E7-transduced Human Breast Epithelial Cells Show Partial Differentiation in Three-dimensional Culture

Kimberly M. Spancake, Christine B. Anderson, Valerie M. Weaver, Norisada Matsunami, Mina J. Bissell, and Raymond L. White

Huntsman Cancer Institute, University of Utah, Salt Lake City, Utah 84112 [K. M. S., C. B. A., N. M., R. L. W.], and Lawrence Berkeley National Laboratory, Berkeley, California 94720 [V. M. W., M. J. B.]

Abstract

Disruption of the retinoblastoma (RB) tumor suppressor pathway is a common and important event in breast carcinogenesis. To examine the role of the retinoblastoma protein (pRB) in this process, we created human mammary epithelial cells (HMEC) deficient for pRB by infecting primary outgrowth from breast organoids with the human papillomavirus type 16 (HPV16) E7 gene. HPV16 E7 binds to and inactivates pRB and also causes a significant down-regulation of the protein. Culturing normal HMEC in a reconstituted basement membrane (rBM) provides a correct environment and signaling cues for the formation of differentiated, acinar-like structures. When cultured in this rBM, HMEC+E7 were found to respond morphologically as normal HMEC and form acinar structures. In contrast to normal HMEC, many of the cells within the HMEC+E7 structures were not growth arrested, as determined by a 5-bromo-2-deoxyuridine incorporation assay. pRB deficiency did not affect polarization of these structures, as indicated by the normal localization of the cell-cell adhesion marker E-cadherin and the basal deposition of a collagen IV membrane. However, in HMEC+E7 acini, we were unable to detect by immunofluorescence microscopy the milk protein lactoferrin or cytokeratin 19, both markers of differentiation expressed in the normal HMEC structures. These data suggest that loss of RB in vivo would compromise differentiation, predisposing these cells to future tumor-promoting actions.

Introduction

The tumor suppressor gene RB can play a significant role in breast carcinogenesis. RB has been shown to be inactivated in 19% of human breast tumors and 25% of human breast carcinoma cell lines (1, 2). Other members of the RB regulatory network have also been reported as having aberrant expression in a significant number of breast carcinomas. For example, cyclin D1, which through regulation of the cyclin-dependent kinase Cdk4 phosphorylates and inactivates pRB, is overexpressed in 35% of breast tumors (3). The p16 protein, also a regulator of pRB activity through inhibition of Cdk4, is absent in 40% of breast tumors, as determined by immunohistochemical analysis (4). Additionally, restoration of pRB expression in human breast cancer cell lines lacking functional pRB has been shown to cause a reduction in the cells’ tumorigenicity (5). These data suggest that disruption of the RB regulatory network is a common and important step in breast carcinogenesis.

In addition to the cellular inactivation of pRB by cyclins and cyclin-dependent kinases, several DNA tumor virus proteins abrogate pRB activity by binding to and functionally sequestering pRB. The HPV16 E7 protein not only inactivates pRB function by binding but also enhances degradation of pRB through ubiquitin-dependent proteolysis (6). HPV16 E7 was shown previously to immortalize early passage HMEC in culture (7), suggesting a critical role in growth regulation for pRB in vivo. Disruption of such fundamental growth control mechanisms should generate cell populations predisposed for transformation. To create a pRB-deficient cell line, we infected primary breast epithelial cell outgrowth with a retrovirus expressing the HPV16 E7 gene (HMEC+E7). In this study, only early passage, G418-resistant, precrisis HMEC+E7 cells were examined. To characterize the impact of this pRB deficiency, we used a sensitive three-dimensional culture assay shown previously to support the formation of spheroid structures that resemble breast acini (8). Culturing human, luminal breast epithelial cells in a rBM provides an environment that supports not only organogenesis but also formation of endogenous BM necessary for differentiation (9). This culture system can also be used to distinguish between normal and cancer cells because of the inability of the transformed cells to organize into acinar structures (10).

We examined the effect of pRB deficiency on structure formation, cell-cell interactions, and differentiation by culturing the cells in a rBM. Our data demonstrate that even in the absence of functional pRB, human breast epithelial cells organize and form morphologically typical acini-like structures. Furthermore, in these pRB-deficient structures, proper localization of E-cadherin and deposition of a collagen IV BM, both markers of organogenesis, were found. However, unlike normal HMEC, the structures formed by HMEC+E7 do not exit the cell cycle and are deficient for the expression of cytokeratin 19 and lactoferrin, two proteins associated with the differentiation of luminal breast epithelial cells. These data suggest that pRB is not necessary for the transmission of signals from the extracellular matrix that convey information necessary for structure formation, but pRB does appear to be necessary for cell cycle withdrawal and expression of some proteins normally present in the differentiated luminal epithelial cell. Our data suggest that the initial loss of RB in vivo would not predispose breast epithelial cells to an apoptotic or immediate transformed fate, but rather loss of pRB activity would contribute to a less differentiated cellular state that, in turn, may eventually lead to a malignant phenotype.

Materials and Methods

Cell Culture and Retroviral Infection. Surgical discard material from reduction mammoplasties was minced with opposing scalps, placed in digestion buffer, and incubated in spinner flasks at 37°C until stroma dissolved (~3–5 h). Digestion buffer contained 1 unit/ml Collagenase D (Roche Molecular Biochemicals, Indianapolis, IN), 2.4 units/ml dispase (Roche Molecular Biochemicals), and 6.25 units/ml DNase (Sigma Chemical Co., St. Louis, MO) in Dulbecco’s PBS. The digested material plus 10% FCS was centrifuged at 800 rpm for 10 min. The resulting pellet was resuspended in wash buffer (11), and the organoids were...
coated with 100 μm stained with 100 ng/ml DAPI (Sigma) or TO-PRO-3 1:750 (Molecular Probes).

Primary epithelial outgrowth was infected with an LXSN retroviral construct containing the HPV16 E7 gene (LXSNI6E7; Ref. 13). HMEC were incubated with LXSNI6E7 in CD3 plus 4 μg/ml Polybrene (Sigma) for 24 h. Viral supernatant was aspirated, and HMEC were cultured in virus-free CD3 for 48 h prior to selection in CD3 containing 50 μg/ml Geneticin (Life Technologies, Inc.). Early-passage HMEC (either passage one or two) and HMEC containing LXSNI6E7 (HMEC+E7) were cultured in a rBM, Matrigel (Becton Dickinson, Bedford, MA), as described previously (10). Briefly, 2.5 × 10^5 HMEC were resuspended as single cells in 300 μl of 10 mg/ml Matrigel per well and plated onto Nunc four-well multidishes coated with 100 μl of Matrigel. Matrigel cultures were overlaid with 50 μl of CD3.

Western Blot Analysis. HMEC and HMEC+E7 cultured in polystyrene flasks were scraped in lysis buffer (PBS containing 0.1% Triton X-100, 0.1% NP40, 0.2 mg/ml Pefablock, 0.01 mg/ml aprotinin, 0.01 mg/ml leupeptin, 20 mM sodium fluoride, and 1 mM sodium orthovanadate) and sonicated for 30 s on ice. HMEC and HMEC+E7 cultured in rBM for 10 days were liberated from the Matrigel by incubation with dispase (Becton Dickinson) for 1 h at 37°C. The acini were then washed with PBS plus 5 mM EDTA, resuspended in lysis buffer, homogenized for 10 s, and sonicated for 30 s on ice. Protein lysates from cells cultured on plastic or in Matrigel were boiled in SDS-PAGE sample buffer for 3 min, and 50 μg of total protein were resolved on 4–12% gradient acrylamide Tris-Glycine gels (Novex, San Diego, CA) by SDS-PAGE. Proteins were transferred to nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Immunoblot analysis was performed as described previously (14). Antibodies were used in the following concentrations: E-cadherin (mouse IgG1, G3–245) 1:400 (PharMingen, San Diego, CA), RB (mouse IgG1, G3–245) 1:400 (PharMingen, San Diego, CA), RB-DEFICIENT BREAST CELLS FAIL TO FULLY DIFFERENTIATE

Fig. 1. Western blot analysis of pRB in normal and HPV16 E7-expressing breast epithelial cells. Protein lysates from HMEC and HMEC+E7 were analyzed by SDS-PAGE and immunoblotting with a monoclonal anti-pRB antibody. pRB is present in the normal HMEC, but the level of expression is reduced in HMEC+E7.

Results

HMEC expressing HPV16 E7 Show a Decrease in pRB Expression. Primary epithelial outgrowth from normal human organoids was infected with a retroviral construct expressing the HPV16 E7 gene. HMEC+E7 were selected by culturing the cells in CD3 growth medium containing Geneticin. To determine the effect of HPV16 E7 expression on pRB, HMEC and HMEC+E7 were examined by Western immunoblot analysis. Fig. 1 shows the expression level of pRB in the normal, parental HMEC when cultured on plastic. In contrast, HMEC+E7 showed a significant decrease in pRB. This down-regulation of pRB is in agreement with previous results demonstrating the ubiquitin-dependent proteolysis of pRB in the presence of HPV16 E7 (6).

HMEC+E7 Form Acinar Structures When Cultured in Extracellular Matrix But Are Not Growth Arrested. Both normal HMEC and HMEC+E7 were cultured in rBM. By 10 days in culture,
HMEC also formed morphologically normal-appearing acinar structures when cultured in rBM (Fig. 2C). The HMEC+E7 acini were similar in size to the parental structures, although DAPI staining revealed somewhat larger and slightly less organized nuclei (Fig. 2D). Additionally, HMEC acini had growth arrested, as determined by minor BrdUrd incorporation similar to levels reported previously (15); however, a significant increase in BrdUrd labeling was observed in HMEC+E7 (Fig. 2E).

**Normal Localization of E-cadherin and Type IV Collagen in HMEC+E7 Acinar Structures.** HMEC+E7 were examined for the expression of proteins shown previously to have distinctive localization patterns characteristic of organogenesis in normal HMEC acini (15). HMEC and HMEC+E7 rBM cultures were fixed, frozen, cut into 5-μm sections, and stained with the indicated primary antibodies and corresponding secondary antibodies. In normal HMEC acinar structures, the cell-cell adhesion protein E-cadherin was localized to points of cell-cell contact (Fig. 3A). This same pattern of E-cadherin expression at points of cell-cell contact was present in the HMEC+E7 structures (Fig. 3B). Collagen IV was basally deposited in a continuous BM surrounding the normal HMEC structures when cultured in rBM (Fig. 3C). HMEC+E7 acini also deposited a basal collagen IV-containing BM (Fig. 3D).

**Lactoferrin and Cytokeratin 19 Are Not Expressed in Structurally Differentiated HMEC+E7.** The proteins lactoferrin and cytokeratin 19 are markers for differentiation in luminal breast epithelial cells (16–18). HMEC and HMEC+E7 were prepared as described above and examined for the expression of lactoferrin and cytokeratin 19. HMEC and HMEC+E7 acinar structures were identified by the presence of the luminal marker cytokeratin 18 (Fig. 4A and B, respectively) and the absence of the myoepithelial marker cytokeratin 14 (data not shown). Lactoferrin is an iron-binding milk protein that was expressed in the normal HMEC acini (Fig. 4C). In contrast, immunofluorescence analysis of HMEC+E7 structures demonstrated the absence of lactoferrin (Fig. 4D). As expected, structures derived from normal HMEC expressed the luminal differentiation marker cytokeratin 19 (Fig. 4E). HMEC+E7 acini, however, were deficient for the expression of cytokeratin 19 (Fig. 4F). These immunofluorescence data were confirmed by Western immunoblot analysis of HMEC and HMEC+E7 acinar structures, which showed barely detectable levels of cytokeratin 19 in HMEC+E7 (Fig. 4G).

**Discussion**

HMEC+E7 are deficient for pRB, a key protein governing cell cycle regulation and differentiation; thus, formation of even a partially “differentiated” structure when cultured in rBM was unexpected. Further investigation showed that although these pRB-deficient cells were capable of organizing into morphologically normal acini, the nuclei of these structures appeared to be larger and slightly less normal HMEC organized into differentiated acini ~55 μm in diameter (Fig. 2A). A 5-μm frozen section of a representative structure stained with DAPI revealed a ring of basally located nuclei (Fig. 2B). HMEC+E7 also formed morphologically normal-appearing acinar structures when cultured in rBM (Fig. 2C). The HMEC+E7 acini were similar in size to the parental structures, although DAPI staining revealed somewhat larger and slightly less organized nuclei (Fig. 2D). Additionally, HMEC acini had growth arrested, as determined by minor BrdUrd incorporation similar to levels reported previously (15); however, a significant increase in BrdUrd labeling was observed in HMEC+E7 (Fig. 2E).
organized. Additionally, HMEC+E7 acini contained many cells that had not growth arrested, unlike the normal HMEC structures. Immunofluorescence analysis of the HMEC+E7 structures revealed normal patterns of E-cadherin and collagen IV localization, indicating that the HMEC+E7 were forming normal cell–cell contacts and were properly polarized. HMEC+E7 acini expressing the luminal marker cytokeratin 18 lacked proteins typically expressed in the differentiated luminal breast epithelial cell. Neither the milk protein lactoferrin nor the luminal differentiation marker cytokeratin 19 were found in the HMEC+E7 acinar structures.

pRB has a significant role in the differentiation of many different cell types, including muscle, neuronal, and erythroid cells (19). Typically, an early event in differentiation is the activation of pRB by dephosphorylation, allowing the cell to arrest in G<sub>0</sub>-G<sub>1</sub>. During muscle cell differentiation, hypophosphorylated pRB associates with the transcription factors MyoD and myogenin, preventing phosphorylation of pRB and the consequent reemergence into the cell cycle (20). pRB has not been shown previously to have a role in directing the differentiation of breast epithelial cells. Because pRB is down-regulated in HMEC+E7, our data suggest that pRB is not necessary during the organization and structure formation of HMEC when cultured in a three-dimensional rBM. However, the absence of pRB does impact the expression of some proteins normally associated with the differentiated phenotype. Interestingly, a similar observation was made during MyoD-induced skeletal muscle differentiation in which cells showed attenuated expression of a late differentiation marker, myosin heavy chain (21). The deficiency of differentiation markers in the HMEC+E7 structures could be attributable to the role of pRB in transcriptional regulation outside its classically described interaction with E2F (reviewed in Ref. 19). Recently, pRB was shown to interact directly with the transcription factor SP1 and increase transcription of the dihydrofolate reductase gene via the SP1 binding site (22). Sequence analysis of human lactoferrin and mouse cytokeratin 19 has revealed SP1 sites in the promoter regions of both these genes (23, 24). Taken together, these data suggest a possible role for pRB in transcriptionally regulating lactoferrin and cytokeratin 19, a control that is abolished by HPV16 E7.

HPV16 E7 protein can also interact with the pRB family members p107 and p130. However, neither p107 nor p130 have been shown to be targeted for ubiquitin-dependent proteolysis by HPV16 E7, as has been established for pRB (25). Additionally, to date no human tumors have been identified that contain mutations in p107 or p130 (reviewed in Ref. 26). Consequently, the roles of p107 and p130 in transformation remain unclear, and inactivation of these proteins could also be contributing to the phenotype observed in the HMEC+E7 structures. Our data demonstrate that breast epithelial cells with down-regulated pRB retain the ability to respond to structure-forming signaling cues from a rBM; however, these acinar structures are not growth arrested and do not express some of the proteins normally associated with differentiation. These data suggest that mutation of pRB alone in vivo in human breast epithelial cells would not cause transformation but rather create a lesser differentiated cellular state. This modified phenotype, in conjunction with additional mutations, growth factor and hormonal modulations, and extracellular matrix perturbations, would likely result in malignancy.

Finally, it should be noted that the retinoblastoma protein has a complex set of functions within cells, and finding conditions that allow separation of these functions has been challenging. Our observations indicate that culture in a three-dimensional matrix affords a new operational definition of pRB activities that distinguishes those functions involved in polarity and initiation of differentiation from those functions that define a fully differentiated cell.

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References

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