Human Papillomavirus Type 16 E6 Inactivation of p53 in Normal Human Mammary Epithelial Cells Promotes Tamoxifen-mediated Apoptosis

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ABSTRACT

Aberrant p53 expression is frequently observed in mammary epithelial cells obtained from women at high risk for developing breast cancer and is a predictor for the subsequent development of malignancy. Tamoxifen has recently been shown to reduce the incidence of noninvasive breast cancer in high-risk women, but the molecular mechanism of tamoxifen chemoprevention in mammary epithelial tissue that does not overexpress the estrogen receptor is poorly understood. We suppressed p53 expression by retroviral-mediated expression of human papillomavirus type-16 E6 protein (HPV-16 E6) in human mammary epithelial cells (HMECs) to develop an in vitro model of tamoxifen chemoprevention in the context of p53 loss. Early passage p53(−) HMEC-E6-transduced cells treated with 1.0 μM tamoxifen rapidly underwent apoptosis. In contrast, early passage p53(+) HMEC-LXSN vector controls treated with 1.0 μM tamoxifen underwent G1-G0-G2/M phase arrest but did not undergo apoptosis. p53(−) HPV-16 E6 cells rapidly acquired resistance to tamoxifen-mediated apoptosis after 10 passages in culture (in the absence of tamoxifen). Both p53(+) and p53(−) HMECs exhibited a low level of estrogen receptor staining and minimal estrogen binding, characteristic of proliferating normal luminal mammary epithelial cells. Tamoxifen-mediated apoptosis in p53(−) HPV-16 E6 cells was not blocked by inhibitors of transcription and protein synthesis. These data suggest that the acute loss of p53 function in HMECs by expression of HPV-16 E6 results in marked sensitivity to tamoxifen-mediated apoptosis but that resistance to apoptosis rapidly develops within 10 passages in vitro. Observations in our model predict a critical role for the early institution of tamoxifen chemoprevention.

INTRODUCTION

The National Surgical Adjuvant Breast and Bowel Project BCPT recently demonstrated that tamoxifen reduced the incidence of breast cancers in high-risk women, thus raising the possibility of breast cancer chemoprevention (1). Tamoxifen reduced the occurrence of ER+ invasive cancers by 69% and reduced the incidence of noninvasive breast cancers by 50% (1). There was no difference in the occurrence of ER− invasive cancers (1). Although the BCPT appears to demonstrate a clear clinical benefit from tamoxifen, several questions remain. Two important questions are (a) did “true” prevention occur or were the benefits of tamoxifen attributable to ablation of preclinical breast cancer? and (b) what was the mechanism by which tamoxifen reduced the incidence of ER-poor noninvasive breast cancers?

Normal proliferating mammary tissue, hyperplastic mammary epithelial cells, and noninvasive breast cancers such as ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS) frequently exhibit low levels of ER expression relative to ER+ invasive cancers. Although it is not surprising that (a) tamoxifen reduced the incidence of ER+ invasive breast cancers in the BCPT and (b) did not prevent the emergence of ER− invasive breast cancers, the exact mechanism by which tamoxifen reduced the incidence of ER-poor, noninvasive breast cancers is unclear.

Tamoxifen has been shown to mediate both growth arrest and apoptosis (2–4). Despite the extensive information related to the antitumor effects of tamoxifen in cancer cell culture, in experimental animals models, and in humans there is little information on how tamoxifen may act in noninvasive mammary epithelial cells. On the basis of information in the model system we have developed, we hypothesize that tamoxifen may not only act through a classic ER-dependent mechanism to eliminate ER+ cancer cells but under certain circumstances, may also act to target ER-poor, noninvasive, abnormal mammary epithelial cells. We hypothesize that this latter mechanism may be important in understanding how tamoxifen chemoprevention reduced the incidence of noninvasive breast cancer during the BCPT.

P53 is a cell cycle “checkpoint” protein important for cell cycle regulation and is functionally inactivated in human cancer at a high frequency (5). p53 mutations are commonly detected in breast cancers and are associated with an increased risk of malignancy (6–8). Overexpression of p53 protein in mammary epithelial cells is frequently detected in women at high risk for the development of breast cancer (9) and is associated with an increased risk of progression to breast cancer in women with benign breast disease (10).

The E6 protein of the cancer-associated HPV-16 binds to p53 and targets it for degradation through the ubiquitin pathway (11–13). Retroviral-mediated introduction of HPV-16 E6 protein into cells provides a model for the isolated loss of p53 function. We used this approach to acutely suppress p53 function in normal HMECs. Because p53 loss is associated with an increased risk of breast cancer, we can use this in vitro system to model tamoxifen chemoprevention in a high-risk setting. We will use this system to test the hypothesis that loss of p53 function in HMECs increases sensitivity to tamoxifen-induced apoptosis. The advantage of this system is that we can test the ability of tamoxifen to prevent the occurrence of ER-poor noninvasive breast cancers in mammary epithelial cells that do not overexpress the ER. The disadvantage of this system is that we do not model the ability of tamoxifen to prevent ER+ breast cancer.

MATERIALS AND METHODS

Materials. Tamoxifen (Sigma, St. Louis, MO; 1 mm stock solution) was prepared in 100% ethanol and stored in opaque tubes at −70°C. Control cultures received equivalent volumes of the ethanol solvent. Tamoxifen stocks were used under reduced light.

Cell Culture. T47-D and MDA-MB-231 were obtained from American Type Culture Collection (Manassas, VA). Normal HMEC strain AG11132 (M.
Stampfer No. 172R/AA7) was purchased from the National Institute of Aging, Cell Culture Repository (Coriell Institute; Ref. 14). HMEC strain AG11132 is a heterogeneous population of normal HMECs derived from reduction mammoplasty (14). Cells were grown in Mammary Epithelial Cell Basal Medium (Clonetics, San Diego, CA) supplemented with 4 μl/ml bovine pituitary extract (Clonetics), 5 μg/ml insulin (UBI, Lake Placid, NY), 10 ng/ml epidermal growth factor (UBI), 0.5 μg/ml hydrocortisone (Sigma), 10−3 M isoproterenol (Sigma), and 10 mM HEPES buffer (Sigma) [Standard Medium]. Phenol red-free Standard Medium was prepared using mammary epithelial cell basal medium lacking phenol red (Clonetics). G418 (Life Technologies, Inc., Grand Island, NY)-containing medium was prepared by the addition of 300 μg/ml of G418 to the above Standard Medium. Cells were cultured at 37°C in a humidified incubator with 5% CO2/95% air. Mycoseptra testing was performed as reported previously (15).

ER Immunostaining. Approximately 5 × 104 cells were plated per well in a 4-chamber slide (Corning) in Standard Medium for 24 h. The cells were fixed in 10% paraformaldehyde in PBS for 20 min at room temperature. Determination of ER subcellular expression by immunocytochemistry was performed as follows. Cells were incubated with 100 μl of a 1:500 dilution of Immunotech mouse antihuman ER (Coulter, France) in PBS with 1% BSA for 40 min at room temperature. Slides were washed three times in PBS at room temperature and then incubated with 100 μl of a 1:200 dilution of Biomouse IgG peroxidase secondary antibody (Vector Laboratories, Burlingame, CA) in PBS at room temperature for 20 min. Slides were washed three times in PBS at room temperature, developed with Elite ABC (Vector Laboratories) as per manufacturer’s recommendations for 20 min at room temperature, washed three times in PBS at room temperature, and counterstained with methyl green.

Estrogen Binding Assays. Approximately 2 × 105 cells were plated per well in a 6-well plate (Corning) in Standard Medium for 24 h. The cells were washed with PBS and grown in phenol red-free Standard Medium for 48 h. Each 6-well plate was dosed in triplicate with one concentration of [2, 4, 6, 7-H]estradiol (Amersham) at concentrations ranging from 39.06 pm to 5.0 nm and [2, 4, 6, 7-H]estradiol plus a 200-fold excess of unlabeled estradiol (Sigma) and incubated at 37°C for 2 h. The media was removed, and cells were washed twice with ice-cold PBS. One ml of 95% ethanol was added per well for 30 min, cells were then scraped and lysed by resuspension with a pipetman, and a 500-μl aliquot taken for counts. To normalize cell number per well, 250 μl of the remaining ethanol lysate was placed in a microfuge tube, spun at 2000 rpm, and the supernatant discarded. The remaining ethanol was evaporated, and the pellet was resuspended in 50 μl of PBS. DNA content in the cell pellet was quantitated using the diphenylamine assay as described previously (1 cell = 7 pg DNA; Ref. 16). The number of binding sites per microgram of DNA was calculated by Scatchard analysis (17).

Retroviral Transduction. The LXSN16E6 retroviral vector containing the HPV-16 E6 coding sequence (generous gift of Denise Galloway, Fred Hutchinson Cancer Research Center, Seattle, WA) has been described previously (18). AG11132 normal HMECs (passage 8) were plated in four T-75 tissue culture flasks (Corning) in Standard Medium and grown to 50% confluency. Transduction conditions for AG11132 cells with LXSN16E6 or the control LXSN vector were as described previously (19). HMEC-P parental cells, p53(−) HMEC-LXSN vector control cells, and p53(+) HMEC-E6 cells expressing HPV-16 E6 were serially passaged in culture as described previously (19).

Cell Synchronization. Approximately 2 × 105 p53(+) HMEC-LXSN or p53(−) HMEC-E6 cells were plated in a T-75 flask (Corning) on Day −5 in Standard Medium and grown for 4 days (Day −1). We previously observed that, on Day −1, >85% of growth factor-depleted cells are in G1-G0 phase, trypsinize without difficulty, and rapidly resume proliferation in the presence of fresh Standard Medium. Cells were synchronized by this method before each experiment.

Northern Blotting. RNA was extracted with guanidine hydrochloride and subjected to Northern blotting in formaldehyde denaturing gels as described previously (16). Ten μg of RNA were loaded per lane. Molecular probes used in the Northern analysis are as follows: the human ER α probe is a 1.7-kb EcoRI and SalI fragment of the PCMV-ER plasmid (generous gift of Geoffrey Green, University of Chicago, Chicago, IL; Ref. 20, 21). The 36B4 probe (700-bp PstI fragment) was used as a loading control (22).

Western Blotting. Preparation of cellular lysates and immunoblotting are as described previously (16). Equal amounts of protein lysates (~100 μg of total protein) were loaded on 10% polyacrylamide gels; the gels were run and then electroblotted (Hoeffer) at 80 mA for 45 min onto Hybond-ECL membrane (Amersham). The membrane was blocked with 20% BSA (Sigma) in PBS overnight at room temperature and then incubated with a 1:100 dilution of mouse antihuman p53 (Oncogene Science Ab-2). The membrane was washed three to five times at room temperature with 250 ml of PBS containing 0.1% Tween 20 and then incubated with either a horseradish peroxidase-conjugated goat antismouse IgG (Jackson ImmunoResearch) at a 1:35,000 dilution or a 1:2000 dilution of horseradish peroxidase-conjugated Protein A (Sigma) for 1 h at room temperature. The blot was washed again, and complexes were detected by using enhanced chemiluminescent Western blotting detection reagents (Amersham) as described by the manufacturer.

Cell Growth Curves. p53(−) HMEC-LXSN vector controls and p53(−) HMEC-E6 cells were plated in duplicate at 1 × 104 cells per well in 12-well tissue culture plates (Corning) on Day −1 and allowed to adhere. On Day 0, the medium was replaced with Standard Medium with 0, 0.1, or 1.0 μM tamoxifen. Untreated controls received an equivalent volume of ethanol solvent (0.01% final concentration). Cells were trypsinized at 24-h time intervals and counted in triplicate.

For estrogen competition experiments, p53(−) HMEC-E6 cells were plated as above. On Day 0 cells were first treated with either 0, 0.1, 1.0, or 5.0 μM estradiol for 1 h and then treated with either 0, 0.1, or 1.0 μM tamoxifen. Untreated controls were treated with either grows or p53(−) HMEC-LXSN cells were surveyed at 0, 1, 2, and 3 and harvested on Day 4. Cells were trypsinized and nuclei were isolated and stained with propidium iodide, as reported previously (23), and analyzed by FACScan. Ten thousand events were collected in list mode fashion, stored, and analyzed on Muticycle AV software (Phoenix Flow Systems, San Diego, CA).

Electron Microscopy. p53(−) HMEC-E6 cells and p53(+) HMEC-LXSN vector control cells were plated on Day −1 in 6-well tissue culture plates (Corning). On Day 0, cells were treated with 0.1 or 1.0 μM tamoxifen. Electron microscopy was used as described previously (24). Approximately 200 p53(−) HMEC-E6 cells were surveyed per data point at 0, 12, and 24 h after tamoxifen treatment for the presence or absence of apoptosis and >200 p53(+) HMEC-LXSN cells were surveyed at 0, 1, 2, and 4 days.

Annexin Staining. Annexin V-FITC/α (Boehringer Ingelheim, Heidelberg Germany) was used as per manufacturer’s recommendation with some modification. Approximately 5 × 105 p53(+) HMEC-LXSN or p53(−) HMEC-E6 cells were plated in T75 flasks (Corning) on Day −1 and allowed to adhere. On Day 0, the medium was replaced with fresh Standard Medium, and tamoxifen was added for a final concentration of 0.1 or 1.0 μM. Untreated controls received an equivalent volume of ethanol solvent (0.01%). Cells were harvested after 24 h (Day 1) and did not exceed 25% confluency. Cells were trypsinized, washed in PBS, and resuspended in binding buffer [10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl2; filtered through a 0.2 μm pore filter]. Cell density was adjusted to 2–5 × 105 cells/ml Five μl of recombinant human Annexin V-FITC/α (BMS306Fa) was added to 195 μl of cell suspension; the mixture was briefly mixed and incubated for 10 min at room temperature in the dark. Cells were washed once and resuspended in 190 μl of binding buffer. Cells were then analyzed by FACScan (23, 25).

Transcription and Protein Synthesis Inhibition. Early passage p53(−) HMECs were treated with either the RNA or protein synthesis inhibitor actinomycin D (Sigma) or cyclohexamide (Sigma) at concentrations of 0, 0.1, 0.5, or 1.0 μM for 18–48 h. To establish an inhibitor concentration that resulted in growth arrest but less than 5% apoptotic or necrotic cells, inhibitor-treated early passage p53(−) HMEC-E6 cells were analyzed as described above by (a) growth curves, (b) protein iodide staining followed by FACScan analysis to determine cell cycle distribution and the percentage of necrotic cells, and (c) Annexin V binding to determine the percentage of apoptotic cells. After the dose response and toxicity curves were obtained, early passage p53(−) HMECs were pretreated with either actinomycin D (0.5 μg/ml Sigma) or cyclohexamide (1.0 μg/ml Sigma) for 3 h. Actinomycin D- and cyclohexamide-treated and untreated controls were then treated with 0, 0.1, and 1.0 μM tamoxifen for 18 h. Cells were analyzed for the presence of apoptosis by Annexin V binding as described above.

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Cytogenetic Analysis of Vector Control and Early Passage HMECs. Cultures of p53(+) HMEC-LXSN (passage 10) and p53(-) HMEC-E6 (passages 10 and 18) were checked for sufficient numbers of dividing cells and exposed to colcemid (Life Technologies, Inc.) at a final concentration of 0.01–0.02 µg/ml for 2–3 h. Subsequently, the cells were released from flasks by trypsinization, exposed to hypotonic solution, and fixed as described previously (26). Chromosome preparations were made, and, after appropriate aging, slides were subjected to SKY, a method that enables simultaneous display of all of the human chromosomes in different colors (27, 28). Additional slides were also stained with DAPI (Vector Laboratories) alone. For SKY, the slides were hybridized with the SKY probe mixture, containing combinatorially labeled painting probes for each of the autosomes and sex chromosomes [Applied Spectral Imaging (ASI), Migdal Haemek, Israel] for 42–45 h at 37°C. The hybridization and detection procedures were performed according to the manufacturer’s (ASI) protocol, and chromosomes were counterstained with DAPI in antifade solution. The multicolor hybridizations were visualized with the SpectraCube SD 200 system (ASI) mounted on the Zeiss Axioplan 2 epifluorescence microscope (Carl Zeiss, Jena, Germany) using a custom designed optical filter (SKY-1; Chroma Technology, Brattleboro, VT). The DAPI images of all of the metaphase cells hybridized with the SKY probe mixture were acquired using a DAPI-specific optical filter. Spectral analysis and classification were performed using SkyView 1.2 visualization and analysis software (ASI, Carlsbad, CA). The assignment of breakpoints in structural abnormalities was made on the comparison of images of SKY-classified chromosomes with the images of the same chromosomes stained with DAPI that were electronically inverted and contrast-enhanced by SkyView 1.2 software. Karyotypic abnormalities were classified according to the recommendations of the International System for Human Cytogenetic Nomenclature ISCN (1995) (29).

HPLC Analysis of Tamoxifen Metabolism. HMEC-P (passage 10), HMEC-LXSN (passage 10), HMEC-E6 (passage 10), and HMEC-E6 (passage 20) cells were treated for 2, 12, and 24 h with 0.1 µM [3H]tamoxifen combined with 0.9 µM unlabeled tamoxifen. The cells were then washed twice with ice-cold PBS, removed from the flask by scraping into 5 ml of ice-cold PBS, and pelleted. The pellet was extracted twice with 1 ml of methanol/ethyl acetate (1:3 v/v). The extracts were combined, dried under a stream of argon, and redissolved using the same solvent. Analysis of the extract was by HPLC using a C18 narrowbore column (Vydac). The gradient used was: (a) 75% solvent A [aqueous 1% triethylamine (Aldrich)/25% solvent B (acetonitrile/1% triethylamine (B&J) containing 1% triethylamine), which was held for 5 min after sample injection; (b) a linear gradient to 80% solvent B over 15 min; and (c) a continuation of 80% solvent B for 10 min. The flow rate was 0.3 ml/min, and 20 µl of extract, containing approximately 50,000 dpm, was injected. Samples were held in amber vials at 4°C and handled under low-light conditions. Unlabeled tamoxifen and 4-hydroxytamoxifen were used as standards and were monitored by UV absorption.

RESULTS

p53 Protein Suppression in HMECs. Retroviral-mediated expression of the HPV-16 E6 protein was used to suppress normal intracellular p53 protein levels in HMECs. Actively dividing, passage 9, AG11132 HMECs were infected with the retroviral vector LXSN16E6 or the control vector LXSN and selected as described in “Materials and Methods.” Western blots demonstrated expression of p53 protein in p53(+) HMEC-LXSN vector controls but not in p53(-) HMEC-E6 cells (passage 11 and 27; Fig. 1).

Comparison of Tamoxifen Sensitivity. p53(-) HMEC-E6 cells and p53(+) HMEC-LXSN vector controls were cultured in Standard Medium containing 0 or 1.0 µM tamoxifen. HPV-16 E6 inhibition of p53 expression was associated with a marked increase in sensitivity of early passage p53(-) HMEC-E6 cells (passage 10) to tamoxifen relative to early passage p53(+) HMEC-LXSN vector controls (passage 10; Fig. 2A). In contrast, late passage p53(-) HMEC-E6 cells (passage 18) were resistant to tamoxifen, and, although p53(+) HMEC-LXSN vector controls (passage 18) demonstrated a decreased rate of proliferation as they neared in vitro senescence, they remained sensitive to tamoxifen-mediated growth arrest (Fig. 2B). Pretreatment with 1.0 or 5.0 µM estradiol failed to block the induction of apoptosis by tamoxifen (Fig. 2C). These observations suggest that the acute loss of p53 function by the targeted expression of HPV-16 E6 in HMECs is associated with markedly enhanced sensitivity to tamoxifen-mediated cytotoxicity relative to vector controls, but that cells rapidly acquire resistance after serial passaging in vitro in the absence of tamoxifen.

FACS analysis confirmed that tamoxifen-treated p53(+)/HMEC-LXSN vector control cells underwent G1-G0-phase arrest in vitro. Treatment of p53(+)/HMEC-LXSN cells with 1.0 µM tamoxifen resulted in a 26, 35, 52, and 61% reduction in the percentage of cells in S phase and an associated 9, 16, 22, and 29% increase in the percentage of cells in G1 phase after 1, 2, 3, and 4 days of treatment, respectively.

Targeted Loss of p53 Expression in Early Passage HMECs is Associated with Sensitivity to Tamoxifen-induced Apoptosis. Early passage (passage 10) p53(-)/HMEC-E6 cells but not p53(+)/HMEC-LXSN vector controls underwent tamoxifen-mediated apoptosis in vitro as evidenced by characteristic morphological changes and by Annexin V binding.

Electron microscopy of tamoxifen-treated p53(-)/HMEC-E6 cells revealed morphological changes characteristic of apoptosis (30). The first morphological changes were observed 3–6 h after treatment. At 6 h, passage 10 p53(-)/HMEC-E6 cells that were treated with 1.0 µM tamoxifen exhibited margination of chromatin (data not shown). After 24 h, 99% of cells exhibited cell shrinkage, margination of chromatin, and formation of apoptotic bodies (Fig. 3 D and E). In contrast, passage 10 p53(+)/HMEC-LXSN did not demonstrate morphological evidence of apoptosis after treatment with 1.0 µM tamoxifen for 1, 2, or 4 days (Fig. 3B and data not shown).

Annexin V exhibits antiphospholipase activity and binds to phosphatidylserine. Cells undergoing apoptosis acquire Annexin V binding sites during apoptosis and provide a convenient method for detection of cells undergoing apoptosis. We used FITC-conjugated Annexin V followed by FACS analysis to detect the presence or absence of apoptosis in cells treated with tamoxifen (25). Ninety-nine-nine % of early passage (passage 10) p53(-)/HMEC-E6 cells treated with 1.0 µM tamoxifen for 24 h demonstrated evidence of apoptosis as demonstrated by Annexin V staining (Fig. 4D). In contrast, early passage (passage 10) p53(+)/HMEC-LXSN vector controls were resistant to tamoxifen-mediated apoptosis in vitro as evidenced by lack of Annexin V binding (Fig. 4B).
passage 10 were pretreated with 1.0 or 5.0 μM estradiol. Growth curves of p53 (+) HMEC-LXSN vector controls and p53 (−) HMEC-E6 cells at passage 10 (A), and passage 18 (B) treated with and without 1.0 μM tamoxifen. Cells were plated on day −1 in Standard Medium in duplicate at 1 × 10^4 cells per well. Cells were reseeded on day 0 with Standard Medium containing 0, 0.1, or 1.0 μM tamoxifen. Untreated controls received an equivalent volume of ethanol (0.1% final concentration). For estradiol-blocking experiments (C), p53 (−) HMEC-E6 cells at passage 10 were pretreated with 1.0 or 5.0 μM estradiol for 1 h. Cells were then treated with Standard Medium containing 0, 0.1, or 1.0 μM tamoxifen. Cells were trypsinized at 24-h time intervals and counted in triplicate. These data are representative of three separate experiments.

The induction of apoptosis by 1.0 μM tamoxifen in early passage (passage 10) p53 (−) HMEC-E6 cells was not blocked by pretreatment with the inhibitors of RNA and protein synthesis actinomycin D (1.0 μg/ml) and cyclohexamide (0.5 μg/ml; Fig. 5). Early passage p53 (−) HMEC-E6 cells, pretreated with actinomycin D or cyclohexamide for 3 h and then treated with 1.0 μM tamoxifen for 18 h, exhibited 88 and 71% Annexin V-positive cells respectively (Fig. 5, E and F). Control cells that were treated with either 1.0 μg/ml actinomycin D or 0.5 μg/ml cyclohexamide (in the absence of tamoxifen) for 21 h demonstrated 5 and 2% Annexin V positive cells, respectively (Fig. 5, A and B).

These observations suggest that (a) tamoxifen-mediated apoptosis in our in vitro system occurs in the absence of p53 expression, (b) HPV-16 E6 suppression of p53 expression in HMECs is associated with induction of tamoxifen-mediated apoptosis, and (c) tamoxifen-mediated apoptosis is independent of RNA and protein synthesis.

**Late passage p53 (−) HMECs Acquire Resistance to Tamoxifen-mediated Apoptosis.** p53 (−) HMEC-E6 cells were passaged in the absence of tamoxifen and tested for the presence or absence of tamoxifen-induced apoptosis at passages 16, 18, and 27. p53 (−) HMEC-E6 cells acquired resistance to tamoxifen at passage 18 as evidenced by lack of morphological feature characteristic of apoptosis (Fig. 3G) and loss of Annexin V binding (Fig. 4F). These observations suggest that serial passaging of HMECs lacking p53 expression results in the rapid acquisition of resistance to tamoxifen-mediated apoptosis.

**ER Expression and Estrogen-binding Assays in p53 (+) and p53 (−) HMECs.** Early and late passage (passage 10 and passage 18 respectively) p53 (+) HMEC-LXSN controls and p53 (−) HMEC-E6 cells were tested for ER expression by Northern analysis (ER-α) and by immunocytochemistry (ER-α). All of the cells exhibited a low level of ER-α mRNA and protein expression (data not shown). There was no difference in ER-α expression between tamoxifen-sensitive and -resistant cells.

p53 (−) HMEC-E6 cells and p53 (+) HMEC-LXSN vector controls were tested for estrogen binding in triplicate. p53 (+) HMEC-LXSN vector control cells, early passage p53 (−) HMEC-E6 cells, and late passage p53 (−) HMEC-E6 cells exhibited low numbers of estrogen-binding sites per cell (Table 1). ER+ T47-D positive control cells exhibited a significantly higher level of estrogen-binding sites relative to ER- MDA-MB-231 negative control cells and HMECs (Table 1). These data show that all HMEC-derived cells exhibit a low level of estrogen binding similar to that of ER− breast cancer cell lines and that there was no difference in estrogen binding in tamoxifen-sensitive and -resistant cells.

**Cytogenetic Analysis of HMECs.** Cytogenetic analysis of p53 (+) HMEC-LXSN vector controls and early and late p53 (−) HMEC-E6 cells with HPV-E6-suppressed p53 expression was performed using both spectral analysis (SKY) and DAPI staining. This was to (a) confirm that p53 (+) HMEC-LXSN control cells had a normal karyotype, (b) verify that early passage p53 (−) HMEC-E6 cells did not contain chromosomal rearrangements, and (c) test whether resistance to tamoxifen-mediated apoptosis correlated with specific chromosomal numeric changes or structural rearrangements.

Twenty early passage p53 (−) HMEC-LXSN vector control cells (passage 10) were analyzed. Nineteen (95%) p53 (+) HMEC-LXSN vector control metaphase cells had a normal diploid karyotype, including 5 cells with random chromosome loss. One cell had trisomy 20 as the sole abnormality. No p53 (+) HMEC-LXSN metaphase cell contained a whole arm translocation, a deletion, or a dicentric chromosome similar to those occurring in late passage p53 (−) HMEC-E6 cells (see next page).

A total of 20 early passage p53 (−) HMEC-E6 metaphase cells (passage 10) were karyotyped. Two cells were analyzed by SKY and
19 cells were analyzed using inverted and contrast-enhanced DAPI staining. The majority of cells (16 cells, 80%) had a normal diploid chromosome content, including 3 cells with random chromosome loss. The remaining four cells were aneuploid but had no structural changes.

In contrast to early passage cells, late passage p53\(^2\) HMEC-E6 (passage 20) were markedly abnormal, with numerical and structural chromosome aberrations. A total of 35 metaphase cells were analyzed: 27 using SKY and 8 using inverted and contrast-enhanced DAPI staining. No cell had a normal diploid karyotype. The majority of cells (28 cells, 80%) were hypodiploid, with the modal chromosome number of 44 (range, 32–84). There were five cells (14%) with a chromosome number greater than 46: two hyperdiploid (49 and 55 chromosomes), two hypotripleloid (61 and 63 chromosomes), and one hypotetraploid (84 chromosomes) cell. Moreover, only one hypodiploid cell did not display any structural chromosome rearrangement. The remaining 34 cells (97%) contained at least one structural aberration (median 3, range 1–10). The predominant types of structural changes were deletions, whole arm translocations, and dicentric chromosomes with breakpoints in the pericentromeric and/or telomeric regions. With the exception of chromosomes 4 and 11, all chromosomes participated in structural changes in at least one cell. However, the involvement of particular chromosomes in aberrations was highly nonrandom. The most frequently rearranged chromosomes were: chromosome 16 (24 cells, 69%), 21 (19 cells, 54%), 12 (16 cells, 46%), 17 (12 cells, 34%), and 20 (12 cells, 34%). In contrast, chromosomes 1, 13, and 19 were each involved in 1 cell (3%) only. The observed structural and numerical chromosome aberrations lead almost exclusively to net loss of genetic material. Fig. 6 shows the frequency with which material from specific chromosome short (p) and long (q) arms was lost because of structural aberrations or whole chromosome loss in the late passage (passage 20) p53\(^2\) HMEC-E6 cells. The most frequent losses involved the following chromosomal arms: 16p (26 cells, 74%), 12p (17 cells, 49%), 21p (17 cells, 49%), and 17p (14 cells, 40%). The high frequency of loss of 16p in late passage p53\(^2\) HMEC-E6 cells suggests that 16p harbors a gene(s)
whose loss may be important in the development of resistance to tamoxifen-induced apoptosis.

**HPLC Analysis of Tamoxifen Metabolism in Tamoxifen-sensitive and -resistant HMECs.** p53(+)-- HMEC-P parental cells (passage 8), p53(+) HMEC-LXSN controls (passage 10), early passage p53(--) HMEC-E6 cells (passage 10), and late passage p53(--) HMEC-E6 cells (passage 20) were treated with 1.0 μM radiolabeled tamoxifen and analyzed by HPLC at 24 h. There was no difference in tamoxifen metabolism in tamoxifen-sensitive and -resistant HMECs (data not shown). All radioactivity was recovered in the tamoxifen peak. Differential sensitivity to tamoxifen was not attributable to differences in tamoxifen metabolism.

**DISCUSSION**

Tamoxifen chemotherapy in ER+ tumors is thought to involve the following mechanism: ligand-bound steroid receptors bind to specific promoter elements and thereby activate or inhibit the expression of target genes. This “genomic” mechanism of tamoxifen action requires the presence of the ER and both transcription and translation. Tamoxifen has been shown to be effective in preventing ER-poor noninvasive breast cancer in women at risk for the development of malignancy, however, the molecular mechanism of tamoxifen action in noninvasive mammary tissue that does not overexpress the ER is not well understood. Therapeutic concentrations of tamoxifen are several orders of magnitude higher than required to inhibit ER function (31), and proliferating luminal mammary epithelial cells typically exhibit low levels of ER expression (32). Furthermore, recent evidence suggests that estrogens and perhaps antiestrogens, may also act through nongenomic, calcium-mediated signaling pathways. Estrogen has been shown to induce extremely rapid increases in intracellular calcium and cAMP (33–35) independent of new RNA and protein synthesis. These observations strengthen the hypothesis that estrogens and antiestrogens may act through nongenomic, as well as genomic, signal transduction pathways.

We developed a model of tamoxifen chemoprevention of ER-poor noninvasive breast cancer to test how tamoxifen may act in HMECs that have acutely lost p53 expression. The p53(--) and p53(+) HMECs used in our experiments exhibited low levels of ER expression and minimal estrogen binding (Table 1) characteristic of proliferating normal luminal mammary epithelial cells. The concentration of tamoxifen (1.0 μM) used in our chemoprevention model is similar to those plasma levels achieved in women receiving 20 mg/day of tamoxifen (36). We observed that tamoxifen rapidly induced apopto-

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**Table 1** Absence of change in estrogen-binding affinity and number of binding sites in tamoxifen-sensitive and -resistant HMECs

<table>
<thead>
<tr>
<th>Cells</th>
<th>Kd’ (nM)</th>
<th>n’ (fmol/μg DNA)</th>
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</thead>
<tbody>
<tr>
<td>T47D (ER+)</td>
<td>2.30</td>
<td>10.59</td>
</tr>
<tr>
<td>MDA-231 (ER-)</td>
<td>2.20</td>
<td>1.51</td>
</tr>
<tr>
<td>p53(+) HMEC-LXSN</td>
<td>2.09</td>
<td>1.49</td>
</tr>
<tr>
<td>p53(--) HMEC-E6 p10</td>
<td>2.20</td>
<td>1.54</td>
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<tr>
<td>p53(--) HMEC-E6 p18</td>
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<td>1.21</td>
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</table>

* Kd’, the binding affinity coefficient for estradiol.

* n’, the number of binding sites per microgram of DNA, calculated by Scatchard analysis.

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Fig. 4. Tamoxifen induces apoptosis in early passage HMECs lacking p53 expression but not in later passage cells nor in vector controls. p53(--) HMEC-E6 HPV 16-E6-transduced cells (passage 10; C, D) and passage 18 (E, F) and p53(+) HMEC-LXSN vector controls (A, B) are treated with (B, D, F) or without (A, C, E) 1.0 μM tamoxifen for 24 h. Detection of apoptotic cells was with FITC-conjugated Annexin V as described in “Materials and Methods.” Ninety-nine % of early passage (passage 10) p53(--) HMEC-E6 tamoxifen-treated cells exhibited evidence of apoptosis as demonstrated by Annexin V positivity (D). In contrast, p53(+) HMEC-LXSN vector control cells and late passage (passage 18) p53(+) HMEC-E6 cells were resistant to tamoxifen-mediated apoptosis as evidenced by lack of Annexin V staining (B, F). These data are representative of three experiments.

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Fig. 5. Tamoxifen-mediated apoptosis in early passage p53(--) HMECs is not blocked by inhibitors of RNA or protein synthesis. p53(--) HMEC-E6 HPV 16-E6-transduced cells (passage 10) were plated on day –1 in Standard Medium in duplicate at 1 × 10⁴ cells per well. On day 0, cells were first pretreated with Standard Medium containing 0.5 μg/ml cyclohexamide (A, C, E) or with 1.0 μg/ml actinomycin D (B, D, F) for 3 h. Cells were then treated with either 0, 0.1, or 1.0 μM tamoxifen for 18 h. Untreated controls received an equivalent volume of ethanol (0.1% final concentration). Cells were trypsinized at 21 h. Detection of apoptotic cells was with FITC-conjugated Annexin V as described in “Materials and Methods.” Cells were counterstained with propidium iodide to analyze DNA content. Both cyclohexamide (E)– and actinomycin D (F)–pretreated early passage (passage 10) p53(--) HMEC-E6 cells treated with 1.0 μM tamoxifen underwent apoptosis as demonstrated by Annexin V positivity. In contrast, p53(--) HMEC-E6 cells treated with either 0.5 μg/ml cyclohexamide (A) or 1.0 μg/ml actinomycin D alone (B) for 21 h exhibited 2 and 5% apoptotic cells, respectively. Pretreated p53(--) HMEC-E6 cells treated with 0.1 μM tamoxifen exhibited 2% apoptosis (C, D, respectively. These data are representative of three experiments.
sis in early passage p53(−) HMEC-E6 cells (first observed at 3 h) and was not blocked by inhibitors of RNA and protein synthesis (Fig. 5), nor was it blocked by estradiol (Fig. 2C). These observations suggest that the induction of apoptosis in p53(−) HMEC-E6 cells by tamoxifen may be activated through a nongenomic signaling pathway that is different from conventional slow-acting, ER-mediated transcription. These observations are clinically relevant because the identification of nongenomic pathways of antiestrogen action may uncover important novel targets for breast cancer prevention strategies.

Abnormal p53 expression in benign breast tissue is associated with the subsequent development of breast cancer and may represent a very early event in breast carcinogenesis (9, 10). We observe that HMECs with normal cellular p53 levels undergo G1-G0-phase arrest but not apoptosis when treated with 1.0 μM tamoxifen (Figs. 3 and 4). In contrast, early passage HMECs with HPV-16 E6-suppressed p53 levels readily undergo apoptosis when treated with 1.0 μM tamoxifen (Figs. 3 and 4). These observations provide evidence that tamoxifen-mediated apoptosis in HMECs occurs in the absence of p53 expression.

There is extensive experimental evidence supporting the role of p53 in promoting apoptosis (37, 38). However, the role of p53 in apoptosis is not unequivocal. Adenovirus-mediated expression of 17-β-estradiol-F1 in human breast and ovarian carcinoma cells lacking p53 results in apoptosis (39), and apoptosis and remodeling of mammary gland tissue during involution proceeds through p53-independent pathways (40). Furthermore, a majority of the observations delineating a requirement of p53 for effective apoptosis have been made in experimentally transformed cells lines or in cancer cell lines. Loss of p53 function confers genetic instability, and studies of p53 function in these model systems may be complicated by mutations acquired subsequent to p53 inactivation. This possibility is supported by our observation that HMECs with HPV-16 E6-suppressed p53 levels rapidly acquire resistance to tamoxifen-mediated apoptosis with in vitro passaging (Figs. 3 and 4). We observed that p53(−) HMEC-E6 exhibited marked chromosomal instability and acquired nonrandom losses of chromosome 16, 12, 21, and 17 within 10 passages of transduction (Fig. 5).

We observe a differential mechanism of action of tamoxifen in normal cells versus early passage HMECs with HPV-16 E6-suppressed p53 expression and hypothesize that the acute loss of p53 expression may sensitize HMECs to tamoxifen-mediated apoptosis. This possibility is supported by the recent observation that the acute loss of p53 expression enhances the sensitivity of fibroblasts to apoptosis induced by chemotherapeutic agents (41–43). Alternatively, it is also possible, that the expression of HPV-16 E6 may result in molecular events independent of the targeted degradation of p53. However, enhanced sensitivity to p53-independent apoptosis by paclitaxel and other chemotherapeutic agents has been observed in normal human fibroblasts expressing HPV-16 E6, human placental cells expressing SV40 T antigen, and mouse embryonic fibroblasts isolated from p53(−/−) transgenic mice (42). These observations support our hypothesis that enhanced sensitivity to apoptosis is attributable to the specific loss of p53 function and not caused by a p53-independent effect of HPV-16 E6.

The ability of tamoxifen to inhibit proliferation has been extensively studied. However, the molecular mechanism by which tamoxifen initiates apoptosis is poorly understood. Activation of apoptosis by tamoxifen in HMECs with HPV-16 E6-suppressed p53 function may be initiated by (a) tamoxifen-induced growth inhibition or (b) DNA damage induced by tamoxifen or (c) may be caused by an indirect mechanism such as the induction of a secondary mediator of apoptosis, e.g., TGF-β. The first possibility is less likely because induction of G1-G0-phase arrest by all-trans-retinoic acid in HMECs with HPV-16 E6-suppressed p53 expression does not result in apoptosis (19). We cannot exclude the possibility that tamoxifen may activate apoptosis by initiating DNA damage. Tamoxifen is thought to primarily act as an ER agonist/antagonist, but there is also evidence that tamoxifen may cause DNA damage. Recently, analysis of the p53 gene in 40 endometrial tumors indicate an excess of G:C→A:T transitions at non-CpG sites in 91% of the tumors arising in women taking tamoxifen relative to 19% in controls (44).

Although the acute inactivation of p53 expression in HMECs is associated with tamoxifen-mediated apoptosis, p53(−) HMEC-E6 cells rapidly develop resistance as they are subsequently passaged in culture in the absence of tamoxifen (Figs. 3 and 4). Acquired resistance to tamoxifen-mediated apoptosis in our in vitro system may be the result of several mechanisms. First, there might be a change in ER expression or a difference in tamoxifen metabolism in late passage p53(−) HMEC-E6 cells. However, there was no difference in ER expression, estrogen binding, or tamoxifen metabolism in tamoxifen-resistant and -sensitive HMECs. Second, there might be a loss of down-stream effector targets of the ER. Third, serial passaging of HMECs lacking p53 function in tissue culture might favor the clonal expansion of cells harboring resistance to apoptosis. We cannot exclude the second possibility; however, we have observed resistance to other mediators of apoptosis in late passage HMECs lacking p53 function (data not shown), which suggests that resistance to tamoxifen apoptosis is attributable to the evolution of a population of cells with generalized defects in the cellular apoptotic machinery.

We observe a preferential loss of chromosome 16p (74%) in late passage p53(−) HMEC-E6 cells correlating with the development of resistance to tamoxifen. Cells were analyzed at the passage in which resistance was first observed, before the potential emergence of a dominant clonal population. The high frequency of chromosome 16p loss suggests that the loss of a particular gene(s) in this region may be critical for the development of resistance to tamoxifen.

One of the most frequent aberrations observed in breast cancer is the loss of material from chromosome 16 (45, 46). However, cytogenetic, restriction fragment length polymorphism, and loss of heterozygosity analyses have primarily identified loss of chromosome 16q.
rather than 16p (45, 47–49). This does not exclude the possibility that there are small deletions involving 16p, not detected by cytogenetic analysis, or point mutations present in genes located on 16p. The role of 16p in determining sensitivity to tamoxifen-mediated growth regulation and apoptosis is unknown. It has been observed that loss of heterozygosity at 16p13 is detected in a majority of benign and malignant microdissected papillary neoplasms of the breast (50). Papillary carcinoma of the breast is a specific category of breast cancer characterized by a papillary, arborescent growth pattern with fibrovascular support. These tumors are typically low grade and among epithelial cells is unknown. It has been observed that loss of p53 is critical for tamoxifen-mediated apoptosis. Observations in our model system, suggesting that tamoxifen may be effective in preventing hormone-independent breast cancers, only if given early during mammary carcinogenesis.

In summary, we developed an in vitro model of tamoxifen chemoprevention of ER-poor noninvasive breast cancer to investigate how tamoxifen might act in HMECs that have acutely lost p53 function. We propose that the acute loss of p53, mediated in our model system, is critical for tamoxifen-mediated apoptosis. Observations in our model system have important clinical implications because they predict a critical role for early institution of tamoxifen chemoprevention before the development of p53 mutations and are consistent with clinical studies that show an association between abnormal p53 expression in breast cancer and a poor response to tamoxifen chemotherapy. By investigating how tamoxifen might target the elimination of abnormal cells in our model system, novel sites of therapeutic intervention can be identified, giving rise to more effective strategies for the prevention of breast cancer.

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Human Papillomavirus Type 16 E6 Inactivation of p53 in Normal Human Mammary Epithelial Cells Promotes Tamoxifen-mediated Apoptosis


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