Advances in Brief

Propagation of Genetically Altered Tumor Cells Derived from Fine-Needle Aspirates of Primary Breast Carcinoma[1]

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Abstract

Because primary breast tumors are diagnosed earlier in the clinic, procurement of sufficient amounts of tumor tissue for in-depth biological characterization is becoming increasingly difficult. We demonstrate here that relatively small numbers of tumor cells within samples of fine-needle aspirates (FNA) can be propagated in culture. Of 25 cases attempted, 12 were passageable, resulting in up to 10^7 viable cells. FNA-derived cultures were evaluated for anchorage-independence, c-*erb*-B2 overexpression, aneusomy, and pattern of allelic loss. In every case examined, the cultured cells closely resembled the original tumor tissue and displayed one or more tumor phenotypes. The incidence of *erb*-B2 overexpressing tumors was similar in passageable and nonpassageable cases (33% versus 31%, respectively). FNAs that are expanded from a wide range of clinical breast material could be useful for functional studies presently limited to rare established cell lines, such as aberrant signal transduction and gene regulation, and for testing potential anticancer vaccines and drugs.

Introduction

Previously reported approaches for the *in vitro* propagation of primary breast tumors, such as (*a*) mechanical disaggregation or (*b*) degradation of connective tissue by enzymatic digestion, have generally required large quantities (up to several grams) of tumor tissue as starting material. Cultures that have grown out using these approaches were relatively normal, which suggests that slow-growing tumor cells may be rapidly overgrown by normal breast epithelium interspersed within the tumor tissue. Occasionally (<1% of the time), primary tumor specimens have yielded continuously proliferating cultures or cell lines (for review, see Refs. 1 and 2). In this study, we demonstrate that tumor cells can be harvested from small quantities of aspirated material present in routine FNAs[3] of primary breast carcinoma. Viable cells within FNA specimens can be expanded, and pure epithelial populations can be obtained that display distinctive characteristics of the malignant specimen from which they are derived.

Materials and Methods

Collection of FNAs. FNAs obtained from 25 consecutive cases of fresh surgical discard tissue of pathologically confirmed, infiltrating primary breast carcinoma at the California Pacific Medical Center and the University of California (San Francisco, CA) were used in this study. The samples were collected from the excised tumor in the Pathology Department, in most cases before the specimen was cut open. This approach was taken because small (1–2 cm) palpable tumors are easier to sample as a whole than the cut tissue. A

23-gauge disposable needle and syringe were attached to a syringe holder and used for aspiration. Cells were removed from the tissue by moving the needle back and forth approximately 20 times through the tumor while pulling back on the syringe plunger to maintain 1–2 ml of suction. At the end of the procedure, the FNA suspension obtained was expelled into a centrifuge tube containing cell culture transport medium. The procedure was repeated until a suspension containing visible sinking fragments was obtained. In all of the cases, a portion of the tissue was available as snap-frozen or formalin-fixed, paraffin-embedded blocks.

Growth Media and Cell Culture. The FNA suspension was centrifuged, and the cell pellet was plated in replicate dishes in MCDB 170 medium containing 0.06 mM calcium (University of California at San Francisco Cell Culture Facility, San Francisco, CA) and supplemented as described earlier (3) with insulin, epidermal growth factor, hydrocortisone (Sigma Chemical Co., St. Louis, Mo.), bovine pituitary extract (Hammond Cell Tech., Alameda, Ca.), and 2% FCS. Upon attachment, contaminating fibroblasts were removed by differential trypsinization as described previously (4). At subconfluence, cells were passaged by routine trypsinization procedures.

Anchorage-independent Growth. An aliquot of the FNA suspension was filtered through a 51-μm nylon filter to remove cell clumps. The filtrate was centrifuged and resuspended in 0.3% agarose (Sigma Chemical) in MCDB 170 growth medium (described above) and then gently layered on a 0.7% base layer of agarose. Cultures were incubated and replenished with growth medium up to 4 weeks.

Immunofluorescence. Expression of *erb*-B2 was determined by immunolocalization of a mouse monoclonal antibody, CB11 (Biogenex, San Ramon, CA) on frozen sections of the original tumor block and compared with tumor-derived cultures at second or third passage as described previously (5). Nonmalignant epithelium within the section served as normal baseline control. The breast cancer cell line BT474 was used as a positive control for *erb*-B2 overexpression.

LOH Analysis. DNA was isolated from tumor cells that were microdissected from H&E-stained sections of tumor tissue and from FNA-derived tumor cultures for analyzing the presence of LOH. Nonmalignant tissue, such as skin or lymph node, or cultured fibroblasts derived from the FNA sample were used as controls to ascertain informative status at specific polymorphic loci. PCR conditions were as described in Deng *et al.* (6), in the Sequence-tagged Sites (STS) map or in the Human Genome Database (GDB), with minor modifications. For PCR amplification at specific chromosomal loci, the following primers were used: (*a*) 3p-*EABMD, EABH, D3S1244, D3S1768, D3S2414, D3S2405*; (*b*) 7q-*GH390*; (*c*) 8p-*D8S322, D8S1121*; (*d*) 11p-*INS.2, TH.2*; and (*e*) 17p-*TP53.5, D17S969, D17S154*.

Samples were scored as positive for LOH if ≥30% reduction was observed in the ratio of the upper:lower alleles compared with control DNA.

FISH. Details of fixation, *in situ* hybridization, and scoring of signals were as described previously (7). Cells at second passage were directly analyzed as adherent monolayers on microscope slides after fixation with Carnoys. Pericentromeric probes to chromosomes 1, 3, 7, 11, 17, and a dual probe for 17/*erb*-B2 (Vysis, Downers Grove, IL) were used to detect changes in copy number. Epithelial cultures that were expanded from reduction mammoplasty-derived "organoids" served as normal, diploid controls. Fluorescence was visualized and recorded using a Zeiss Axioskop microscope and an Optronics CCD camera. Data were collected from cases where unequivocal signal interpretation was possible on 100 cells.

Results

Characteristics of Cultured Epithelial Cells Isolated from FNAs.

Fine-needle aspiration biopsy is an established method for the diagnosis of breast carcinoma. Two important factors that determine the cellular yield of the FNA are: (a) the cellularity of the tumor sample; and (b) the skill of the person performing the procedure. Whether tumor cells present in the FNA will propagate in culture is largely dependent upon the number of viable, proliferation-competent cells in the sample.

The FNA samples in this study displayed different levels of cellularity. In addition to epithelial cells, the aspirates contained RBCs, lymphocytes, and mesenchymal cells to varying degrees (Fig. 1a). The hematopoietic cells remained in suspension and rapidly degenerated and lysed.Contaminating fibroblasts were removed by differential trypsinization after initial cell attachment and growth of the culture, to yield pure epithelial populations (Fig. 1b).

Vital staining at the time of receipt (within 4–6 h after surgery) in three randomly selected cases demonstrated a range of 20–80% viable cells and a total count of 10^3 to 10^5 nonhematopoietic, presumably mostly epithelial, cells in the FNA sample.

Growth Potential.

As summarized in Table 1, almost one-half (12 of 25) of the FNA cases that were initiated in culture displayed vigorous epithelial growth. All of these samples were proliferative beyond the second passage and were subcultured 3–7 times. In one case, a continuously proliferating population presently at passage 60 was isolated. During the first three passages, the cultures proliferated relatively rapidly—doubling in number every 48–72 h. After this stage, cell numbers increased slowly, doubling at 10–12 days. Generally, cell yields ranging from 10^6 to 10^7 cells per sample were

Table 1 *Growth potential of epithelial cells isolated from FNAs of primary breast carcinoma*

Extent of growth	% cases	n
No attachment or growth	40	10
Epithelial patches, not passageable	12	3
Passageable, 2–7 times	44	11
Infinite growth	4	1

achieved, which enabled the additional in-depth characterization described below.

In 10 of 25 of the FNA specimens seeded in culture, there was no cellular attachment or growth. This suggested that either (a) the sample did not contain a sufficient number of viable or proliferation-competent cells or (b) the sample primarily consisted of anchorage-independent cells. The latter seems unlikely as no further growth of the initial suspension was observed.

In another three cases, although small patches of <50 epithelial cells were observed during the first few days of culture, there was no appreciable growth, and the culture did not progress further during 2 weeks of observation.

To ascertain that epithelial cells cultured from FNA-derived tumor tissue displayed phenotypes of transformed cells, they were tested for anchorage-independent growth and aneusomy. In addition, fixed cultures and tumor tissue were compared for alterations in *erb*-B2 expression and analyzed for the presence of LOH. The following observations, summarized in Table 2, confirm the growth and expansion of bona fide tumor cells in culture:

Anchorage-independent Growth.

Of eight samples seeded directly into agar, seven tumors developed visible colonies over a period
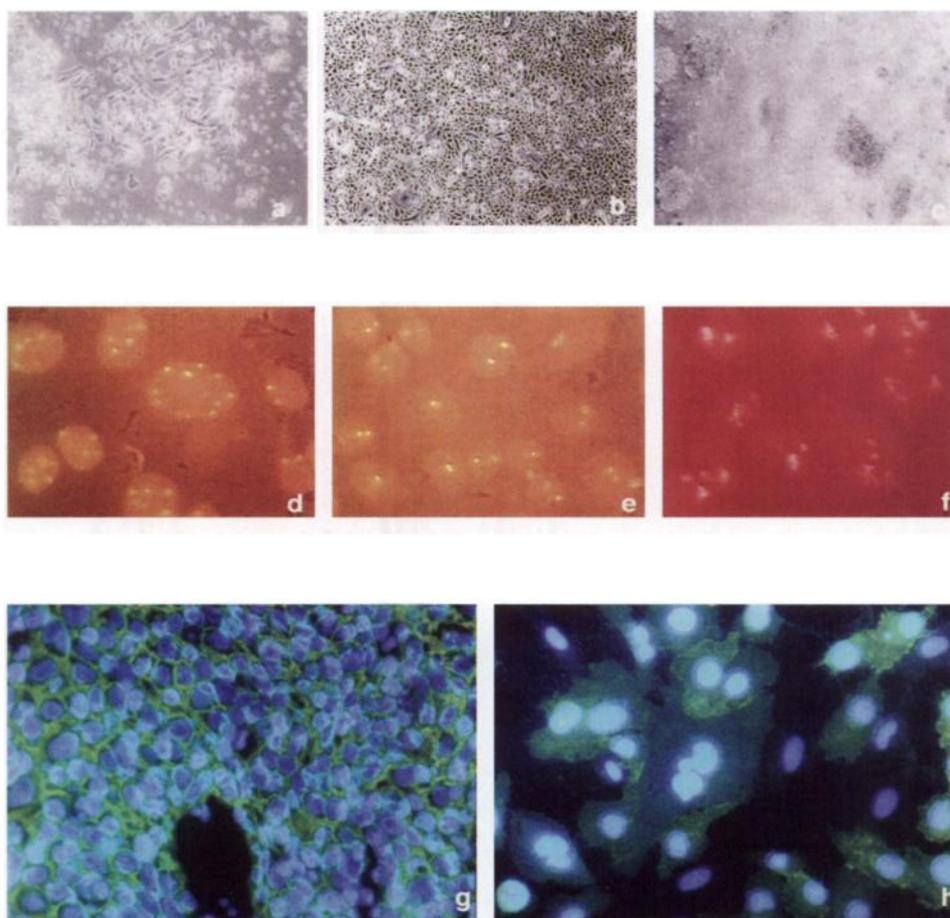

Fig. 1. *a-c*, growth and morphology. *a*, morphological complexity of an FNA sample at 24 h after plating. Note the population of attached cells with relatively undefined morphology and the large numbers of lymphocytes in suspension. *b*, the FNA sample after 10 days in primary culture. Note the pure population of epithelial cells. *c*, anchorage-independent clonal growth of FNA-derived epithelium. *d-f*, FISH analysis of FNA cultures. *d*, second passage epithelial cells with a pericentromeric probe to chromosome 11. Note the presence of multiple signals in the nuclei. *e*, a pericentromeric probe to chromosome 17 showing diploid signal in a tumor culture at passage 2. *f*, multiple copies of the *erb*-B2 gene located on chromosome 17 in the culture shown in *e*. *g-h*, the immunofluorescenct localization of erb-B2 protein. *g*, a frozen section of primary breast tumor tissue. *h*, the FNA-derived epithelial culture of tumor tissue shown in *g* at passage 3. Note the strong localization of antibody in cells in the tissue section and in culture. *a*, ×100; *b* and *c*, ×40; *d-h*, ×400.

Table 2 *Tumor-associated phenotype of FNA-derived epithelial cultures*

Specimen	Anchorage-independence[a]	Aneusomy[b] at chromosome	erb-B2 expression		LOH	
			Tumor tissue	Cell culture	Tumor tissue	Cell culture
G382	ND	1, 3, 7	+++[c]	++	8p, 17p	No LOH
G383	ND	3	+++	+++	8p	No LOH
G386	N	3, 17				
G395	ND		+	+	3p	No LOH
G396	ND	1	+	+	3p, 8p, 17p	8p, 17p
G430	Y		+	+	3p	3p
G432	Y		+	+	3p, 8p	3p, 8p
S257	Y	1, 7, 11	+++	+++	7q, 17p	7q, 17p
S324	Y				No LOH	No LOH
S329	Y					
S331	Y		−	−	No LOH	No LOH
S337	Y		−	−	8p, 17p	No LOH

[a] Anchorage Independent Growth (AIG) resulting in ≥50 cells/colony within 2–4 wks. ND, not done; N, no AIG observed; Y, AIG observed.

[b] A total of 100 cells was examined with each probe. Aneusomy shown was displayed by ≥30% of the cell population. Presence of aneusomy in ≥16% cells is significantly different from diploid control cells derived from one reduction mammoplasty specimen by Fisher's exact test at $p < 0.01$.

[c] +++, ≥50% strong positive cells; ++, ≥50% weak positive cells; +, scattered positive cells; −, negative.

of 2–4 weeks. In contrast, repeated attempts with epithelial cells from five independent reduction mammoplasty samples failed to show anchorage-independent growth. The phenotype of colonies that developed in soft agar varied from tumor to tumor. An example in Fig. 1c shows a loosely arranged, irregular-shaped mass in which the individual cells are clearly visible. Other samples gave rise to relatively symmetrical spheres of tightly packed cells in which, although growth was evident, the total number of cells in the colony could not be assessed accurately. These seven cases were also proliferative on a plastic substrate (Table 2).

Aneusomy. Using FISH with pericentromeric probes to chromosomes 1, 3, 7, 11, and 17, we found that epithelial cells that grew out of the FNA samples displayed a variety of numerical changes. In five cases described here, multiple chromosome copies ranging from 3–5 were observed in ≥30% of cells for at least one of the five chromosomes tested. For example, in specimen S257T, at third passage >75% of the cultured cells contained 3–5 copies of chromosome 1 and chromosome 11 (Fig. 1d), whereas, for chromosome 17, >90% of the culture was diploid (Fig. 1e). As shown in Fig. 1f with a dual probe, although this sample had no apparent alteration in chromosome 17 copy number, it displayed amplification or several copies of the erb-B2 gene located on 17p. This finding demonstrates that in some cases even when the number of whole chromosome copies was unaltered, anomalies in copy number existed at the gene level.

Expression of erb-B2. Cell preparations optimal for immunofluorescent localization of the erb-B2 gene product were available for nine independent cultures. As shown in Table 2, the number of widely positive (+++ and ++), scattered positive (+), and negative cases among tumors that were proliferative in culture was 3 of 9, 4 of 9, and 2 of 9, respectively. Concordant expression of the erb-B2 protein in the original tumor and in the tumor-derived culture was seen in all of the cases (Fig. 1, g and h). Nonmalignant breast tissue was negative for the expression of erb-B2 protein, whereas the BT474 cell line (known to be amplified for the erb-B2 gene) showed strong immunofluorescence.

erb-B2 status of the 13 cases that did not result in passageable cultures was also determined by immunostaining the frozen sections of the tumor block. The number of tumors expressing strong immunoreactivity in the majority of cells (+++ and ++) was similar in both sets (33% in passageable tumors, 31% in nonpassageable tumors).

LOH Analysis. In 10 cases in which the FNA sample had yielded proliferative epithelial cultures, normal control DNA of the patient from tissues such as skin or lymph node or from cultured fibroblasts expanded from the FNA sample was evaluated for informative or

heterozygous status at 14 polymorphic loci on chromosome 3, 7, 8, 11, and 17. DNA of tumor cells microdissected from cryopreserved tissue blocks and from the FNA-derived epithelial cultures was compared at the informative loci for each specimen (Table 2).

In 4 of 8 cases in which LOH was present in the tumor tissue, cells cultured from the FNA samples of the tumors displayed loss of the same allele. As illustrated in Fig. 2, the microdissected tumor tissue showed partial LOH, possibly caused by contamination with normal stromal cells or by tumor heterogeneity. In contrast, the FNA culture
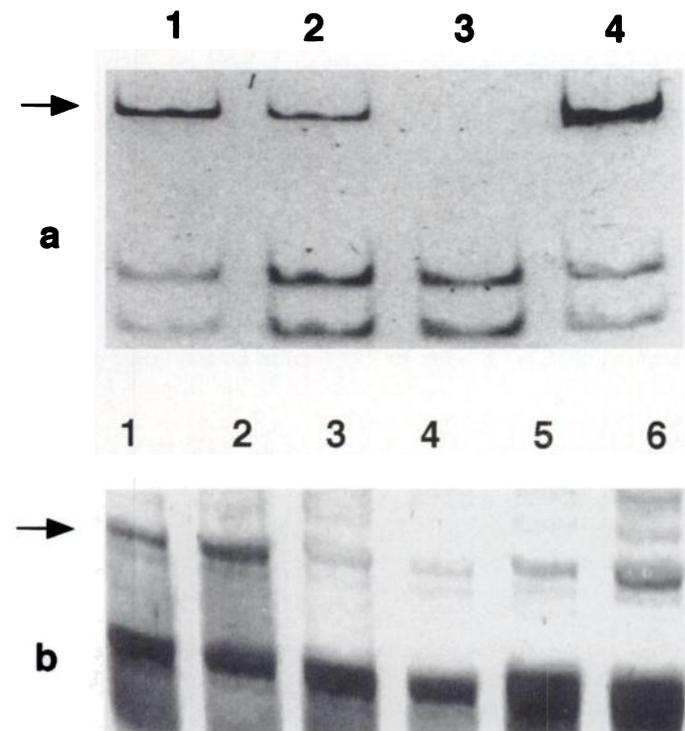

Fig. 2. LOH Analysis. a, LOH at 7q31 in DNA from case S257T (RFLP locus G390). Lane 1, microdissected normal breast lobule; Lane 2, microdissected tumor tissue; Lane 3, FNA-derived epithelial culture; Lane 4, FNA-derived fibroblast culture. Arrow, upper allele is lost in tumor tissue and tumor-derived cultured epithelium whereas tumor-derived cultured fibroblasts display a normal restriction fragment length polymorphism (RFLP) pattern. Note the relatively complete loss of allele in the epithelial culture as compared with the microdissected tumor tissue. b, LOH at 17p in DNA from case G396 (microsatellite locus D17s1541). All of the samples are shown in duplicate runs. Lanes 1 and 2, FNA-derived fibroblast culture; Lanes 3 and 4, microdissected tumor tissue; Lanes 5 and 6, FNA-derived epithelial culture. Arrow, upper allele is lost in tumor tissue and cultured epithelium.

showed considerably greater loss of allele, demonstrating the relative purity of the cultured population of cells.

In two cases, LOH was not observed either in the tumor tissue or in the tumor culture. At the same loci, another four cases displayed LOH in the tumor tissue but not in the tumor culture. However, two of these cultures were aneusomic, one was anchorage independent, and one case displayed erb-B2 overexpression in culture (Table 2). These findings may reflect the presence of widespread tumor heterogeneity. In other words, variation between tumor tissue and cell culture may represent the sampling of different areas of the tumor. Alternately, the culture may be enriched in tumor cells that display alterations favoring growth *in vitro*. Consequently, those cells harboring unfavorable alterations are outnumbered.

Discussion

Fine-needle aspiration, first used by Martin and Ellis (8), is a relatively noninvasive method for obtaining samples from solid breast lumps for pathological characterization. In the last 2 decades, this procedure has become widely accepted in the United States. In addition to morphological analysis for the detection of neoplasia, in recent years cytological smears of breast cancer FNA have served in independent studies toward the determination of estrogen and progesterone receptors (9), growth fraction (10), p53 expression (11), and erb-B2 protein expression (12) by indirect immunolocalization. In assays such as PCR analysis for allelic loss (13) or for telomerase expression (14), which are not based on a cell-by-cell evaluation, the results are largely dependent upon the cellular composition of the FNA sample. We have demonstrated here that from an admixture of cell types in FNA samples, pure viable populations of tumor epithelium can be derived and expanded by several orders of magnitude for comprehensive analyses of multiple biological parameters.

Efforts during the past 2 decades to improve the success rate of primary-breast-tumor cell culture has led to a number of approaches that include improved media formulations (15, 16), optimization of tumor tissue dissociation (5), and a culture system simulating the tumor microenvironment *in vivo* (17). The high frequency at which we have successfully propagated FNA samples in this report may be closely related to the mechanics of the sampling process. As the beveled tip of the collection needle shears through the tumor, it dislodges many small fragments of tissue. Because invasive tumor cells are less cohesive and easier to aspirate from the tissue than elements embedded in the stroma, such as normal breast ducts and lobules or components of the vasculature, the sample is relatively enriched in malignant epithelium.

In a previous study (18), it was demonstrated that upon mechanical disaggregation of tumor tissue *ex vivo*, cells that are relatively loosely associated will "spill out." Such populations, although predominantly aneuploid, do not exclude vital dyes and are presumably nonviable. Our observations on vital staining of FNA-derived samples were similar. These results may be of particular relevance to studies of gene expression using sensitive PCR and array-based methodology, in which the maintenance of cellular integrity and viability is important for ensuring full representation of labile molecules such as RNA. Toward this end, one obvious approach can be the *in vitro* expansion of viable cells in the original sample.

An important goal of this study was to determine how closely epithelial cultures propagated from FNA samples resembled the original tumor tissue. In this regard, we have predominantly found concordance between the tissue block and the cultures both for overexpression of erb-B2 protein as well as loss of heterozygosity at previously reported loci (reviewed in Ref. 19). Additional support for the assumption that FNA-derived epithelial cultures represent primary

tumor cells is provided by the presence of aneusomy and the ability for anchorage-independent growth.

Propagation of the viable tumor cells present in FNAs or other more recent forms of automated core-needle biopsy for palpable lesions [as well as from image-directed automated core-needle biopsy for non-palpable lesions (20)] could facilitate the functional analysis of limited tissue. Small ($\leq$1-cm) tumors—even if sampled at the bench after surgical excision—could provide adequate material for expansion in culture without compromising conventional pathological diagnosis.

From a patient care perspective, the possibility of an in-depth analysis on the FNA sample of a lesion before tumor excision could lead to more effective therapeutic strategies that might replace surgery or serve as a surgical adjuvant. Furthermore, experimental manipulation of viable tumor cells that involve gene transduction may offer additional treatment options such as gene therapy and tumor vaccines.

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