Progression of Hormone-dependent Adenocarcinoma Cells to Hormone-independent Spindle Carcinoma Cells in Vitro in a Clonal Spontaneous Rat Mammary Tumor Cell Line

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ABSTRACT

A hormone-dependent, clonal carcinoma cell line, designated RM22-F5, was derived from a malignant mammary mixed tumor spontaneously arising in an outbred old female Wistar rat. These cells expressed keratin and desmosomal protein and formed epithelial monolayers in a growth factor and hormone-supplemented medium (LHC-8) containing 10% fetal bovine serum (E-type cells). Cells subcultured for 6 to 8 passages in RPMI 1640 medium containing 10% fetal bovine serum without supplements appeared to be fibroblastic and expressed vimentin (F-type cells). The shift to a fibroblast-like morphology was associated with a more malignant phenotype which included rapid, hormone-independent growth and invasive sarcoma-like character in nude mice. F-type cells were no longer able to express their original epithelial phenotype in LHC-8 medium. Cytogenetic analysis revealed that both E- and F-type cells had essentially the same karyotype. Analysis of PCR-amplified DNA further demonstrated a point mutation of the H-ras-1 gene at codon 12 and loss of the normal H-ras-1 allele in both cell types. Genetic tagging of E-type cells with the neomycin-resistance gene resulted in the generation of F-type cells with neomycin resistance in RPMI 1640 medium, suggesting that F-type cells are a malignant variant of E-type cells arising during in vitro culture. Somatic cell fusion between E- and F-type cells revealed that with most hybrid clones tested, the fibroblast-like phenotype was greatly suppressed. These results suggest that an irreversible phenotypic transition, representative of tumor progression from hormone-dependent adenocarcinoma to more malignant hormone-independent spindle carcinoma cells, is a recessive event and may involve loss of a suppressor function.

INTRODUCTION

Many tumors change phenotype with time and acquire more aggressive and malignant characteristics. This acquisition of alternative phenotypic traits in an expanding tumor population is termed "tumor progression" (1). Studies of human breast cancers and rat mammary neoplasms suggest that mammary tumors progress through multiple steps (2). A progression from ER-positive to ER-negative cells occurs after various endocrine treatments in both human breast cancers and rat mammary tumors as tumor cells acquire growth autonomy (3, 4). Epithelial to fibroblastic cell progression with acquired vimentin expression has also been reported in human breast cancers. Vimentin expression of breast cancers is correlated with the more malignant phenotype such as poor histological grade, higher growth rate, and increased invasiveness (5). Vimentin is expressed in a subset of ER-negative human breast cancer cell lines, but not by any of the ER-positive cell lines (6). Therefore, acquisition of vimentin expression of tumor cells may be a late event, subsequent to the loss of ER in mammary tumor progression (7).

Several genetic changes including activation of oncogenes and inactivation of tumor suppressor genes have been reportedly associated with mammary tumor progression. In human breast cancers, amplification of c-myc and c-erbB2 genes occurs in advanced cases at a significantly high frequency (8, 9). Loss of one H-ras-1 allele correlates with the lack of ER and/or PR and the occurrence of distant metastases (10). Recently, a correlation between loss of heterozygosity at chromosomes 11p and 17p13.3 and lymph node metastasis was also reported (11). In chemically induced rat mammary tumors, the H-ras-1 gene is reportedly involved not only in initiation but also in progression as a result of mutation and amplification together with alterations in chromosome 1 (12). However, the mechanism of mammary tumor progression at the genetic level still remains unclear.

Several investigators have developed hormone-independent spindle cell tumors from hormone-dependent mammary carcinoma using in vivo selection in castrated mice and rats (13, 14). Isolation of drug-resistant variants showing loss of ER and acquisition of vimentin expression has also been reported in a human breast cancer cell line (15). However, to our knowledge, no reproducible in vitro models for mammary tumor progression from hormone-dependent carcinoma to hormone-independent spindle carcinoma are available.

We previously isolated a hormone-dependent adenocarcinoma cell line from a malignant mammary mixed tumor (16). In the present study, we demonstrate that this adenocarcinoma progresses to a hormone-independent spindle cell carcinoma expressing vimentin and exhibiting a more invasive phenotype both in vitro and in vivo. The results obtained from somatic cell fusion studies suggest that progression to spindle cell carcinoma is a recessive event and therefore, may involve the loss of suppressor function.

MATERIALS AND METHODS

Animals. All animals used in this study were 6-week-old female athymic nude mice of the KSN strain (Shizuoka Laboratory Animal Center, Hamamatsu, Japan).

Cell Culture. RM22 cells were isolated from a primary mammary tumor arising spontaneously in a 22-month-old outbred female Wistar rat maintained in the animal colony of the Gerontology Research Center (National Institute on Aging). RM22-F5 cells were obtained from RM22 cells by single-cell dilution cloning as reported previously (16). RM22-F5 E-type and F-type cells were maintained in LHC-8 medium (Bio-Fluids, Rockville, MD) containing 10% FBS (E medium) and RPMI 1640 medium with 10% FBS (F medium), respectively. LHC-8 medium used here consisted of LHC-basal medium (Bio-Fluids) supplemented with calcium, phosphoethanolamine, insulin, EGF, dexamethasone, transferrin, triiodothyronine, and a trace amount of pituitary extract.

Immunofluorescence Microscopy. Cells were grown on Lab-Tek chamber slides (Nunc, Naperville, IL) for immunofluorescence microscopy. After...
fixation with methanol at 20°C for 2 min, the cells were washed with PBS and incubated for 30 min at room temperature in PBS containing 1% BSA. The cells were then treated for 1 h with rabbit antiserum to bovine keratin (Dakopatts, Carpinteria, CA) and mouse monoclonal antibody to human vimentin (Sigma, St. Louis, MO). The cells were washed with PBS and incubated for 30 min with FITC-conjugated goat anti-rabbit IgG (Sigma) and FITC-conjugated goat anti-mouse IgG (Sigma) and rinsed with PBS. Slides were viewed with a × 40 objective using an Axioplan universal microscope (Carl Zeiss, Oberkochen, Germany). For flow cytometric analysis, the cells were stained in suspension with antibody to vimentin and subjected to FACSscan (Becton Dickinson, Mountain View, CA).

**Cell Growth Analysis.** Growth rates of E-type and F-type cells in vitro were examined both in E and F medium. Cells (1 × 10^6) were seeded onto 35-mm culture dishes, and cell number was determined with a hemocytometer after 3 days. Growth factor responsiveness was determined in LHC-9 basal medium containing 10% FBS without any supplements and RPMI 1640 medium with 1% FBS (control media) for E-type and F-type cells, respectively. The cells were plated at 1 × 10^6/well in control medium and control medium supplemented with various growth factors and hormones, and the number of cells was counted in triplicate every day. Concentrations of growth factors and hormones used to supplement the medium were: insulin (5 μg/ml), EGF (10 ng/ml), dexamethasone (1 μM), prolactin (5 μg/ml), 17β-estradiol (2 μM), and progesterone (0.2 μg/ml). All of the reagents were obtained from Sigma except rat prolactin (Rat-PRL-B-7) which was a gift from the National Institute of Diabetes and Digestive and Kidney Diseases (Bethesda, MD).

**Assay for ER and PR.** ER and PR were measured in the cytosolic fractions from the tumors that developed in intact female nude mice after s.c. injection of E-type and F-type cells. The dextran-coated charcoal assay method for ER was used (20). In vivo invasion was assessed by examination of the number of visible parietal nodules. In vivo invasion was assessed by examination of the number of macroscopic lung metastases was determined by counting visible parietal nodules. In vivo invasion was assessed by examination of the number of macroscopic lung metastases.

**Transfection and Cell Fusion.** E-type cells were transfected using the lipofectin method ( Gibco-BRL, Gaithersburg, MD) with the plasmid pRSV-neo which encodes the neomycin-resistance gene. F-type cells were transfected with the plasmid pSV2hygro, which encodes the hygromycin B-resistance gene. Five independent clones were obtained from E-type cells transfected with pRSVneo and four independent clones were obtained from F-type cells transfected with pSV2hygro using G-418 (0.5 mg/ml; Sigma) and hygromycin B (0.5 mg/ml; Wako, Osaka, Japan) selection, respectively. One clone from E-type cells (E1-neo) and one clone from F-type cells (F4-hygro) were used to produce hybrid cells. E1-neo and F4-hygro were maintained in E medium containing G-418 (0.5 mg/ml) and hygromycin B (0.5 mg/ml), respectively. Cells were harvested and a total of 1.2 X 10^6 cells were cocultured in 60-mm dishes at a 1:5, 1:1, and 5:1 E:F ratio in E media for 14 h. The medium was removed and fresh medium (LHC-8, 0.1 mg/ml BSA) was added to the cultures for 24 h. The cells were harvested with trypsin-EDTA, washed with PBS, and resuspended in PBS.

**RESULTS**

We characterized a hormone-dependent, clonal carcinoma cell line, RM22-F5, which was isolated from a spontaneous mammary tumor in an old Wistar rat (16). This cell line formed a typical epithelial monolayer with acinar-like structures (E-type cells) in LHC-8 medium containing 10% FBS plus growth supplements (E medium) (Fig. 1A). When the cells were cultured in RPMI 1640 medium supplemented only with 10% FBS (F medium), small numbers of fibroblast-like cells appeared around epithelial clusters as early as the first passage (Fig. 1B). After subsequent passage, fibroblast-like cells (F-type cells) became predominant and almost completely replaced E-type cells within eight passages (Fig. 1C). This morphological change was accompanied by alterations in intermediate filament and desmosomal protein expression. Immunofluorescence microscopy showed that E-type cells stained intensely for keratin (Fig. 2A) and desmosomal protein (Fig. 2C), but expressed vimentin only weakly (Fig. 2E). In contrast, F-type cells were negative for keratin and desmosomal protein (Fig. 2D and/or 2C), but strongly positive for vimentin (Fig. 2F).

To determine whether this phenotypic transition was reversible, we examined the phenotype of cloned F-type cells on reculture in E medium. Both fibroblast-like morphology and strong vimentin expression of F-type cells remained essentially unchanged even after prolonged culture (data not shown). E-type cells became heterogeneous in morphology to some extent after prolonged culture in E medium (data not shown). This observation prompted us to examine the possibility that variant subpopulations are generated after a single-cell dilution...
Fig. 1. Changes in morphology of RM22-F5 cells on plastic after subculture in F medium. A, monolayer of parental E-type cells grown in E medium, exhibiting a typical epithelial morphology; B, tumor cell morphology after passage 1 in F medium. Fibroblast-like cells appear around epithelial clusters (arrowheads). C, tumor cell morphology after passage 8 in F medium, showing multilayered growth of fibroblast-like cells (F-type cells). × 160.

Fig. 2. Immunofluorescent localization of keratin (A and B), desmosomal protein (C and D), and vimentin (E and F) in E-type (A, C, and E) and F-type cells (B, D, and F). Note partial or complete disappearance of keratin and desmosomal protein staining but the marked increase in vimentin staining in F-type cells. × 640.
cloning of E-type cells. Flow cytometric analysis of vimentin expression after a second single-cell cloning revealed that vimentin fluorescence of these clones (Fig. 3, B-E) was increased compared to fluorescence of parental E-type cells (Fig. 3A) even in E medium. In some clones, additional peaks with higher vimentin fluorescence was apparent, indicating generation of fibroblast-like variants in the doubly-cloned E-type cells during single-cell cloning.

Histologically, the original tumor from which RM22-F5 cells were derived was a malignant mammary mixed tumor consisting of both carcinoma-like and sarcoma-like components (Fig. 4A). Tumors that developed in nude mice after s.c. injection of E-type cells were well-differentiated adenocarcinomas with sarcoma-like elements in limited areas (Fig. 4B). In contrast, F-type cells produced sarcoma-like tumors without any epithelial element in nude mice (Fig. 4C), indicating that the transition from E-type to F-type cells was also irreversible in vivo.

The growth rates of E-type and F-type cells in different media were compared. In E medium, no significant difference in growth rates was observed between E-type and F-type cells. In F medium, however, the growth rate of F-type cells was about 3-fold higher than that of E-type cells (Fig. 5A), indicating a selective growth advantage of F-type cells in the F medium. Data for growth responsiveness of these cells to hormones and growth factors are shown in Fig. 5B. Insulin (P < 0.01), EGF (P < 0.05), dexamethasone (P < 0.001), 17β-estradiol (P < 0.05), and progesterone (P < 0.05) alone significantly stimulated growth of E-type but not F-type cells.

Growth of E-type cells in ovariectomized adult female nude mice was significantly slower than in intact mice with a P < 0.05 (Fig. 6A). In contrast, tumor growth of F-type cells in nude mice as determined by tumor volume was 10-fold higher than E-type cells and was not affected by ovariectomy (Fig. 6B). Histological analysis revealed that the tumor growing after injection of E-type cells in ovariectomized nude mice was mainly sarcoma-like rather than carcinoma, and the remaining small carcinoma component was completely lost after subsequent transplantation into castrated mice (data not shown), indicating that E-type cells are ovary dependent and regress after estrogen ablation with concomitant replacement by ovary-independent sarcoma-like tumor cells. Direct measurement of the levels of cytosol ER and PR in these cell lines by ligand binding assay indicated a low but measurable level of ER in only E-type cells. No significant PR was detected in either cell type (Table 1).

Tumorigenicity, invasiveness, and metastatic abilities of the cells are summarized in Table 2. Both types of cells produced tumors in intact female nude mice with 100% incidence (data not shown). F-type cells were more highly invasive than E-type cells in in vitro invasion assays. Consistent with this result, only F-type cells could invade the peritoneal cavity through the abdominal muscle layer on s.c. injection into the abdominal flank of mice. Therefore, F-type cells are more aggressive than E-type cells. Interestingly, however, the incidence of spontaneous lung metastases after s.c. injection was significantly lower for F-type than for E-type cells in both nude mice and Wistar rats. No lymph node metastases were observed with either cell type in nude mice.

To examine genetic differences between these two types of cells, cytogenetic and mutational analyses were carried out. E-type cells were hyperdiploid and characterized by a modal chromosome number of 44 with two extra chromosomes 12 and 19. No discernible differences were detected in F-type cells at this level of resolution (data not shown). For analysis of H-ras-1 mutations, DNA amplified by PCR from E-type and F-type cells were subjected to SSCP analysis to screen for mutations within exons 1 and 2 of the H-ras-1 oncogene. Primer pairs H1-U/H2-D amplified a 495 base-pair fragment containing exons 1 and 2. DNA amplified from E-type and F-type cells showed the same mobility shift compared to normal amplified rat liver DNA. In these lines, no normal products were detected, indicating probable loss of the normal H-ras-1 allele (Fig. 7A). Primer H1-U/H1-D amplified a 123 base-pair fragment containing exon 1 and primer H2-U/H2-D amplified a 145 base-pair fragment spanning exon 2 of the H-ras-1 gene. SSCP analysis using these primer pairs demonstrated that mutations of H-ras-1 in E-type and F-type cells were located in exon 1 (data not shown). To identify the H-ras-1 exon 1 mutation that led to the observed mobility shift in the SSCP analysis, we subsequently performed slot blot hybridization using H-ras-1 probe.

Fig. 3. Flow cytometric analysis of vimentin expression of parental E-type cells (A) and four doubly cloned E-type cells (B-E) in E medium. Vimentin fluorescence of doubly cloned cells is shown as a rightward shift in mean channel of fluorescence (MCF) compared to fluorescence obtained for parental E-type cells. Additional peaks with higher vimentin fluorescence are observed in some doubly cloned cells (D, E).
codon 12-specific oligonucleotide probes. The results showed that a single-point mutation at codon 12 (G—A transition) was observed in common (Fig. 7B). No mutations of p53 exons 5—8 were observed in either type of cell (data not shown).

To determine the clonal origin of F-type cells directly, E-type cells were genetically tagged with the neomycin-resistance gene and allowed to progress to F-type cells by changing the medium to F medium. Analysis of DNA from the resultant F-type cells by PCR using specific primer pairs for the neomycin-resistance gene demonstrated that a 320 base-pair fragment could be PCR amplified from both E-type and F-type cells (Fig. 7C), indicating that F-type cells are generated from E-type cells in vitro.

To further examine the genetic background of this phenotypic transition, a somatic cell fusion study was carried out. Most of the hybrids generated between E-type and F-type cells were found to exhibit an epithelial monolayer and stained positive for keratin (Fig. 8A). This epithelial phenotype was not attributed to the fusion event itself, since fusion between F-type cells did not result in the formation of hybrids with epithelial morphology. The hybrid nature of these cells was confirmed by PCR analysis using specific primer pairs for the neomycin-resistance gene and the hygromycin B-resistance gene. DNAs amplified from these hybrid cells by PCR yielded both a 320 base-pair band and a 482 base-pair band on agarose gels, corresponding to the neomycin-resistance and the hygromycin B-resistance genes, respectively (Fig. 8B), indicating that all of the hybrids were genuine hybrids containing both sets of parental genomes. The characteristics of the hybrid clones tested are summarized in Table 3. Four hybrids with near-hexaploid chromosomes ranging from 110 to 130 of the modal chromosome number formed typical epithelial monolayers and strongly stained for keratin, similar to the parental E-type cells. Five hybrid clones containing near-tetraploid chromosomes ranging from 84 to 99 also exhibited an epithelial phenotype judging from epithelial-like morphology and keratin staining, but two of these clones showed a somewhat intermediate phenotype between E-type and F-type cells such as elongated morphology and moderate keratin staining, as compared to hybrids with hexaploid chromosomes. Occasionally, some hybrid clones (clones 21 and 24) showed fibroblast-like morphology rather than epithelial. These hybrids had near-triploid chromosomes, suggesting that they are fibroblast-like revertants generated during short-term culture, probably resulting from loss of a copy of parental chromosomes.

**DISCUSSION**

We have found that RM22-F5, a clonal hormone-dependent mammary adenocarcinoma cell line established from a spontaneous mammary mixed tumor, undergoes striking changes in cell morphology from an epithelial to a fibroblast-like phenotype expressing vimentin within six to eight passages after culturing in the absence of growth factors and hormones in vitro. This morphological shift to a spindle shape was associated with a more malignant phenotype, such as loss of hormone dependency, acquisition of higher growth rate, and formation of invasive sarcoma-like tumors in nude mice.

It has been reported that a transition from epithelial to mesenchymal or fibroblast-like phenotype (EMT) is induced in some carcinoma cell lines by growth factors such as acidic FGF and interleukin 6 (23, 24). Scatter factor (hepatocyte growth factor) and culture on Type I collagen can also induce EMT-like changes in some epithelial cell lines (25, 26). In the present study, however, we have not observed such EMT-like changes of E-type cells in response to treatment with acidic FGF, basic FGF, and hepatocyte growth factor or by plating on Type I collagen (data not shown). E-type cells cultured in the conditioned medium prepared from F-type cells exhibited no changes in cell morphology. Furthermore, in contrast to EMT, this phenotypic transition is irreversible. Rat mammary epithelial cell lines convert at low frequency in culture to elongated cells that possess myoepithelial characteristics (myoepithelial differentiation; Ref. 27). This conversion is an irreversible process and the resultant myoepithelial-like cells show fibroelastic morphology with vimentin expression similar to the phenotypic transition observed in this study. F-type cells,
Table 1 Concentration and dissociation constants for ER and PR in the cytosol of E-type and F-type RM22-F5 tumor tissues

<table>
<thead>
<tr>
<th>Tumor tissue</th>
<th>Concentration (fmol/mg protein)</th>
<th>$K_d$ ($\times 10^{10}$ M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E type</td>
<td>13.1</td>
<td>2.36</td>
</tr>
<tr>
<td>F type</td>
<td>ND</td>
<td>ND*</td>
</tr>
<tr>
<td>Normal uterus</td>
<td>181.1</td>
<td>1.54</td>
</tr>
</tbody>
</table>

* ND, not detectable.
Table 2. Invasion and metastasis of E-type and F-type cells in vitro and in vivo

<table>
<thead>
<tr>
<th>Cell line</th>
<th>In vitro invasion (cells/field)</th>
<th>Peritoneal invasion in nude mice</th>
<th>Lung metastasis in nude mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>E type</td>
<td>54 ± 7.1f</td>
<td>0/8</td>
<td>8/8 (7–36)f 6/11 (1–20)</td>
</tr>
<tr>
<td>F type</td>
<td>172 ± 20.5e</td>
<td>5/8</td>
<td>4/8 (2–4) 3/11 (1–8)</td>
</tr>
</tbody>
</table>

a In vitro invasion assay was performed as described in “Materials and Methods,” stained with Diff-Quick, and counted.

b To match the tumor size, necropsy of mice bearing E-type and F-type tumors was performed 2 months and 1 month after s.c. injection of the cells into the abdominal flank, respectively. At necropsy, peritoneal invasion through the abdominal muscle layer was examined.

c At necropsy, lungs were removed and fixed in Bouin’s solution and then macroscopic lung metastases were counted.

d Values represent means ± SD of cells per high-power field as determined from 10 representative fields from three replicate filters.

The rapid and spontaneous generation of spindle variants observed both in vitro and in vivo seems to be difficult to explain by classical spontaneous mutations. One explanation for this is that E-type cells are genetically unstable. In fact, unlike E-type cells, F-type cells have

Fig. 7. Analysis of endogenous H-ras-1 gene and exogenously introduced neomycin-resistance gene in both E-type and F-type cells. A, SSCP analysis of PCR-amplified H-ras-1 exons 1 and 2 from tumor cell DNAs. Lane 1, normal rat liver; Lanes 2 and 3, DNAs from E-type and F-type cells, respectively. Arrows, mobility shift of the band. B, slot blot hybridization using 32P-labeled H-ras-1 codon 12-specific oligonucleotide probes to identify H-ras-1 mutations. G to A transition is observed in both cell types. C, PCR analysis of neomycin-resistance gene in E-type and F-type clones. Lane 1, DNA from E-type cells; Lanes 2–4, DNAs from three independent F-type clones obtained from E-type cells transfected with the neomycin-resistance gene after single-cell cloning in F medium.

Fig. 8. Characteristics of hybrid clones produced from the fusion of parental E1-neo and F4-hygromycin-resistant cells. A, morphology of E-F hybrid clone 9, showing a typical epithelial monolayer (left). × 160. Immunofluorescent localization of keratin in hybrid clone 9. Strong keratin expression is seen (right). × 640. B, PCR analysis of the neomycin-resistance and hygromycin-resistance genes of E-F hybrid clones. All hybrids exhibit 320 (left) and 482 base-pair bands (right) corresponding to the neomycin-resistance and hygromycin B-resistance genes, respectively. Lane 1, clone 2; Lane 2, clone 4; Lane 3, clone 7; Lane 4, clone 8; Lane 5, clone 9; Lane 6, clone 10; Lane 7, clone 11; Lane 8, clone 12; Lane 9, clone 13; Lane 10, clone 21; Lane 11, clone 24.

Conversion of squamous cell carcinomas to the spindle cell carcinomas. On the other hand, Tomita et al. (31) reported that progression of a human colonic adenocarcinoma cell line to a less differentiated and more malignant variant appears to be a dominant event. Our results obtained from cell fusion studies seem to be consistent with the former finding. In the present study, however, a small number of F-type cells appear to be already present at early passages (seven to eight passages) after single-cell cloning. Furthermore, carcinoma-like E-type cells often grew in association with spindle carcinoma in nude mice tumors that developed after s.c. injection of E-type cells. Thus, the rapid and spontaneous generation of spindle variants observed both in vitro and in vivo seems to be difficult to explain by classical spontaneous mutations. One explanation for this is that E-type cells are genetically unstable. In fact, unlike E-type cells, F-type cells have...
were less metastatic to the lung than slow growing and more rapidly growing and highly invasive clones with spindle shape. Therefore, these findings. Interestingly, however, F-type cells are less metastatic to the lung than E-type cells. Similar results were obtained from Lewis lung carcinoma (3LL)-derived cloned cell lines, where rapidly growing and highly invasive clones with spindle shape were less metastatic to the lung than slow growing and more differentiated clones (34). Thus, the metastatic ability of these tumor cells may be regulated independently of their growth rate or invasiveness.

Several cultured cell lines have been reported to be useful for studying tumor progression. These cell lines were obtained either from different sites (primary and metastatic sites) of the same patient (35), from a cell line after long-term culture (31), or by selection after treatment with carcinogens (36), chemotherapeutic agents (15), or by transfection of oncogene (37) and growth factor potentials of tumor cells. Cancer Res., 49: 4713—4719, 1979


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