Tumor Progression in Four Mammary Epithelial Cell Lines Derived from the Same Patient

Vimla Band, Deborah Zajchowski, Karen Swisshelm, Douglas Trask, Victoria Kulesa, Craig Cohen, James Connolly, and Ruth Sager

Division of Cancer Genetics, Dana-Farber Cancer Institute [V. B., D. Z., K. S., D. T., V. K., R. S.], and Department of Pathology, Beth Israel Hospital (J. C.), Boston, Massachusetts 02115, and Department of Medicine, Louisiana State University, New Orleans, Louisiana 70112 (C. C.)

ABSTRACT

Two primary and two metastatic cell lines with distinct phenotypes and genotypes have been established from a patient diagnosed as having infiltrating and intraductal mammary carcinoma (21T series). All four lines can be cultured in the same medium, DFCI-1, which also supports long-term growth of normal epithelial cells. Therefore, we have been able to compare normal and tumor cells at the cellular and molecular levels. The mammary origin of the 21T series was confirmed by using antibodies against the human milk fat globule antigen-2 epitope. The two primary tumor lines (21NT and 21PT) are both immortal and aneuploid, although only 21NT is tumorigenic in the nude mouse assay. The two populations derived from the metastatic pleural effusion (21MT-1 and 21MT-2) each exhibit distinct characteristics in morphology and growth factor requirements. The erbB2 gene is amplified and overexpressed in all of these cell lines compared to normal epithelial cell controls. These four tumor cell lines from a single patient represent a mammary tumor progression series that has been established in long-term cell culture.

INTRODUCTION

Breast cancer is a leading cause of the cancer-related deaths of women in North America and Europe. The availability of normal cells from reduction mammoplasty operations and diseased tissue samples from biopsies and mastectomies provides a unique opportunity for studying human cancer. The importance of growing normal and tumor-derived cells for comparative studies of gene expression, drug resistance, surface antigens, or mechanisms of cell cycle control to look for systematic biochemical differences and prognostic markers can hardly be overstated. While some information can be obtained from fixed or frozen tissues, they are not useful for in-depth studies.

Nonetheless, research with tumor-derived human mammary epithelial cells has been a neglected field. Cells from mammary carcinomas have been among the most difficult human tumor-derived cells to culture (1–4) and have grown poorly as xenografts in the nude mouse (5). Successful long-term growth of tumor cells from primary breast tumor explants has been rare (6–9), and the majority of breast cancer-derived cell lines have been of metastatic origin (4, 10, 11).

As described in this paper, we have found that successful isolation of tumor cells requires not only a good supportive growth medium but also manipulations to separate tumor cells from stromal components and from normal mammary epithelial cells present in the same specimens.

In this paper we describe the isolation and partial characterization of four distinctive tumor populations from the same individual, two primary and two metastatic in origin. The tumor cells were isolated and grown in a new medium developed in our laboratory, DFCI-1 (12). The four lines, called the 21T series, are compared for morphology, growth factor requirements, karyotype, expression of several relevant genes, and tumorigenicity in the nude mouse. These properties as well as keratin expression (13) provide the basis for ranking these four lines in order of increasing malignancy. Thus the 21T series is a cell culture system for the investigation of tumor progression and for use in screening for tumor suppressor and enhancer genes using closely matched pairs of cells.

MATERIALS AND METHODS

Source of 21T Series Cells. 21NT and 21PT tumor cell lines were each derived from a different tissue sample from a 36-year-old woman with infiltrating and intraductal carcinoma who underwent a mastectomy (patient 21). Histopathological examination revealed that the tumors were of nuclear grade III with 3 positive lymph nodes. The original tumor was estrogen- and progesterone-receptor negative. The patient received combined therapy with cytoxan, Adriamycin, and fluorouracil. About 1 year later, she developed metastases to the lung with pleural effusion from which we obtained 21MT-1 and 21MT-2 cell lines. The patient expired from respiratory failure 1 month after the diagnosis of a pleural metastasis.

Source of Other Cells. The normal mammary epithelial cells used in this study are all derived from reduction mammoplasties: 184 (3), 76N, and 81N (12). 21N are apparently normal cells derived from the 21NT specimen which could only be cultured for <5 passages before senescing. 21NF are fibroblasts derived from the same sample. Other breast tumor cell lines, SKBR-3 and BT-20, are from the American Type Culture Collection. MCF-7 was from the Michigan Cancer Foundation.

Isolation of Cells from Primary Tumors. Culture conditions were essentially as described previously (12). In brief, tissue was chopped and cultured in D medium in tissue culture flasks (Falcon). After 1 week both fibroblast and epithelial cell colonies were observed. The fibroblasts were removed by selective trypsinization at room temperature. Once the culture was free of fibroblasts, two types of epithelial cells were observed. Most of the cells had morphological features typical of normal epithelial cells; in addition a second distinct cell type making colonies of 5–10 cells, and probably representing tumor cells, was also observed. These tumor-like cells were selectively expanded by two methods: (a) clonal isolation to separate tumor cell colonies from the normal epithelial cells; and, alternatively (b) serum selection to enrich for tumor cells, since normal epithelial cells differentiate and cease to grow in media containing 10% FCS (DFCI-1 medium has 1% FCS).

Isolation of Cells from a Pleural Effusion. The 21MT cell line was established as previously described (14). The initial passages were morphologically diverse, but by passage 30 (approximately 100 population doublings), two cell types predominated, which we call 21MT-1 and 21MT-2. These cells are morphologically distinct and also differ in their sensitivity to trypsin. 21MT-1 cells are easily released from the culture substratum within 2 min of trypsin treatment at 37°C, whereas 21MT-2 cells are released only after 5–10 min.

Media. All four 21T lines grow in DFCI-1 medium (12) and also in α-MEM supplemented with 10% FCS, I, EGF, and HC (14). In this study, normal cells and tumor cell lines were grown in D medium, 3

Received 4/27/90; accepted 8/15/90.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by NIH Grant CA39814.

2 The abbreviations used are: FCS, fetal calf serum; HMFG, human milk fat globule; BPE, bovine pituitary extract; EGR, epidermal growth factor; EGF-R, EGR receptor; HC, hydrocortisone; I, insulin; TGF-a, transforming growth factor a; D, DFCI-1 medium; α-MEM, α-minimal essential medium; cDNA, complementary DNA.
were kept for 6-8 months. Some tumors were excised for further study.

Mice were observed weekly and tumor-negative mice were used either unfixed (HMFG-2) or after fixation with formalin (erbB2 and EGF R) as described before (12).

Cell Proliferation. Cells were grown in the test media for at least three passages before carrying out growth experiments. Cells were washed once with solution A (a balanced salt solution) (15) and treated with 0.025% trypsin and 0.01% EDTA. Trypsin digestion was stopped with soybean trypsin inhibitor (0.0375% in phosphate-buffered saline), and cells were counted in a Coulter Counter (Coulter Electronics, Hialeah, FL). Cells (5 x 10⁴) were plated in 35-mm dishes (Falcon) with soybean trypsin inhibitor (0.0375% in phosphate-buffered saline), and 10 cells analyzed.

For immunofluorescence staining, exponentially growing cells were used either unfixed (HMFG-2) or after fixation with formalin (erbB2 and EGF R) as described before (12).

RESULTS

Isolation of Cell Lines from Primary Tumors

After fibroblasts were selectively removed from early passage cultures of primary tumors, two epithelial cell types were observed in both 21NT and 21PT cell cultures: (a) normal, spindle-shaped epithelial cells (Fig. 1, A and D) and (b) tumor-like polygonal cells which are loosely arranged and heterogeneous in cell size (Fig. 1, B and C). Fig. 1E shows the distinct morphologies of normal and tumor cells. When the cultures were grown in medium containing 10% FCS, normal cells differentiated and stopped dividing, whereas tumor cells continued to grow (Fig. 1F). Initially, both 21NT and 21PT grew slowly (doubling time of 5 days) but, within 10 passages, the growth rate had increased to approximately 40 h doubling time in both D and supplemented α-MEM medium. Morphologically, 21NT and 21PT are similar (Fig. 1, B and C). These cells have been in continuous culture for more than 2 years.

Isolation of Cell Lines from Pleural Effusion

In later passages of 21MT (>30), two morphologically distinct cell types were predominant and they were separated by differential trypsinization (Fig. 2A). 21MT-1 cells are highly...
TUMOR PROGRESSION IN MAMMARY CELLS

Fig. 2. Morphology of 21MT-1 and 21MT-2 cells grown in DFCI-I medium. A 21MT sample contains both 21MT-1 and 21MT-2 (arrows) in low power (A). B and C are × 230 magnification of 21MT-1 and 21MT-2 cell lines.

Fig. 3. Fingerprint analysis of 21T series. Southern blots using 5 μg of DNA from 21NT (lane 1), 21PT (lane 2), 21MT (lane 3), and 76N (lane 4) were hybridized with probes D2S44 and D14S13. DNA size markers (Lifecodes Corporation) ranging from 34.679 kb to 0.872 kb (lane M).

Fig. 4. Growth of 21T series in DFCI-I (A) and α-MEM-supplemented (B) media. Cells (5 × 10⁴) were seeded/35-mm dish. At the indicated time periods, cells were harvested and counted in a Coulter Counter. Points, means of at least 3 dishes.

Fig. 5 shows that normal reduction mammoplasty-derived 76N cells as well as 21N cells, derived from the 21NT mastectomy sample, do not react (0–7% positive cells) with HMFG-2 antibodies. On the other hand, 21NT, 21PT, 21MT-1, and 21MT-2 cells show very strong reactivity (65–87% positive cells) with HMFG-2 antibodies.

Cytogenetics. Cytogenetic analyses of 21NT and 21PT at passages 9–12 showed that both karyotypes were exceedingly rearranged so that derivative or marker chromosomes could not be identified. Both populations have a mode of 54–55 chromosomes with 6–24 marker chromosomes (Fig. 6). Unlike different individual (lane 4) are different. The probability of finding an identical allelic pattern for these two different individuals is <1% (21).

Characterization of Tumor Cells

Growth in High Serum. As seen in Fig. 4, all four tumor-derived cell lines (21NT, 21PT, 21MT-1, and 21MT-2) grew well in both low serum-containing medium D (doubling time of about 30 h) as well as high serum-containing α-MEM supplemented with EGF, HC, and insulin (doubling time of 27–34 h). Their ability to grow in high serum is similar to other tumor cell lines (10, 11) and in sharp contrast to normal cells, which do not grow in high serum (12, 22).

Reactivity to HMFG-2 Antibodies. We and others have previously shown that HMFG-2 antibodies specifically bind to mammary tumor epithelial cells but not to normal epithelial cells grown in culture (12, 14, 23). We therefore examined the expression of HMFG-2 epitope on 21T series cell lines using HMFG-2 antibodies.

Independent confirmation that the 21T cell lines were derived from the same patient was obtained by “fingerprint analysis.” As shown in Fig. 3, the restriction fragment polymorphisms detected by hybridization with probes D2S44 and D14S13 are identical for samples 21NT (lane 1), 21PT (lane 2), and 21MT (lane 3). The alleles detected in the normal 76N strain from a heterogenous in size and pile on top of each other. These cells grow in three-dimensional clusters and do not form a confluent monolayer (Fig. 2B). On the other hand, the 21MT-2 cell line contains homogenous polygonal cells which grow as a monolayer. These cells are morphologically very similar to the cells derived from the primary tumors, 21NT and 21PT (compare Fig. 2C with Fig. 1, B and C). 21MT-1 and 21MT-2 have been in continuous culture for 2 years.

We have succeeded in establishing cultures of 21NT and of 21PT and 21MT cells twice from the original tumor biopsy and pleural effusion, respectively. These cells do not have detectable levels of Mycoplasma as tested by BRL Mycotect kit.

Fingerprint Analysis

As shown in Fig. 3, the restriction fragment polymorphisms detected by hybridization with probes D2S44 and D14S13 are identical for samples 21NT (lane 1), 21PT (lane 2), and 21MT (lane 3). The alleles detected in the normal 76N strain from a different individual (lane 4) are different. The probability of finding an identical allelic pattern for these two different individuals is <1% (21).

Characterization of Tumor Cells

Growth in High Serum. As seen in Fig. 4, all four tumor-derived cell lines (21NT, 21PT, 21MT-1, and 21MT-2) grew well in both low serum-containing medium D (doubling time of about 30 h) as well as high serum-containing α-MEM supplemented with EGF, HC, and insulin (doubling time of 27–34 h). Their ability to grow in high serum is similar to other tumor cell lines (10, 11) and in sharp contrast to normal cells, which do not grow in high serum (12, 22).

Reactivity to HMFG-2 Antibodies. We and others have previously shown that HMFG-2 antibodies specifically bind to mammary tumor epithelial cells but not to normal epithelial cells grown in culture (12, 14, 23). We therefore examined the expression of HMFG-2 epitope on 21T series cell lines using HMFG-2 antibodies.

Fig. 5 shows that normal reduction mammoplasty-derived 76N cells as well as 21N cells, derived from the 21NT mastectomy sample, do not react (0–7% positive cells) with HMFG-2 antibodies. On the other hand, 21NT, 21PT, 21MT-1, and 21MT-2 cells show very strong reactivity (65–87% positive cells) with HMFG-2 antibodies.

Cytogenetics. Cytogenetic analyses of 21NT and 21PT at passages 9–12 showed that both karyotypes were exceedingly rearranged so that derivative or marker chromosomes could not be identified. Both populations have a mode of 54–55 chromosomes with 6–24 marker chromosomes (Fig. 6). Unlike
Fig. 5. Expression of HMFG-2 antigenic determinant on normal and tumor cells. Exponentially growing 76N (A), 21N (B), 21NT (C), 21PT (D), 21MT-1 (E), and 21MT-2 (F) cells were treated with monoclonal antibody against HMFG-2 antigenic determinant and with control antibody P3 (28). Primary antibodies were localized using fluorescein isothiocyanate-labeled secondary antibody. Fluorescence of cells with HMFG-2 is shown as a rightward shift in comparison to fluorescence obtained by control antibodies. The percentage of fluorescence-shifted cells is given in each histogram.

Fig. 6. Representative karyotypes of 21NT and 21PT. A, 21NT, 55.XX, with multiple rearranged chromosomes: del(1)(q31—qter), der3, der16, +19mars. B, 21PT, 55.X, del(1)(q31—qter), +18mars. including 2 ring chromosomes.

21NT, however, 21PT cells contain about 20% near-diploid cells, which contain rearranged chromosomes. 21MT cells at early passages had only 25 unrearranged chromosomes and 34 marker chromosomes (14). A common aberration seen in all three cell lines is deletion of 1q, 1q31 to 1qter.

Tumorigenicity of Cells. 21NT cells differ from 21PT cells in their capacity to produce tumors in nude mice. 21NT cells were tumorogenic in 9 of 30 mice, whereas 21PT cells failed to grow (0 of 25 mice). We have previously reported that 21MT cells produce tumors in 90% of the mice (14). The latency of tumor formation in 21NT cells, however, was much longer (approximately 6 months) than 21MT cells (approximately 3 months).

Growth Factor Requirement. In order to determine the growth factor requirements for these tumor cell lines, they were grown for at least 3 passages in the respective media before growth rates were determined. As shown in Table 1, 21MT-1 cells grow in D1 medium, which lacks growth factors but contains 1% FCS, whereas 21NT, 21PT, and 21MT-2 cells fail to grow. However, addition of EGF to D1 medium supported growth of these three cell lines (Table 1).

The growth capability of 21T cells was assessed in a completely defined medium, D2, which lacks the two undefined factors, BPE and FCS. 21NT, 21PT, and 21MT-2 cells proliferate in this medium (doubling time 37–53 h), whereas 21MT-1 cells plate but do not grow (Table 1). Thus, the 21MT-1 cells absolutely require growth factors or components found in 1% FCS which are not provided in D2 medium.

To identify the minimal essential growth factor supplements for the growth of 21NT, 21PT, and 21MT-2, these cells were grown in a minimal unsupplemented medium, D3, with either addition of EGF alone or with EGF, HC, and insulin. As shown in Table 1 these cells do not grow well in EGF-supplemented D3 medium (doubling time 75 to 88 h). When further supplemented with insulin and HC, they grow as well as in D2 medium. These experiments emphasize the similarities in the
21NT, 21PT, and 21MT-2 cell lines which all require EGF, insulin, and hydrocortisone for their proliferation. In contrast, 21MT-1 cells require 1% serum.

RNA and Protein Analyses. In order to understand the molecular basis for the difference in growth factor requirements between the 21MT-1 and the other 21T cell lines, expression of various genes at the mRNA level was quantitated. Steady state levels of mRNA isolated from the 21T cells were analyzed for transcripts expressed from genes which have been reported to be involved in breast cancer (i.e., EGF R, erbB2, myc, Rb, and M, 52,000 cathepsin D). Since 21MT-1 cells lack the EGF requirement for growth in culture which is essential for 21NT, 21PT, and 21MT-2, EGF R expression was quantitated. Like the normal mammary epithelial cells, the 21T series contain high levels of EGF R mRNA. The amount of this mRNA decreases approximately 5-fold in 21MT-1 (Fig. 7, lane 5) and 2- to 3-fold in the other 21T series relative to the normal cells (lanes 3, 4, and 6). Preliminary analysis of EGF R protein levels by immunostaining are consistent with the RNA levels (data not shown).

Since TGFα is a ligand for the EGF receptor, we examined expression of its mRNA. Levels of TGFα mRNA approximately equal to that found in normal cells were reproducibly observed in 21NT, 21PT, and 21MT-2 (Fig. 7). In contrast, TGFα mRNA expression in 21MT-1 was reduced 5- to 10-fold in comparison to the normal cells (compare lane 5 with lanes 1 and 2).

As shown in Fig. 8A, all of the 21T series overexpressed erbB2 mRNA when compared with normal 76N or 81N cells. Both primary tumor-derived cell lines (21NT and 21PT, lanes 3 and 4) as well as one metastatic effusion derivative (21MT-2, lane 6), produced approximately 50-fold more erbB2 than the normal cells, and the expression level in 21MT-1 (lane 5) was elevated nearly 500-fold.

Southern analysis of genomic DNA isolated from the same normal and tumor lines revealed that erbB2 gene amplification occurred in both primary and metastatic cells but not in fibroblasts cultured from the same patient (Fig. 8B). The number of copies of the erbB2 gene was 2- to 3-fold higher in 21MT-1 than in the other 21T cell lines and may account for the higher expression detected in 21MT-1 cells relative to the three other erbB2 mRNA. No gross erbB2 gene rearrangements were noted in HindIII-, EcoRI-, or BamHI-digested genomic DNA samples (Fig. 8B and data not shown). In contrast, the ploidy of the EGF R gene is unaltered in the 21T series cells (Fig. 8B and other data). In agreement with other reports (24, 25), we find that the BT-20 cell line contained an EGF R gene amplification of approximately 5-fold (lane 9).

erbB2 protein levels were assessed by immunofluorescence staining. As shown in Fig. 8C, the relative erbB2 antigen expression in the 21T series is higher than that in normal cells. Higher amounts of immunoreactive protein are also detected in 21MT-1 compared to the other 21T cells, which is consistent with the increased erbB2 mRNA levels.

No significant differences were observed in the levels of mRNA expression for c-myc, Rb, and M, 52,000 cathepsin D in normal and 21T series (data not shown).

DISCUSSION

The 21T series described here has been established from primary and metastatic mammary tumor specimens obtained from the same patient. Establishment of the four cell lines was made possible by the use of DFCI-1 medium (12), which allowed outgrowth of both normal and tumor epithelial cells but not fibroblasts from the explant cultures of tumor specimens. Other factors contributed to our ability to establish these lines: (a) physical separation of cell types based on distinct morphologies of normal and tumor cells; (b) selection methods, by cloning or growth in high serum; and (c) careful trypsinization and low split ratios at early passages.

The mammary tumor cell origin of these lines was demonstrated by their expression of the surface epitope recognized by the HMFG-2 antibody (12) and by their retention of rhodamine-123, another phenotype correlated with tumorigenicity (12, 14). Their identity as tumor cells is supported by their loss of senescence, enabling indefinite growth in culture, and by their tumor-forming ability in the nude mouse assay. The inability of 21PT cells to form tumors in the nude mouse assay is an unexplained result. Subtractive hybridization between 21NT and 21PT cells may permit identification of a single gene responsible for this difference in tumor-forming ability.

Karyotypic analyses have also pinpointed differences among the 21T cell lines. The primary tumor-derived lines have been found to contain more normal chromosomes and fewer markers than the cells of metastatic origin. Only the 21PT cells have a small percentage of near-diploid cells. In addition, only the 21MT lines have been found to contain a consistent population of double minute chromosomes (14). Because only 5% of the 21MT cells exhibit double minute chromosomes, it has been difficult to determine their identity.

The most striking morphological differences are seen in the 21MT-1 cells, which grow in tight three-dimensional clusters, whereas the other 21T cell lines grow as monolayers. The 21MT-1 cells are also distinctive in their requirement for 1% FCS, a requirement not satisfied by elevated amounts of other growth factors. Thus, they may require a novel factor not previously identified. The other three cell lines need additional EGF, even in the presence of 1% serum. The different requirements of 21MT-1 and the other T lines are not readily explained.

---

3 V. Band and R. Sager, unpublished observations.
by expression of the EGF receptor, which is slightly decreased at the mRNA level in all four tumor cell types compared to normal cells, or by altered expression of its ligand TGFα, which is unexpectedly decreased only in the 21MT-1 cells.

The erbB2 gene (also called neu or HER-2) is amplified and overexpressed at the mRNA and protein levels in all four cell lines, but 21MT-1 cells produce higher levels than the other 21T lines. Since interactions between the EGF receptor and erbB2 protein have been reported (26), it is possible that the EGF independence of 21MT-1 cells may result from the very high levels of erbB2 protein made by these cells.

In a previous report (13) we have shown that 21N cells differed from the 21T series in their keratin profile (13). 21N cells express keratins K5, K6, K14, and K17, whereas the 21T series express K8, K18, and K19. In this respect 21N cells resemble mammary epithelial cells and 21T cells resemble the established tumor cell lines. It is noteworthy that normal cells immortalized by transfection with human papillomavirus DNA (HPV 16 or HPV 18) (27) express an intermediate keratin phenotype, including a reduced level of K5 and K14 and increased levels of K8 and K18 compared to normals (13). Thus immortalization captures these shifting keratins as the cells are progressing from normal to tumorigenic.

In summary, four tumor-derived cell lines have been established in long-term cell culture, derived from a single patient. The two lines (21PT, 21NT) derived from primary tumors are similar, but only the 21NT cells are tumorigenic. This finding suggests that the 21PT cells may be at an earlier stage of progression than the 21NTs. The two lines derived from a pleural effusion differ considerably. The 21MT-2 line resembles the primaries in its growth factor requirements and gene expression. The 21MT-1 cells are probably the most malignant of the series, as judged by the most elevated erbB2 expression, altered cell surface properties, and loss of the elevated EGF requirement of the other lines.

The analysis of this progression series has just begun. The cells provide material for ongoing and future studies of differences between members of the series, based on biochemical studies of altered phenotype and genetic studies of novel genes.
that are differentially expressed during tumorigenesis and pro-
gression.

ACKNOWLEDGMENTS

We gratefully acknowledge R. Derynk, A. Ullrich, and S. Aaronson for gifts of probes and M. Stampfer for 184 cells. We thank Stephanie Budd for the preparation of the manuscript, Michele Leonard for technical assistance, and Kevin Trimpe for help in immunostaining.

REFERENCES

Tumor Progression in Four Mammary Epithelial Cell Lines Derived from the Same Patient

Vimla Band, Deborah Zajchowski, Karen Swisshelm, et al.


Updated version

Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/50/22/7351

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/50/22/7351.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.