Evidence for the Adenoma to Carcinoma Sequence

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ABSTRACT

The purpose of this study was to establish an \textit{in vitro} model for tumor progression in colorectal carcinogenesis, by transforming the premalignant human colonic PC/AA adenoma cell line to the malignant phenotype. A rare clonogenic variant AA/C1 (colony-forming efficiency (CFE) on plastic of 1.05%) was isolated from the diploid PC/AA adenoma cell line (C. Paraskeva, S. Finerty, and S. Powell, Int. J. Cancer, 41: 908-912, 1988). AA/C1 was aneuploid and when treated with 1 mm sodium butyrate for 14 days gave rise to the AA/C1/SB cell line which had an increased CFE on plastic (6.13%) although the cells remained anchorage dependent and nontumorigenic. After exposure of these AA/C1/SB cells to the carcinogen /V-methyl-A"-nitro-/V-nitrosoguanidine (C. Paraskeva, S. Finerty, and S. Powell, Int. J. Cancer, 41: 908-912, 1988). AA/C1 was aneuploid and when treated with 1 mm sodium butyrate for 14 days gave rise to the AA/C1/SB cell line which had an increased CFE on plastic (6.13%) although the cells remained anchorage dependent and nontumorigenic. After exposure of these AA/C1/SB cells to the carcinogen \textit{N-methyl-N'-nitro-N-nitrosoguanidine} an anchorage-independent cell line was isolated (AA/C1/SB10). On continuous \textit{in vitro} passage, the CFE in agarose of AA/C1/SB10 has increased to 17.3% and the cells have become tumorigenic producing adenocarcinomas in athymic nude mice. AA/C1, AA/C1/SB, and AA/C1/SB10 cell lines have common chromosomal abnormalities including a pericentric inversion of chromosome 1 with deletion of part of the short arm and monosomy for chromosome 18. This \textit{in vitro} progression provides the first reported experimental evidence for the adenoma to carcinoma sequence in the human colon, and the cytogenetic evidence suggests that it is relevant to \textit{in vivo} carcinogenesis.

INTRODUCTION

Carcinoma of the human colorectum is an excellent example of the complex multistage nature of cancer development. Most colorectal cancers are thought to develop from premalignant adenomas (benign tumors sometimes called polyps) in what is called the adenoma-to-carcinoma sequence (1). The isolation of a premalignant adenoma-derived epithelial cell line designated PC/AA from a patient with the rare hereditary disease familial adenomatous polyposis (2) has provided the opportunity to study tumor progression \textit{in vitro}. This cell line, although normal diploid at early passage, has become immortal and shown signs of tumor progression with continuous \textit{in vitro} passage (3, 4) but remained anchorage dependent and nontumorigenic. In order to study tumor progression in human colorectal carcinogenesis, however, it is necessary to be able to characterize cellular phenotypes representing both the premalignant and malignant stages, and we have described several possible \textit{in vitro} and \textit{in vivo} markers for such studies (reviewed in Refs. 4 and 5). Previously, \textit{in vitro} multistage carcinogenesis has mostly been studied in rodent systems, (for example, Refs. 6 and 7). However, although there are relatively few reports describing carcinogen-induced transformation of human epithelial cells, Rhim \textit{et al.} (1986) have described the neoplastic transformation by MNNG\textsuperscript{3} of human keratinocytes previously immortalized with SV40 (8). In this paper we report multiple steps in the transformation of the premalignant adenoma cell line PC/AA to a tumorigenic phenotype. The potent carcinogen MNNG and the naturally occurring fatty acid sodium butyrate were used in these transformation experiments. Sodium butyrate was used because it has been proposed to have a possible role in tumor promotion in human colorectal carcinogenesis (4). This is the first report of experimental evidence for the adenoma-to-carcinoma sequence in the human colon.

MATERIALS AND METHODS

Conditioned Medium. Confluent monolayers of Swiss 3T3 feeder cells were grown under standard culture conditions as described by Paraskeva \textit{et al.} (2), except that in this investigation the cells were grown in 5% CO\textsubscript{2} in air and not in 10% CO\textsubscript{2}. They were passaged at a 1:10 split ratio once a week. The medium to be conditioned was Dulbecco’s modified Eagle’s medium (Flow Laboratory) supplemented with 10% FBS (batch selected), glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 \mu g/ml), which was put on the confluent 3T3 cell layers for 24 h. After conditioning, the medium was filtered through a 0.2-\mu m filter (Nalgene) and supplemented with further FBS to bring the concentration up to 20%, and hydrocortisone sodium succinate (1 \mu g/ml) and insulin (0.2 units/ml) were also added.

Culture Conditions of AA/AA. The PC/AA cell line was derived from a single large colonic tubular adenoma of 3-4 cm in diameter, that exhibited only mild dysplasia (2). Unless stated otherwise the PC/AA cells were grown under standard culture conditions described by Paraskeva \textit{et al.} (2, 1984) at 37°C in 5% CO\textsubscript{2} in air.

Isolation of the Clonogenic Variant, Designated AA/C1. Although PC/AA was routinely passaged with dispase at a 1:4 split ratio, at passage 50 the cells were trypsinized to single cells (0.1% Diffco trypsin containing 0.1% EDTA) and seeded into six T25 collagen-coated flasks at 1 \times 10\textsuperscript{5} cells/flask. They were grown under standard culture conditions but in the absence of 3T3 feeders. The cultures were medium-changed twice a week, and routinely examined microscopically. After 6 weeks only one large colony (>1000 cells) had grown in any of the six flasks. This colony was trypsinized and re-plated into a collagen-coated flask and 3T3 feeders were added. These cells were then routinely passaged using trypsinization as described above, grown with 3T3 feeder cells on plastic (the flasks were no longer coated with collagen), and designated AA/C1. As the cells had retained the ability to be passaged with trypsin, a clonogenic variant had been isolated. From passage 61 (this is equivalent to 50 passages with dispase and 11 from trypsinized cells) the culture conditions of the AA/C1 cells were changed, 3T3 feeder cells were no longer used, and the cells were grown in 20% FBS-conditioned medium on plastic. Further cell lines, designated AA/C1/SB and AA/C1/SB10, were isolated from the AA/C1 cells. The derivation of these lines is described in “Results.”

Culture Conditions of AA/C1, AA/C1/SB, and AA/C1/SB10 Cells. These cells were grown, unless otherwise stated, in 20% FBS-conditioned medium in 25-cm\textsuperscript{2} plastic tissue culture flasks (not collagen-coated). Routine serial passage at a 1:6 split ratio was carried out using 0.1% trypsin containing 0.1% EDTA.

Chromosone Preparations. Chromosomes were prepared according to standard procedures and identified by G-banding (9).

Animal Experiments. Cultures were tested for tumorigenicity by s.c. injection into athymic ICRF (Imperial Cancer Research Fund) nu/nu mice (Table 1). A cell line was recorded as nontumorigenic if the mice remained tumor free 6 months after injection. The method used was described by Paraskeva \textit{et al.} (2). Briefly, dispase solution (a neutral protease; Boehringer, prepared in growth medium at 2 units/ml) was added to the flasks for 30 min, the cells were then removed as sheets, mechanically broken up, and injected as cell clumps. However, the cell...
lines that could be passed by trypsinization were injected as single cell suspensions, although some lines were injected both as single cells and cell clumps (Table 1 and Fig. 1). The number of cells injected per mouse is shown in Table 1, inoculations were always checked for viability by parallel growth in vitro.

Verification of the Origin of the Cells. To confirm that the AA/C1 cell line was derived from the original PC/AA line isoenzyme analysis was carried out, (3). The origin of the AA/C1, AA/C1/SB, and AA/C1/SB10 cell lines from PC/AA was further verified by DNA fingerprinting (result not shown) using a HinF1 digest and the minisatellite probe 6.3 (10). The fingerprint results were also supported by the finding of a marker chromosome 1 common to the AA/C1, AA/C1/SB, and AA/C1/SB10 cell lines (refer to Fig. 2).

Sodium Butyrate Treatment. AA/C1 cells (passage 59), 10⁴/flask, were grown under standard conditions for 1 week after trypsinization (approximately 50% confluent). The cells were then treated continuously with 1 mM sodium butyrate (in 20% FBS standard medium) for 14 days which included twice weekly medium changes. Growth of the cells under these conditions resulted in a reduction in the cell yield of about 50% as determined by observation under phase contrast and cell counts (data not shown). The sodium butyrate-treated cells were then allowed to grow to confluence and routinely passaged under standard growth conditions.

MNNG Treatment. AA/C1 and AA/C1/SB cells (both at passage 64) were seeded at 10⁶ cells/flask and allowed to grow until 60–80% confluence (8–10 days). The cells were then treated with a range of MNNG concentrations; 0 (dimethyl sulfoxide control), 0.1, 1.0, and 10 μg/ml for 24 h, and allowed to recover without passaging under standard growth conditions. The stock solution of MNNG was stored at a concentration of 5 mg/ml in dimethyl sulfoxide at -20°C.

Determination of Colony-forming Efficiency on Plastic. A single cell suspension was obtained after trypsinization, and an appropriate number (1 × 10⁴–5 × 10⁵) of cells were added to 25-cm² flasks with 4 ml of conditioned medium. The cells were allowed to grow for 14 days under standard growth conditions, the colonies were then stained with methylene blue. Only colonies greater than 50 cells were counted.

Determination of Colony-forming Efficiency in Soft Agar. The test for anchorage-independent growth was carried out as reported by Paraskeva et al. (3): 5 × 10⁵ cells were suspended in 1.5 ml of 0.33% agarose (Sea Plaque; Miles, Slough, United Kingdom) in the appropriate medium and seeded over 5 ml of a base layer of 0.5% agarose in 5-cm Petri dishes.

Histology. For cultured cells, dispase solution was added to the flasks for 30 min, the epithelial cells were removed as sheets and fixed in formaldehyde. For tumor sections, mice were sacrificed by cervical dislocation, the tumor dissected out and immediately fixed in formaldehyde. Paraffin wax blocks were then prepared of both and the morphology of the cells examined by staining with hematoxylin & eosin. The mucin distribution was studied by using an Alcian Blue counter stained with periodic acid-Schiff.

RESULTS

Transformation of the Adenoma Cells (See Fig. 3 for Summary). The cell line PC/AA at early passage was diploid. On continuous passage it became aneuploid but retained a very low colony-forming efficiency on plastic (<0.00001%, Ref. 1 and Table 2). A clonogenic variant AA/C1 was isolated from PC/AA at passage 50, which had a colony-forming efficiency on plastic of 1.05%. The CFE of AA/C1 was therefore more than 1000-fold greater than that of the PC/AA cells of equivalent passage, although the AA/C1 cells were still anchorage dependent and nontumorigenic. At passage 59 (this is equivalent to 50 passages with dispase and nine passages from the time the cell line AA/C1/SB was obtained) the AA/C1 cells were treated with 1 mM sodium butyrate as described above and the cell line AA/C1/SB was obtained. These cells were distinguishable from the parent AA/C1 line as they had a higher colony-forming efficiency on plastic (Table 2), although they remained anchorage dependent and nontumorigenic. Furthermore, those cells pretreated with 1 mM sodium butyrate were insensitive to the inhibitory effects of further treatment with the same concentration, and less sensitive to higher concentrations of sodium butyrate (Fig. 4). This phenotypic change represented a stable event, as the AA/C1/SB cells maintained an increased CFE on plastic throughout the duration of study (for more than 15 passages).

In an attempt to transform cells to an anchorage-independent phenotype both AA/C1 and AA/C1/SB cells were treated with

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**Table 1** Tumorigenicity of human colorectal cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Passage no. when tested</th>
<th>No. of mice inoculated</th>
<th>Age* (wk)</th>
<th>No. of cells injected/mouse</th>
<th>Time observed (mo)</th>
<th>No. of tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA/C1</td>
<td>57</td>
<td>6</td>
<td>3–4</td>
<td>1 × 10⁸</td>
<td>&gt;6</td>
<td>Tumor free</td>
</tr>
<tr>
<td>AA/C1/SB</td>
<td>67–75</td>
<td>10</td>
<td>4–6</td>
<td>0.5–1 × 10⁹</td>
<td>&gt;6</td>
<td>Tumor free</td>
</tr>
<tr>
<td>AA/C1/SB10</td>
<td>65–66</td>
<td>7</td>
<td>4–5</td>
<td>1 × 10⁹</td>
<td>&gt;6</td>
<td>Tumor free</td>
</tr>
<tr>
<td>AA/C1/SB10 (picked from agarose)</td>
<td>70–71</td>
<td>7</td>
<td>4–5</td>
<td>1 × 10⁹</td>
<td>&gt;6</td>
<td>Tumor free</td>
</tr>
</tbody>
</table>

* Age of mice when inoculated.

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**Fig. 1.** Relationship between in vitro passage number of AA/C1/SB10 cells and their colony-forming efficiency in agarose. **O**, passage number at which the anchorage-independent growth of the cells was tested. Represented are the passage numbers that were tested for tumorigenicity by s.c. injection in nude mice; **I**, cells injected as a single cell suspension; **I**, cells injected as clumps. The number of cells per inoculum is given in Table 1. **I**, passage number at which the AA/C1/SB cells were treated with 10 μg/ml of the carcinogen MNNG. The carcinoma cell line HT29 (a positive control), at passage 182, had a CFE in agarose of 26.5% (result not shown.)
HUMAN COLONIC EPITHELIAL CELL TRANSFORMATION

Fig. 2. G-Banded karyotype of AA/C1/SB10 cells at passage 72: 71, XY. The pericentric inversion between p32 and q23 on chromosome 1 and the loss of the short arm fragment distal to band p32, as well as the single chromosome 18, are shown.

Progression of the PC/AA adenoma cell line in vitro.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC/AA (early passage)</td>
<td>Diploid, non clonogenic</td>
</tr>
<tr>
<td>50 passages with dispase</td>
<td></td>
</tr>
<tr>
<td>PC/AA (late passage)</td>
<td>Aneuploid, non clonogenic</td>
</tr>
<tr>
<td>Trypsinization to single cells</td>
<td></td>
</tr>
<tr>
<td>AA/C1</td>
<td>Aneuploid, clonogenic</td>
</tr>
<tr>
<td>Sodium butyrate treatment (1mM NaB)</td>
<td></td>
</tr>
<tr>
<td>AA/C1/SB</td>
<td>Aneuploid, clonogenic, increased resistance to NaB</td>
</tr>
<tr>
<td>Carcinogen treatment (10ug/ml MNNG)</td>
<td></td>
</tr>
<tr>
<td>AA/C1/SB10</td>
<td>Aneuploid, clonogenic, increased CFE on plastic</td>
</tr>
<tr>
<td>Anchorage independent</td>
<td></td>
</tr>
<tr>
<td>in vitro passage</td>
<td></td>
</tr>
<tr>
<td>AA/C1/SB10</td>
<td>Tumorigenic in nude mice</td>
</tr>
</tbody>
</table>

Fig. 3. Schematic representation of the progression of the adenoma cell line PC/AA to the tumorigenic cell line AA/C1/SB10.

Table 2 Colony-forming efficiency of the human colorectal cell lines on plastic

<table>
<thead>
<tr>
<th>Cell line</th>
<th>CFE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC/AA (early passage)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>PC/AA (late passage)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>AA/C1</td>
<td>1.05 ± 0.30</td>
</tr>
<tr>
<td>AA/C1/SB</td>
<td>6.13 ± 0.81</td>
</tr>
<tr>
<td>AA/C1/SB10</td>
<td>6.59 ± 0.73</td>
</tr>
</tbody>
</table>

* Taken from Ref. 2.
+ Taken from Ref. 3.
' Mean ± SD of triplicate results.

A range of concentrations of the carcinogen MNNG (0.1–10 μg/ml). The purpose of treating both cell lines was to determine whether the change resulting from the sodium butyrate treatment rendered the cells more susceptible to transformation by MNNG. The toxicity of the carcinogen treatment was assessed by determining the colony-forming efficiency of the cells on plastic immediately after the 24-h exposure to MNNG. The toxicity of the MNNG was found to be comparable for the two cell lines, AA/C1 and AA/C1/SB. The immediate CFE of both cell lines treated with 10 μg/ml MNNG was undetectable in this assay (Table 3). The controls and all treatment groups were left to recover without passaging under standard growth conditions before testing for anchorage independence. This recovery period was 4 weeks for all cells except those cells treated with 10 μg/ml MNNG. Because of the high toxicity of the 10-μg/ml treatment, the recovery period for these cells was longer (6–8 weeks). This was due to the small number of viable cells left in the flasks after exposure to this MNNG concentration. When tested for anchorage independence, the only cells able to...
was then exposed to further concentrations of sodium butyrate for 14 days as shown, and the cell yield compared with that from the nonpretreated AA/C1 cells at passage 60. •, AA/C1/SB cell number; O, AA/C1 cell number. Error bars, standard deviation of triplicate flasks.

Table 3 Colony-forming efficiency on plastic of the AA/C1 and AA/C1/SB cell lines after 24-h treatment with the carcinogen MNNG

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MNNG concentration (µg/ml)</th>
<th>Relative plating efficiency (%)</th>
<th>Relative colony-forming efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA/C1/SB</td>
<td>Control</td>
<td>0.1</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>4.47 (±0.58)</td>
<td>85.5</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>3.82 (±0.19)</td>
<td>73.0</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>&lt;0.0001*</td>
<td></td>
</tr>
<tr>
<td>AA/C1</td>
<td>Control</td>
<td>1.60 (±0.18)</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>1.29 (±0.16)</td>
<td>80.9</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.86 (±0.14)</td>
<td>53.5</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>&lt;0.0001*</td>
<td></td>
</tr>
</tbody>
</table>

*The parallel 10-µg/ml treatment flasks were left to recover without trypsinization (refer to text), and a sufficient number of cells were able to grow under these conditions to give rise to the AA/C1/SB10 and AA/C1/10 cell lines.

Table 4 Anchorage-independent growth of PC/AA and the cell lines AA/C1 and AA/C1/SB after carcinogen treatment

<table>
<thead>
<tr>
<th>Cell line</th>
<th>CFE % in agarose</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC/AA</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AA/C1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AA/C1/0.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AA/C1/10</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AA/C1/SB</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AA/C1/SB 0.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AA/C1/SB 1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AA/C1/SB 10</td>
<td>0.16</td>
</tr>
</tbody>
</table>

*The number at the end of the cell line name represents the treatment concentration of MNNG (µg/ml).

grow in agarose were the AA/C1/1/SB cells treated with the high concentration of 10 µg/ml MNNG (Table 4). The AA/C1/SB cells treated with 0.1 and 1.0 µg/ml MNNG did not grow in agarose (passage 67). Furthermore, none of the original AA/C1 cells treated with the carcinogen formed colonies in agarose, even those treated with 10 µg/ml MNNG (passage 69).

The anchorage-independent line was designated AA/C1/ SB10. The colony-forming efficiency of these cells on plastic was not significantly different from the AA/C1/1/SB cells of equivalent passage, and remained elevated when compared to the AA/C1 cell line, (Table 2). After 12 successive passages the CFE on plastic of the anchorage-independent AA/C1/ SB10 cell line was 10.70% whereas that of the anchorage-dependent AA/C1/1/SB line was 7.84%.

Anchorage-independent Growth. The first detectable colony-forming efficiency in agarose of the cell line AA/C1/ SB10 was 0.16% at passage number 65. However, this colony-forming efficiency increased substantially with successive passages and has so far reached 17.3% of passage 82, as illustrated in Fig. 1. Approximately 20 individual colonies were picked from agar at passage 68 and grown in 2-ml wells (on plastic) in 3T3-conditioned medium under standard culture conditions. These colonies were then trypsinized and the cells were combined in a T25 plastic flask and grown to confluence under standard conditions. The cells were designated “agar-picked.” When the colony-forming efficiency in agarose of these “agar-picked” cells was measured, it was found not to be significantly different from the AA/C1/1/SB10 cells of equivalent passage number (data not shown); therefore the agar-picked colonies did not have a growth advantage in agar over the parental cells.

It is of interest to note that the AA/C1/1/SB cell line (not treated with the carcinogen) has become anchorage independent at late passage and that the colony-forming efficiency in agarose increased from <0.00001% at passage 66 to 4.61% at passage 76. However, there have been no detectable colonies (<0.00001%) of the untreated AA/C1 cells in agarose at either early (passage 56) or late passage (passage 71).

Tumorigenicity. The cell lines under investigation have been regularly injected into nude mice and monitored over the following 6-month period for tumorigenicity (Table 1). All AA/C1 and AA/C1/SB lines, injected both as single cell suspension and clumps of cells, did not form tumors in nude mice.

The AA/C1/1/SB10 cells, due to their increasing CFE in agarose, have been injected into nude mice at specified intervals during the progression of these cells, (as shown in Fig. 1). All animals given injections of cells below passage 70 remained tumor free.

A proportion of mice injected with AA/C1/1/SB10 cells at passage 73 and higher have developed small (approximately 0.5 cm²) but persistent tumors at the site of injection which have not increased in size and have not developed into progressively growing tumors (Table 1). However, a number of mice injected with AA/C1/SB10 cells (either as a single cell suspension or cell clumps) have developed large progressively growing tumors (>1 cm², refer to Table 1). Therefore, to summarize, from a total of 36 mice injected with AA/C1/SB10 cells from passage 73, 10 have developed small persistent tumors and 11 have gone on to develop large progressively growing tumors at the site of inoculation. Furthermore, the number of mice which develop progressive tumors is related to the passage number of the cells injected; those mice inoculated with the later passage numbers are more likely to develop the progressive tumors.

The static tumors produced when AA/C1/1/SB10 cells were injected into nude mice were moderately to well differentiated. The colonic epithelial cells displayed some glandular organization (Fig. 5), and although few goblet cells could be detected, the luminal spaces did contain significant amounts of mucin as revealed by Alcian Blue staining (Fig. 5). The progressive tumors (>1 cm²) were found to be heterogeneous, with areas of moderately to well-differentiated cells as well as large areas of poorly differentiated cells. These poorly differentiated areas showed little or no glandular formation and very little mucin production.

Morphology of Cell Lines Grown in Vitro. The parental diploid cell line PC/AA displayed glandular organization and...
produced mucin and goblet-like cells in vitro (2), characteristics that are partially retained by the AA/C1 and AA/C1/SB cell lines. However, unlike these anchorage-dependent adenoma cell lines (PC/AA, AA/C1, and AA/C1/SB), the anchorage-independent AA/C1/SB10 cells are unable to organize and differentiate in vitro. They do not form glandular-like structures in culture, there are no true goblet cells and little mucin production.

Karyotypic Analysis of the Cell Lines. PC/AA, at early passage was diploid and at late passage (passage 50–60), after subculture in the presence of 3T3 feeders, became aneuploid (2). The karyotypes from 10 different spreads from each of the cell lines AA/C1, AA/C1/SB, and AA/C1/SB10 (between passages 66 and 70) showed that all three cell lines were aneuploid and although complex shared some common chromosomal abnormalities. A typical karyotype of AA/C1/SB10 is shown in Fig. 2. The common abnormalities include the presence of one to two copies of an abnormal chromosome 1 as well as one to two normal copies of chromosome 1. The abnormal chromosome 1 has undergone a complex rearrangement involving breakpoints at bands p32 and q23, followed by a pericentric inversion with loss of the short arm fragment distal to band p32. Other notable abnormalities include up to six copies of chromosomes 7, 9, and 13 and monosomy of chromosome 18. Further molecular characterization of these cells are now in progress. The presence of the deleted chromosome 1 in all three cell lines acts as a marker chromosome and further verifies the origin of the anchorage-independent cell line.

DISCUSSION

The development of colorectal cancer is a complex multistage process which is thought to involve up to six separate events (11). Although in colorectal carcinogenesis there is a clear premalignant stage, the adenoma, it is complicated by a number of histological states of adenoma which represent premalignant cell populations of varying malignant potential. For example, in general small tubular adenomas of less than 1 cm in diameter have a low malignant potential, whereas large villous adenomas have a high malignant potential and the tubular villous adenomas fall somewhere between the two (1).

The aim of this investigation was to provide an in vitro model to study the sequential changes involved in the progression from colonic adenoma to carcinoma. An analysis of the precancer stages is essential in understanding the complex initiation and promotional events involved in the development of the adenomas and their progression and conversion to malignancy.

In previous work we have exploited the different in vitro

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Fig. 5. High-powered section of a glandular structure from a moderately to well-differentiated tumor formed in an athymic nude mouse after s.c. injection of AA/C1/SB10 cells. A, section (×100) stained with H&E, showing the glandular organization of the xenograft. B, section (×100) stained with Alcian Blue/periodic acid-Schiff. Arrow, positive material within the luminal spaces of glandular-like structures. Similar glandular organization was seen both in the static and progressive tumors.
growth characteristics of the adenoma versus malignant cell types to distinguish the intermediate stages of cancer development (3). Potential markers to study tumor progression in vitro include: escape from senescence (in vitro immortalization), aneuploidy, clonogenicity, resistance to the inhibitory effects of sodium butyrate, anchorage-independent growth, and tumorigenicity in athymic nude mice (reviewed in Ref. 5). The PC/AA adenoma cell line, which at early passage is normal diploid and nontumorigenic, was chosen for this investigation, the results of which are summarized in Fig. 3. Successive in vitro passage with dispase resulted in an immortal cell line which although aneuploid remained nonclonogenic and nontumorigenic (3). Taking advantage of our previous observation that colorectal cancer cell lines will grow clonally (after single cell genic (3)), we deliberately selected for a clonogenic variant of late passage PC/AA designated AA/C1 (Table 2). We argued that a clonogenic variant of late passage PC/AA may represent a later stage in tumor progression and that the adenoma cells are acquiring certain characteristics of cancer cells. AA/C1 however remained anchorage dependent and nontumorigenic.

We have previously reported that the colorectal carcinoma cell lines PC/JW and HT29 are more resistant to the growth-inhibitory effects of sodium butyrate than the PC/AA adenoma cells (4). The AA/C1 cell line was therefore treated with sodium butyrate in an attempt to isolate cells with increased resistance to sodium butyrate. It was interesting to note that only these treated cells were later susceptible to transformation by the carcinogen MNNG and that even those non-sodium butyrate-treated AA/C1 cells exposed to 10 μg/ml MNNG were unable to form colonies in agarose. Although the mechanism by which sodium butyrate exerted its effect on the cells is unclear, the resistance of the AA/C1/SB cells to sodium butyrate treatment, their increased CFE on plastic and their susceptibility to transformation by the carcinogen implies a stable change in the cellular phenotype. Further experiments are in progress to determine whether this change resulting from the sodium butyrate treatment represents a relevant step in the progression of the clonogenic AA/C1 cell line towards the malignant phenotype.

It has been argued that because adenoma cells are more sensitive to its inhibitory effect than carcinoma cells (4) sodium butyrate may select for cells with an increased malignant potential by a process analogous to that proposed for 12-O-tetradecanoylphorbol-13-acetate-induced carcinogenesis in mouse skin (12). If sodium butyrate is important in the control of growth and differentiation in the colon, it is possible that any cell which, in vivo, can escape this control may be able to proliferate unchecked. In vitro, at concentrations greater than 0.5 mM sodium butyrate can inhibit cell growth in culture perhaps through the induction of terminal differentiation. Therefore, if cells are present that are resistant to this inhibitory signal, treatment with sodium butyrate may result in the selective outgrowth of an increasingly abnormal cell population.

The malignant conversion of benign tumors is thought to require further genetic changes in the tumor cell. These changes could result from an inherent instability in the genome of the premalignant cells, from spontaneous mutation more likely to occur in the expanding cell population of proliferating benign cells, or by additional exposure to exogenous genotoxic agents (reviewed by Yuspa and Poirier (13)). Therefore, although the sodium butyrate-treated AA/C1 cells showed signs of progression through passing alone (see "Results"), the use of the carcinogen MNNG resulted in the isolation of cells which exhibit a transformed anchorage-independent phenotype with a very high colony-forming efficiency in agarose (up to 17.3% so far).

The transformed AA/C1/SB10 cells have become tumorigenic in nude mice. The earlier passage AA/C1/SB10 cells produce predominantly small but persistent tumors at the site of inoculation. A positive correlation between frequency of tumor formation and increasing passage number of the cells injected has been observed. This could suggest that the actual number of malignant cells in the inoculum increased with successive passage in vitro, perhaps through selection of the more abnormal cells or as a result of further events occurring in vitro which are involved in the conversion of the cells to the malignant phenotype. Significantly, when injected with the later passage AA/C1/SB10 cells, a greater proportion of the mice have gone on to develop progressive tumors (see "Results"), indicating a relationship between increasing passage number and frequency of progressive tumor formation. In summary, with successive in vitro passage, AA/C1/SB10 cells are capable of forming large progressively growing adenocarcinomas in nude mice and are therefore tumorigenic. It is important to note that previously adenoma cell lines have been shown to be completely nontumorigenic in nude mice (2, 5, 14).

It is unclear why most of the earlier tumors produced by the AA/C1/SB10 cells from passage 70 did not grow beyond a certain size in the nude mice (Table 1). A possible explanation is that this limited growth is due to an immune response against the human tumor cells since a lymphocytic infiltrate was observed surrounding them. It may be related to the ability of the cells to produce angiogenesis factor, as tumor cells unable to produce this factor have been shown to form tumors of limited size. Another possibility could be that the AA/C1/SB10 cell inoculum contained a subpopulation of nontumorigenic cells which are able to restrict the growth of neighboring malignant cells (15, 16). However, the later passage AA/C1/SB10 cells are able to escape some or all controls. Successive in vitro passage of the AA/C1/SB10 cell line has resulted in either the selective outgrowth of more aggressively growing cells leading to fewer nontumorigenic cells in the inoculum or the emergence of a malignant phenotype resistant to normal growth constraints and/or able to evade any immunological response.

In summary, after carcinogen treatment, the AA/C1/SB cells first became anchorage independent and then, after continuous in vitro passaging, tumorigenic. Similar in vitro progression has previously been reported for rodent cells both after carcinogen treatment (17, 18), and in the case of spontaneous transformation (19). It is of interest that sodium butyrate-treated AA/C1 cells did eventually develop an anchorage-independent phenotype without carcinogen treatment after continuous in vitro passage (see "Results"), although they have not yet formed tumors in nude mice (investigated up to passage 75). Furthermore, the colony-forming efficiency in agarose of these cells was significantly lower than that of the AA/C1/SB10 cells of equivalent passage. It is possible that the carcinogen may have accelerated the progression of the cells that would eventually occur after the sodium butyrate treatment through in vitro passaging alone.

It has been shown that anchorage independence is not a universal marker for the malignant potential of a cell line and its ability to produce tumors. Several reports indicate that human cells treated with carcinogens form colonies in agarose or agar, but fail to produce tumors in mice (20-22). Furthermore, Weissman et al. (23) reported that when suppression of tumorigenicity was induced by the introduction of a normal
chromosome 11 into Wilms tumor cells the cells remained anchorage independent. It is further complicated by the observation that some tumorigenic cell lines do not grow in agarose at all although they do form progressive tumors in athymic mice (2, 24). Therefore, the two phenotypes, anchorage independence and tumorigenicity, can in some cases be separated and the question remains as to whether the ability of human cells to grow in agar after carcinogen exposure represents a specific stage in transformation, and whether a further event is required to complete the malignant conversion.

This study of the transformation of human cells in culture further emphasizes their stability as the progression of the PC/AA cell line in vitro to the malignant phenotype has involved multiple steps. The isolation of the tumorigenic AA/C1/SB10 cells from the PC/AA adenoma cell line represents the first example of the malignant progression of human colonic adenoma cells in vitro. Furthermore, the cytogenetic abnormalities related to this progression are consistent with those frequently observed in in vivo carcinomas (25), indicating that this in vitro progression is relevant to the in vivo situation. These cells are now being used to study the cellular and molecular properties that may represent the many premalignant phenotypes as well as the malignant one.

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Neoplastic Transformation of a Human Colonic Epithelial Cell Line: *In Vitro* Evidence for the Adenoma to Carcinoma Sequence

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