Tamoxifen but Not 4-Hydroxytamoxifen Initiates Apoptosis in p53(-) Normal Human Mammary Epithelial Cells by Inducing Mitochondrial Depolarization*

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Despite the widespread clinical use of tamoxifen as a breast cancer prevention agent, the molecular mechanism of tamoxifen chemoprevention is poorly understood. Abnormal expression of p53 is felt to be an early event in mammary carcinogenesis. We developed an in vitro model of early breast cancer prevention to investigate how tamoxifen and 4-hydroxytamoxifen may act in normal human mammary epithelial cells (HMECs) that have acutely lost p53 function. p53 function was suppressed by retrovirally mediated expression of the human papillomavirus type 16 E6 protein. Tamoxifen, but not 4-hydroxytamoxifen, rapidly induced apoptosis in p53(-) HMEC-E6 cells as evidenced by characteristic morphologic changes, annexin V binding, and DNA fragmentation. We observed that a decrease in mitochondrial membrane potential, mitochondrial condensation, and caspase activation preceded the morphologic appearance of apoptosis in tamoxifen-treated early passage p53(-) HMEC-E6 cells. p53(-) HMEC-E6 cells rapidly developed resistance to tamoxifen-mediated apoptosis within 10 passages in vitro. Resistance to tamoxifen in late passage p53(-) HMEC-E6 cells correlated with an increase in mitochondrial mass and a lack of mitochondrial depolarization and caspase activation following tamoxifen treatment. We hypothesize that an early event in the induction of apoptosis by tamoxifen involves mitochondrial depolarization and caspase activation, and this may be important for effective chemoprevention.

Apoptosis, or programmed cell death, is critical for embryogenesis and for normal tissue homeostasis (1). Deregulated apoptotic signaling is felt to contribute to human cancer and autoimmune disorders (2, 3). Chemotherapeutic agents are also felt to exert many of their cytotoxic effects by induction of apoptosis, and chemotherapy resistance frequently correlates with resistance to apoptotic signaling (4).

Apoptosis is morphologically characterized by specific structural changes including margination of chromatin, cell shrinkage, and formation of apoptotic bodies (5). There is much evidence that apoptotic signaling activates highly regulated and specific proteolysis mediated by caspases (6). Caspases are a highly conserved family of aspartic acid-specific proteases that are synthesized as zymogens and are converted to active heterodimers by proteolytic cleavage (7, 8). Activated caspases are thought to be responsible, in part, for cellular changes that occur during the execution phase of apoptosis such as DNA fragmentation, chromatin condensation, and formation of apoptotic bodies (9). A large body of evidence supports a cascade model for effector caspase activation; a proapoptotic signal culminates in release of mitochondrial cytochrome c, resulting in activation of initiator caspases that, in turn, activate effector caspases, resulting in cellular disassembly (9).

Recent evidence suggests that mitochondria play a central role in apoptosis as integrators of cellular apoptotic signal transduction and in amplification of the apoptotic response (10). Disruption of mitochondrial electron transport and energy metabolism is recognized as an early event in apoptosis and precedes the appearance of morphologic changes characteristic of apoptosis (10). Mitochondrial dysfunction is characterized by an increase in mitochondrial membrane permeability and loss of membrane potential (ΔΨm). Associated with this decrease in ΔΨm, cytochrome c is translocated from the intermembrane compartment of the mitochondria to the cytosol. Cytosolic cytochrome c forms an essential part of the “apoptosome,” composed of cytochrome c, Apaf-1, and procaspase-9 (11). This results in activation of procaspase-9 and that, in turn, activates other caspases, such as caspase-3, to orchestrate the execution phase of apoptosis (10).

The estrogen agonist/antagonist tamoxifen is a triphenylethylen that has been shown to act as both a chemotherapeutic agent for the treatment of breast cancer and, more recently, as a breast cancer chemoprevention agent. The Breast Cancer Prevention Trial demonstrated a 45% reduction in breast cancer incidence among the participants who took tamoxifen, 6 years after its inception (12). This was the first study to demonstrate that a chemopreventive agent could reduce the incidence of breast cancer. However, many questions surround the results from the Breast Cancer Prevention Trial. 1) Did "true" chemoprevention occur or were the benefits of tamoxifen due to ablation of preclinical breast cancer? 2) Tamoxifen has been shown to induce both growth arrest and apoptosis (13–15). Did tamoxifen act as a cytostatic or cytotoxic agent in the Breast Cancer Prevention Trial?
p53 is a critical regulator of cell cycle control, and the high frequency with which p53 is functionally inactivated in early human breast cancer attests to its key role in preventing mammary carcinogenesis (16–18). Approximately 50% of all primary node-negative breast cancers have deleted or mutated p53, and individuals with germ line heterozygous mutations in p53, or Li-Fraumeni’s syndrome, demonstrate an increased risk of breast cancer (19–21). Furthermore, aberrant expression of p53 in mammary epithelial cells is a predictor of risk for the subsequent development of breast cancer. 1) The accumulation of p53 protein (but not c-ErbB-2) in benign breast lesions is a significant predictor for the subsequent development of breast cancer (22, 23). 2) p53 protein is frequently overexpressed (36%) in benign mammary epithelial cells obtained from high-risk women but not observed (0%) in low-risk women (22). 3) Aberrant expression of p53 in the setting of mammary hyperplasia is a significant predictor of the subsequent development of breast cancer in high-risk women (23). These observations suggest that loss of p53 function may be an early event in breast carcinogenesis.

Although abnormal expression of p53 predicts a poor response to tamoxifen chemotherapy, little is known about the fate of normal human mammary epithelial cells (HMECs)1 that acutely lose p53 function during tamoxifen chemoprevention. We sought to model tamoxifen chemoprevention in normal HMECs that have acutely lost p53 function. p53 function was suppressed utilizing retrovirally mediated introduction of human papillomavirus type 16 (HPV-16) E6 protein (24). The E6 protein of the cancer-associated HPV-16 binds to p53 and targets it for degradation through the ubiquitin pathway (25, 26) and thus p53(−) HMECs provide a model for the isolated loss of p53 function. HMEC strain AG11132 was established from normal tissue obtained at reduction mammoplasty, has a limited life span in culture, and fails to divide after ~20–25 passages. AG11132 cells exhibit a low level of estrogen receptor staining, characteristic of normal mammary cells. AG11132 was at passage 8 at the time of receipt. Cells were grown in mammary epithelial cell basal medium (Clonetech, San Diego, CA) supplemented with 4 μg/ml heparin (Clonetech) and 1 nM insulin (Upstate Biotechnology Inc., Lake Placid, NY), 10 ng/ml epidermal growth factor (Upstate Biotechnology Inc.), 0.5 μg/ml hydrocortisone (Sigma), 10−5 M isoproterenol (Sigma), 10 mM HEPES buffer (Sigma) (Standard Medium). Cells were cultured at 37 °C in a humidified incubator with 5% CO2, 95% air. Mycoplasma testing was performed as reported previously (33).

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

Retroviral Transduction—The LXSN16E6 retroviral vector containing the HPV-16 E6 coding sequence (provided by D. Galloway) has been constructed as previously described.1 AG11132 normal human mammary epithelial cells (passage 9) were plated in four T-75 tissue culture flasks in Standard Medium and grown to 50% confluency. Transducing virions from either the PA317-LXSN16E6 or the control PA317-LXSN (without insert) retroviral producer line were added at a multiplicity of infection of 1:1 in the presence of 4 μg/ml Polybrene (Sigma) to log phase cells grown in T-75 flasks. The two remaining T-75 flasks were not infected with virus. After 48 h the 2 flasks containing transduced cells and 1 flask with untransduced cells were selected with Standard Medium containing 300 μg/ml G418. Cells were continued in G418 containing medium for 1 week, until 100% of control untransduced cells were dead. The 4th flask of unselected, untransduced parental control cells was passaged in parallel with the selected, transduced experimental and vector control cells. Parental AG11132 cells are designated HMEC-P, and transduced cells expressing the HPV-16 E6 construct are designated p53(−) HMEC-E6, and vector control clones are designated p53(+) HMEC-LXSN.

Western Blotting—Preparation of cellular lysates and immunoblotting were performed as described previously (34, 35). Equal amounts of protein lysates (~100 μg of total protein) were loaded on 10% polyacrylamide gels, and the gels were run and then electroblotted (Hoeffer) at 80 mA for 45 min onto Hybond-ECL membrane (Amersham Pharmacia Biotech). The membrane was blocked with 20% bovine serum albumin (Sigma) in PBS overnight at RT and then incubated with a 1:100 dilution of mouse anti-human p53 (Oncogene Science Ab-2). The membrane was washed three to five times at RT with 250 ml of PBS containing 0.1% Tween and then incubated with either a horseradish peroxidase-conjugated goat anti-mouse 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 RT. The blot was washed again, and complexes were detected by ECL Western blotting Detection Reagents (Amersham Pharmacia Biotech) as described by the manufacturer.

High Performance Liquid Chromatography (HPLC) Analysis of Tamoxifen Metabolism—p53(+) HMEC-P parental cells (passage 10), p53(−) HMEC-LXSN vector controls (passage 10), p53(−) HMEC-E6 (passage 10), and p53(−) 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 narrow bore column (Vydac). The gradient used was as follows: 1) 75% solvent A (aqueous 1% triethylamine (Aldrich)), 25% solvent B (acetonitrile (Burdick & Jackson) containing 1% triethylamine (Aldrich)) that was held for 5 min after sample injection; 2) a linear gradient to 80% solvent B over 15 min; and 3) a continuation of 80% solvent B for 10 min. The flow rate was 0.3 ml/min, and 20 μl of extract, containing ~50,000 dpm, was injected. Samples were held in

1 The abbreviations used are: HMEC, human mammary epithelial cells; HPV-16, human papillomavirus type 16; PBS, phosphate-buffered saline; ECL, enhanced chemiluminescent detection; FACS, fluorescence-activated cell sorting; RT, room temperature; FITC, fluorescein isothiocyanate; HPLC, high performance liquid chromatography; NAO, n-nonyl acetate orange.

2 E. C. Dietze, L. E. Caldwell, S. L. Grupin, M. Mancini, and V. L. Seewald, unpublished observations.
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amber vials at 4 °C, handled under low light conditions, and monitored with an on-line scintillation detector (Packard Instrument Co.). Unlabeled tamoxifen and 4-hydroxytamoxifen were used as standards and monitored by UV absorption. 

Growth Curves—p53(+)- HMEC-LXSN vector controls and p53(-) HMEC-E6 cells were plated in duplicate at 1 × 10^6 cells per 12-well tissue culture plates on Day -1 and allowed to adhere. On Day 0 the medium was replaced with Standard Medium with or without 1.0 μM tamoxifen or 4-hydroxytamoxifen. Untreated controls received an equivalent volume of ethanol solvent (0.1% final concentration). Cells were trypsinized at 24-h time intervals and counted in triplicate.

Detection of Apoptosis—Annexin V-Staining—Annexin V-FITC (a gift from Prof. J. Engelsheim, Heidelberg, Germany) was used as per manufacturer’s recommendation with some modification. Approximately 5 × 10^6 p53(+) HMEC-LXSN or p53(-) HMEC-E6 cells were plated on T-75 flasks on Day -1 and allowed to adhere. On Day 0 the medium was replaced with fresh Standard Medium, and tamoxifen or 4-hydroxytamoxifen was added for a final concentration of 1.0 μM. Untreated controls received an equivalent volume of ethanol solvent (0.1%). Cells were then harvested at 0, 1, 6, 12, 18, or 24 h after treatment. Cells were trypsinized, washed in PBS, resuspended in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl_2, filtered through a 0.2-μm pore filter). Cell density was adjusted to 2–5 × 10^6 cells/ml. Five μl of recombinant human Annexin V-FITC and 1 μl of caspase-3 substrate (DEVD-AFC; 50 μM final concentration) was added to 195 μl of cell suspension, and 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 binding buffer. Cells were analyzed by FACSscan as described below.

Diphenylamine Assay—2.5–5.0 × 10^6 cells were plated per T-25 flask and were grown in Standard Medium. Tamoxifen or 4-hydroxytamoxifen stock was added directly to the media on Day 0 to bring the final concentration to 1.0 μM. Confluency did not exceed 70%. Cells were trypsinized 0, 6, 12, 15, or 24 h after treatment, washed in cold PBS, pelleted, and then resuspended in 100 μl of lysis buffer (5 mM Tris, pH 8.0, 20 mM EDTA, 0.5% Triton X-100) on ice for 15 min. The lysate was spun at 12,000 rpm for 30 min in a refrigerated microcentrifuge. The supernatant was removed and transferred to a second microcentrifuge tube. Both tubes were placed on ice, and 1.0 ml of 0.5 N perchloric acid was added to the nuclear pellet in the first tube and vortexed. Five hundred μl of 1 N perchloric acid (Day 1) was added to the cytoplasmic fraction in the second tube and vortexed. Both tubes were spun at 12,000 rpm for 15 min. The supernatants were then discarded, and 1.0 ml of 0.5 N perchloric acid was added to the pellets. The tubes were heated to 70 °C for 20 min to hydrolyze the DNA, then cooled to RT, and 1.0 ml of diphenylamine solution (1.5 g of diphenylamine (Aldrich) in 100 ml of glacial acetic acid, to which was added 1.5 ml of sulfuric acid and 0.1 ml of 1.0 M diphenylamine solution) was added on the day of assay. The mixture was incubated at 37 °C for 15 min. The color developed in 10 min was measured by absorbance at 5386 nm (36).

Transmission Electron Microscopy—p53(-) HMEC-E6 cells and p53(+) HMEC-LXSN vector control cells were plated on Day -1 in 6-well tissue culture plates. On Day 0 cells were treated with 1.0 μM tamoxifen or 4-hydroxytamoxifen for 0, 1, 3, 6, 12, 18, and 24 h. Cells were then fixed in half-strength Karnovsky’s fixative (37) for 6 h, rinsed in 0.1 M sodium cacodylate buffer, and post-fixed in 1% collidine-buffered osmium tetroxide. Dehydration in graded ethanol and propylene oxide was followed by infiltration and embedding in Epon 812. Approximately 70–90-nm sections were stained using saturated aqueous uranyl acetate and lead tartrate.Photographs were taken using a JEOL 100 SX transmission electron microscope operating at 80 kV. Approximately 200 cells were surveyed per data point following treatment for the presence or absence of apoptosis.

Caspase Assays—Activated caspase-3 and -9 were detected utilizing the ApoAlert™ Caspase Fluorescent Assay Kit (CLONTECH). p53(-) HMEC-E6 cells and p53(+) HMEC-LXSN vector control cells were plated on Day -1 and treated with 1.0 μM tamoxifen or 4-hydroxytamoxifen in duplicate. Cells were trypsinized and counted, and 1 × 10^6 cells were pelleted and frozen at −80 °C. On the day of assay, the pellet was resuspended in 150 μl of binding buffer (CLONTECH), and incubated on ice for 10 min. Cell lysates were centrifuged for 3 min at 4 °C to remove debris, and lysates were then transferred to a new microcentrifuge tube. Fifty μl of 2× Reaction Buffer (CLONTECH), with 10 μl of 1 μl diethiothreitol, and 5.0 μl of either 1.0 mM caspase-3 substrate (DEVD-AMC; 50 μM final concentration) or 5 μM caspase-9 substrate (LEHD-AMC; 250 μM final concentration) was added to each tube and incubated for 1 h at 37 °C. To confirm the

RESULTS

p53 Protein Suppression in HMECs—Retrovirally mediated expression of the HPV-16 E6 protein was utilized to suppress normal intracellular p53 protein levels in HMECs. Western blots were performed on p53(+) HMEC-LXSN vector controls (passages 10 and 18) and p53(-) HMEC-E6 transduced cells (passages 10 and 18) to determine the relative levels of p53 protein expression. Expression of p53 protein was observed in p53(+) HMEC-LXSN vector controls but was not detectable in HMECs (Fig. 1).

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

FIG. 1. p53 protein expression is suppressed in HMECs transduced with HPV-16 E6. p53(+) HMEC-LXSN vector controls (LXSN) (passages 10 and 18) and p53(-) HMEC-E6 transduced cells (E6) (passages 10 and 18) are analyzed for p53 protein expression as described under “Experimental Procedures.” Equal amounts of protein lysate were loaded per lane. An unknown 45-kDa protein band was used as a loading control.

The correlation between protease activity and product formation, either 1.0 μl of caspase-3 inhibitor (DEVD-CHO) or 2.0 μl of caspase-9 inhibitor (LEHD-CHO) was added to the reaction mixture of an induced sample and incubated for 1 h at 37 °C before adding the caspase-3 or caspase-9 substrate, respectively. Samples were read in a Shimadzu RF-1501 spectrophotometer at a 400 nm excitation filter and a 505 nm emission filter (caspase-3) or 380 nm excitation filter and a 460 nm emission filter.

Assessment of Mitochondrial Changes—Mitochondrial transmembrane potential was measured by rhodamine 123 (Molecular Probes) (38) and JC-1 red fluorescence (CLONTECH) (39). JC-1 mitochondrial aggregate formation was measured by JC-1 green fluorescence (CLONTECH) (39). Relative mitochondrial mass was measured by flow cytometry using 1-nonyl acridine orange (NAO; Molecular Probes) (40). For rhodamine 123 staining, 1 × 10^6 cells/ml were incubated at 37 °C in 0.5 mg/ml rhodamine 123. For JC-1 staining, 1 × 10^6 cells incubated with 10 μg/ml JC-1 for 10 min at 37 °C and were analyzed for red and green fluorescence. For NAO staining, 1 × 10^6 cells were resuspended in 1.0 ml of 1.0 μM NAO in PBS. Fluorescence of individual nuclei and whole cells was performed using a FACScan flow cytometer equipped with an argon-ion laser at 488 nm and 250 milliwatts light output and Lysis II software (Becton Dickinson Immunocytometry Systems). Forward and side scatter were used to establish size gates and exclude cellular debris. The excitation wavelength was 488 nm. The observation wavelengths were 530 nm for green fluorescence and 585 nm for red fluorescence. The red and green JC-1 fluorescence emissions from each cell were separated and measured using the standard optics of the FACScan. Ten thousand events were collected in list mode fashion, stored, and analyzed on Multicycle AV software (Phoenix Flow Systems).

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radiolabeled tamoxifen and analyzed by HPLC at 24 h. There was no difference in tamoxifen metabolism in p53(−) or p53(+) HMECs (data not shown). No tamoxifen was metabolized to 4-hydroxytamoxifen, and all radioactivity was recovered in the tamoxifen peak.

*p53(−) HMEC-E6 Cells Exhibit Increased Tamoxifen Cytotoxicity*—p53(−) HMEC-E6 cells and p53(+) HMEC-LXSN vector controls were cultured in Standard Medium containing 1.0 μM tamoxifen or 4-hydroxytamoxifen. 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 cytotoxicity relative to early passage p53(+) HMEC-LXSN vector controls (passage 10) (Fig. 2A). In contrast, early passage p53(+) HMEC-E6 cells and p53(+) HMEC-LXSN controls treated with 1.0 μM 4-hydroxytamoxifen exhibited similar cytotoxicity (Fig. 2A). Late passage p53(−) HMEC-E6 cells (passage 18) were resistant to both tamoxifen and 4-hydroxytamoxifen (Fig. 2B). Although p53(+) HMEC-LXSN vector controls (passage 16) demonstrated a decreased rate of proliferation as they neared in vitro senescence, they remained sensitive to tamoxifen and 4-hydroxytamoxifen-mediated growth arrest (Fig. 2B). These observations suggest that the acute loss p53 function by the targeted expression of HPV-16 E6 in HMECs is associated with enhanced sensitivity to tamoxifen-mediated cytotoxicity but not that of 4-hydroxytamoxifen. p53(−) HMEC-E6 rapidly acquire resistance to both tamoxifen and 4-hydroxytamoxifen cytotoxicity after serial passaging in vitro.

Tamoxifen but Not 4-Hydroxytamoxifen Induces Apoptosis in Early Passage p53(−) HMEC-E6 Cells—We investigated the mechanism by which tamoxifen might potentiate increased cytotoxicity in early passage p53(−) HMEC-E6 cells. We observed that early passage p53(−) HMEC-E6 cells treated with tamoxifen underwent apoptosis as evidenced by annexin V binding, characteristic morphologic changes, and by the presence of fragmented cytoplasmic DNA. Cells were trypsinized and counted in triplicate. These data are representative of three separate experiments.

**Fig. 2.** Acute suppression of p53 protein expression in HMECs is associated with increased tamoxifen but not 4-hydroxytamoxifen cytotoxicity. Growth curves of p53(+) HMEC-LXSN vector controls passage 10 (A) and passage 16 (B) and p53(−) HMEC-E6 cells at passage 11 (A), and passage 18 (B) treated with and without 1.0 μM tamoxifen (TAM) and 4-hydroxytamoxifen (4-OH) are shown. Cells were plated on Day −1 in Standard Medium in duplicate at 1 × 10⁴ cells per well and treated on Day 0 with 0 or 1.0 μM tamoxifen or 4-hydroxytamoxifen. Untreated controls received an equivalent volume of ethanol (0.1% final concentration). Cells were trypsinized and counted in triplicate. These data are representative of three separate experiments.
with tamoxifen. Ninety nine percent of early passage p53(-) HMEC-E6 cells (passage 10) treated with 1.0 μM tamoxifen for 24 h demonstrated evidence of apoptosis as demonstrated by annexin V staining (Fig. 3D). In contrast, early passage p53(-) HMEC-E6 cells did not undergo apoptosis by this measure when treated with 1.0 μM 4-hydroxytamoxifen (Fig. 3E). In addition p53(+) HMEC-LXSN vector controls (passage 10) and late passage p53(-) HMEC-E6 cells (passage 18) and are treated with 1.0 μM tamoxifen (TAM) (B, D, and G) or 1.0 μM 4-hydroxytamoxifen (OHT) (E) for 24 h. Untreated control cells (A, C, and F) received an equivalent volume of ethanol. Detection of apoptotic cells was with FITC-conjugated annexin V as described under “Experimental Procedures.” These data are representative of three experiments.

Electron microscopy of tamoxifen-treated early passage p53(-) HMEC-E6 cells (passage 10) revealed morphologic changes characteristic of the effector phase of apoptosis including margination of chromatin, cell shrinkage, and formation of apoptotic bodies (5). Margination of chromatin was the first morphologic change detected and was observed 12 h after treatment with 1.0 μM tamoxifen (Fig. 4E). After 24 h, 99% of cells exhibited cell shrinkage, condensed chromatin, and formation of apoptotic bodies (Fig. 3D). In contrast, early passage p53(-) HMEC-E6 cells treated with 1.0 μM 4-hydroxytamoxifen did not exhibit evidence of apoptosis by morphologic criteria (data not shown). In addition, neither p53(+) HMEC-LXSN vector controls (passage 10) nor late passage p53(-) HMEC-E6 cells (passage 21) demonstrated morphologic evidence of apoptosis after treatment with 1.0 μM tamoxifen for 24 h (Fig. 4, B and H, and data not shown).

The diphenylamine assay measures endonuclease-fragmented DNA present in the cytoplasm of apoptotic cells (42). Time points were obtained from 0 to 24 h after treatment of the target cells with either 1.0 μM tamoxifen or 4-hydroxytamoxifen. Early passage p53(-) HMEC-E6 cells (passage 12) treated with tamoxifen demonstrated increased DNA fragmentation starting at 18 h (Fig. 5B). The percentage of fragmented DNA for tamoxifen-treated early passage p53(-) HMEC-E6 cells was 17% at 18 h and 56% at 24 h. In contrast, early passage p53(-) HMEC-E6 cells treated with 4-hydroxytamoxifen did not demonstrate fragmented DNA (Fig. 5B). In addition, no significant increase in DNA fragmentation was observed in p53(+) HMEC-LXSN vector controls (passage 12) and late passage p53(-) HMEC-E6 cells (passage 18) treated with 1.0 μM tamoxifen for 24 h (Fig. 5, A and B).

These observations indicate that tamoxifen but not 4-hydroxytamoxifen induces apoptosis in early passage p53(-) HMEC-E6 cells. Expression of HPV-16 E6 appears to sensitize HMECs to apoptosis as p53(+) HMEC-LXSN cells do not undergo apoptosis. Resistance to tamoxifen-mediated apoptosis develops rapidly (within 10 passages) as apoptosis is not observed in tamoxifen-treated late passage p53(-) HMEC-E6 cells.
**Mitochondrial Ultrastructure in Tamoxifen-sensitive and -resistant HMECs**—Changes in mitochondrial ultrastructure correlated with the development of resistance to tamoxifen-induced apoptosis in p53(-) HMEC-E6 cells. Mitochondria in untreated p53(+) HMEC-LXSN cells (passage 10) appear similar to those in untreated early passage p53(-) HMEC-E6 cells (passage 10) (Fig. 6, A and C). Electron micrographs depict oval-shaped mitochondria with a finely granular matrix. Cristae are few in number. In contrast, mitochondria in apoptosis-resistant, late passage untreated p53(-) HMEC-E6 cells (passage 21) are increased in number, are longer, and exhibit a branched morphology (Fig. 6G).

Sequential changes in mitochondrial ultrastructure were identified in tamoxifen-treated early passage p53(-) HMEC-E6 cells undergoing apoptosis but not in tamoxifen-treated p53(+) HMEC-LXSN cells or in late passage p53(-) HMEC-E6 cells. One hour after treatment with 1.0 μM tamoxifen the mitochondrial matrix in early passage p53(-) HMEC-E6 cells (passage 10) is condensed, and the cristae are well formed, transversing the mitochondrion (Fig. 6D). Six hours following treatment with tamoxifen, mitochondria in early passage p53(-) HMEC-E6 cells exhibit further morphologic changes. Mitochondria are small, and the outer mitochondrial membrane appears indistinct, and internal structures are obscured by highly electron-dense material (Fig. 6E). Overall, these changes are consistent with mitochondrial matrix condensation and volume loss. After 6 h of treatment, nuclear chromatin is normal, and there is no morphologic evidence of apoptosis (data not shown). At 12 h, a majority of cells exhibited margination of chromatin and mitochondrial condensation (Fig. 4E and data not shown). In contrast, tamoxifen-treated p53(+) HMEC-LXSN vector controls (passage 10) and late passage p53(-) HMEC-E6 cells (passage 21) do not exhibit changes in mitochondrial morphology after 24 h of treatment (Fig. 6, B and H). These observations indicate that mitochondrial matrix condensation, starting at 1 h, precedes execution phase morphologic changes in early passage p53(-) HMEC-E6 cells treated with tamoxifen but not 4-hydroxytamoxifen.

**Caspase Activation in Tamoxifen-sensitive and -resistant HMECs**—Caspases are thought to be responsible, in part, for cellular changes that occur during apoptosis such as DNA fragmentation, chromatin condensation, and formation of apoptotic bodies. Effector caspases are constitutively expressed in their inactive form and are activated through intracellular caspase cascades. We investigated the relationship between caspase-9 and caspase-3 activation in tamoxifen-treated, apoptosis-sensitive and -resistant HMECs.

Recent evidence suggests that apoptosis can be triggered by inducing mitochondrial release of cytochrome c. The initiator caspase, caspase-9, is activated by mitochondrial release cytochrome c and that in turn activates caspase-3 (9). Activation of caspase-9 was observed in apoptosis-sensitive, early passage p53(-) HMEC-E6 cells (passage 11) treated with 1.0 μM tamoxifen but not 1.0 μM 4-hydroxytamoxifen for 6 h (Fig. 7A). In contrast, p53(+) HMEC-LXSN vector controls (passage 12) and apoptosis-resistant, late passage p53(-) HMEC-E6 cells (pas-
three separate experiments.

Electron microscopy during tamoxifen-induced apoptosis were
used to examine the mitochondrial abnormalities observed by staining with NAO, a fluorescent dye that specifically binds to the mitochondrial inner membrane independent of the transmembrane potential. To measure mitochondrial potential \( (\Delta \Psi_m) \), cells were stained with rhodamine 123 (38) or the J-aggregate forming cationic dye JC-1 (39). JC-1 is a dye that normally exists as a monomer emitting green fluorescence. JC-1 is taken up by mitochondria and in response to the mitochondrial membrane potential forms multimers, which then emit red fluorescence (39). Decreasing mitochondrial transmembrane potential results in a decrease in JC-1 red fluorescence and an increase in JC-1 green fluorescence.

The relative mitochondrial mass was similar in p53(+) HMEC-LXSN cells (passage 12) and early passage p53(−) HMEC-E6 cells (passage 11) (1.0 \( \pm 0.05 \) and 0.92 \( \pm 0.07 \), respectively) (Table I). These data are consistent with the observation made by electron microscopy that there is a qualitative increase in the number of mitochondria in apoptosis-resistant p53(−) HMEC-E6 cells relative to apoptosis-sensitive p53(−) HMEC-E6 cells and p53(+) vector controls (Fig. 4). These observations suggest that an increase in mitochondrial mass may be associated with the development of resistance to tamoxifen-induced apoptosis.

As measured by rhodamine 123 and JC-1 red fluorescence, \( \Delta \Psi_m \) did not decline in p53(+) HMEC-LXSN or late passage p53(−) HMEC-E6 cells (passage 19) after treatment with 1.0 \( \mu \text{M} \) tamoxifen for 6 h, and there was no change in JC-1 green fluorescence in late passage p53(−) HMEC-E6 cells treated with 1.0 \( \mu \text{M} \) tamoxifen for 6 h (Fig. 9, G and H). However, \( \Delta \Psi_m \) declined by 17–20% after early passage p53(−) HMEC-E6 cells (passage 11) were treated with 1.0 \( \mu \text{M} \) tamoxifen (Table I). JC-1 red fluorescence decreased 32–66% after 3 and 6 h of tamoxifen treatment (data not shown). Increased JC-1 green fluorescence was observed in these cells at 1, 3, and 6 h (Fig. 9, B–D) in association with the decreased JC-1 red fluorescence. In contrast, treatment of early passage p53(−) HMEC-E6 cells treated with 1.0 \( \mu \text{M} \) 4-hydroxytamoxifen showed no change in either rhodamine 123 and JC-1 red fluorescence (data not shown) or JC-1 green fluorescence (Fig. 9, E and F). p53(+) HMEC-LXSN vector controls (passage 12) and apoptosis-resistant p53(−) HMEC-E6 cells (passage 19) did not undergo apoptosis when treated with tamoxifen and did not exhibit a decline in \( \Delta \Psi_m \). Changes in \( \Delta \Psi_m \) observed at 1 h in tamoxifen-treated early passage p53(−) HMEC-E6 cells (passage 11) correlate with the detection at 1 h of mitochondrial condensation by electron microscopy (Fig. 6) and caspase-9 activation (Fig. 7).

To Compare the Base-line Values of \( \Delta \Psi_m \) in apoptosis-sensitive and -resistant cells, \( \Delta \Psi_m \) was normalized to mitochondrial mass (\( \Delta \Psi_m/\text{NAO fluorescence} \)). The normalized values of \( \Delta \Psi_m \) were decreased in both untreated apoptosis-sensitive (passage 11) and untreated apoptosis-resistant (passage 19) p53(−) HMEC-E6 cells relative to p53(+) HMEC-LXSN vector control cells (passage 12) (Table I). The decreased base-line \( \Delta \Psi_m \) and normal mitochondrial content in apoptosis-sensitive p53(−) HMEC-E6 cells correlates with sensitivity to tamoxifen-induced apoptosis.

**DISCUSSION**

Apoptosis is a dynamic process that involves initiation by a pharmacologic or DNA-damaging agent, activation of proteolytic enzymes, and execution of characteristic morphologic changes. Mitochondria serve as sensors and amplifiers of the apoptotic process (43). Diverse apoptotic stimuli converge at the mitochondria resulting in activation of the caspase proteolytic cascade that ultimately leads to cellular disassembly (9).
In this study, we present evidence that the acute suppression of p53(−) function in HMECs, mediated by HPV-16 E6, results in increased sensitivity to tamoxifen-induced apoptosis via a signaling pathway that involves mitochondrial depolarization and caspase activation. In contrast, the related metabolite, 4-hydroxytamoxifen fails to induce either mitochondrial depolarization or caspase activation and subsequently does not induce apoptosis.

We observe that early passage p53(−) HMEC-E6 cells treated with tamoxifen for 12–24 h exhibit morphologic and biochemical changes characteristic of the execution phase of apoptosis. These apoptotic changes are preceded by mitochondrial matrix condensation starting at 1 h (D). At 6 h after tamoxifen treatment, mitochondria exhibit increased mitochondrial matrix condensation (E) that is evident also at 24 h (F). Mitochondria in apoptosis-resistant, late passage p53(−) HMEC-E6 cells are increased in number and exhibit a branching morphology (G). There is no evidence of mitochondrial condensation in late passage p53(−) HMEC-E6 cells after 24 h treatment with 1 μM tamoxifen. Magnification is ×20,000.

In this study, we present evidence that the acute suppression of p53(−) function in HMECs, mediated by HPV-16 E6, results in increased sensitivity to tamoxifen-induced apoptosis via a signaling pathway that involves mitochondrial depolarization and caspase activation. In contrast, the related metabolite, 4-hydroxytamoxifen fails to induce either mitochondrial depolarization or caspase activation and subsequently does not induce apoptosis.

We observe that early passage p53(−) HMEC-E6 cells treated with tamoxifen for 12–24 h exhibit morphologic and biochemical changes characteristic of the execution phase of apoptosis. These apoptotic changes are preceded by mitochondrial matrix condensation, mitochondrial depolarization (∆Ψm), and caspase-9 activation, all first observed 1 h after tamoxifen treatment. p53(−) HMEC-E6 cells rapidly developed resistance to tamoxifen-induced apoptosis after about 10 passages in vitro. Resistance to tamoxifen was associated with an increase in mitochondrial mass, the appearance of a branching mitochondrial morphology, and lack of membrane depolarization and caspase activation following tamoxifen treatment. Taken together, these observations suggest a critical role for mitochondrial signaling in mediating sensitivity to tamoxifen-induced apoptosis.

To date, most studies have investigated tamoxifen action using human breast cancer cell lines that contain complex chromosomal rearrangements and are a poor model for chemoprevention. Although the importance of p53 as a tumor suppressor is well documented, little is known about the fate of normal human cells that acutely lose p53 function in the context of tamoxifen chemoprevention. A majority of cellular studies investigating the role of p53 in tamoxifen sensitivity 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. We observe that p53(−) HMEC-E6 cells are genetically unstable and acquire major chromosomal rearrangements and deletions within 10 passages of transduction in vitro.

It has been observed recently that the acute loss of p53 function results in enhanced sensitivity to apoptosis in normal human fibroblasts expressing HPV-16 E6, human placental cells expressing SV40 T antigen, and mouse embryonic fibroblasts isolated from p53−/− transgenic mice (44–46). We compared base-line mitochondrial membrane potential (∆Ψm) standardized to mitochondrial mass (Mm) in p53(+) and p53(−) HMECs to investigate whether a decrease in base-line ∆Ψm/Mm might correlate with sensitivity to tamoxifen-induced apoptosis. We observed that ∆Ψm/Mm in apoptosis-insensitive p53(+)/HMEC-LXSN controls was significantly increased relative to apoptosis-sensitive early passage p53(−)/HMEC-E6 cells. This observed base-line mitochondrial membrane depolarization may provide a mechanism for the increased sensitivity of early passage p53(−) HMECs to apoptotic stimuli. Interestingly, the development of tamoxifen resistance in late passage p53(−) HMEC-E6 cells was not the result of increased apoptosis.

3. K. Mrózek and V. L. Seewaldt, unpublished observations.
Recently it has been observed that there is an increase in intracellular calcium and cAMP accumulation of 4-hydroxytamoxifen in primary breast cancers that have acquired resistance to tamoxifen chemotherapy (49).

In addition, significantly higher levels of 4-hydroxytamoxifen, relative to tamoxifen, were observed in the plasma samples taken from the acquired resistance group (49). These observations lead the investigators to hypothesize that perhaps the appearance of increased levels of 4-hydroxytamoxifen could account for tamoxifen resistance and are consistent with observations in our in vitro system.

Although the tamoxifen metabolite, 4-hydroxytamoxifen, is detected in the plasma and tissue of women treated with tamoxifen, we do not detect 4-hydroxytamoxifen in tamoxifen-treated HMECs by HPLC. This observed lack of tamoxifen metabolism allows us to assess independently the ability of tamoxifen to induce apoptosis in early passage p53(-) HMEC-E6 cells. Since 4-hydroxytamoxifen has a higher affinity for the estrogen receptor than does tamoxifen, we expected that if apoptosis was initiated via an estrogen receptor-derived signal, 4-hydroxytamoxifen would exhibit equal or increased ability to induce apoptosis relative to tamoxifen (18, 30). We observe, however, that whereas tamoxifen is able to induce apoptosis in p53(-) HMEC-E6 cells, 4-hydroxytamoxifen induces growth arrest alone.

It is hypothesized that patients treated with tamoxifen may exhibit de novo or acquired resistance through changes in drug metabolism. Recently it has been observed that there is an accumulation of 4-hydroxytamoxifen in primary breast cancers that have acquired resistance to tamoxifen chemotherapy (49).
Tamoxifen Initiates Apoptosis in p53(−) HMECs

Table I

| Treatment          | E6(E) | LXSN | E6(L) |
|--------------------|-------|------|-------|
| NAO                | 0-h Tam | 1.00 ± 0.05 | 0.92 ± 0.07 | 1.48 ± 0.09 |
| JC-1 red           | 0-h Tam | 1.00 ± 0.04 | 1.28 ± 0.03 | 1.50 ± 0.05 |
| JC-1 red/NAO       | 0-h Tam | 0.66 ± 0.06 | 1.30 ± 0.04 | 1.47 ± 0.06 |
| Rhodamine          | 0-h Tam | 1.00 ± 0.05 | 1.49 ± 0.07 | 1.01 ± 0.08 |
| Rhodamine/NAO      | 0-h Tam | 0.88 ± 0.06 | 1.23 ± 0.06 | 1.59 ± 0.04 |

Mitochondrial potential (∆Ψm) is measured by rhodamine 123 staining and by JC-1 (red) fluorescence and normalized to mitochondrial mass, measured by NAO staining. Mitochondrial mass in apoptosis-resistant, late passage p53(−) HMEC-E6 cells (passage 21) is relative to p53(+) HMEC-LXSN vector controls (passage 11). Base-line mitochondrial potential normalized to mitochondrial mass (JC-1 red (∆Ψm)/NAO or rhodamine (∆Ψm)/NAO fluorescence) in p53(−) HMEC-E6 cells is relative to p53(+) HMEC-LXSN vector controls. Fluorescent values are reported relative to ethanol-treated controls. Reported values represent the average of three separate experiments. Tam, tamoxifen.

Acknowledgments—We are indebted to Judy Goombridge and Franque Remington for the preparation of electron microscopy specimens.

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FIG. 9. Decreased JC-1 aggregates are observed in tamoxifen-treated early passage p53(−) HMEC-E6 cells. Early passage p53(−) HMEC-E6 cells (passage 11) (E6/E) treated with 1.0 μM tamoxifen (TAM) for 0, 1, 3, and 6 h (A–D, respectively) and 1.0 μM 4-hydroxytamoxifen (OHT) for 6 and 12 h (E and F). Apoptosis-resistant, late passage p53(−) HMEC-E6 cells (passage 19) (E6/L) treated with (H) and without (G) 1.0 μM tamoxifen for 6 h.

and 4-hydroxytamoxifen are structurally and functionally very similar but differ in their affinity for calmodulin (53). Calmodulin has been shown recently to be a mediator of apoptosis in several cell systems (54, 55), and calcium/calmodulin-dependent protein kinase has been shown to regulate apoptosis through the death-associated protein kinase-2 (31, 57). We speculate that apoptosis mediated by anti-estrogens in HMECs with low levels of estrogen receptor expression may involve a calcium-mediated signaling pathway, perhaps modulated by calmodulin binding.

Cumulatively, the data presented in this study support the hypothesis that tamoxifen-mediated apoptosis in early passage p53(−) HMEC-E6 cells rapidly occurs via a mitochondrial signaling pathway, requiring mitochondrial membrane depolarization and the activation of caspase-3 and caspase-9. Whereas tamoxifen readily induces apoptosis in early passage p53(−), the tamoxifen metabolite 4-hydroxytamoxifen does not induce mitochondrial changes and hence does not induce apoptosis. These data suggest a need to investigate the relative contributions of genomic and nongenomic mechanisms in the induction of apoptosis by tamoxifen, an activity that is likely to support the development of novel pharmacologic agents for breast cancer chemoprevention.
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