attaches poorly and does not proliferate once attached. The cell clumps attached to culture surfaces pretreated with rat tail collagen (collagen I) [105 ± 10 cell patches (SD) at day 2] and collagen IV (80 ± 9 cell patches at day 2) but did not attach to surfaces pretreated with fibronectin or laminin (Table 1).

Some of the cell clumps that attached to the collagen coated surfaces spread onto the surfaces; however, none of these attached and spread cells proliferated to any extent and cultures of adherent cells were not obtained.

Proliferation. Because of the fact that the cell clumps are not able to be dissociated into viable single cells, it has not been possible to do standard proliferation studies. Pulse labeling with BUdR has shown that 20% of cells are labeled after 4 h.
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Table 2 Immunochemical and histochemical staining of LIM1863

<table>
<thead>
<tr>
<th>Stain</th>
<th>Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periodic acid-Schiff</td>
<td>2%+ goblet cells, some cytoplasmic staining*</td>
</tr>
<tr>
<td>Aminopeptidase N</td>
<td>Luminal membrane and apical cytoplasm</td>
</tr>
<tr>
<td>Dipeptidyl peptidase IV</td>
<td>Luminal membrane</td>
</tr>
<tr>
<td>Sucrase-isomaltase</td>
<td>Luminal membrane</td>
</tr>
<tr>
<td>Maltease-glucoamylase</td>
<td>Negative</td>
</tr>
<tr>
<td>Large intestinal mucus antigen</td>
<td>70%+ goblet cells and generalized cytoplasmic staining</td>
</tr>
<tr>
<td>Small intestinal mucus antigen</td>
<td>5%+ cytoplasmic staining</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>Luminal membrane</td>
</tr>
<tr>
<td>CEA</td>
<td>Cytoplasm of all cells</td>
</tr>
<tr>
<td>Antineuropeptide*</td>
<td>Negative</td>
</tr>
<tr>
<td>Chromogranin</td>
<td>Negative</td>
</tr>
</tbody>
</table>

* Staining was assessed on frozen sections using a 3-layer immunoperoxidase technique.
* See "Results."

Fig. 7. Three panels from a time-lapse video of LIM1863 organoids in culture. The organoid increases in size from t0 (A) to 36 h (B) and divides by fission (C, 3.5 days). × 200.

(Fig. 6). The whole organoids have been studied by time lapse recording and the organoids have been shown to divide by fission (Fig. 7).

Histochemistry and Immunochemistry. Staining results are summarized in Table 2. Goblet cells stained strongly for mucus by PAS-diastase and PAS staining was also present in the lumen of the organoids and in the cytoplasm of many cells (Fig. 8). The antimucus monoclonal antibody LIMA stained more cells with cytoplasmic staining being present in 70% of cells. Material in the lumen of the organoids also stained. In contrast, a monoclonal to the small intestinal mucus antigen stained only 5% of cells. The luminal membrane stained strongly for the brush border peptidases, dipeptidyl peptidase IV and aminopeptidase N (Fig. 9) and for the disaccharidase, sucrase-isomaltase. There was no staining for the other disaccharidase, maltase-glucoamylase. Alkaline phosphatase staining was present on the luminal membrane. The presence of these enzymes has been confirmed using biochemical assays (results not shown). All of the cells stained strongly for CEA.

No staining of endocrine granules was seen using any of the peptide antisera (bombesin, cholecystokinin, glucagon, neuropeptide Y, substance P, somatostatin, or vasoactive intestinal peptide) or with an antiserum to chromogranin.

Culture Studies. The cell line grows readily in serum-free
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media. Two such media have been used, HITES and RPMI 1640 supplemented with ITS.

Cultures have been grown for 2 months in HITES or RPMI 1640 and ITS and have been passaged regularly. No morphological or histochemical changes have been noted in cells grown for long periods in either of these serum-free media.

Karyotype. A study of 13 G-banded metaphases revealed a near triploid modal count (approximately, 80 chromosomes with much random loss). There was frequent tetrasomy of chromosomes X, 7, 11, 12, 14–16, and 19, multiple chromosome 20 (5–6 copies), and frequent disomy of chromosomes 8, 18, 21 and 22. In addition, consistent structural rearrangements were del 8p (1–3 copies), 8p+ (1 copy), 9p+ (2 copies), iso (13q) (4 copies), 17p+ (2 copies), and a marker chromosome (Fig. 10).

Xenograft Studies. The LIM1863 cells produced a tumor in 1 of 6 nude mice given injections of 0.05 ml of packed cell clumps. The cultural characteristics and morphology of the cell line established from the xenografted tumor were identical to those of the parent line. The histology of the xenografted tumor was the same as that of the original tumor from the patient.

DISCUSSION

Although a number of colon carcinoma cell lines have shown some degree of differentiation in vitro none has shown the degree of differentiation described here. The LIM1863 cell line consistently yields a degree of morphological differentiation similar to that seen in the intestinal crypt. Both columnar cells complete with a brush border and goblet cells are present, arranged around a central lumen into which mucus is secreted. The cells are also functionally mature and express alkaline phosphatase and a number of small intestinal brush border enzymes on the luminal membrane. This consistent maturation of cells over the life span of this culture (21 months) with a continuous shedding of dead cells suggests that these cells are very similar to the cells of the normal intestinal crypts and argues for the presence of an as yet unidentified precursor cell which has many of the properties of the intestinal stem cell (34–36) or of a committed progenitor cell.

Other colon carcinoma cell lines have been described that exhibit apparent differentiation in vitro. Caco2 cells form a polarized monolayer when fully confluent. This differentiation

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Fig. 10. Representative G-banded karyotype of colon cancer cell line LIM1863. 83, xxx, 3n[+x, +3, +7, -8, +11, - - -13, +14, +15, +16, - - -17, -18, +19, + + +20, -21, -22, +3 x 8p, +8p+, +2 x 9p+, +4 x iso(13q), +2 x 17p+, +mar]. J, rearranged chromosomes; mar, consistent marker chromosome.
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is spontaneous and the cells develop a brush border and synthesise brush border enzymes. Mucus containing cells were not described in this cell line (9). Other colon carcinoma lines have also been shown to contain cells capable of forming brush borders (8, 15), and some cell lines have been shown to be capable of fluid transport (9, 16, 17). In addition, we have previously described a cell line that contains both cells with brush borders and cells containing mucus (14). However the LIM1863 is unique in the degree of morphological maturation and the ability of the cells to organize into a structure characteristic of the intestinal crypt.

The organoids of LIM1863 are not totally representative of the intestinal crypts because endocrine cells have not been found in these cultures either by histochemical staining or electron microscopy. The failure to detect endocrine cells in this cell line may indicate that the endocrine lineage diverges from the absorptive cell-goblet cell pathway very early in the differentiation pathway of the crypt stem cell. Alternatively the lack of endocrine cells may support the alternate argument that these cells arise from a separate precursor cell (37). Further studies on this cell line may provide definitive evidence to validate the unitary theory proposed by Cheng and Leblond (35).

The presence of small intestinal brush border enzymes in cell lines derived from colonic tumors has been reported previously (38) and probably represents a reexpression of fetal function because these enzymes have been shown to be present in the fetal colon (38).

Little is known about the influences that act on the intestinal stem cell and its progenitors. In the bone marrow a range of growth and differentiation factors are known that influence the differentiation of the stem cell leading to the different cell lineages that are known to arise from this stem cell. The proportion of columnar and goblet cells is known to vary in the different regions of the colon (39, 40); however, nothing is known about the factors that control this pattern of differentiation. The fact that LIM1863 cells grow readily in a simple serum-free medium containing only insulin, transferrin, and selenium suggests that these cells may be capable of autonomous growth and are either producing the factors required for their proliferation, differentiation, and organization or are independent of these factors. This cell line therefore has potential as a model system in which the hormones that are believed to influence proliferation, differentiation, and organization of the gut may be studied.

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