Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 1805–1809, March 1985
Medical Sciences

Invasiveness and ploidy of human mammary carcinomas in short-term culture

(denuded human amnions/diploid malignancies/DNA content/karyology/tumor heterogeneity)

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Communicated by Melvin Calvin, November 2, 1984

ABSTRACT **Invasiveness and ploidy were examined in cultures of human epithelial cells derived from nonmalignant breast tissue, primary breast carcinomas, and breast cancer effusion metastases. Successful short-term culture was achieved from approximately 70% of the primary breast cancers. These primary cancers were essentially diploid by flow cytometry and karyotype in contrast to the effusion metastases, which were mostly aneuploid. The diploid tumor cells retained their malignant phenotype in culture as demonstrated by invasion into a denuded human amnion basement membrane. In contrast, epithelial cells cultured from nonmalignant mammary tissue did not invade the amnion. We suggest that the diploid carcinoma cultures may be useful for investigating the essential differences between normal and malignant cells and may complement information derived from studies of tumor cell lines with grossly aberrant karyotypes.**

The biology of primary breast cancers may differ substantially from other primary cancers such as lung cancer (1) because it is often associated with long remission periods or surgical cures (2). Most primary breast carcinomas grow slowly *in vivo* and, therefore, have rarely yielded dividing cells for karyotypic analysis. The DNA content of such tumors can be measured in interphase cells by flow cytometry or microspectrophotometry. By these techniques, 30–40% of breast cancer specimens are reported as diploid or tetraploid (possibly in G_2 phase), with an additional 20% of specimens containing both diploid and aneuploid cells and the remainder appearing totally aneuploid (refs. 3–12; for review, see ref. 13). Poor prognosis and dedifferentiation, measured as loss of estrogen receptors, are associated with aneuploidy (7–12).

We have developed techniques that permit primary mammary carcinomas to be cultured readily (14–17), and in these cultures we found that cells from many breast carcinomas were essentially diploid (18). Cells cultured in this manner have been shown to be bona fide mammary epithelium by a number of markers, including morphology, ultrastructure, presence of secretory domes (17), keratin (19), distinctive fibronectin distribution (20), and presence of mammary epithelial antigens (14). It has been more difficult to find criteria that distinguish epithelial cultures of carcinomas from those of nonmalignant mammary tissues (13). Tumor-derived cultures were distinguished from cultures of nonmalignant tissue by the presence of a tumor-specific antigen (19); however, this criterion can no longer be used since appropriate antiserum is no longer available. To confirm that the diploid cells cultured from primary carcinomas indeed represent malignant cells, we investigated the phenotype of invasiveness because it is perhaps the most important single criterion by which human solid malignancies can be diagnosed. We used a recently developed *in vitro* assay for invasion utilizing denuded human amnions (21, 22). Here we show that the diploid tumor cells retained their malignant phenotype in culture by being capable of invasive growth. In contrast to the primary tumors whose viable cultured cells were diploid, cultured cells from most effusion metastases were aneuploid.

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MATERIALS AND METHODS

Human Mammary Epithelial Cell Culture. Techniques for obtaining and growing human mammary epithelial cells have been reported in detail (14–17). Briefly, nonmalignant breast tissue was dissected away from skin, grossly fatty areas and, in mastectomy specimens, from any hard nodular areas suspicious for occult malignancy. Tumor masses were dissected free of all grossly evident normal stroma prior to mincing and disaggregation. At this stage, a portion of the isolated tissue was evaluated histologically. For culture we used only those tumor specimens where nonmalignant epithelial cells were not detected histologically and nonmalignant specimens where no areas of occult tumor were present. The tissue was digested with collagenase and hyaluronidase at 37°C with gentle rotation until epithelial clumps appeared to be free of attached stroma. The clumps collected by filtration with polyester screen filters were used to initiate primary cultures on tissue culture plastic. Enzymatic digestion was not necessary for the pleural effusions. In some cases, cells were cryopreserved with 10% dimethylsulfoxide and 10% fetal calf serum prior to culturing. After approximately 1 week in primary culture, when numerous mitotic figures were present in the cultures, cells were dissociated with trypsin and either seeded into flasks for flow cytometry or karyotypic analysis or onto lucite chambers for invasion assays. After trypsinization, cells were considered "second passage." In most cases, there was no clear-cut evidence of morphologic heterogeneity and subsequent selection of some populations in second passage.

Cells were grown in a medium designed for human mammary epithelial cells that consisted of the following components: 30% Dulbecco's modified Eagle's medium (DME medium), 30% Ham's F-12 medium, 30% conditioned medium from the human fetal intestine epithelial cell line Hs741nt (23) and the human bladder epithelial cell line Hs767B1 (23), 9% conditioned medium from human myoepithelial cell line Hs578Bst (24), 0.5% fetal calf serum, 10 μg of insulin per ml (Sigma), 5 ng of epidermal growth factor per ml (Collaborative Research, Waltham, MA), 10 nM triiodothyronine (Sigma), and 1 ng of cholera toxin per ml (Sigma). Conditioned medium was prepared from confluent cultures incubated for 1–3 days with equal volumes of DME medium and Ham's F-

12 medium containing 5% fetal calf serum and 5 μg of insulin per ml.

Cell lines were grown as follows: BT549 (25) in 50% DME medium/50% F-12 medium containing 5% fetal calf serum and 10 μg of insulin per ml; MDA231 (26) in 50% DME medium/50% F-12 medium containing 10% fetal calf serum; and MCF7 (27) in DME medium containing 10% fetal calf serum and 10 μg of insulin per ml.

Invasion Assays. Cells (2×10^5) were seeded onto prepared amnions stretched and clamped between the two halves of a lucite chemotaxis chamber (detailed in refs. 21 and 22) dividing the chambers into two compartments, upper and lower. Cells were seeded onto the upper chamber in 0.2 ml of medium, and the chamber was placed in a plastic container with 10 ml of medium bathing the other side of the amnion. One hour later, an additional 1 ml of medium was added to the upper chamber. The chambers were incubated at 37°C for a week with one medium change. The chambers were immersed in 10% formalin, and the coded specimens were sent to one of the authors (L.A.L.). Paraffin sections, stained with hematoxylin/eosin, were scored blindly for invasion of the membrane.

Isolation and preparation of human amnion basement membrane have been described in detail (21). Briefly, normal-term placentas were obtained within 3 hr after delivery. Any placentas that showed a yellow color, tore easily, or had signs of chorioamnionitis were discarded. The amnion was separated from the chorion manually, starting at the periphery and working toward the insertion of the umbilical cord, where it was cut. The amnion membrane was aseptically peeled away from the chorion by blunt dissection and immediately rinsed two to three times with phosphate-buffered saline supplemented with penicillin (100 units/ml), streptomycin (100 units/ml), and fungizone (5 μg/ml) ("supplemented phosphate-buffered saline"). Blood and mucous were wiped from the stromal surface between each wash (the final wash was clear). The outer portions of amnion were denuded by treatment with 0.01% ammonium hydroxide (20 min at 25°C), followed by gentle wiping with a sterile gauze. The membrane was then rinsed several times and stored at 4°C in supplemented phosphate-buffered saline until used.

DNA Content by Flow Cytometry. For flow studies of cultures derived from nonmalignant tissue and primary carcinomas, second passage cells were used. Malignant effusions were studied in primary culture except for specimen 523M, which was assayed at second and third passage. Cells were trypsinized, pelleted, and resuspended in 0.5–1.0 ml of medium to a final concentration of approximately 10^6 cells per ml. The suspension was placed in a sterile test tube and gently agitated at 37°C for 1 hr in the presence of 10 μM Hoechst 33342 (Calbiochem–Behring). DNA content was then examined on a fluorescence-activated cell sorter (modified Becton-Dickerson FACS II) at a wavelength of 351 nm using a neutral density 2.0 filter. DNA content of each sample was compared to the same normal specimen (437E, not shown) each day and was based upon analysis of at least 10^4 cells.

Chromosome Analysis. Colcemid solution (GIBCO Diagnostics) at 10 μg/ml was added to the culture fluid to a final concentration of 0.5 μg/ml, and the flask was incubated at 37°C for 4–5 hr. Culture fluid and trypsinized cells from a 25-cm^2 tissue culture flask were centrifuged and resuspended in 6–8 ml of 0.38% potassium chloride added drop by drop for the first 0.5 ml and incubated for 15 min at 37°C. Five or six drops of freshly prepared Carnoy's solution [methanol/glacial acetic acid, 3:1 (vol/vol)] were added, and the cells were centrifuged, gently resuspended in 2 ml of Carnoy's solution,

Table 1. Invasion of human amnions by cultured normal and malignant epithelial cells

Specimen	Age, yr	Pathology	Invasion assay result
	Nonmalignant tissues		
Reduction mammoplasties (second passage)			
337EL	59	Focal duct ectasia	Benign
356E	21	Normal	Benign*
399E	20	Normal	Benign
446E	30	Normal	Benign
527E	NA	Normal	Benign
Nonmalignant tissue from patients with carcinoma (second passage)			
Breast tissue peripheral to carcinoma			
469P	28	Ductal hyperplasia	Benign
524P	70	Fibrocystic and fibroadenomatous	Benign
Breast tissue contralateral to carcinoma			
478C	65	Fibrocystic	Benign†
Normal skin from cancerous breast			
559SK	68	Normal breast skin	Benign
	Mammary carcinomas		
Primary carcinomas (second passage)			
192T	42	Infiltrating lobular	Invasive
335T	58	Infiltrating lobular	Benign†
343T	48	Infiltrating ductal	Invasive
407T	43	Infiltrating ductal	Invasive
469T	28	Infiltrating ductal	Invasive
559T	68	Inflammatory	Invasive in focal areas
Established cell lines			
BT549	NA	Primary adenocarcinoma	Benign†
MDA231	51	Pleural effusion	Invasive
MCF7	NA	Pleural effusion	Invasive

NA, not available.
*Pleomorphism, nuclear abnormalities, foci of piled up cells.
†Cells appear to be degenerated.

and refrigerated for 24–72 hr. Finally, the suspension was centrifuged, the supernatant was removed, and the cell pellet was dispersed and resuspended in a small volume of fresh Carnoy's solution. The cell suspension was dropped from a height of 45–60 cm onto clean, chilled, wet slides held at approximately a 30° angle. They were dried rapidly on a moderate warming tray. Within 20–72 hr after the cell suspension was dropped, the slides were heated in an oven for 10 min at 90°C prior to staining. GTG bands were elicited with 0.1% trypsin solution in Dulbecco's phosphate-buffered saline in a modification of Seabright's technique. Slides were scanned at a magnification of ×125. Countable cells were analyzed at ×1250. During this process, an attempt was made *not* to avoid less-than-perfect cells so that abnormal cells would not be eliminated by subjectivity in the selection process.

RESULTS

Invasion of Human Amnions. Table 1 summarizes results of invasion of human amnion membranes by cultured mammary epithelial cells from different sources. Of eight nonmalignant mammary specimens tested, none were invasive. Included among these were nonmalignant breast tissues from patients with carcinomas and from patients undergoing cosmetic procedures (reduction mammoplasties). In one case, skin epithelium from a cancerous breast was cultured in an identical manner to the mammary tissue; the normal skin epithelium was noninvasive. In contrast, five of six primary carcinoma specimens tested were invasive. By χ^2 analysis, the tumor-derived cultures were statistically different from the nonmalignant cultures with a P value of <0.01. Included among the invasive cultures were three histologic types of breast cancer—lobular, infiltrating ductal, and inflammatory. Where tumor and nonmalignant tissue were obtained from the same patient, only the cultured epithelium from malignant tissue was invasive (specimen 469P vs. specimen 469T). Differences between the nonmalignant and malignant specimens were not related to age of the donors; the age range of the tumor sources was included in the age range of the sources of nonmalignant specimens. Except where indicated as a footnote to Table 1, the tumor and normal cells appeared equally healthy on the membrane. In the single case of tumor in which no invasion was seen (specimen 335T), the cells appeared degenerated as if their proliferation were incompatible with the membrane. Similar results have been seen with established breast cancer cell lines. Cells from the line BT549 (25), which were noninvasive, appeared to degenerate on the denuded amnion. Two other cell lines, MDA231 (26) and MCF7 (27), were both invasive. Typical appearances of invasive and noninvasive cultures are illustrated in Fig. 1.

To date, we have been unsuccessful in our attempts to reculture the primary carcinoma cells that invade through the amnion because the short-term cultures have only limited proliferative capacity. Therefore, karyotypic analysis of those cells that have invaded the amnion has not been possible.

Analysis of DNA Content and Karyotype. In several of the tumors, a dual approach to DNA analysis was attempted. Table 2 summarizes the DNA content by flow cytometry of cultured normal and malignant mammary epithelium. In all cases tested, the primary carcinomas were diploid and indistinguishable from nonmalignant cultures. In contrast, four of five metastases were aneuploid. Karyotypic analysis (Table 3) of an aneuploid pleural effusion, 576M, showed a chromosomal mode of 59–60, with both structural and numerical abnormalities. In contrast, chromosome analysis of three invasive primary carcinoma cultures and one noninvasive primary carcinoma revealed that, in all cases, the cultures were predominantly diploid. Only a few nonclonal chromosomal abnormalities were detected (Table 3). These tumors are representative of a much larger series of cultured primary breast

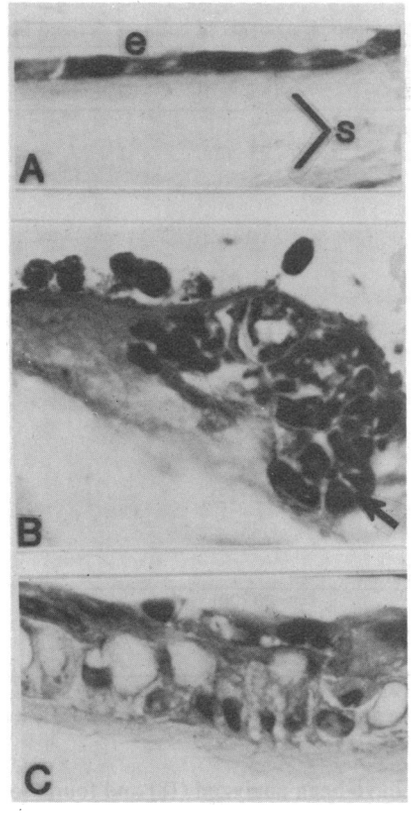

FIG. 1. Invasion of human amnion extracellular matrix by cultured human breast carcinoma cells. (*A*) Specimen 524P: absence of invasion. The mammary cells (e) adhere and grow on the basement membrane surface but do not invade the underlying stroma (s). (×400.) (*B*) Specimen 469T: invasive. The mammary tumor cells penetrate the basement membrane and invade (arrow) the amnion interstitial stroma. (×400.) (*C*) Specimen 192T: invasive. The mammary tumor cells have invaded and replaced the majority of the amnion stroma. (×400.)

Table 2. DNA content by flow cytometry of cultured normal and malignant mammary epithelial cells

Specimen	Ploidy value of G_1 peaks
Nonmalignant	
337E	2
356E	2
399E	2
469E	2
Primary carcinoma	
192T	2
343T	2
407T	2
469T	2
Malignant effusions	
486M (ascites)	4.4
521M (ascites)	2.7, 2.9, 3.0*
524M (pleura)	No distinct peak (broad range from 2 to 4)†
576M (pleura)	2.4
600M (pleura)	2.0, 2.0
Established cell lines	
BT549	2.5
MDA231	2.3

*Assays done on three different primary cultures.
†Assays done on cells at second and third passages.

Table 3. Chromosome analysis of cultures derived from breast cancer

Specimen*	Total (banded)	46,XX or random loss	Tetraploid range	Aneuploid
Primary carcinoma				
192T	23 (2)	17	2	4 41,XX,−6,−8,−9,−9,−14,−19,−20,+t(8;14;19) 47,XX,+C 47,XX,+D 49,XX,Gp+,+C,+G−,+G−
192T†	11 (11)	11	0	—
335T	10 (10)	8	0	2 44,XX,−12,−15,−19,+M?(8p;7q) 44,XX,4p−,−4,−13,−14,−22, +M_1 (large submetacentric), +M_2 (medium metacentric), +M_3 (tiny metacentric)
407T	10 (10)	10	0	—
469T	12 (12)	10	0	2 46,XX,14q+ 46,XX,1q+
Effusion metastases				
576M	20 (20)	1	0	19 61,XX,+3B,+7,+8,+8,+14,+22,−1,−17,+M1(inv ins 1), + variable number of D, F, and marker chromosomes

*Unless otherwise indicated, studies were done on cells at second passage.
†To exclude the possibility that aneuploid cells were lost after passage, primary organoid cultures 7 days after seeding were treated directly with colcemid.

cancers that have been analyzed (18) and found to be essentially diploid.

DISCUSSION

In a previous study, we found that the vast majority of cells in short-term cultures of 15 different primary breast carcinomas were diploid (18). Only an occasional cell within these cultures was found with minimal nonclonal karyotypic deviations from normality. Here we show that similar short-term diploid cultures of breast carcinomas retain their capacity for invasiveness in an *in vitro* assay. Nonmalignant cells, similarly cultured, were not invasive in the same assay. These findings help confirm that the cells cultured from primary carcinomas indeed represent malignant cells and indicate that malignant cells (at least in the breast) need not have gross karyotypic rearrangements. We suggest that such cells may be more informative than tumor cell lines with grossly aberrant karyotypes for investigating some of the critical early changes involved in the acquisition of malignant characteristics.

In contrast to the primary carcinomas, the malignant effusion-derived cultures were aneuploid (Table 2). Because the malignant effusions do not grow as readily in culture as primary carcinomas (28), it was more difficult to examine their karyotypes. Therefore, we utilized flow cytometry to determine the DNA content of the slowly proliferating malignant effusion cultures. In one case, aneuploidy was verified by cytogenetic analysis and the population showed distinctive clonal karyotypic aberrations.

The observation that cells cultured from primary breast cancers are diploid while those from malignant effusions are aneuploid raises interesting questions about the relationship between ploidy and malignant progression. Studies of breast carcinomas prior to culture generally report that 60–70% of specimens are composed of aneuploid cells at least partially, if not completely (3–12). Unfortunately, we do not know whether the specimens from which we cultured diploid tumor cells originally contained aneuploid cells. It is likely that at least some of the specimens did, since our success rate in culturing primary carcinomas is significantly higher (23 of a consecutive series of 32 specimens or 72%) than the 30–40% of specimens expected to be diploid (by DNA content). However, to establish this point conclusively, it will be necessary to measure DNA content on the original specimens.

If aneuploid cells are present in the original primary carcinomas but not after culture, it will be necessary to explain why they are lost from primary carcinomas but not from metastases. One possibility is that the aneuploid cells within primary tumors are viable *in vivo* but are preferentially lost during isolation or culture, while the aneuploid cells within metastases have gained the ability to survive under the experimental conditions described. An alternate possibility is that the majority of aneuploid cells within primary breast cancers are nonviable even *in vivo*. Other studies using flow cytometry suggest that tumor-cell subpopulations that are enriched in aneuploid cells are largely nonviable by dye-exclusion analysis (29, 30). Therefore, we hypothesize that minor subpopulations of viable aneuploid cells may be responsible for malignant effusions. We suggest that these metastatic subpopulations can be generated from diploid, invasive cells by a process of genetic instability similar to that originally proposed by Nowell (31). Most of the aneuploid cells generated by this process are probably nonviable; however, an occasional cell could retain viability while gaining the capacity for metastatic growth, thus becoming the source of metastases for that patient. The time of development of these aneuploid cell populations within the primary tumor or its metastases may vary among individual tumors. Whichever alternative proves correct, the fundamental point presented here is that diploid cells within breast cancers are capable of invasive growth.

We gratefully acknowledge the excellent technical assistance of Mr. A. Hiller, Mr. S. Sylvester, and Mrs. S. Bornstein. We thank the Pathology Departments of Peralta–Merritt and Providence hospitals (Oakland, CA) and Dr. M. Lippman and his colleagues (National Cancer Institute, Bethesda, MD) for providing specimen material. We also thank Dr. L. Herzenberg (Stanford University, Stanford, CA) for use of his flow cytometry facility. This work was supported in part by Grants CA34193 and CA34192 from the National Cancer Institute to H.S.S.; M.C.H. was supported by a Damon

Runyon–Walter Winchell Cancer Fund Postdoctoral Fellowship (DRG 713) in the laboratory of Dr. R. Schimke (Stanford University, Stanford, CA).

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