 Specific Cytogenetic Abnormalities in Two New Human Colorectal Adenoma-derived Epithelial Cell Lines

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ABSTRACT

Two new epithelial cell lines from sporadic human colorectal adenomas designated S/AN and S/RG are reported. S/AN was from a villous adenoma and S/RG from a tubular adenoma. Both cell lines have extended growth capacities in vitro reaching passages 18 and 15, respectively, so far and show no signs of senescence. S/AN and S/RG have retained in vitro the ability to form mucin-producing goblet-like cells.

Every cell of S/AN has a deletion on the short arm of chromosome 1 and one normal copy of chromosome 1. S/AN is also monosomic for chromosome 18. The majority of cells of S/RG only have one normal copy of chromosomes 6, 7, 14, 17, 18, and 22. S/RG also has several marker chromosomes. Although aneuploid S/AN and S/RG are non-tumorigenic in athymic nude mice, these cytogenetic abnormalities are insufficient for the fully tumorigenic phenotype. The common abnormality for S/AN and S/RG is monosomy for chromosome 18, indicating that this is a central and important step in colorectal carcinogenesis. Our cytogenetic analysis of the adenoma cell lines suggests at least two possible routes by which premalignant colonie cells can develop and progress to malignancy.

S/RG, unlike most other adenoma cell lines, is clonogenic. Aneuploidy, clonogenicity, and extended in vitro growth capacity may therefore be useful in vitro markers for adenoma cell lines with a relatively high malignant potential.

INTRODUCTION

Cancer development in the large intestine, which is one of the major sites for human malignant disease, is a good example of the multistage nature of cancer. Most colorectal cancers are thought to develop from adenomas (premalignant tumors sometimes referred to as polyps) in what is called the adenoma carcinoma sequence (1). The adenomas are themselves derived from the normal epithelium lining the intestine.

Understanding the cellular and molecular events involved in the progression of adenomas to carcinomas could be greatly assisted by the analysis of colorectal adenoma cell lines. Although there are numerous human colorectal cancer cell lines (2–5), there are relatively few colorectal adenoma epithelial cell lines. Friedman et al. (6) have described a primary culture system for colorectal adenomas. We have previously described the serial passage in vitro of colorectal adenomas and reported the first isolation of three adenoma epithelial cell lines (7), one of which became immortal and showed signs of tumor progression in vitro (8, 9). More recently, Willson et al. (10) described new conditions for the growth of adenoma cell lines. In this paper we report the isolation and characterization of two new sporadic colorectal adenoma cell lines which will be very valuable for further studies in colorectal carcinogenesis.

MATERIALS AND METHODS

Medium and Standard Culture Conditions. Growth medium and culture conditions have been described previously (7, 11). Briefly, most adenoma cell cultures cannot be passaged by routine trypsinization since they only grow from clumps of cells and not from single cells (7). Instead dispase (a neutral protease; Boehringer) prepared in growth medium at 2 units/ml was used. Dispase solution was added to cell monolayers and removed the epithelium as sheets of cells and not as single cells (7).

Growth medium was Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum (batch selected), hydrocortisone sodium succinate (1 µg/ml), insulin (0.2 units/ml), 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 µg/ml). Cells were grown on collagen-coated T25 flasks in the presence of Swiss 3T3 feeder cells as described previously (7).

Isolation of Two New Sporadic Adenoma Cell Lines. Two new adenoma-derived epithelial cell lines, designated S/AN and S/RG, were isolated using techniques described previously (7). S/AN and S/RG were derived from isolated (sporadic) adenomas from patients with no known history of colorectal cancer. S/AN was derived from a 59-yr-old male from a single colonic villous adenoma of 1 to 2 cm in diameter with moderate dysplasia. S/RG was isolated from a 59 year old female from a single colonic tubular adenoma of 1 to 2 cm in diameter with moderate dysplasia.

Growth from Single Cells (Clonogenicity). Cells were tested for clonogenicity as described previously (7). Briefly, a single cell suspension was prepared using 0.05% Difco trypsin plus 0.05% EDTA. Cells were counted using a counting chamber. The appropriate number of cells was seeded onto collagen-coated T25 flasks in standard growth medium in the presence of 3T3 feeders as described previously (7). Cells were tested for clonogenicity at cell densities ranging from 1 × 10^4 to 1 × 10^6 cells/T25 flask. In some cases the 3T3 feeders were omitted to determine the 3T3 feeder dependency.

Animal Experiments. Cultures were tested for tumorigenicity by s.c. injection into athymic ICRF (Imperial Cancer Research Fund) nu/nu nude mice as described previously (7).

Closin in Soft Agar. The method used similar to that described by Macpherson and Montagnier (12) except that agarose (sea plaque; Miles Laboratory) was used.

Chromosome Preparation. Chromosomes were prepared according to standard procedures, and G-banding and C-banding were used to identify chromosomes (13, 14).

Mycoplasma Testing. Upon routine fluorescent Hoechst 33258 staining, as described by Chen (15), cultures were found to be negative for Mycoplasma contamination.

RESULTS

Characterization of Two New Adenoma Epithelial Cell Lines. Primary epithelial cultures were routinely derived from sporadic adenomas using techniques described previously for adenomas from familial polyposis coli patients (7). Of 12 independently obtained primary cultures, 2 gave extended in vitro growth giving rise to epithelial cell lines, designated S/AN and S/RG. A wide range of success was obtained with the other 10 cultures which could be passaged between 2 and 9 times with the mode being 4 passages. The success with routine passaging depended on the quality (for example, how necrotic) and size of tissue obtained at surgery, and therefore precise projections regarding success rates are very difficult.

S/AN and S/RG have a characteristic epithelial morphology which was evident in primary culture and has been retained
ABNORMAL KARYOTYPES IN COLONIC ADENOMA CELL LINES

Fig. 1. Phase-contrast photographs of cell cultures of adenoma epithelial cell lines of S/AN at passage 9 (a) and S/RG at passage 5 (b). Note fibroblastic cells surrounding epithelial colony in (a) are 3T3 feeders. Bar, 25 µm.

with in vitro passage. The cells exhibit a closely packed polygonal morphology typical of epithelial cells (Fig. 1). Both S/AN and S/RG had an ultrastructure characteristic of colonic epithelium, notably the formation of mucin droplets and microvilli (Fig. 2).

As expected, both cell lines also stained positively with Alcian blue, which is a histological stain for acid mucopolysaccharides, and with the monoclonal antibody, LE61 (16), which reacts with keratin 18 filaments of simple epithelia (results not shown). Both S/AN and S/RG have an extended in vitro growth capacity. S/AN has reached passage 18 and shows no signs of senescence. It is passaged at a 1:2 split ratio every 4 to 5 wk. S/RG has reached passage 15 and again shows no signs of senescence. S/RG grows relatively quickly and is routinely passaged at a 1:2 split ratio approximately every 2 wk. Both are currently being tested for in vitro immortality.

Growth from Single Cells (Clonogenity) and Anchorage-independent Growth. S/AN, like most previously tested colorectal adenoma epithelial cell lines, was nonclonogenic and could not be passaged using a standard trypsinization protocol (7, 8). At passage 8, S/AN was seeded after trypsinization to single cells at 1 x 10⁶ cells per collagen-coated T25 flask and had a CFE of <0.0005 even in the presence of 3T3 feeders. S/RG, however, at passage 9 could be grown clonally and, when seeded after trypsinization to single cells at 5 x 10⁶ cells per collagen-coated T25 flask, had a CFE of 0.56% in the presence of 3T3 feeders. In the absence of 3T3 feeders this dropped to <0.0005%. Under these conditions even when seeded at 1 x 10⁶ per T25 flask, all previously tested adenoma cell lines and cell cultures had CFE of <0.0005% with or without 3T3 feeders (7, 8).

Neither S/AN nor S/RG was anchorage independent. Seeding a total of 2 x 10⁶ cells of each cell line into agarose resulted in them both having a CFE of <0.00001%.

Tumorigenicity. S/AN and S/RG were both nontumorigenic in athymic nude mice. For S/AN at passage 12, a total of six 3- to 4-wk-old nude mice was given injections of 1 x 10⁷ cells/mouse, and all the mice have remained tumor free for at least 180 days. For S/RG a total of nine nude mice was given injections of 1 x 10⁷ cells/mouse. Five 3- to 4-wk-old mice were given injections of S/RG at passage 8 and these have been tumor free for at least 150 days. Four mice at 5 wk old were given injections of S/RG at passage 9, and these have been tumor free for at least 120 days. These results are consistent with previous studies showing colorectal adenoma-derived cell lines to be nontumorigenic in athymic nude mice (7, 8, 10), whereas colorectal cancer cell lines are normally tumorigenic in athymic nude mice (2-4, 7). It should be added, however, that although the majority of colorectal cancers are tumorigenic, there are reports of colorectal cancer-derived established cell lines being nontumorigenic in athymic nude mice (17).

Chromosome Analysis of Sporadic Adenoma S/AN. S/AN was examined cytogenetically at passages 10 and 12 and was found...
to be aneuploid. Karyotypes of 19 S/AN cells were examined by G-banding, and the modal chromosome number was found to be 45. All 19 cells had a deletion on the distal end of the short arm (p) of chromosome 1 and one copy of chromosome 1 which appeared normal (Fig. 3). S/AN is therefore monosomic for this region on the short arm of chromosome 1. From the morphology of the deleted chromosome it could not be determined whether the Ip monosomy involved a terminal deletion at p32 or p33, or whether it involved an interstitial deletion (p32 p36). The latter is quite possible as the terminal regions of the chromosome are unusually stable. Eighteen of the 19 cells with a chromosome 1 deletion had only one copy of chromosome 18, and thus the majority of cells were monosomic for chromosome 18. The few metaphases with less than 45 chromosomes showed random loss of individual chromosomes.

Chromosome Analysis of Adenoma Cell Line S/RG. S/RG was examined cyogenetically at passage 7 and was found to be highly aneuploid. Karyotypes of 15 cells were examined by G-banding, and the modal chromosome number was found to be 43. A modal karyotype is shown in Fig. 4. In this cell only one normal copy of chromosomes 6, 7, 14, 17, 18, and 22 is present. The patient was female, but no normal X chromosomes could be determined with certainty, although there are two nonassigned “C group” chromosomes d and e whose banding patterns were unclear. The marker chromosome b is considered to be derived from chromosome 6 with a deletion in the long arm; the origin of markers a and c could not, however, be determined. The chromosome abnormalities described above were present in the majority of cells karyotyped. Of these 15 cells, 14 had only one normal copy of chromosome 6. Eleven cells had only one normal copy of chromosomes 6, 7, and 2; two cells were missing both normal copies of chromosome 7. Fourteen of the 15 cells had only one normal copy of chromosome 17, and one cell had both copies missing. Fourteen of 15 cells had only one normal copy of chromosome 18, and one cell had both copies missing. Fourteen of 15 cells had only one normal copy of chromosome 17, and one cell had both copies missing. Nine of the 15 cells have all five marker chromosomes a, b, c, d, and e. Three of the cells had the four marker chromosomes a, b, c, and d. Two of the cells had the four marker chromosomes b, c, d, and e. One of the cells had the four marker chromosomes a, b, d, and e.

**DISCUSSION**

Two new adenoma-derived lines, S/AN and S/RG, are reported. S/AN has a deletion on the short arm of chromosome 1 and one normal chromosome 1. Abnormalities of chromosome 1 have been previously reported in a variety of cancers (18). In determining the importance of chromosome changes in cancer development, an important question is at which stage of carcinogenesis are the chromosome changes occurring (19). For example, do they occur during the adenoma stage when the adenomas are tubular or as they become villous or at the adenoma-to-carcinoma interface? Reichman et al. (20) described a duplication of part of the long arm of chromosome 1 in a small number of cells from a colonic villous adenoma. Willson et al. (10) reported a villous adenoma-derived established cell line VACO 235, with a deletion of the short arm of chromosome 1 and an isochromosome 1 (q).

We have previously reported a familial polyposis coli adenoma-derived cell line, designated PC/AA, which at early passage was normal diploid (7). Two immortal aneuploid variants of PC/AA both had independent abnormalities of chromosome 1, leading us to conclude that abnormalities of chromosome 1 may be involved in in vitro immortalization (8, 21). Recently, Sugawara et al. (22) from experiments using somatic cell hybrids between immortal Syrian hamster cells and normal human fibroblasts concluded that human chromosome 1 may be involved in cellular senescence. It is of interest therefore that S/AN, which has an extended in vitro growth capacity, has a deletion on the short arm of chromosome 1. Abnormalities involving both the loss of one copy of the short arm of chromosome 1 and/or the presence of an extra copy of the long arm of chromosome 1 have been implicated in tumor progression (8, 18, 21, 23). The precise role of chromosome 1 in tumor progression, however, remains unclear, and this has recently been discussed in detail (21).

Any model of human colorectal carcinogenesis needs to take into account reports that frequent loss of alleles on chromosome 5 (24), chromosome 17 (25–27), chromosome 18 (26, 27), and chromosome 22 (28) is involved in colorectal carcinogenesis. The majority of the cells of S/RG have only one normal copy of chromosomes 6, 7, 14, 17, 18, and 22, the latter three chromosomes being implicated in colorectal carcinogenesis. S/RG...
AN as well as having a deletion on the short arm of chromosome 1 is also monosomic for chromosome 18. The common abnormality for both S/RG and S/AN is therefore monosomy for chromosome 18. The villous adenoma-derived cell line VACO 235, previously reported by Willson et al. (10), has a deleted chromosome 18. Monosomy for chromosome 18 therefore appears to be an important and central step in the sequential development of colorectal cancer. Further analysis of the adenoma cell lines with polymorphic DNA probes for chromosome 18 markers is necessary to determine whether homozygosity for chromosome 18 alleles is occurring in these premalignant tumors or as previously reported occurs at the cancer stage (26, 27).

S/RG is unlike most other adenoma cultures tested (7, 8) in that even at early passage it is clonogenic. Aneuploidy and/or clonogenicity may therefore be good in vitro markers for adenomas with a relatively high malignant potential. In support of this is that S/RG was derived from a relatively large tubular adenoma and S/AN from a relatively large villous adenoma, both of which have significant malignant potentials (1). Also of interest is our previous work with the PC/AA adenoma cell line which at early passage is normal diploid, 3T3 feeder dependent, and nonclonogenic (7). A 3T3 feeder-independent variant of PC/AA, designated PC/AA/FI, which is aneuploid and immortal in vitro has also become clonogenic (21).

PC/AA, although normal diploid and nonclonogenic at early passage, was from a large adenoma (3 to 4 cm in diameter), and therefore not all large adenomas are necessarily aneuploid and clonogenic (7). However, of 15 independently derived familial polyposis coli cultures, PC/AA was the only cell line to immortalize in vitro, and no cell cultures derived from small adenomas (<1 cm in diameter) immortalized in vitro (8), indicating that the acquisition of in vitro immortality is associated with a relatively late stage in the adenoma carcinoma sequence when adenomas are large and/or villous (8). In vitro immortality, as well as aneuploidy and clonogenicity, may therefore be useful in vitro parameters to study the multistage pathways of colorectal carcinogenesis.

Colorectal carcinogenesis is a complex multistage process in that allele loss on a number of chromosomes has been implicated (24, 25, 27, 28) as well as activation of the c-K ras oncogene (29, 30). The possible involvement of so many loci in colorectal carcinogenesis and the reports that not all colorectal tumors show the same patterns of allele loss and ras gene activation argue that colorectal carcinogenesis is multipathway as well as multistage. Our cytogenetic analysis of the adenoma cell lines suggests at least two possible routes by which premalignant colonic cells can develop and progress to malignancy.

It is unclear why S/RG, which was from a tubular adenoma, is clonogenic and displays more chromosomal abnormalities than S/AN, which was from a villous adenoma. Villous adenomas have a greater malignant potential than tubular adenomas (1). That increased chromosomal abnormalities do not always correlate with a greater malignant potential was emphasized by Willson et al. (10) who reported that a villous adenoma-derived cell line had more chromosomal abnormalities than two colorectal cancer cell lines. One possible explanation for the in vitro behavior of S/RG is that a highly dysplastic subpopulation of cells which was not detected histologically in the original S/RG tubular adenoma has been selected for in cell culture. However, consistent with S/AN being derived from adenoma cells with a higher malignant potential than S/RG is our report that the S/AN cell line has a c-k-ras gene mutation in codon 12 (arginine substitution for glycine), whereas no ras gene mutations were detected in the S/RG cell line (31). These results clearly emphasize the complex multistage and multipathway nature of colorectal carcinogenesis.

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