Growing colorectal tumors: minimizing microbial and stromal competition and assessing *in vitro* selection pressures *

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Abstract

Development of primary colorectal cancer cell lines is hampered by contamination from regional microbes, overgrowth of stromal cells, and purported genetic drift from selection pressures *in vitro*. We initiated 32 primary adenocarcinomas, 3 recurrences and 6 distant metastases in cell culture. Twelve cell lines from eleven tumors were generated (26.8%) overall. Nine of 32 primary tumors yielded 10 cell lines, 5 were lost to contamination, 13 were overwhelmed by stromal cells, and 5 demonstrated no growth. Addition of isobutyl methyl xanthine (IBMX) to culture limited fibroblastoid growth. There was no association between tumor location ($p = 0.535$, mid-P), degree of differentiation ($p = 0.850$, mid-P) or clinicopathologic stage ($p = 0.400$, mid-P), and the ability of cells to become established in culture. The majority of cell lines had similar nuclear DNA content and expression of cell-surface antigens compared with their parent tumors. Microbial contamination and stromal cell overgrowth present the greatest obstacle to capturing a representative bank of colon tumors *in vitro*.

Introduction

Colorectal cancer cell lines are utilized routinely for research. The benefits of a purified, easily expandable source of tumor cells are obvious. However, critics contend *in vitro* studies neglect tumors lost during the process of culture, and fail to consider the ongoing selection pressures of cell culture through multiple passages which may alter the biology of tumor cells (Oh et al., 1999; Park and Gazdar, 1996). We therefore set about to analyze the process of primary colorectal cancer cell culture with particular interest to: (1) variables affecting successful tumor growth, and (2) comparison of the biology of cell lines and parent tumors.

Methods

Specimen procurement

Colorectal cancer specimens from patients at Dartmouth-Hitchcock Medical Center were collected aseptically at operation. After expeditious pathologic processing 1 cm² pieces of tumor were placed into sterile tissue-culture media. Determinations regarding histologic variables were made during standard pathologic assessment. Tumor clinicopathologic stage (TNM stage) was assigned according to guidelines set forth by the American Joint Committee on Cancer. In total, 32 primary adenocarcinomas, 3 recurrences and 6 distant metastases were sampled (Table 1).

Cell culture

Microbial contamination was controlled by washing tissue culture specimens in 70% ethanol for 2–5 sec,

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Table 1. Procurement data, culture results and survival

Case no.	Tumor location	Specimen class	Degree of differentiation	TNM stage ^a	Culture result ^b	Survival (days)	Follow-up status
1	Liver	Metastasis	Poorly	4	stroma	540	DOD
2	Rectum	Primary	No record	2	cont	1601	DOC
3	Rectum	Primary	Well	3	no	1717	NED
4	Sigmoid	Primary	Moderately	3	stroma	1462	DOC
5	Liver	Metastasis	No record	4	no	1019	DOD
6	Left colon	Primary	Well	1	stroma	1761	NED
7	Right colon	Primary	Well	1	cont	1561	NED
8	Right colon	Primary	No record	2	cont	689	DOD
9	Liver	Metastasis	No record	4	yes	597	DOD
10	Right colon	Primary	Well	2	stroma	1475	NED
11	Left colon	Primary	Moderately	3	yes (2)	280	DOD
12	Rectum	Primary	Moderately	2	Stroma	745	DOC
13	Bladder	Metastasis	No record	4	no	159	DOD
14	Sigmoid	Primary	Well	2	yes	1469	NED
15	Right colon	Primary	Moderately	2	stroma	1646	NED
16	Sigmoid	Primary	Well	2	stroma	1640	NED
17	Rectum	Primary	No record	1	stroma	1622	NED
18	Rectum	Recurrence	Moderately	2	no	306	DOD
19	Right colon	Primary	Well	4	yes	691	DOD
20	Sigmoid	Primary	Moderately	2	stroma	1631	NED
21	Sigmoid	Primary	Moderately	3	yes	25	DOD
22	Sigmoid	Primary	Moderately	1	no	1639	NED
23	Sigmoid	Primary	Poorly	4	yes	398	DOD
24	Sigmoid	Primary	Well	2	no	1509	NED
25	Right colon	Recurrence	Poorly	4	stroma	429	DOD
26	Rectum	Primary	No record	3	no	1555	NED
27	Sigmoid	Primary	Moderately	2	stroma	1432	NED
28	Right colon	Primary	Moderately	4	cont	354	DOD
29	Right colon	Primary	Poorly	4	no	146	DOD
30	Spleen	Metastasis	Moderately	4	yes	901	DOD
31	Rectum	Recurrence	Moderately	4	no	1222	DOD
32	Sigmoid	Primary	Moderately	3	cont	1472	NED
33	Liver	Metastasis	No record	4	stroma	551	DOD
34	Right colon	Primary	Moderately	2	yes	1443	NED
35	Right colon	Primary	Moderately	2	stroma	1429	NED
36	Right colon	Primary	Moderately	2	yes	959	DOD
37	Right colon	Primary	Moderately	1	no	1323	NED
38	Left colon	Primary	Moderately	2	stroma	882	DOD
39	Sigmoid	Primary	Moderately	2	yes	508	DOC
40	Right colon	Primary	Well	3	yes	1138	NED
41	Right colon	Primary	Moderately	4	stroma	524	DOD

^a TNM staging: (American Joint Committee on Cancer). 1 – invasion of submucosa or muscularis propria; 2 – invasion of all layers or adjacent organs; 3 – metastasis to regional lymph node(s); 4 – metastasis to distant organs; i = incomplete resection.

^b 'stroma' = stromal overgrowth; 'no' = no cell line; 'cont' = contamination; 'yes' = cell line; 'DOD' = dead of disease; 'DOC' = dead other cause; 'NED' = no evidence disease.

Table 2. Ploidy results for cell lines and parent tumors

Sample ^a	Population A			Population B			Population C		
	Mode ^b	(Range)	% cells	Mode	(Range)	% cells	Mode	(Range)	% cells
T9	3.06	(2.22–3.84)	80.7	5.40	(4.18–6.55)	14.0	None		
CL9	2.48	(1.89–3.00)	57.1	4.36	(3.11–5.78)	39.1	None		
T11	1.05	(0.83–1.22)	39.4	1.53	(1.23–3.01)	53.3	None		
CL11	1.06	(0.89–1.28)	75.8	2.13	(1.83–2.33)	14.8	None		
CL11m	None			1.61	(1.22–2.11)	71.3	3.38	(2.33–4.45)	21.3
T14	1.04	(0.89–1.22)	30.4	1.93	(1.44–2.44)	41.1	3.54	(2.56–4.23)	25.9
CL14	None			1.96	(1.78–2.67)	86.6	3.24	(3.22–4.45)	9.2
T19	1.09	(1.00–1.23)	81.6	1.84	(1.56–2.40)	18.4	None		
CL19	0.93	(0.89–1.11)	73.3	1.84	(1.83–2.11)	17.8	None		
T21	1.66	(1.55–1.89)	37.6	3.34	(2.00–3.50)	57.8	None		
CL21	1.67	(1.33–2.44)	12.3	2.92	(2.67–4.00)	58.5	6.19	(4.45–6.90)	25.5
T23	1.00	(0.89–1.44)	85.9	1.68	(1.67–2.44)	11.3	None		
CL23	None			2.10	(1.78–2.67)	75.4	3.27	(2.67–6.01)	21.8
T30	0.90	(0.89–1.39)	92.2	None			None		
CL30	None			1.71	(1.56–1.89)	82.5	2.28	(2.23–3.45)	15.6
T34	1.07	(0.89–1.44)	84.5	2.27	(1.55–2.33)	14.4	None		
CL34	1.14	(1.06–1.34)	74.8	2.40	(1.39–2.45)	25.2	None		
T36	1.03	(0.89–1.33)	19.4	1.89	(1.67–3.00)	59.2	3.80	(3.34–5.34)	19.4
CL36	None			2.09	(1.33–2.22)	18.2	4.73	(2.67–6.45)	66.7

^a Abbreviations: T = tumor, CL = cell line.

^b The modal DNA Index and range are indicated for each distinct population of cells.

in PBS for 1 min and twice in antibiotic/antimycotic solution (6 units/ml penicillin, 6 μ g/ml streptomycin and 12 ng/ml amphotericin B; Sigma, St. Louis, MO) for 1 min. After tissues were minced and dissociated in growth medium, cell cultures were initiated *in vitro* in Ham's Nutrient Medium F12/Dulbecco's Modified Eagle's Medium (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (Sterile Systems, Logan, UT), 2 units/ml penicillin, 2 μ g/ml streptomycin and 4 ng/ml amphotericin B. Cell cultures were incubated in 5% CO₂ at 37 °C, and medium was changed every 3–5 days, as needed. Methods used to enhance tumor growth and minimize stromal overgrowth included: (1) differential trypsinization to separate stromal cells from adherent tumor colonies (0.05% trypsin/0.53 mM EDTA for 3–5 min (Gibco, NY) with deactivation of trypsin and removal of stromal cells with Hanks balanced salt solution (HBSS) washes before refeeding), and (2) inclusion of 0.5 μ M isobutyl methyl xanthine (IBMX; Sigma, St. Louis, MO) in growth medium to suppress fibroblast growth (Logsdon and Williams, 1986). When cultures did not attach spontaneously within 10–14 days, they were replated on rat-tail collagen or basement membrane (Matrigel, Collaborative Research, Waltham,

MA) substrates. A cell line was considered established once a uniformly, continuously growing population of tumor cells was obtained free of benign stromal cells. Cell lines were cryopreserved in liquid nitrogen. Influence of tumor location, grade and TNM stage on *in vitro* growth was measured by mid-P test.

Immunohistochemistry

To demonstrate common features with adenocarcinomas from which they were derived, cell lines and tumors were stained for expression of: (1) human carcino-embryonic antigen (CEA), a glycoprotein product found in gastrointestinal tumors (mouse monoclonal IgG, 1:100 dilution, BioGenex, San Ramon, CA); and (2) human mucin-like carcinoma associated antigen (MCA) a non-specific marker of adenomatous differentiation of gastrointestinal and other solid carcinomas (mouse monoclonal IgG, undiluted, AMAC, Inc., Westbrook, ME), using a Cadenza Automated Immunostainer (Shandon, Pittsburgh, PA). Formalin-fixed pellets of scraped cells from cell lines and formalin-fixed, deparaffinized tissue blocks from parent tumors were cut at 4–5 μ m thickness. Primary antibodies were applied for 55 min. Using a StrAvis-

Gen MultiLink Immunodetection Kit (BioGenex, San Ramon, CA), secondary antibodies (biotinylated anti-immunoglobulins) were applied for 30 min, followed by horseradish peroxidase-labeled streptavidin for 30 min and DAB (3,3'-diaminobenzidine tetrahydrochloride) substrate solution for 5 min. Counterstain (1 min) and bluing agents (2 min) were added according to manufacturer's instructions.

Ploidy

Imprints were made by applying glass slides to solid tumors. Cell lines were dissociated using 0.05% trypsin/0.53 mM EDTA, and 4×10^4 cells were deposited on a glass slide using a Cytospin centrifuge (Shandon, Pittsburgh, PA). Slides were allowed to air-dry and were fixed in 10% neutral buffered formalin for 30 min. Tumor samples and calibration-control rat hepatocytes (Cell Analysis Systems/Becton Dickinson, Elmhurst, IL) were stained by the Feulgen method (Quantitative DNA Staining Kit, Cell Analysis Systems/Becton Dickinson, Elmhurst, IL). Nuclear DNA content was analyzed on the CAS Model 200 Image Analysis System with the QDA v. 3.0 software module (Cell Analysis Systems/Becton Dickinson, Elmhurst, IL) (Zuna et al., 1993). DNA index was calculated as the ratio of the mean optical density for each major tumor population to that of calibration-standard cells (rat hepatocytes) stained at the same time.

Results

Overview

We established 12 cell lines from 11 human colon adenocarcinomas of varying stage and location, and determined whether demographic, anatomic or histologic variables influenced the likelihood of tumors to propagate *in vitro*. We characterized cell-surface antigen expression by immunohistochemistry, and nuclear DNA content by optical methods, to verify each cell line's derivation from its parent carcinoma and to ascertain the degree of biologic similarity.

Cell culture

Overall, continuous cell lines were generated from 26.8% of colorectal adenocarcinomas. Nine of the 32 primary colonic tumors yielded 10 cell lines, 5 were lost to contamination, 13 were overwhelmed by

stromal cells and 5 demonstrated no growth. None of the recurrences demonstrated *in vitro* growth. Two of the metastatic tumors yielded cell lines, 2 contained only fibroblasts and 2 were not viable.

The addition of IBMX to culture medium limited fibroblastoid growth, which disappeared from all cell lines by 2 months. All cases of microbial contamination resulted from fungus or yeast. Morphology and behavior in cell culture varied. Most populations initially grew in radially-oriented colonies and retained colonic structural organization with papillary and epithelial characteristics (Figure 1). After several months *in vitro*, all cell lines grew as a confluent monolayer. Morphologic heterogeneity was observed in several cases. Two distinct cell lines, one predominantly epithelioid (CL11) and the other mucoid (CL11m), were developed from case 11 and have been analyzed separately. Several cell lines routinely sloughed large numbers of non-viable cells. Population doubling times ranged from 2 days to several weeks, but accelerated with later passages. From their inception, 2 cell lines (cases 23 and 36) demonstrated rapid doubling and marked morphologic heterogeneity without colony formation.

No TNM stage 1 tumors became established cell lines, but similar results were obtained for TNM stage 2–4 tumors. There was no significant association between tumor location ($p = 0.535$, mid-P), degree of differentiation ($p = 0.850$, mid-P) or TNM stage ($p = 0.400$, mid-P), and the ability of cells to become established in culture (Table 1).

Immunohistochemistry

Expression of tumor-associated antigens CEA and MCA was measured in the first 10 cell lines and 9 parent tumors. The degree of immunoreactivity was determined by counting immunoperoxidase stained cells per 150 histologically recognizable tumor cells in 3 widely separated low-power fields. Mean intensity of labeling was estimated on a 0–4+ scale. All tumors showed CEA immunoreactivity without staining of stromal elements, and each cell line demonstrated CEA immunoreactivity of similar intensity and distribution to that of its parent tumor (Figure 2, Table 3). Based on cellular morphology and immunohistochemical analyses, no stromal cells were identified in any cell line. Since we were unable to identify a reliable positive marker for stromal cells, we relied on morphology and the absence of immunoreactivity to epithelial markers as evidence for stromal identity.

Figure 1. Two representative tumor cultures, 470X magnification. (a) *In vitro* passage #12, 10 months after initiation, developed from a well-differentiated T3N0M0 sigmoid adenocarcinoma (CL14). (b) *In vitro* passage #4, 3 months after initiation, derived from a moderately-differentiated T3N1M0 sigmoid adenocarcinoma (CL21).

Cultures of normal tissue taken at a distance from the tumor were unavailable; thus, there could be no comparison between cultured normal colonic epithelium and tumor cells by immunohistochemistry. MCA labeling was 0–1+ in 7 of 10 cell lines and parent tumors, whereas 2 cell lines and parent tumors showed more intense (2+) staining. By immunohistochemical staining, cell lines retained the ability to express antigens present in parent tumors.

Ploidy

Nuclear DNA content was determined in the first 10 cell lines and 9 parent tumors. A minimum of 100 cells from at least 5 high-power fields were analyzed. Cell lines from cases 9, 14, 19, 21 and 34 demonstrated DNA content similar to the dominant populations of corresponding parent tumors. Each cell line derived from tumor 11 demonstrated distinct ploidy suggesting separate subpopulations of the original tumor. Cell lines from cases 23, 30 and 36 showed increased DNA ploidy compared to parent tumors (Table 2).

Figure 2. Immunohistochemical staining of carcinoembryonic antigen (CEA) in case 14, 160X magnification. (a) Parent tumor demonstrates cytoplasmic and membrane staining of tumor cells (arrows) but not of stromal cells (asterisk). (b) Uniform staining of the corresponding cell line.

Discussion

In this study, we established continuous cell lines from 9 primary and 2 metastatic colorectal tumors in culture and characterized nuclear DNA content and expression of surface antigens associated with colonic tumors. The 26.8% success rate of establishing colon cancer cell lines compares favorably with other reports (Leibovitz et al., 1976; Park et al., 1987; McBain et al., 1987; Gazdar and Oie, 1986). We have found that use of alcohol washes, IBMX and differential trypsinization, all improve the yield of cell lines from primary colon cancer cultures.

Ethanol and antibiotic/antimycotic washes were utilized to minimize microbial contamination. None of the metastatic colonic adenocarcinomas developed microbial contamination in culture, compared with 15.6% of primary tumors exposed to the fecal stream. Stromal competition presented the greatest challenge to successful tissue culture, with competing stromal cells overwhelming tumor cells in 37.5% of primary cultures. In some cases, differential trypsinization eliminated fibroblasts from slowly growing tumors that formed tightly adherent colonies. The addition of IBMX to media impaired the ability of fibroblasts to reattach after enzymatic dissociation and conferred

Table 3. Immunohistochemistry results

Cas ^a	CEA		MCA	
	Intensity	% cells	Intensity	% cells
T9	2+	53	1+	<1
CL9	2+	59	1+	<1
T11	3+	>95	1+	<1
CL11	3+	>95	1+	<1
CL11m	3+	62	1+	<1
T14	4+	>95	2+	<1
CL14	4+	>95	2+	<1
T19	2+	>95	0	0
CL19	2+	>95	0	0
T21	4+	>95	0	0
CL21	4+	>95	0	0
T23	3+	>95	2+	30
CL23	3+	>95	2+	20
T30	3+	>95	1+	40
CL30	3+	>95	1+	5
T34	3+	>95	0	0
CL34	4+	>95	0	0
T36	2+	>95	1+	20
CL36	2+	>95	1+	<1

^a Abbreviations: T = tumor, CL = cell line.

a growth advantage to tumor cells. In one case, a single colony was manually separated from surrounding fibroblasts and developed into a tumor cell line. More often, however, efforts to segregate cell types physically resulted in loss of tumor cells.

Analyses of morphology, ploidy and immunohistochemical staining verify the derivation of these cell lines from their parent tumors and suggest biologic similarity in most cases. Ploidy data also suggest the observed variations between cell lines and parent tumors relate to differential expansion of subpopulations of tumor cells, which may have growth advantage *in vitro* by nature of their ability to attach and propagate, rather than genetic drift. In most cases, the predominant subpopulation(s) from the parent tumor was/were expressed in the cell line.

Critics who reject the merits of *in vitro* research based on the concept of 'genetic drift' will find little support in these ploidy and antigen expression data. Whereas the 'drop-out' of sub-populations of

colorectal tumor cells during the process of culture is a true manifestation of *in vitro* selection, competition from benign cells and microbes remain a more significant barrier to creation of primary colorectal cancer cell lines.

Growth of colorectal tumors in culture is time consuming and challenging. Although relationships may exist between early *in vitro* behavior and the ultimate biologic aggressiveness of individual tumors, valuable prognostic associations will not be possible unless the difficulties in capturing these tumors in culture are overcome.

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