Transformation of Human Mammary Epithelial Cells by Oncogenic Retroviruses

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ABSTRACT

We have introduced viral oncogenes into human mammary epithelial cells through the use of murine retroviruses. A continuous cell line (184A1N4) derived from benzo(a)pyrene treatment of normal breast epithelial cells was used as a recipient for the ras, mos, and T-antigen oncogenes. Each of these oncogenes enabled the 184A1N4 cells to grow in a selective medium, thus demonstrating the potential utility of these cells for oncogene detection and isolation. 184A1N4 cells transformed by T-antigen were nontumorigenic in athymic mice, but v-ras transformants were weakly tumorigenic. Transformants bearing both the T-antigen and ras oncogenes were strongly tumorigenic, however. The karyotype of these double transformants shows a high degree of stability. These results demonstrate the stepwise acquisition of the fully malignant phenotype by normal human epithelial cells in vitro.

INTRODUCTION

The use of DNA-mediated oncogenic transformation of rodent fibroblasts in vitro has contributed greatly to our understanding of tumorigenesis in general and oncogene function in particular (1). However, the identity and function of oncogenes involved in most human cancers remain unclear. It has been shown that some human oncogenes are not active in rodent cells (2) and that the activity of some oncogenes is dependent on host cell type (3). Thus progress in this area may be dependent on the development of in vitro models for various human cancers that utilize human cancer progenitor cells as oncogene recipients. A consistent problem with this approach has been the difficulty of maintaining human cells in culture (4). This is particularly true for human epithelial cells, the progenitors of most cancers. As a result of progress in the culture and establishment of human mammary epithelial cells (5–7), we have developed a model system for breast carcinoma that encompasses several sequential stages of transformation and utilizes both in vitro and in vivo assays for oncogene function.

MATERIALS AND METHODS

Cell Culture. 184 cells were maintained in MCDB-170 prepared as described (5, 7). 184A1N4 cells were maintained in FM medium, a 1:1 mixture of Ham's F-12 and DME supplemented with fetal calf serum (0.5%), insulin (10 µg/ml), hydrocortisone (0.1 µg/ml), and EGF (5 ng/ml). Retroviral transformants were selected and maintained in DME plus 10% FCS.

Viruses. HaMSV was produced from TK minus rat fibroblasts (Rat 2 cells) that were transfected with a genomic clone (pBVX) of HaMSV (KP6 cells) and infected with amphotropic (American Type Culture Collection VR-884) or xenotropic helper virus (NZB No. 00510, a gift of J. Levy, University of California, San Francisco). The focus-forming titer of both pseudotypes was 1 to 5 × 10⁴ pfu/ml on Rat2 cells. MoMSV was produced from CCCB1 cells (cat fibroblasts transformed by MoMSV) that were infected by xenotropic helper virus (titer, ~1 × 10⁴ pfu/ml on Rat2). Xenotropic EVXT virus was produced by infecting ectropic EVXx2 (8) with xenotropic helper virus and passaging the resulting virus first in CV1 monkey cells and subsequently in Rat2 cells. The focus-forming titer of EVXT was 100 to 1000 pfu/ml on Rat2 cells and <5 pfu/ml on 3T3 (mouse) cells.

Retroviral Infections. Virus-containing culture medium from producing cell lines was harvested at near confluency and centrifuged (15 min at 5000 × g) or filtered (0.45 µm) to remove cells. Recipient cells were pretreated with 4 µg/ml of Polybrene in culture medium for 20 min prior to infection. The cells were incubated for 24 h in the virus-containing medium after removing the Polybrene solution.

Anchorage Independence. Anchorage-independent growth was determined by suspending 10⁴ single cells in 5 ml of a 1.25% methocel solution made up in 1:1 DME-Ham's F-12 with 0.5% FCS, 10 µg/ml of insulin, 0.1 µg/ml of hydrocortisone, 5 ng/ml of EGF, 10⁻⁴ M triiodothyronine, and 10⁻⁷ M prostaglandin E₂. Sixty-mm Petri dishes were seeded for each cell type and fed once a week. After 3 wk, colonies greater than 100 µm in diameter were counted and sized using a calibrated ocular grid.

Tumorigenicity. Groups of athymic mice (BALB/c-nu/nu; Charles River Laboratories) for each cell line were given s.c. injections of 4 × 10⁶ cells diluted in Dulbecco's phosphate-buffered saline. Two tumor-bearing mice from each group were sacrificed at 20 days postinoculation for histopathological examination. Tumor sections from each animal were prepared and stained with eosin and hematoxylin as described (9).

Karyology. The variant cell line 184A1N4 responded to our routine procedures for well-tumor chromosome preparations (10). Clone 184A1N4-T-ras-C9 did not respond to the hypotonic treatment with a 1:1 mixture of 0.4% sodium citrate and 0.4% KCl, regardless of the time of exposure. The chromosomes remained clumped in a gelatinous mass. Exposure to 0.4% KCl alone solved this problem, and the procedure was otherwise as referred to above. The chromosomes were G-banded by the trypsin-Giemsa method (11).

RESULTS

Culture and Establishment of Human Mammary Epithelial Cells. Normal epithelial cells (184) were cultured from reduction mammoplasty tissue of Specimen Donor 184, a 21-yr-old with no detectable epithelial cell pathology. The tissue was digested to epithelial organoids as previously described (6, 7) and grown up to 18 passages in a serum-free medium (MCDB170) especially formulated for normal HMEC (5). These cells display characteristics typical of mammary epithelial cells: epithelial specific keratin; pattern of cell associated fibronectin; human milk fat globule antigens; and the mammary-specific enzyme thioesterase II (5). An immortal human cell line (184A1) was established from 184 cells after exposure to benzo(a)pyrene (6). This line retains the normal mammary epithelial characteristics mentioned above, requires anchorage for growth, and does not form tumors in nude mice. Thus it is not likely that 184A1 has been malignantly transformed. This line does, however, express several abnormal properties that distinguish it from the normal parental cells including aneuploidy (a majority are near diploid), altered surface antigen expression, and altered differentiation state. A variant of this line...
184A1, designated 184A1N4, was obtained by adapting 184A1 cells to grow in a less complex medium (FM) containing 0.5% fetal calf serum supplemented with epidermal growth factor, hydrocortisone, and insulin. The growth of this line is inhibited by high levels of serum (10%) and the absence of added growth factors. In contrast to the parent line, the majority of the 184A1N4 cells contain a nearly trisomic chromosome complement (see below).

Retroviral Transformation of 184A1N4 Cells. We have shown that 184 and 184A1N4 cells take up and transiently express transfected DNA as efficiently as 3T3 cells (12). In contrast, our attempts to obtain stable transformation of 184 and 184A1N4 cells by DNA transfection of the neomycin resistance gene were not successful. The 184 and 184A1N4 cells did, however, produce G418-resistant colonies in response to a xenotropic retrovirus that contained a neomycin resistance gene (data not shown). Thus retrovirus vectors were used to introduce oncogenes into these cells in subsequent experiments.

Three oncogenes (v-mos, v-Ha-ras, and SV40 T-antigen) were used to transform 184 and 184A1N4 cells via retroviral infection. The products of these oncogenes are structurally unrelated to one another and localize to distinct cellular compartments: nucleus (T-antigen); cytoplasm (mos); or inner plasma membrane (ras) (13). The ras and mos oncogenes were introduced by the spontaneously arising oncogenic murine retroviruses HaMSV (14) and MoMSV (15), respectively; and T-antigen was introduced by the recombinant virus EVX-T (8). Virus stocks were prepared from rat (EVX-T and HaMSV) or cat (MoMSV) cells as xenotropic (EVX-T and MoMSV) or amphotropic (HaMSV) murine leukemia virus pseudotypes.

Unlike 184 and 184A1N4 cells, many human breast cancer-derived cell lines (e.g., MCF-7 and MDA-MB-231) will grow in minimal medium (DME) supplemented with high levels (5 to 10%) of fetal calf serum. Thus we sought to use this medium as a selection for oncogene transformation, in a manner similar to that described for the selection of rodent fibroblasts transformed by viral oncogenes (16). To determine whether oncogenes could induce 184A1N4 cells to grow in DME plus FCS, we infected these cells with the EVX-T, HaMSV, MoMSV, or xenotropic helper viruses and transferred to the selective medium (DME+FCS). When subcultured into DME+FCS after reaching saturation density, the infected or helper virus-infected 184A1N4 cells stopped growing after one or two cell divisions. The growth of these cells remained arrested for 6 wk but resumed growth when returned to FM medium. In contrast, cells infected with HaMSV or MoMSV continued to proliferate without noticeable interruption after transfer to this medium (Fig. 1). Most of the cells infected with EVX-T failed to grow, but 2 to 5 colonies per 90-mm plate appeared after 3 wk in DME+FCS. The low level of proliferation probably reflects the low titer of the EVX-T virus as compared to the HaMSV or MoMSV stocks. The 184A1N4-ras and 184A1N4-mos cells displayed a similar compact morphology and grew to high density within a monolayer (Fig. 1). The 184A1N4-T cells, however, grew to relatively low density and formed organized "swirling" patterns on the plate (Fig. 2). The observation that three different oncogenes induced growth in DME+FCS suggests that this selection may be generally useful for the detection of oncogene expression in human mammary epithelial cells. In addition to the single oncogene transformants described above (184A1N4-ras, 184A1N4-mos, and 184A1N4-T), double transformants were obtained by infecting 184A1N4-mos and 184A1N4-T with the high-titer amphotropic HaMSV stock. Infection of the 184A1N4-T cells with the Ha-ras virus resulted in a loss of organized growth patterns and the appearance of small clumps of cells growing above the monolayer (Fig. 2). In contrast, superinfection of 184A1N4-mos cells with HaMSV had no obvious effect on cell morphology (Fig. 2).

Normal 184 cells infected with HaMSV or MoMSV showed little or no ability to grow in DME+FCS and senesced when grown in MCDB170. In contrast, 184 cultures infected with EVX-T virus produced 10 to 100 colonies per 10^6 cells that were capable of extended growth (up to 30 doublings) in DME+FCS. We were unable, however, to obtain continuous cell lines from these transformants in spite of attempts to culture them in a variety of media including 184-conditioned MCDB170. Accordingly, we limited this study to the characterization of the 184A1N4 transformants. However, these results suggest that the ras, mos, and T-antigen genes are unable to directly immortalize 184 cells at easily detectable frequencies.

Expression of p21-ras and T-Antigen in 184A1N4 Transformants. The induction of viral transforming proteins was observed by immunoprecipitation of 32P-labeled proteins with monoclonal antibodies (Fig. 3). This method detects viral rather than cellular p21 (ras) protein because the viral mutation at position 59 (glutamine to threonine) is required for autophosphorylation (17). T-antigen was detected only in the 184A1N4-T and 184A1N4-T-ras cells, and p21 was detected in only the 184A1N4-ras and 184A1N4-T-ras cells. The levels of p21 and T-antigen in the 184A1N4-T-ras double transformant were nearly equal to the levels observed in the single transformants (184A1N4-ras and 184A1N4-T). In addition, the 184A1N4-T cells were tested for T-antigen immunofluorescence using the monoclonal antibody Pab 419. The cells were found to be uniformly positive for nuclear fluorescence (data not shown).

Anchorage Independence. The 184A1N4 transformants were tested for their ability to form colonies in methocel (Table 1). All of the oncogene-containing retroviruses except MoMSV (mos) conferred some degree of anchorage independence on the 184A1N4 line. No colonies were observed when the 184A1N4 cells were infected with helper virus alone. The plating efficiencies observed were generally low (0.01 to 0.1%) but were comparable to that observed for the human breast tumor-derived cell line MDA-MB-231. Superinfection of the 184A1N4-T line with the Ha-ras virus resulted in a marked increase in efficiency, especially when larger (>150 μm) colonies are considered. This suggests that the effects of the ras and T-antigen proteins are complimentary in this assay. In contrast, preinfection of 184A1N4 cells with MoMSV (mos) did not enhance the ability of HaMSV to induce these cells to grow in methocel.

Tumorigenicity in Nude Mice. The 184A1N4 transformants were tested for their ability to form tumors in athymic mice after 7 to 15 passages in culture (Table 2). Three clones of 184A1N4-T-ras out of three tested produced large rapidly growing tumors in these mice at 100% efficiency. Clone A was obtained by cloning in soft agar, and clones C9 and D10 were obtained by dilution to one cell per well. Similar results (large tumors in 6 of 6 mice) were obtained for a 184A1N4-T-ras mass culture injected at 4 passages after the HaMSV infection. The histopathological type of these tumors ranged from carcinomatous to undifferentiated (Table 2). The 184A1N4-T cells and helper virus-infected cells produced no tumors in this assay. The 184A1N4-mos, 184A1N4-ras, 184A1N4-mos-ras, and MDA-MB-231 cells produced tumors at relatively low frequency. These tumors were small (5 to 8 mm) compared to the 184A1N4-T-ras tumors with the exception of 184A1N4-ras-mos, which produced large (17-mm) tumors, and they were histopathologically similar to the 184A1N4-T-ras tumors ex-
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Fig. 1. 184A1N4 cells growing in FM medium were mock infected or infected with murine retroviruses containing either the ras (HaMSV) or mos (MoMSV) genes. Three days after infection the cells were sub cultivated in DME plus 10% FCS. A-C, crystal violet-stained cultures showing selective growth of transformed cells in DME+FCS. After infection with HaMSV (B), MoMSV (C), or mock infection (A), cells were subcultured by serial dilution (1:3 with 10,000 cells in the first well) into 10 cm² wells of 6-well cell culture plates. Cells were stained 10 days after subculturing. D-F, phase-contrast photomicrographs showing morphology and relative growth of HaMSV (E), MoMSV (F), or mock infected cells (D) 7 days after subculturing 1:10 in DME+FCS.

cept for 184A1N4-mos tumors, which appeared to be well-differentiated adenomas. Thus the ras gene compliments the T-antigen gene by increasing tumor frequency and both the T-antigen and mos gene by increasing tumor size. One or more mice from each group were examined for evidence of metastasis and for tumor histology (Table 2). One of six mice receiving 184A1N4-T-ras injections was found to have a metastatic lesion in the spleen, and one of one 184A1N4-ras-mos mice was found to have metastatic lesions in the spleen, lymph nodes, and lung. No evidence of metastasis was found in the 184A1N4-mos- or 184A1N4-ras injected mice. The MDA-MB-231 line produced lesions in the lung and mediastinum. In each case the histology of the metastasis closely matched that of the primary tumor. Although the amphotropic HaMSV strain used here is capable of inducing lymphosarcomas of mouse origin when introduced to athymic mice via other infected human epithelial cells, no histological evidence of such tumors was seen in this study, probably as a result of relatively low virus production by 184A1N4 cells. An explant of a primary tumor from one 184A1N4-T-ras-A-injected mouse was cultured and found by metaphase chromosome analysis to consist of human cells (data not shown).

Karyology of 184A1N4 and 184A1N4-T-ras-D10. To determine whether chromosomal alterations accompanied the malignant transformation of 184A1N4, this line and the malignant 184A1N4-T-ras-D10 line were subjected to karyotypic analysis. The parent line (184A1) to 184A1N4 is a near-diploid cell line, and the following stemline was observed at the earliest passage (lip) examined: 45,XX,-6,del(3q)(pter→q27),del(12q)(pter→q14). These specific aberrations remained stable in subsequent passages, although a few other chromosomes participated in new aberrations, and a subpopulation (<10%) of near-trisomic cells appeared. In contrast, 16 members of the 184A1N4 chromosomal complement were trisomic, 3 disomic, 2 tetrasomic, and one pentasomic. In addition, one new aberration was consistently present in all the cells analyzed; t(8q;16)(q13;p13) disomic (Fig. 4). The malignant line 184A1N4-T-ras-D10 is separated from the 184A1N4 line by 2 separate viral infections, a growth selection, and a cloning by dilution (about 75 doublings). The significant karyotypic changes for this cell line are: a numerical increase for about half the members of the complement; the disappearance of the translocation (8q;16); and the gain of a 22q+ chromosome (Fig. 5). The deleted 12q chromosome of 184A1 was also absent in over 50% of the cells analyzed. Other chromosomal aberrations were variously present, but none of these was clonally represented. In addition, five cells from another clonal isolate (184A1N4-T-ras-C9) were karyotyped (data not shown). These cells revealed identical karyotypes, both numerically and for aberrations, and differed only slightly in numbers of individual chromosomes from clone D10. Clone C9, like clone D10, had lost the “parental” translocation (8q;16) and contained the 22q+ chromosome. In summary the malignant cell line 184A1N4-T-ras is a karyotypically stable, near-tetraploid line that is relatively free of additional clonal chromosomal aberrations as compared to the nonmalignant line from which it was derived.

DISCUSSION

We have developed a model system for the malignant transformation of HMEC. As described previously (6) the progression to malignancy begins with the emergence of extended life

\[ J. \text{ Kopplin and B. Zimmerman, personal communication.} \]
cultures from benzo(a)pyrene-treated normal epithelial cells and continues with the rare emergence of continuous lines from these cultures. We have obtained transformants from one of these HMEC lines through the use of oncogenic retroviruses. Our analysis of the transformants demonstrates the stepwise acquisition of the fully malignant phenotype by normal HMEC in vitro (Table 3). We believe this system will be useful in defining the elements of growth control for HMEC and potentially for identifying somatic mutations that are involved in the development of mammary carcinomas.

In recent years our understanding of tumorigenesis has been greatly increased by the use of in vitro transformation assays to identify oncogenes that are active in avian or rodent cells (1). It has been more difficult, however, to extend these studies to human systems for a variety of reasons including the resistance of human cells to DNA transfection and the problem of identifying the appropriate transformed phenotypes (18, 19). Nevertheless it has been shown that several types of human cells, including epithelial cells, become transformed in response to viral oncogenes (20–23). We have chosen to introduce oncogenes into HMEC via murine retroviruses. The exclusive use of retrovirus rather than DNA virus vectors (e.g., SV40 and adenovirus) avoids possible host cell alteration that may result from DNA virus replication in the infected cells (10, 24–27) and allows the stable transformation by mos or ras of a large...
Fig. 4. Karyotype of the 184A1N4 cell line. Twenty cells were karyotyped, and all the cells had the same chromosomal constitution except for chromosomes 10, 18, 20, 21, and X. For chromosome 10, 6 cells had two no. 10s, +iso(10q); for chromosome 18, 5 cells were trisomic; and for chromosomes 20 and 21, the cells varied from the representative karyotype by ±1 chromosome. For the X chromosome 4 cells had 2 Xs, + Xq. The two translocation chromosomes involving no. 16 have tentatively been identified as t(18q23;16)(q13;p13), and they were present in all 20 cells karyotyped.

Fig. 5. Karyotype of the 184A1N4-T-ras-D10 cell line. This karyotype was prepared by W. D. Peterson at Children's Hospital of Michigan using the trypsin-Giemsa method (11). The total number of chromosomes ranged from 83 to 90 in 39 cells counted, with the most frequent number being 89 (14 cells). The chromosomes showing the most fluctuation in number of members were: 4; 5; 15; 16; and X. The deleted 12q chromosome was found in only three of nine cells karyotyped.

portion of the culture, thus minimizing selection and extensive passaging prior to characterization.

In contrast to transformation assays that depend on focus formation to identify transformants, we have used a simple medium-dependent growth selection. This selection appears to have a broad specificity for oncogene detection as the oncogenes tested here are very different in structure, localization, and putative function. Preliminary results suggest that the basis for this selection is complex, depending on both repression of growth by high serum levels and the absence of multiple growth factors from the selective medium. Thus our attempts to simplify the selection have not been successful. An alternative in
vitro assay for transformation of HMEC, growth in methocel, appeared to be more oncogene specific, determining transformation by T-antigen and v-Ha-ras, but not by v-mos (Table 1).

Carcinogenesis is a multistep process, and in rodent systems this is reflected by the ability of certain oncogenes to complement one another in producing the malignant phenotype (28). Although single oncogenes were sufficient to induce growth of 184AIN4 in DME+FCS, complementation between two oncogenes (T-antigen and ras) was required to produce the fully malignant phenotype. This complementation was also seen in the effect of these oncogenes on growth in methocel. Similar observations have been made for human keratinocytes (21) and amniocytes (10), suggesting that these cells have similar requirements for malignant transformation.

In vivo and in vitro changes of human cells to malignancy are associated with the presence of clonal chromosomal aberrations. These aberrant chromosomes vary in number and structural complexity, often resulting in unidentifiable "new" chromosomes (22, 29–31). Thus it is surprising that karyotype aberrations of the 184AIN4-T-ras-D10 and D9 lines revealed only one clonally represented aberration (22q+) not present in the parent line. In fact, the most prominent marker chromosome in the 184AIN4 complement was lost in most of the 184AIN4-T-ras-D10 and C9 cells. Furthermore, the karyotypes of these lines are numerically stable as compared to the parent line. These observations lend support to the notion that the transgene expression rather than by secondary alterations of the 184AIN4 genome and that v-Ha-ras p21 and SV40 T-antigen do not induce chromosomal aberrations in the 184AIN4 line.

Table 3 Phenotypes of 184-derived cells

<table>
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<th>Cell type</th>
<th>Established growth</th>
<th>Growth in DME+FCS</th>
<th>Growth in methocel</th>
<th>Tumors in nude mice</th>
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* Differentiated adenoma (benign).

**REFERENCES**

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