Spontaneous Fusion with, and Transformation of Mouse Stroma by, Malignant Human Breast Cancer Epithelium

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Abstract

Adenocarcinoma cells from the pleural effusion of a patient with breast cancer were injected into the mammary glands of nude mice and grown into solid tumors. A cell line derived from these tumors expressed α-smooth muscle actin but not human cytokeratin 7, indicating “activated” stroma of mouse origin. Cells in mitosis exhibited mainly polyplody mouse karyotypes, but 30% had mixed mouse and human chromosomes, among which 8% carried mouse/human translocations. Nuclei of interphase cells were 64% hybrid. Hybrid mouse/human nuclei were also detected in the primary xenograft. Thus, synkaryons formed in the solid tumor by spontaneous fusion between the malignant human epithelium and the surrounding normal host mouse stroma. The transformed stroma-derived cells are tumorigenic with histopathologic features of malignancy, suggesting a new mechanism for tumor progression. (Cancer Res 2006; 66(16): 8274-9)

Introduction

The epithelial cell subpopulation and its genetic alterations have traditionally driven our understanding of the biology and metastatic behavior of adenocarcinomas (1). Recently, however, studies have focused on the stromal microenvironment at the tumor boundary and its possible role in tumor progression (1). Stromal “activation” or desmoplasia is observed in many human malignancies including breast cancers (2–4). It has been defined as stroma that exhibits reactivation of fetal-like phenotypic properties with reexpression of cytokines, growth factors, and their receptors; changes resembling ones normally observed in wound healing (5).

In malignancies, such changes contribute to altered epithelial cell morphology, accelerated cell migration, extracellular matrix remodeling, and angiogenesis (1, 6, 7). Whether “activated” stroma is genetically transformed has been under debate. Multiple studies report that stromal enzymes and growth factors, although reexpressed in malignancies (8–11), are not otherwise different from normal patterns observed during neoplastic tissue remodeling (10). Therefore, it has been proposed that epigenetic mechanisms are involved in these changes (1, 4, 12). However, some recent studies have reported the loss of heterozygosity in DNA isolated from microdissected tumor stroma, with changes that are either similar to (13, 14) or distinct from (15, 16) the genetic modifications found in the adjacent malignant epithelium. This would suggest genetic mechanisms for stromal remodeling. Tumor-associated vascular endothelial cells with cytogenetic abnormalities have also been reported (16), and both benign (e.g., fibroadenoma) and malignant (e.g., metaplastic carcinomas and sarcoma phyllodes) tumors are known to arise in, or resemble breast stroma (17). These studies also suggest the possibility that juxtatumoral stroma can be genetically different from normal stroma. However, the mechanisms for such genetic changes remain unclear. One proposal posits that carcinogenic events that alter the epithelium simultane- ously alter the stroma (1, 15). Another proposes that cell-cell fusion, with generation of hybrid variants, can contribute to tumor cell diversity (18). We now report direct evidence using an experimental model system, for the malignant transformation of normal host stroma induced by fusion with malignant epithelium. The hybrid cells thus generated are capable of forming highly aggressive tumors.

Materials and Methods

Pleural effusion and cell culture. The patient, a 65-year-old female, presented with a 3 cm, poorly differentiated infiltrating ductal carcinoma with involved lymph nodes. The tumor was estrogen receptor (ER)–positive, progesterone receptor (PR) unknown, HER2 unknown. After surgery, she received six cycles of Cytoxan, Adriamycin, and 5-fluorouracil, followed by radiation therapy and 5 years of Tamoxifen. Three years after completing Tamoxifen therapy, the patient presented with a malignant pleural effusion and underwent thoracentesis. Immunohistochemistry of the pleural cells showed them to be ER (0%), PR (7%), and HER2 (0%). An Institutional Review Board–approved tissue acquisition protocol and patient-informed consent were obtained to acquire blood and tissue for research purposes.

Immunohistochemistry. Cells were fixed and stained as previously described (19). Cells were stained with anti-α-smooth muscle actin (Novacastra, United Kingdom), or anti-cytokeratin 7 (DAKO, Carpinteria, CA). HeLa human cervical carcinoma cells served as controls. Nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI).

Immunohistochemistry. Tumors were excised and processed as previously described (19). Five-micrometer sections were cut, fixed, and stained as described. Immunohistochemistry for ER used the monoclonal antibody 1D5 (DAKO).

Xenografts: BC6 cells and creation of BJAZ cell line. Pleural effusion cells were passaged once in vitro in the presence of penicillin/streptomycin for 72 hours to remove debris. Four million adherent or floating cells (named BC6 cells) in 100% Matrigel (BD Biosciences/Clontech, Mountain View, CA) were separately xenografted into each fourth mammary gland of ovariectomized nu/nu mice, supplemented with a 17β-estradiol (E)
releasing pellet to yield circulating hormone levels of 145 ± 47 pg/mL, and grown into tumors for 12 weeks. One tumor was excised, and a representative portion was fixed, paraffin embedded, sectioned (5 μm), and stained with H&E. Cells in the remaining tumor segment were dispersed without enzymes using a pestle and 45 μm nylon mesh, and cultured in 10% FCS containing MEM (20). A permanent cell line, BJZ3, was established.

**Fluorescent BJ3Z cells.** BJ3Z cells were transduced with ZsGreen expressing viruses and highly green fluorescent cells were isolated by flow cytometry to yield "BJ3Zg" cells. One million cells were injected bilaterally into the fourth mammary gland of ovariectomized nude mice and tumor size was measured biweekly for 11 weeks. Fluorescent intravital imaging used an Olympus (Melville, NY) SZ-61 dissecting microscope coupled to an Olympus C-5050 digital camera, under white light or a 470 nm excitation filter and 515 nm viewing filter incorporated into the imaging equipment. Fluorescence imaging of BJ12 histologic (5 μm) sections were done using a Nikon (Melville, NY) Eclipse E600 microscope with 480/30 nm excitation and 535/40 nm emission filters at 200× magnification.

**Karyotyping and fluorescence in situ hybridization.** Cells in the log phase of growth were fixed and labeled as previously described (21). Metaphase spreads from BJ3Z cells were hybridized with human or mouse Cot-1 DNA labeled with SpectrumGreen or SpectrumRed as described (22).

**Confocal microscopy.** Images were captured with a Zeiss (Thornwood, NY) LSM 510 NLO laser scanning confocal microscope using a Zeiss 63× Apochromat oil immersion lens (numerical aperture, 1.4; working distance, 0.09 mm) with Z-step sizes of 1.0 to 0.5 μm and 1,024 × 1,024 resolution (~0.05 μm pixels).

**Results**

A female patient originally diagnosed and treated for an ER-positive primary breast cancer underwent thoracentesis after presenting 8 years later with a pleural effusion. An aliquot of the pleural fluid was removed for clinical assays at which time the tumor cells were reported to be ER-negative, but with some PR positivity. The remaining tumor cells were precipitated and

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**Figure 1.** Breast cancer cells from a pleural effusion, their growth as a mouse tumor, and isolation of an immortalized stromal cell line (BJZ3) derived from the xenograft. A. BC6 pleural effusion cells (adherent or floating cells) were separately xenografted into each fourth mammary gland and grown into tumors for 12 weeks. Both cell types yielded similar tumors. B, one tumor was excised and stained with H&E. Histopathologic examination shows a poorly differentiated adenocarcinoma with signet ring features (arrow) in a desmoplastic stromal background (*); magnification, ×200. C, mouse and human cells were stained for mouse Cot-1 (red) or human Cot-1 (green); magnification, ×200. D, immunohistochemistry for ER with the monoclonal antibody 1D5. Cells in the remaining tumor segment were dispersed, cultured, and a permanent cell line, BJZ3, was established. E, BJZ3 cells at passage 15; magnification, ×40. BJZ3 cells were stained with (F) anti-α-smooth muscle actin (SMA) or (G) anti-cytokeratin 7 (CK7). HeLa human cervical carcinoma cells served as controls. Nuclei were counterstained with DAPI.
incubated in vitro for 72 hours in the presence of penicillin/streptomycin to remove debris. Four million adherent or floating cells from the effusion (named BC6 cells), in 100% Matrigel, were then implanted into the mammary glands of 6-week-old, ovariectomized nu/nu mice, and supplemented with a 17β-estradiol releasing pellet (23) in view of the possible hormone dependence of the patient’s disease.

Solid tumor xenografts (Fig. 1A) of variable size were removed 12 weeks later, sectioned for histopathologic examination and immunohistochemical studies, and/or suspended and cultured in vitro. Histology of a representative tumor (Fig. 1B) shows a poorly differentiated adenocarcinoma (arrow), morphologically consistent with the original diagnosis of a poorly differentiated infiltrating ductal carcinoma of the breast, in a desmoplastic stromal background (asterisk). The tumors contained both mouse and human cells as shown by Cot-1 staining for mouse (red) or human (green) DNA (Fig. 1C). Many cells were ER positive (Fig. 1D), suggesting that the receptors were reexpressed in the estrogenized solid tumor microenvironment.

Several cell lines were obtained from the solid tumors, one of which, BJ3Z, is shown in Fig. 1E. Their characteristic spindle-shaped fibroblastic morphology suggests that the cells are of
stromal origin; probably mouse. This assignment was confirmed by presence of α-smooth muscle actin, a marker of myofibroblasts associated with reactive stroma (Fig. 1F), and by the absence of human cytokeratin 7 (Fig. 1G). In addition, BJ3Z cells were positive for stromal cell-derived factor 1 and vimentin (data not shown), further indices of activated stroma.

Because the mouse stroma supporting growth of the human tumor xenograft in vivo was presumed to be normal at the outset, immortalization of mouse BJ3Z cells in culture after a period of crisis is evidence that they had become transformed. Indeed, analysis of 100 metaphase spreads and 13 karyotypes confirmed the mouse assignment, and showed that 70% of cells were polyploid, averaging 72 to 83 (4n) chromosomes (Fig. 2A). However, distinct human chromosomes were detected among the mouse chromosomes in 30% of spreads and karyotype analyses of these cells revealed distinct human banding patterns (Fig. 2B, bottom row). Dual-color fluorescence in situ hybridization (FISH) of 42 metaphases showed 26 cells (62%) with all green (mouse) painted chromosomes (Fig. 2C, a) and 16 (38%) with a combination of red (human) and green (mouse) chromosomes (Fig. 2C, b). These

![Figure 3](image3.png)

**Figure 3.** Hybrid mouse/human interphase nuclei are present in cells and solid tumors. A, interphase nuclei from BJ3Z hybridized with mouse DNA labeled with Cot-1 SpectrumGreen and human Cot-1 DNA labeled with SpectrumRed. Arrows, hybrid cells. B, interphase nuclei from a solid tumor xenograft showing a hybrid nucleus. A representative Z stack image using a red and green filter. Z-stack images were exported as a movie that can be viewed as Supplementary Material (Movie S1).

Figure 4.

BJ3Z stromal cells are tumorigenic. BJ3Zg cells were injected bilaterally into the fourth mammary gland of ovariectomized nude mice and grown into tumors for 11 weeks. A, total tumor burden per mouse, in two mice (BJ11 and BJ12). B, (top) fluorescent whole body imaging using: (a) white light and (b) a 470 nm excitation filter and 515 nm viewing filter; (bottom) fluorescent intravital imaging under: (c) white light or (d) above filters. C, fluorescence imaging of BJ12 histologic sections; magnification, ×200. D, H&E-stained section of BJ12 tumor showing: (a) a spindle cell tumor aggressively infiltrating normal mammary adipose tissue and entrapping a mammary duct (*); magnification, ×100; (b) exhibiting cytologic pleomorphism, brisk mitotic rate (white arrow, mitotic figure), atypical (multipolar) mitoses (black arrow) and single cell necrosis (black arrowhead); magnification, ×400.
16 synkaryons averaged 75 to 89 chromosomes which included the recurrent presence of human chromosomes 3, 5, 8, der (11;14), 15, 17, 18, 19, 20, 21, der(X)(X:7) among the polyploid mouse complement. A few of these (8%) were found to have mouse or human translocations (Fig. 2D, a, b, arrows). Two-hundred consecutive interphase BJ3Z nuclei (Fig. 3A) showed an even higher proportion (64%) of synkaryons than did the mitotic cells, suggesting a decrement in cell division rate among the hybrid cells. Indeed, karyotype analysis of later passage cells shows genetic drift, a decrease in the average number of chromosomes (72-83 versus 60-73 in early versus late passage, respectively), and gradual deletion of the human chromosomes (data not shown).

To determine whether the mouse stroma/human epithelial cell fusions occurred in vitro or were present in the solid tumor xenografts prior to culture, tumors taken directly from mice were isolated by flow cytometry, and 1 million cells were injected in vitro deletion of the human chromosomes (data not shown). Indeed, karyotype analysis of later passage cells shows genetic drift, a decrease in the average number of chromosomes (72-83 versus 60-73 in early versus late passage, respectively), and gradual deletion of the human chromosomes (data not shown).

A representative Z-stack image shows blue DNA including red and green chromosomes, confirming the existence of hybrid cells in the solid tumor. A movie showing a three-dimensional view of a cell containing both mouse and human DNA is available online at the journal’s web site for viewing as Supplementary Material (Movie S1).

To determine whether the mouse mammary stromal cell line was tumorigenic, BJ3Z cells were fluorescently tagged by transduction with retroviruses expressing ZsGreen. Bright green cells were isolated by flow cytometry, and 1 million cells were injected bilaterally into the fourth mammary gland of ovariectomized nude mice. Tumors grew slowly initially, then underwent accelerated, exponential growth. Figure 4A shows the growth pattern and average bilateral tumor area in two different mice. Figure 4B (a and b) shows a tumor that fluoresces green in a mouse, and Fig. 4B (c and d) shows a small, green fluorescent tumor in the fourth mammary gland detected after necropsy and dissection. These data confirm that the tumors developed from the injected exogenous stromal cells and not from endogenous normal mouse stroma. Histology and fluorescence microscopy confirm the BJ3Z origin of the tumors (Fig. 4C). Histopathological examination shows a biologically aggressive spindle cell tumor with features of high-grade malignancy, including aggressive infiltration of mammary tissue (Fig. 4D, a) and cytologic pleomorphism with brisk mitotic rate, atypical multipolar mitoses, and single cell necrosis (Fig. 4D, b). The histopathologic features of the tumors indicate that these new stromal-derived cells are highly aggressive.

Discussion

Inherent in malignant progression is the acquisition of cell subpopulations exhibiting cytogenetic abnormalities characterized by aneuploidy and chromosomal rearrangements. Multiple mechanisms can give rise to aneuploid cells and genomic instability, including mutation of key genes, or cell cycling defects with associated inactivation of tetraploid checkpoints (24). Because spontaneous fusion between cells of the same species in vivo is almost impossible to detect, little is known about this process. Exceptions involve cell fusion as part of normal development including fusion of egg and sperm, formation of muscle cell syncytia, or the syncytiotrophoblast of pregnancy (24, 25). However, spontaneous cell fusion between phenotypically distinct cells may represent an understudied mechanism for the generation of aneuploidy and diversity among cancer cells. There are rare reports of in vivo tumor cell fusion. One study published >30 years ago described the formation of highly aggressive tumors arising from human/hamster cell hybrids (26). Another, using chimeric mice, reported the formation of tumors that contained markers of both parental strains (27). The mechanisms for such “illicit” cell fusion (28) in vivo are unknown. In vitro, such fusion can be caused by a variety of agents including viruses (28), fusogenic proteins, and chemicals like polyethylene glycol (18).

The presence of hybrid cells in the solid tumors we describe here is evidence of a spontaneous fusogenic event between the normal stroma of the mouse mammary gland and the malignant human tumor cells embedded within them. In this regard, we observe rapid (<10 days) colonization by blood vessels and stroma of the Matrigel-encased tumor cells in mice (data not shown). Although we cannot definitively comment on the frequency of such fusogenic events, the fact that it was detected the first time the primary human tumor cells were implanted into mice suggests that the phenomenon may be quite common. Because the primary tumor cells were derived from a metastatic pleural effusion, their aggressiveness was already established. We speculate therefore, that similar fusogenic events may be especially prevalent at metastatic sites, in which case, they would involve the normal stroma of the distant host organ and the malignant epithelial cells that arrive there.

Our data prompt us to hypothesize that through fusogenic events, malignant epithelial cells can acquire the ability to genetically transform their stromal microenvironment. Previous studies have shown that activated stroma is involved in tumorigenesis, and that within malignant lesions, it supports angiogenesis and extracellular matrix remodeling (29, 30). We propose that established tumors, activated stroma may also be genetically transformed, enhancing aggressiveness and metastatic potential because it is composed of hybrid cell variants expressing properties that differ from those of either parental cell (18, 31, 32). That is, when genetic alterations are induced in stroma by its malignant epithelial partner, the resultant tumor—now a mixture of transformed cell types—will augur a poor prognosis for the patient. Indeed it is possible that highly aggressive metaplastic breast cancers, exhibiting mixed epithelial/mesenchymal origins of unknown etiology, arise by such a mechanism.

In sum, cell fusion can be an engine of genetic variability that even within a tumor generates cells with new, possibly more aggressive properties; a hallmark of cancer progression. Such a mechanism could also explain the outgrowth of drug-resistant cell subpopulations and suggests that even for adenocarcinomas, therapies might need to target stromal cells. This is the first demonstration, to our knowledge, of genetic transformation of stroma by malignant epithelium in a solid tumor.

Acknowledgments

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References

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