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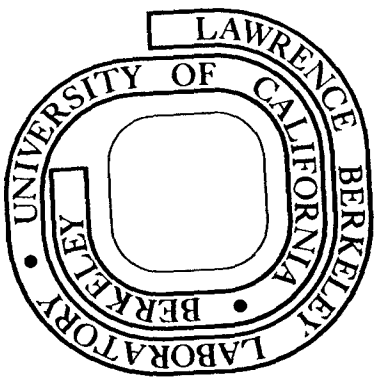

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INTRODUCTION

Human carcinoma cells grown in tissue culture are important for many different fields of research. Biological and biochemical studies on the nature of malignancy as well as searches for etiologic agents are often limited by the absence of proper cell substrates. Clinically, the cells are needed for immunotherapy regimens as well as for evaluating chemotherapy protocols. Unfortunately, establishment of human carcinoma cells in culture has been a relatively rare event (1, 2). The major obstacle to progress in this area has been that fibroblastic cells from connective tissue proliferate rapidly in culture and overgrow the epithelial cells. In the past few years, we have developed simple, easily reproducible methods for isolating epithelial cells (3) and have applied these techniques to the problem of culturing human carcinomas (4). Our results suggest that at least some types of carcinomas can now readily be cultured.

Many altered growth properties have been attributed to tumor cells in culture including abnormal morphology, growth to high saturation density, colony formation on contact-inhibited monolayers and tumorigenicity in immunosuppressed animals (5,6). Karyotypically the tumor-derived cultures show various aberrations in chromosome number and pattern (6). However, it is not known whether these criteria will be applicable to the vast majority of primary carcinomas. Alternatively, these criteria may apply only to the occasional extremely virulent cancer cells which previously were the only types isolated. In this paper, we describe the in vitro properties of a number of carcinoma cell lines.* We have found that each tumor specimen had a unique combination of aberrant properties.

*Nomenclature used conforms with Fedoroff (7) a "cell line" arises from a primary culture at the first subculture while an "established cell line" is one which has demonstrated the potential to be subcultured indefinitely."

METHOD

Cell Culture

The growth medium used was Dulbecco's modification of Eagle's medium (Gibco, Grand Island, N.Y., USA, No. 196G) containing 4.5 g/l glucose, supplemented with 10% fetal calf serum and 10 g/ml insulin (Calbiochem, San Diego, California, USA).

Electron Microscopy

Monolayer cultures were fixed *in situ* with 2.5% glutaraldehyde in 0.1N sodium cacodylate buffer (pH 7.3) and post-fixed in Dalton's chromeosmium followed by 2% ethanolic uranyl acetate. The specimens were dehydrated in a graded ethanol series, cleared with propylene oxide, and embedded in Epon 812. Thin sections were post-stained with lead citrate and uranyl acetate and examined in the Seimens Elmiskop 101 electron microscope operating at 80 V.

The assays determining saturation density, morphology, karyology, growth on monolayers and tumorigenicity have been described (8). Methods for karyological observations following conventional staining, fluorescent staining for Q-banding or trypsin-Giemsa staining for G-banding have also been described.

RESULTS

The techniques used to culture epithelial cells have been described (3,4). Briefly, cultures were initiated by incubating minced tissue fragments in medium containing 1 mg/ml collagenase (Worthington Biochemical Corp., Freehold, N.J.). After incubation (37°C) for 18-24 hours followed by gentle pipetting, many clusters of epithelial cells remained undispersed among the singly dispersed connective tissue cells. The visible cell clusters which were allowed to settle in the flask were diluted in growth medium without collagenase and distributed to additional culture flasks in amounts providing 2 or 3 clusters/cm^2. These cell clusters attached and grew out slowly to form monolayer islands of epithelial cells which were soon surrounded by rapidly growing, motile fibroblast cells. The fibroblasts were removed by differential trypsinization; the strong lateral adhesions between epithelial cells resisted the brief exposure to trypsin:versene. When rinsed and refed, these epithelial cell islands often continued to grow into areas freed of fibroblasts, forming a confluent monolayer of polygonal cells in a mosaic-like growth pattern.

Successful outgrowth of mixed epithelial and fibroblastic cells was obtained from 50 (83%) of 60 carcinoma tissues dissociated by collagenase as described above. From these 50 mixed cultures, 18 cell lines of pure epithelial morphology were isolated by selective detachment of fibroblasts with trypsin:versene. In addition, 7 other lines of epithelial cells were selectively grown from apparently normal tissues of cancer patients and three lines were established from intestine of surgically aborted fetuses (4).

Successful establishment of epithelial lines varied depending on the tissue of origin. While 17 of 28 carcinomas from steroid independent tissues were successfully cultured, only one epithelial carcinoma line was established from steroid dependent tissues such as breast. Similar results were obtained with specimens of noncancerous tissue where only steroid hormone independent tissues were successfully cultured (Table 1).

The source of each cell line is listed in Table 2. All of the cell lines displayed a flat polygonal or cuboidal shape typical of epithelial cells (7). By electron microscopy, most of the lines showed ultrastructural features (11-13) of secretory epithelium including tonofibrils, microvilli and desmosomal junctions. While it is likely from the morphological and ultrastructural features described in previous publications that epithelial rather than fibroblastic cells had in fact been cultured, these criteria do not distinguish normal from malignant epithelial cells. To determine whether carcinoma cells had in fact been cultured, all of the cell lines were characterized for a number of growth properties usually associated with in vitro transformation including morphology, nuclear and mitochondrial ultrastructure, saturation density, growth rate, karyotype, ability to grow on contact inhibited monolayers as well as tumorigenicity in immunosuppressed mice.

In a previous publication (4) we tentatively described the epithelial cell lines by 3 morphological categories; normal, abnormal and very abnormal, based on the following characteristics observed with the light microscope:

Normal Cells - Size, shape and staining properties of cells and nuclei are nearly uniform.
Abnormal Cells - Some variation in size, shape and staining of cells and nuclei; increased numbers of multinucleated cells and giant nuclei.
Very Abnormal Cells - Gross alterations in size, shape and staining of cells; bizarre nuclear and nucleolar shapes.

We found that the four cultures established from noncancerous tissues all had normal morphology, while all of those established from tumor specimens showed some degree of morphological aberrancy. The cell lines derived from tumor tissue showed a continuum of morphological abnormalities ranging from slightly abnormal to grossly aberrant.

Unlike cell lines "transformed" in vitro we have also reported (4) that the population doubling times of the carcinoma-derived lines were slow compared to normal fibroblasts and even in some cases compared to those epithelial cell lines derived from noncancerous tissues (4). The saturation density of the lines also varied widely; only in a few cases were the tumor-derived lines significantly higher than those derived from normal tissue (4).

Most of the tumor-derived cultures also showed abnormal nuclear ultrastructure not observed in the non-tumor cell lines. A representative nucleus which has many of the markers associated with tumor cells (2) is illustrated in Fig. 1A. This micrograph illustrates sharply dentate nuclear margins. The heterochromatin was clumped and marginated. A single fibrillar nuclear body and several perichromatin granules occupied the periphery of the nucleus. The single nucleolus was

comprised of a loose nucleolema network. For comparison, Fig. 1B illustrates the nuclear ultrastructure of a non-tumor cell line. All of the tumor cell lines showed nuclear abnormalities. While each abnormal nucleus did not show all of the properties described for Fig. 1A at least two abnormal markers were observed.

Many abnormal mitochondria were also observed in the tumor-derived cells, but not in those lines established from noncancerous tissue. Fig. 1C illustrates typical abnormal metochondria. They were distinctive by their round, distended shape, electron transparant matrix, and abnormal cristae; often, myelin figures were observed within them. Mitochondria from a nonmalignant cell are illustrated in Fig. 1D.

Table 3 describes the karyology of the cultures. Karyological data served first, to monitor for species specificity and concurrence of chromosomal sex of the cell lines with that of their respective donor in order to detect possible intraspecies cell line contamination. Second, further detailed analysis of chromosonal analysis by banding was initiated when grossly altered karyotypes were signaled by conventional staining methods.

The three normal fetal cell lines could only be sexed by chromosome analysis as records failed to indicate the "donors" sex. Strain 614T revealed some cells lacking the expected Y chromosome, but this could be accounted for by the concomitant hypodiploidy in some of the cells which probably involved the loss of a Y chromosome in these cells. Non-conformance of chromosomal and donor's sex was seen only in the male-derived 766T cells all of which were devoid of a Y chromosome.

One of four of the normal lines exhibited some chromosome imbalance by the presence of pseudodiploidy (the diploid number of chromosome, 46 is present, but clearly one or more chromosomes are morphologically unlike the normal karyotype). This, is of course, entirely possible under "normal" circumstances if a parent contributes a morphologically aberrant chromosome to the offspring and may have no relationship to somatic aberrantions associated with disease.

8 of 11 of the tumor-derived cell lines exhibited various indices of altered karyotypes including also pseudo-diploidy. Clearly numerically aberrant karyotypes such as those of 578T, 766T are also structurally altered or "marked" by aberrant chromosomes. 761T is remarkable for its high incidence of polyploidy, a signal of aberrant karyology. Among numerically normal karyotypes one, 675T was clearly aberrant having cells with 3 number 7 chromosomes as revealed by banding using the trypsin-Giemsa technique. Banding studies on the remaining three lines with numerically normal karyotypes is incomplete.

The growth properties of the cultures on contact inhibited epithelial or fibroblastic monolayers are shown in Table 4. The lines derived from noncancerous tissue did not grow on either monolayer. Some carcinoma-derived lines were able to grow on the monolayers. There was no obvious correlation between ability to form colonies on monolayers and type of carcinoma. In most cases, the efficiency of colony formation was similar on the epithelial cells and on the skin fibroblasts.

Four of the tumor-derived cell lines produced carcinomas when inoculated into mice immunosuppressed with antithymocyte serum (Table 5). As described previously (8), the tumors were small and subsequently regressed. Histologic examination of the nodules revealed carcinomas rather than host infiltrations of lymphocytes or fibroblastic reactive tissue.

Table 6 summarizes the in vitro properties of the tumor-derived lines indicating which properties are aberrant. No obvious pattern emerges. Each tumor line appears to have a unique combination of abnormal properties.

DISCUSSION

One of the major problems in studying the biology of human tumor cells has been overgrowth by rapidly dividing normal fibroblasts. We have demonstrated that these fibroblasts routinely can be removed by differential trypsinization resulting in purely epithelial cultures. From many of these epithelial cultures, cell lines can be established which can subsequently undergo numerous subcultures. In general, successful lines of epithelial cells have only been established from those tissues (both normal and carcinomatous) that are not dependent on steroid hormones. These observations suggest that the hormone milieu provided by fetal calf serum may be inappropriate for the growth of hormone dependent cells and that most carcinomas of steroid dependent organs maintain their hormone dependence. Attempts to culture such cells by varying the steroid hormone concentrations are in progress.

The cell lines derived from carcinomas were characterized for a number of growth properties associated with in vitro transformation. Most of the tumor-derived lines showed some alteration in morphology and nuclear ultrastructure suggesting that tumor cells rather than normal epithelial cells surrounding the tumor had in fact been cultured. Many of these tumor cell lines were not aberrant in other growth properties such as saturation density, growth on monolayers or tumorigenicity in immunosuppressed mice. Each tumor cell line possessed a unique combination of aberrant properties. Whether any of these patterns correlate with prognosis for the patient remains to be determined.

Some of the tumor-derived lines showed grossly altered karyology while others were apparently numerically normal; however banding studies on these lines is incomplete. Karyotypically normal or only slightly altered cells have previously been reported to be highly tumorigenic (14, 15). While we cannot state precisely which patterns are associated with tumors or which aberrations if any are found in specific tumors, we can now say that contrary to the earlier established hypothesis (16) that bona fide tumor cell lines do not by definition always consist of highly altered heteroploid cells. We can confirm to some extent the work of others (17) which indicates that metastatic tumors consist, in general, of cells with more aberrant karyotypes than those of primary lesions particularly in numbers of chromosomes. However, we clearly see tumor cells with close to normal karyotypes which by other criteria must be considered malignant. This leads to the supposition that neoplasia may well involve changes at gene loci only and not of entire genomes.

Finally, one major difficulty with the epithelial cell lines is that they grow slowly in culture. Now that pure epithelial culture are available it is possible to perform experiments to optimize the growth rate by varying media. While these cultures are difficult to grow, they are not impossible. However, with present technology, these lines will be most useful as substrates for experiments where only small numbers of cells are required.

SUMMARY

The isolation and long-term cultivation of either normal human epithelial cells or carcinoma cells has been a rare event. In most cases, fibroblastic stromal tissue overgrows the epithelial carcinoma cells. Therefore, the few tumor cultures that have been established may not be representative of all tumor cells, but rather of a rare specimen capable of overgrowing normal fibroblasts. Utilizing techniques developed for the isolation of murine epithelial cells, epithelial cell lines have been isolated from 28 human specimens. There was a high success rate for culturing specimens from some types of tissues including kidney, bladder, rectum and various metastases. All of the cultures have been characterized for a number of properties associated with *in vitro* transformation including karyology, morphology, ultrastructure, growth rate, growth on contact inhibited monolayers, and tumorigenicity in immunosuppressed mice. Although specimens from metastatic lesions tended to express a higher number of transformed properties than those derived from primary lesions, each specimen had a unique combination of aberrant properties. These experiments suggest that cells constituting at least some types of carcinomas can be readily cultured. Such cultures are potentially useful to the cancer clinician developing new modes of immunotherapy and chemotherapy as well as to the tumor cell biologist.

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Table 1 - SUCCESS RATE IN CULTURING EPITHELIAL CELLS

Type of Specimen		No. Cultures Containing Epithelial Cells / No. Specimens Cultured
Carcinoma-Derived Cell Lines		
Steroid Hormone Independent		
Colon-Rectum		4/6
Kidney		2/4
Liver		1/1
Lung		0/2
Metastatic		6/11
Small Intestine		0/1
Stomach		0/1
Transitional Cell		4/5
	TOTAL	17/28 (61%)
Steroid Hormone Dependent		
Adrenal		0/1
Breast		1/20
Metastatic Breast		0/1
Prostate		0/2
	TOTAL	1/24 (4%)
Nontumor-Derived Cell Lines		
Steroid Hormone Independent		
Bladder		1/2
Colon		1/2
Kidney		5/5
Liver		0/1
	TOTAL	7/10 (70%)
Steroid Hormone Dependent		
Vagina		0/1
Vas Deferens		0/1
Uterus		0/1
Thyroid		0/2
	TOTAL	0/5 (0%)

TABLE 2 - SOURCES OF CELL LINES

Designation	History of Patients and Biopsy Specimens			
	Sex	Age (Years)	Race[1]	
Carcinoma-Derived Cell Lines				
614T	M	59	C	rectal carcinoma extended to bladder
675T	information unknown			colon carcinoma
785T	M	58	-	colon carcinoma
761T	M	65	C	transitional cell carcinoma-kidney
789T	F	67	C	transitional cell carcinoma-ureter
703T	M	55	C	liver carcinoma
578T	F	74	C	carcinosarcoma of breast
700T	M	61	C	carcinoma (prob. colon, intestine or pancreas) metastatic
766T	M	64	C	carcinoma of pancreas with metastasis
696T	M	43	O	adenocarcinoma metastatic to bone-primary unknown
806T	F	82	C	recurrant bladder carcinoma originating as a transitional cell carcinoma of ureter metastatic to bladder or primary bladder trans. cell carcinoma
Nontumor-Derived Cell Lines				
677Int	-	3-4 mo.	-	familial history-immune disorder
680Int	-	3-4 mo.	-	familial history of Wiscot Aldredge Syndrome
74Int	-	3-4 mo.	-	therapeutic abortion, no known abnormalities
767Bl	M	45	C	normal bladder mucosa from patient with prostate carcinoma

TABLE 3 - KARYOLOGY OF CULTURES

Cells (Earliest Passage Studied)	Chromosome No. Range	Mode	Y-Chromosome[1]	Remarks[3,4]
Carcinoma-Derived Cell Lines				
Intestine-Rectum-Colon				
614T (6)	35-46		±	Hypodiploid, c.s.
675T (5)	41-100	46	+	Pseudodiploid, banded trisomy No. 7
785T (14)	44-48	46	N.D.	Banding inc.
Transitional Cell				
761T (9)	45-92	46	+	38% polyploid
789T (11)	45-47	46	-	Banding inc.
Liver				
703T (6)	43-47	46	+	Pseudo diploid, c.s., banding inc.
Breast				
578T (4)	31-58	58	-	4-5 banded mrks.
Metastatic				
700T (6)	50-62	60	-	Hyperdiploid, c.s.
766T (11)	60-62	60	-	Hyperdiploid, banded marker
696T (5)	38-43	43	+	Hypodiploid, c.s.
806T (8)	79-88	85	N.D.	Hypotetraploid, c.s.
Nontumor-Derived Cell Lines				
Fetal Intestine				
677Int (6)	44-48	46	-[2]	Banding inc.
680Int (10)	44-47	46	-[2]	Banding inc.
74Int (19)	45-48	46	-[2]	Banding inc.
Adult Bladder				
767B1 (8)	45-48	46	+	Pseudodiploid, c.s., Banding inc.

[1] + or -, presence or absence of Y chromosome in metaphases, by fluorescence.
[2] Donor's sex unknown, sex of fetuses determined by karyotype
[3] Based on clearly altered karyotype, by conventional staining (c.s.) or on basis of alterations in banding pattern by trypsin-Giemsa technique.
[4] Abbreviations: c.s. = conventional staining; inc. = incomplete; N.D. = not done.

Table 4 - COLONY FORMATION ON CONTACT-INHIBITED MONOLAYERS

Cell (Passage)	Number Colonies 1,2	
	74 Int. (Epithelial)	392 Sk (Fibroblastic)
Carcinoma-Derived Cell Lines		
Intestine-Rectum-Colon		
614T (13)	0	0
675T (9)	3	0
785T (18)	0	0
Transitional Cell		
761T (19)	0	0
789T (16)	0	0
769T (12)	56	11
Breast		
578T (28)	800	520
Metastatic		
700T (9)	330	120
766T (16)	35	30
696T (16)	0	0
NonTumor-Derived Cell Lines		
Fetal Intestine		
677Int (7)	0	0
680Int (13)	0	0
74Int (20)	0	0
Adult Bladder		
767Bl (17)	0	0

[1]

[2]No. of colonies formed/60mm dish with 10^3 cells plated.
[2]Representative Experiment Shown.

TABLE 5 - TUMORIGENICITY IN IMMUNOSUPPRESSED MICE[1]

Cells (Passage)	No. Animals With Tumors / No. Animals Inoculated	Histology
Carcinoma-Derived Cell Lines		
Intestine-Rectum-Colon		
675T (7,8)	5/14	very tiny, reactive tissue only
785T (17)	0/3	
682T (10)	1/3	carcinoma
Transitional Cell		
761T (17,18)	0/6	
789T (15)	2/4	carcinoma
769T (12)	5/6	carcinoma
Breast		
578T (28)	1/14	too small, no histology available
Metastatic		
700T (9)	7/7	
766T (16)	2/2	carcinoma
696T (16)	0/6	
Nontumor-Derived Cell Lines		
677Int (9)	1/6	very small, benign
680Int (13,21)	0/7	
74Int (15,23)	1/13	too small, no histology available
Adult Bladder		
767B1 (9,10,17)	1/14	too small, no histology available

[1] 3 to 6 x 10^6 cells inoculated per animal.

TABLE 6 - SUMMARY OF PROPERTIES OF CARCINOMA-DERIVED LINES[1]

Cells	Cellular Morphology[2]	Ultrastructure[2] Nuclear	Ultrastructure[2] Mitochondrial	Sat. Density[2]	Growth on Monolayers Epithelial	Growth on Monolayers Fibroblastic	Tumorigenicity	Karyotype
Intestine-Rectum-Colon								
675T	+	+	+	0	$\frac{+}{0}$	0	0	+
785T	+	NT	NT	0		0	0	I
Transitional Cell								
761T	+	+	+	0	0	0	0	+
789	+	+	+	0	0	0	+	I
Liver								
703T	+	+	+	+	NT	NT	+	I
Breast								
578T	+	+	+	NT	+	+	0	+
Metastatic								
700T	+	+	+	0	+	+	0	+
766T	+	+	+	0	+	+	+	+
696T	+	+	+	0	0	0	0	+
806T	+	NT	NT	0	NT	NT	NT	+

[1]Abbreviations
+ = Property observed only in tumor-derived cells
0 = Property observed in nontumor-derived cells
NT = Not tested
I = Evaluation incomplete
[2]From reference 4

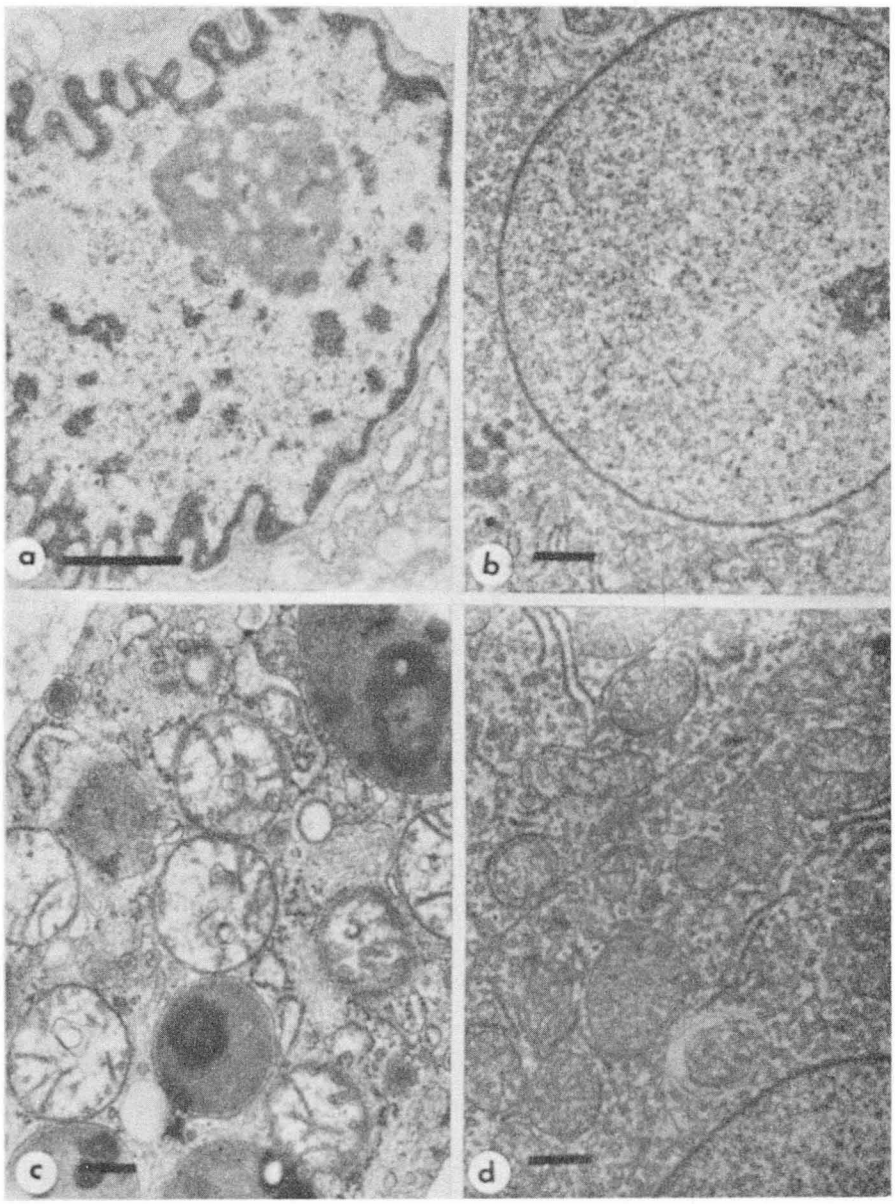

FIG. 1. (a) Typical abnormal nucleus from 76IT (marker is 0.5 micron). (b) Typical normal nucleus from 767B1 (marker is 1.0 micron). (c) Typical abnormal mitochondria from 76IT (marker is 0.5 micron). (d) Typical normal mitochondria from 767B1 (marker is 1.0 micron).

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