Characterization of Quantitative Mucin Variants from a Human Colon Cancer Cell Line

Shih-Fan Kuan, James C. Byrd, Carol B. Basbaum, and Young S. Kim

Gastrointestinal Research Laboratory, Veterans Administration Medical Center, and Departments of Medicine (J. C. B., Y. S. K.) and Pathology (S. K., Y. S. K.) and Anatomy (C. B. B.), School of Medicine, University of California, San Francisco, California 94121

ABSTRACT

Colonial mucins are high molecular weight glycoproteins produced by goblet cells of colonic epithelium. Some studies have indicated that patients with colon carcinomas that produce high amounts of mucin have a poorer prognosis than patients whose tumors produce low amounts of mucin. However, the role of mucin in affecting the behavior of colon cancer cells is not well understood. To further elucidate the relationship between cellular mucin content and the growth characteristics and morphology of tumor cells, we utilized a replica plating technique and immunoscreening method to identify and purify variant clones of the human colon cancer cell line LS174T that produce high and low levels of mucin. This procedure enabled us to isolate two high mucin-containing variants (HM3 and HM7) and one low mucin-containing variant (LM12). These variants exhibited different morphology. Both high mucin variants tended to form cell aggregates and suspended cells with adjoining mucoid threads. The low mucin variant formed spread monolayers on the substratum with the formation of cell processes. Metabolic labeling using [3H]glucosamine demonstrated that high mucin variants synthesized 2-fold more mucin in the cell layer and secreted 3-fold more mucin into the culture medium than the low mucin variant. The colony-forming efficiency in semisolid agar for these variants positively correlated with their mucin content. High mucin variant cells when injected into athymic nude mice formed tumors 2-fold larger than those of the parental cells while the low mucin variant formed tumors only one-half as large as those of the parental cell line. These mucin variants should provide a useful model for understanding the biological behavior of mucinous colon cancer cells in vivo and in vitro.

INTRODUCTION

Mucinous colorectal cancer is a histologic subtype which constitutes 10 to 20% of all cases of colorectal cancer (1-6). Clinically, patients with mucinous colorectal cancer usually present with disease in a more advanced stage, have a lower resectability rate, and have a higher postoperative recurrence rate (2). Symond and Vickery (3) reported that patients with mucinous colorectal cancer have a lower 5-year survival rate (34%) than patients with nonmucinous cancer (53%). Conflicting reports, however, contend that mucinous differentiation correlates with prognosis only in certain stages of disease (4, 5). Other investigators were unable to find a correlation between mucin production and prognosis of patients (6). This contradiction raises the possibility that mucinous colorectal cancers are heterogeneous. Indeed, Umpleby et al. (2) have found that only cancers with high mucin content (more than 80% of tumor volume) are significantly different from nonmucinous cancer (2).

If mucin is a factor indicating a worse prognosis for patients with colorectal cancer, then colon cancer cells producing higher amounts of mucin might be expected to manifest more malignant phenotypes. To test this hypothesis, we used a somatic cell cloning method to isolate high and low mucin-containing variant clones from a human mucinous colon cancer line, LS174T, which has been well characterized by others (7-9). The high mucin variants exhibited markedly different morphology at both the cellular and the subcellular levels. Furthermore, the high mucin variants appeared to have greater colony-forming efficiency in soft agar and larger tumor size in athymic nude mice than the low mucin variant.

MATERIALS AND METHODS

Cell Line and Cell Culture. LS174T, a mucin-producing colon cancer line, was a gift from Dr. B. D. Kahan (7). Cells were grown in DMEM supplemented with 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate. Cultures were maintained at 37°C in a humidified 5% CO2 atmosphere. Cells in their eighth passage were used for replica plating cloning.

Antiserum Production. To produce sufficient quantities of antigen to prepare antiserum, xenografts were obtained by injecting LS174T cells s.c. into athymic nude mice. Mucin was purified according to the method of Podolsky and Isselbacher (10). Briefly, tumor xenografts were homogenized and centrifuged, and the supernatants were applied to a column of Sepharose CL-4B. The void volume fractions were dialyzed, lyophilized, redissolved in PBS, and rechromatographed on the same column. The second void volume fractions were then subjected to nuclease digestion and CsCl density gradient ultracentrifugation. The fractions with 1.3-1.5 g/ml density were pooled and designated purified mucin.

Two New Zealand White rabbits were given i.m. and s.c. injections of 0.5 mg purified LS174T xenograft mucin in Freund’s complete adjuvant. After 3 weeks a booster of 0.1 mg purified mucin in Freund’s incomplete adjuvant was administered, and after an additional 2 weeks serum was collected and used in the immunoassays.

Preliminary immunoassay experiments show that antigenicity is lost after protease treatment and reduction and alkylation, which suggests that a protein component of mucin is required for the antigenicity of this antiserum. These details will be published elsewhere.

Isolation of Mucin Variants. The replica plating technique of clonal selection has been described previously (11), and Fig. 1 depicts this method as it was used in the present study. The parental LS174T cells in their eighth passage were well trypsinized to make single cell suspensions. Approximately 500 cells were seeded into each culture plate (10 cm diameter; Falcon, Oxnard, CA) in 15 ml of DMEM. After incubation for 1 day at 37°C, each plate was examined under a microscope to document that a single cell suspension had been made. The cells were then overlaid with 2 polyester cloths (PeCap HD7-1; Tetko, Elmsford, NY). The cloths were weighted down by a monolayer of sterile 4-mm glass beads. Cultures were maintained in the incubator for 4 weeks, during which time the cells grew into the cloths. Culture medium was changed once a week. After 4 weeks, the upper cloths were discarded and the cells were washed with PBS. The cells on each plate were then trypsinized to single cell suspensions. The cells were replated in the lower chamber and cultured for an additional 2 weeks. After this time, plates were examined under a microscope to determine which plates had grown cells. Several plates were picked and the cells were maintained in DMEM. This process was repeated until subclones of high and low mucin variants were obtained. The final subclones were designated HM3 and HM7 for the high mucin variants and LM12 for the low mucin variant. These subclones were free of contamination as determined by the immunoperoxidase reaction using anti-mucin antiserum.

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The abbreviations used are: DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; PAS, periodic acid-Schiff reagent.

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2 To whom requests for reprints should be addressed, at G. I. Research Lab. (151 M2), VA Medical Center, 4150 Clement Street, San Francisco, CA 94121.

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and the lower cloths were transferred to another 10-cm bacteriological culture dish (Falcon) containing 15 ml of fresh medium. The master plates were briefly rinsed, covered with fresh medium, and kept in an incubator.

The cloths were rinsed several times with PBS through a Buchner funnel on a suction flask. Then, the cloths were briefly fixed with cold acetone and covered with 10 ml PBS containing rabbit anti-mucin antiserum (1:500 dilution) and bovine serum albumin (3 mg/ml) (Calbiochem, La Jolla, CA). The reaction was allowed to stand at room temperature for 1 h. The cloths were then rinsed three times with PBS through a Buchner funnel and incubated for 1 h more with 10 ml PBS containing 125I-protein A (10^6 cpm/ml) which was radiolabeled from protein A (Zymed Laboratories, Burlingame, CA) by the chloramine-T method (12). Finally, the cloths were thoroughly washed and allowed to air-dry. Autoradiograms were then developed from Kodak X-O-mat film exposed to the cloths. Following this, the cloths were stained with Coomassie blue (0.05% Brilliant Blue G in 10% acetic acid) for 1 h and destained with methanol:water:acetic acid (45:45:10) until the colonies were stained appropriately as dark blue spots. The phenotypes of individual colonies were assessed by superimposing the autoradiograms onto stained cloths and comparing the intensities of images and protein stains. The colonies exhibiting dark spots on autoradiograms but relatively faint protein stains were thus identified as potential high mucin variants and were isolated by local trypsinization of the master plates. The reverse applied for the selection of low mucin variants. Control experiments were carried out under the same conditions except that preimmunized rabbit serum was substituted for rabbit anti-mucin antiserum.

Morphology and Growth Properties in Vitro. Each variant line was maintained in standard culture conditions and expanded to the desired cell number. They were observed daily under a phase-contrast microscope and photographs were taken. For the study of growth properties, 2 x 10^5 cells were seeded into 16-mm wells of cluster culture plates (Costar, Van Nuys, CA). Duplicate wells were trypsinized each day and the cell number was counted with a Coulter Counter.

Metabolic Labeling of Cells. Parental and variant cells were grown in 25-cm² culture flasks until confluent. The medium was then replaced with low glucose DME H-16 medium (Grand Island Biological Co., Grand Island, NY) supplemented with 10 μCi/ml [3H]glucosamine through a Buchner funnel and incubated for 1 h more with 10 ml PBS containing 125I-protein A (10^6 cpm/ml) which was radiolabeled from protein A (Zymed Laboratories, Burlingame, CA) by the chloramine-T method (12). Finally, the cloths were thoroughly washed and allowed to air-dry. Autoradiograms were then developed from Kodak X-O-mat film exposed to the cloths. Following this, the cloths were stained with Coomassie blue (0.05% Brilliant Blue G in 10% acetic acid) for 1 h and destained with methanol:water:acetic acid (45:45:10) until the colonies were stained appropriately as dark blue spots. The phenotypes of individual colonies were assessed by superimposing the autoradiograms onto stained cloths and comparing the intensities of images and protein stains. The colonies exhibiting dark spots on autoradiograms but relatively faint protein stains were thus identified as potential high mucin variants and were isolated by local trypsinization of the master plates. The reverse applied for the selection of low mucin variants. Control experiments were carried out under the same conditions except that preimmunized rabbit serum was substituted for rabbit anti-mucin antiserum.

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Fig. 4. Morphology of parental LS174T (A), LM12 (B), HM3 (C), and HM7 (D) in cell culture. SA, spheroid aggregate; CM, cell monolayer; MT, mucoid thread; CP, cell processes. × 400.

(specific activity, 40 Ci/mmol; ICN Biomedicals, Inc., Irvine, CA). Cell number was determined from duplicate flasks. After a 24-h labeling period, flasks were chilled on ice and the medium was quickly removed. The cell layer was rinsed with PBS and the washes were combined with the medium. The cell monolayers were scraped with a rubber policeman and the cell pellets were sonicated. The cell homogenates were subjected to ultracentrifugation (100,000 × g, 1 h) and the supernatants were designated as cytosol fractions. Both the medium and the cytosol fraction were then dialyzed against distilled water at 4°C for 48 h. The dialysates were lyophilized and redissolved in sample buffer.

Gel Filtration of Labeled Glycoproteins. The medium and cytosol fractions equivalent to the same number of parental and variant cells were subjected to gel filtration in a Sepharose CL-4B (Pharmacia, Uppsala, Sweden) column with dimensions of 1 x 26 cm. The void volume and included volume were determined with blue dextran and [3H]glucosamine, respectively. Samples (100 μl) were applied to the column and eluted with 10 mM Tris-HCl buffer, pH 8. Fractions of 0.8 ml were collected and 0.1-ml aliquots were counted in a scintillation counter.

Chemical and Enzymatic Degradation. Aliquots of medium and cytosol fractions were analyzed subsequent to the following treatments. Reductive cleavage of O-glycosidic linkages by β-elimination was done in 50 mM NaOH:1 M NaBH₄ at 50°C for 48 h. Trypsin (25 μg/ml) digestion was done at 37°C for 24 h in 50 mM Tris-HCl, pH 8 (8). Digestion with chondroitinase ABC at a concentration of 5 units/ml was carried out in enriched Tris buffer (250 mM Tris-HCl:176 mM NaC₂H₄O₂:250 mM NaCl, pH 8.0) at 37°C for 15 h (8). Hyaluronidase was used at a concentration of 10 units/ml at 37°C for 16 h in 0.1 M acetate buffer, pH 5.0 (13). Heparitinase digestion using 5 units/ml was carried out in 0.1 M Tris-HCl, pH 7.2, for 24 h (14). Trypsin, chondroitinase ABC, heparitinase, and hyaluronidase (from Streptomyces) were all obtained from Sigma (St. Louis, MO).

Immunoprecipitation and Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. For immunoprecipitation, lyophilized [3H]glucosamine-labeled material secreted into the medium by the same number of
cells from each cell line was dissolved in 1 ml buffer A (1% Nonidet P-40:0.5 M NaCl:10 mM Tris-HCl, pH 8). The sample was incubated overnight at 4°C with 2 µl of anti-mucin antiserum and 100 µl of a 10% (w/v) solution of protein A-bearing Staphylococcus aureus (Zymed). Bacteria were sedimented by centrifugation at 12,000 x g for 5 min and then washed twice with buffer A. Washed bacteria were resuspended in 60 µl sample buffer and heated at 100°C for 3 min. The solubilized proteins in the supernatant were applied to a linear gradient of 3–10% polyacrylamide gel with 0.1% sodium dodecyl sulfate and examined by gel electrophoresis as described by Laemmli (15). The gel was stained, dried and autoradiographed with Kodak XAR-2 film. The autoradiogram was then scanned with a densitometer. The following standards were used for molecular weight determination: laminin, myosin, β-galactosidase, phosphorylase, bovine serum albumin, and ovalbumin.

Growth in Soft Agar. Bacteriological dishes (35 mm; Falcon) were precoated with 1 ml DMEM containing 10% FCS and 0.5% agar (Bacto-agar; Difco, Detroit, MI). This agar underlayer was allowed to solidify before use. Two ml DMEM containing 10% FCS, 0.3% agar, and 1 x 10^4 cells were overlaid onto the precoated dishes. After a 24-h incubation at 37°C in 5% CO2:95% air, all dishes were examined under a microscope to ascertain that single cell suspensions were obtained. The number of colonies larger than 0.2 mm diameter was determined using a phase-contrast microscope after 4 weeks growth. The colony-forming efficiency (CFE) was determined by the formula

\[
\text{CFE} = \frac{\text{No. of colonies}}{\text{No. of cells seeded}} \times 100\%
\]

Tumorigenicity in Nude Mice. Six- to 8-week old male NCR-/nu-nuathymic nude mice were obtained from Simonsen Laboratories, Inc. (Gilroy, CA). Single cell suspensions of parental and variant cells were prepared by brief trypsinization, and viability was determined by trypan blue exclusion. Viable cells (1 x 10^4) in 0.1 ml DMEM were inoculated s.c. into the right anterior flank area of each mouse. Tumors were
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Table 1 Radioactivity of [3H]glucosamine-labeled glycoproteins eluted in the void volume (V_o) and included volume (V_t) of Sepharose CL-4B column

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>V_o (cpm/4 x 10^6 cells)</th>
<th>V_t (cpm/4 x 10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LS174T</td>
<td>132,105</td>
<td>134,650</td>
</tr>
<tr>
<td>LM12</td>
<td>85,195</td>
<td>106,360</td>
</tr>
<tr>
<td>HM3</td>
<td>272,690</td>
<td>196,770</td>
</tr>
<tr>
<td>HM7</td>
<td>276,510</td>
<td>189,605</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>Cytosol</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>130,400</td>
<td>492,030</td>
</tr>
<tr>
<td></td>
<td>89,665</td>
<td>469,175</td>
</tr>
<tr>
<td></td>
<td>128,015</td>
<td>500,190</td>
</tr>
<tr>
<td></td>
<td>134,535</td>
<td>474,545</td>
</tr>
</tbody>
</table>

| Experiment 1 | 132,425                  | 500,510                  |
| LM12        | 79,210                   | 100,785                  |
| HM3         | 265,110                  | 200,490                  |
| HM7         | 275,385                  | 210,560                  |

| Experiment 2 | 135,850                  | 485,110                  |
| LM12        | 135,210                  | 82,660                   |
| HM3         | 85,195                   | 100,785                  |
| HM7         | 136,995                  | 490,010                  |

Fig. 6. Sepharose CL-4B profiles of [3H]glucosamine-labeled glycoproteins from medium (A) and cytosol (B) of parental LS174T cells. V_o, void volume; V_t, total volume.

Table 2 Colony-forming efficiency in soft agar

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<th>Size of colonies</th>
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<tr>
<td>LS174T</td>
<td>1.2, 1.0</td>
<td>Medium</td>
</tr>
<tr>
<td>LM12</td>
<td>0.2, 0.3</td>
<td>Small</td>
</tr>
<tr>
<td>HM3</td>
<td>4.4, 4.8</td>
<td>Large</td>
</tr>
<tr>
<td>HM7</td>
<td>9.0, 11.2</td>
<td>Large</td>
</tr>
</tbody>
</table>

* The 2 sets of numbers represent results obtained from 2 different experiments.

Fig. 8. Immunoprecipitation of radiolabeled mucin. Equal numbers of cells from LM12 (Lane A), HM3 (Lane B), HM7 (Lane C), and LS174T (Lane D) were metabolically labeled with [3H]glucosamine and the labeled medium was precipitated by anti-mucin antibody. The immunoprecipitates were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 3–10% polyacrylamide gel. K, thousands.

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measured with a caliper every other day after their appearance and tumor volume was calculated using the formula

\[ \text{Volume} = \frac{\text{Length} \times (\text{width})^2}{2} \]

At the end of each experiment (about 4 weeks) animals were sacrificed and autopsies were done to examine for possible metastasis to internal organs. The tumors were removed and fixed in 4% formaldehyde in saline for histological examination.

Histology and Electron Microscopy. The formalin-fixed xenografts obtained from mice given injections of parental and variant cells were embedded in paraffin and cut into 5-μm thin sections. Hematoxylin-eosin and PAS stains were done for each tumor. Electron microscopic examination of cultured cells was also performed, as described (16).
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RESULTS

Replica Plating Cloning. Approximately 20,000 colonies from the LS174T parental cell line were screened using the replica plating method (Fig. 1). Preliminary studies demonstrated that transfer efficiency of LS174T from master plates to the polyester cloths was nearly 100% (data not shown). All colonies on cloths can be stained with anti-mucin antiserum (Fig. 2). The preimmunized normal rabbit serum did not stain the colonies on top of the monolayer. In other areas, suspended cells connected with mucoid threads are seen. Low mucin variant LM12 forms well-spread monolayers with prominent cell processes (Fig. 4B). Mucoid threads with suspended cells were never observed in this variant. Cell aggregates were observed only in highly confluent flasks of the LM12 variant. In contrast, HM3 and HM7 variants grew as cell aggregates rich in mucoid threads even in sparse culture with low cell density (Fig. 4, C and D). Cell processes were rarely seen in these high mucin variants. At very high cell density, the morphology of high and low mucin variants could not be distinguished because cells tended to pile up. Dome formation was observed in the parental cell line as well as in all three mucin variants when cultures were confluent. This phenomenon supports the epithelial origin of these cell lines. Although the LM12 variant exhibited slightly more domes than the HM3 and HM7 variants, there was no consistent relationship between the abilities to form domes and to secrete mucins.

Transmission electron micrographs demonstrated the typical morphology of enterocytes including microvilli (Fig. 5). Cells contained both mucinous secretory granules and lipid droplets. Both HM3 and HM7 variants exhibited numerous and prominent mucinous granules with variable electron density (Fig. 5, C and D). These correspond to PAS-positive granules seen in semithin (0.5-μm) Epon sections. In contrast, LM12 variant cells contained fewer mucinous granules (Fig. 5B). Parental LS174T cells were intermediate in mucin granule content (Fig. 5A).

The growth curves showed that both parental and variant cells had similar doubling times (data not shown). High mucin variants achieved slightly higher cell densities than low mucin variant probably because of their ability to form cell aggregates.

Mucin Production in Parental and Variant Cells. The synthesis of high molecular weight mucin in the cell layer and its secretion into the medium were estimated by measuring the amount of [3H]glucosamine-labeled glycoproteins excluded on Sepharose CL-4B. Fig. 6 shows the gel filtration profiles of newly synthesized glycoproteins labeled with [3H]glucosamine from the medium (Fig. 6A) and the cytosol (Fig. 6B) fractions of parental LS174T cells. The mucinous nature of the high molecular weight (>10^6) glycoprotein peak in the void volume is supported by the following evidence: (a) the excluded peak shifts to the included volume after alkali-borohydride treatment, which preferentially cleaves the O-glycosidic linkage found in mucin (Fig. 7B); (b) the resistance of this excluded peak to trypsin digestion (Fig. 7C) is consistent with the high content of carbohydrate side chains in mucin; (c) its insensitivity to hyaluronidase, heparitinase, and chondroitinase treatment (Fig. 7, D to F) argues against the presence of glycosaminoglycans or proteoglycans in this fraction. The high and low mucin variants were subjected to the same procedure of cell labeling and gel filtration as above and the void volume and included volume were pooled separately. The radioactivities from each variant were compared on the basis of cell number (Table 1). High mucin variants had over 3-fold more [3H]glucosamine-labeled mucin secreted into the medium than the LM12 variant. A less pronounced difference (approximately 2-fold) was observed when the mucin in the cell layer (cytosol) was compared. The mucin synthesis and secretion of the parental cells were closer to those of the low mucin variant, consistent with the observation that most cells in the parental line exhibit the morphology of the low mucin variant.

Examination of immunoprecipitated [3H]glucosamine-labeled mucin by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed a diffuse high molecular weight band which only barely penetrated the running gel and stayed on the top of 3-10% gradient gel (Fig. 8). The molecular weight was estimated ranging from 400,000 to greater than 1 million. Approximately 50-70% of the total radioactivity can be precipitated from the parental and variant cell lines. The completeness of immunoprecipitation has been checked by retrograding the supernatants, in which case less than 1% of the residual radioactivity was precipitated. Densitometric scanning demonstrated a 2- to
3-fold greater intensity for mucin bands from high mucin variants than that from low mucin variant.

Colony-forming Efficiency in Soft Agar. HM3 and HM7 variants had colony-forming efficiencies ranging from 4.4 to 11.2% while that of LM12 variant was only 0.2% (Table 2). The colony-forming efficiency of parental cells was intermediate between the high and low mucin variants. Furthermore, the size of colonies also differed among the variants (Table 2). Both HM3 and HM7 formed large almost opaque colonies which could be visualized with the naked eye. The colonies were so tight and compact that light could hardly pass through them when observed under a phase contrast microscope. In contrast, low mucin variant LM12 formed only a few tiny and loose colonies while most cells remained single.

Tumorigenicity in Athymic Nude Mice. Viable cells (10⁶) from the parental or variant cells were injected s.c. into the flank area of nude mice with five mice for each group. Tumors became visible between the seventh and tenth days after inoculation. The take rate of both the parental and variant cells was 100%. However, there were dramatic differences in tumor size. Fig. 9 compares the mean values of tumor volume from each group of five mice. LM12 variant cells reached a mean tumor size of only 0.3 cm³ at the end of 4 weeks. In contrast, HM7 and HM3 reached mean sizes of 2.4 cm³ and 1.8 cm³, respectively. The mean tumor size of LS174T parental line was 1.0 cm³ at the termination of the experiment. With the exception of one mouse given injections of HM7 cells that died of overwhelming tumor burden at the 24th day postinoculation, all other mice survived until sacrifice. Autopsies from mice given injections of HM3 and HM7 cells showed the presence of well-encapsulated s.c. tumors filled with whitish, chalky, and mucoid materials. Those tumors formed by LM12 cells were small and solid with less mucoid substance. No gross tumor metastasis was observed for either the parental or the variant cells. Histologically, xen-
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Fig. II. Section of xenografts from nude mice given injections of LS174T (A), LM12 (B), HM3 (C), and HM7 (D) cells. PAS, × 200.

Oxografts from the parental cell lines showed moderately well-differentiated tumors (Fig. 10A) with glandular structures filled with PAS-positive material (Fig. 11A). Histological sections from HM3 and HM7 xenografts demonstrated well-differentiated tumors with prominent glands (Fig. 10, C and D) and strong PAS staining inside gland lumens (Fig. 11, C and D). In contrast, LM12 xenografts contained cord-like structures indicating a grade of poor differentiation (Fig. 10B). Some glands were empty and without any PAS-positive material (Fig. 11B).

DISCUSSION

Colorectal cancer is one of the leading causes of cancer death in this country (17) and several factors appear to affect the prognosis of patients with this malignancy (18). Clinical stage is the most important prognostic factor (19). The histology of tumors including the cellular type and tumor grade is also an important determinant (1). Mucinous carcinoma is a subtype characterized by cells with high capacity for producing mucin. Although it constitutes only 10–20% of all cases of colorectal cancer, patients with this subtype usually have poor prognosis (2–5). However, whether the presence of mucin per se is an independent prognosis factor or is interrelated with other as yet undetermined variables is not known. For the most part, this is due to a lack of any experimental system for examining the potential role of mucin in colon cancer cell behavior. In this paper, we now report the establishment of an experimental model for studying the biological properties of colon cancer cells that vary in mucin content.

Cell cloning has long been used for the determination of cell heterogeneity in vitro. Limiting dilution followed by screening is the conventional strategy of cell cloning (20). However, this procedure is time consuming and tedious. Recently, replica plating has proved to be a quick, sensitive, and versatile method
of cell cloning (11, 21–23). It can be adjusted to several tools of screening including enzyme assay (21), isotopic incorporation (22), and antiserum binding (23). Using this technique, we were able to screen 20,000 colonies in 2 days and finish the cloning procedure within 4 weeks.

When the mucin variants were compared with respect to morphology, both high mucin variants tended to form cell aggregates whereas the low mucin variant formed well-spread monolayers. In the parental cells, the spheroid aggregates tended to pile on the monolayer (Fig. 4A). Such a topographic distribution of high and low mucinous cells is compatible with the observation of Augeron and Laboisse (24) that colon cancer cells induced to secrete mucin by butyrate treatment were arranged into basal layers of undifferentiated cells and a superficial layer of mucus-secreting cells. The reason why mucinous cancer cells assume different morphology in vitro can only be speculated upon at present. It is possible that the high concentration of mucin present in the medium of high mucin variant may reduce the cell-cell interaction. Alternatively, the cell shape may be a regulatory factor of mucin biosynthesis and secretion. Indeed, Hand et al. (9) have found that LS174T cells grown in suspension or spheroid culture, which may change the spatial configuration of cells, demonstrated a 2- to 7-fold increase of a mucin antigen.

The amounts of mucin produced by these variants were quantitatively measured by gel filtration and immunoprecipitation of [3H]glucosamine-labeled glycoproteins. Sepharose CL-4B patterns showed that high mucin variants incorporated 2- to 3-fold more [3H]glucosamine into cell layer mucin than 3-fold more [3H]glucosamine into the secreted mucin than the low mucin variant. Immunoprecipitation revealed the same trend as gel filtration. When cells were injected into nude mice, they maintained the relative abilities of mucin production as in cell culture system. This has been semiquantitatively determined by PAS stain. High mucin variants also exhibited more PAS-positive materials than low mucin variant.

The growth of cancer cells in soft agar measures their ability to grow without attachment to solid substratum (25–27). The mechanism for why high mucin variants have higher efficiency of colony formation in soft agar is not well understood at present. The secretion of mucin into soft agar may provide the cells with a "sticky" milieu as a substratum for growth. Alternatively, the high mucin variant cells filled with mucinous granules may exhibit a round shape which facilitate cell growth without substratum.

Many factors are involved in the tumor formation in athymic nude mice (28). These include the cell-doubling time in vivo, angiogenesis around the tumor, and host immunity against tumor. There is evidence suggesting that mucin is a substance that inhibits the cellular immunity in colon cancer patients (4). It is possible that mucin may also inhibit lymphocytes of athymic nude mice and subsequently enhance the tumor size. Whether high mucin variants have increased angiogenic ability or in vivo doubling time is not known from present study. However, the secretion of large amounts of mucin is apparently a factor responsible for the larger tumor size in high mucin variants because the xenografts from the latter exhibit larger areas of PAS-positive material.

Tumor heterogeneity of colon carcinoma has been reported in several systems (29). Brattain et al. (30) isolated three cell lines from a colon tumor using Percoll gradient. These three lines differ in the morphology, carcinoembryonic antigen content, tumorigenicity in nude mice (30), and chemotherapeutic responses (31). Dexter et al. (32) selected two clones from a colon cancer cell line by difference of morphology. They found these two clones also differ in karyotypes, mucin content, cloning efficiency in soft agar, histology of nude mice xenografts (32), and response to X-irradiation or chemotherapeutic drugs (33). These studies have shown that clonal populations that differ in various phenotypes can be isolated from a heterogeneous parental colon cancer cell line. However, no study to date has attempted to isolate clones based on mucin content. In this report, we have isolated three clones with different levels of mucin production and secretion from a mucin-producing colon cancer cell line and found that the high mucin variants exhibited high colony-forming efficiency in soft agar and larger tumor size in athymic nude mice. It is not known whether mucin heterogeneity is the direct cause of difference in tumor size. This cannot be tested prior to the availability of specific inhibitors of mucin biosynthesis.

The present model should provide a useful system for investigating the interrelationship between mucin content and several biological properties of colon cancer cells such as tumorigenicity, invasion, metastasis, immunogenicity, and chemotherapeutic susceptibility.

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Shih-Fan Kuan, James C. Byrd, Carol B. Basbaum, et al.


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