Effects of Tamoxifen on Telomerase Activity in Breast Carcinoma Cell Lines

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BACKGROUND. The authors tested the effects of the antiestrogenic agent tamoxifen on telomerase activity and cell proliferation in MCF-7 and MDA-MB-231 breast carcinoma cell lines. MCF-7 cells belong to a known estrogen receptor positive cell line, whereas MDA-MB-231 cells, previously thought to be estrogen receptor negative, are now shown to contain estrogen receptor-β.

METHODS. Both cell lines were grown in the presence of tamoxifen 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} M for 10 days. Cells in separate flasks were harvested daily for determination of total cell number, protein was extracted for determination of telomerase activity, and RNA was extracted for reverse transcriptase–polymerase chain reaction analysis to measure expression levels of the telomerase components (the RNA component and the catalytic subunit) and estrogen receptors.

RESULTS. Total cell counts and telomerase activity levels of both cell lines with 10^{-8} M tamoxifen treatment were lower than control cells and other tamoxifen treatments. Changes in the expression of individual telomerase components correlated with telomerase activity. Estrogen receptor status did not correlate with telomerase activity.

CONCLUSIONS. Tamoxifen strongly affected both cell count and telomerase activity within the 10^{-8} M concentration of both cell lines. Cells were able to overcome drug inhibition at all other doses after 4 days. Telomerase activity and cell proliferation were correlated in both cell lines and depended on drug concentration. Tamoxifen showed long term effects on cell proliferation of the MCF-7 cells. ***Cancer* 1999;85:1523–9.** *© 1999 American Cancer Society.*

KEYWORDS: telomerase, tamoxifen, breast carcinoma, estrogen receptor.

The fight against breast carcinoma has spawned many different treatment options as well as improved means of early detection. Mokbel and Ghilchik suggested studying the effects of tamoxifen, one of the most widely used treatment options, on expression of the enzyme telomerase,[1] both a useful biomarker for breast carcinoma and a potential target. This study deals with the effects of tamoxifen on telomerase activity in estrogen positive and negative cells.

Tamoxifen is a hormonal agent (antiestrogen) touted as the endocrine treatment of choice for all stages of breast carcinoma.[2] It is normally recommended for women who are postmenopausal or older than 50 years whose tumors have a positive estrogen receptor status. One mechanism of action for tamoxifen is to compete with estrogen by binding to the estrogen receptors to inhibit cancer cell growth and hopefully to prevent breast carcinoma recurrence. This agent is also known to work through growth factors and the immune system to provide some benefit even to patients whose tumors are not estrogen sensitive.[3]

Tamoxifen has been included in several major studies, including

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TABLE 1
Primer Sets for Reverse Transcriptase–Polymerase Chain Reaction

Human telomerase RNA component (HTR)
Forward (13–34) 5′-AGTTCGCTTTCCTGTTGGTGGGG-3′
Reverse (102–81) 5′-TCAGGTTTGGGGGTTCACAAGC-3′
Reverse transcriptase catalytic subunit (RT)
Forward (103–126) 5′-TTTCTGGAGCTGCTTGGGAACCAC-3′
Reverse (409–388) 5′-TGAACTTCTTGGTGTTCCTGAG-3′
Estrogen receptors[19] (ER-α)
Forward (1060–1083) 5′-CAGGGGTGAAGTGGGGTCTGCTG-3′
Reverse (1543–1520) 5′-ATGCGGAACCGAGATGATGTAGC-3′
β_2 microglobulin
Forward (1477–1504) 5′-GTGGAGCATTCAGACTTGTCTTTCAGCA-3′
Reverse (3537–3511) 5′-TCACTCAATCCAAATGCGGCATCTTCA-3′

the National Surgical Adjuvant Breast and Bowel Project (NSABP), with over 13,000 patients enrolled from the U.S. and Canada. The project was terminated early due to reports that the agent reduces the incidence of breast carcinoma in high risk patients by up to 45% despite other reports that this agent also increased the risk of endometrial carcinoma.[4] Women in the study were unblinded to their treatment arms, and patients in the placebo group were given the opportunity to switch to tamoxifen. The impact of this announcement was furthered by a later announcement by the Early Breast Cancer Trialist's Collaborative Group,[5] who combined 55 independent trials with 37,000 overall patients showing that tamoxifen therapy given to women with estrogen receptor positive or unknown estrogen receptor status, regardless of age or menopausal status, significantly improved overall survival over a 10-year period.

Telomeres are essential portions of eukaryotic linear chromosomes consisting of repeating DNA sequences in which the human repeat sequence is (TTAGGG)n. Telomeres stabilize the chromosome, prevent degradation of DNA, and act as a signal for cell senescence.[6,7] Due to an "end replication" problem, DNA polymerase is not able to completely copy the 3′ end of the telomere, and this results in an estimated loss of 50–200 bp during each round of replication. Eventually, these telomeres reach a critical length and the cell dies. This problem is alleviated in some cells by the enzyme telomerase.[8,9] Telomerase is a ribonucleoprotein that basically "replaces" the lost telomeric DNA with new repeats. Telomerase has a specialized reverse transcriptase activity that allows it to use its RNA template to form new DNA repeats onto the shortened end of a telomere. Telomerase activity has been recognized as a useful tool in the early detection of cancer (detectable in over 85% of all tumors), and semiquantitative measurements have also shown a correlation with prognosis in a few tissue types.[10] Portions of the telomerase enzyme have been recently sequenced: the RNA component that acts as a template for new telomeric repeats (HTR),[11] a telomerase-associated protein of yet-unknown function (TAP),[12] and the catalytic subunit of the enzyme that confers the reverse transcriptase activity (RT).[13]

There have been several key articles describing telomerase activity in breast carcinomas. Hiyama et al. and others have detected telomerase activity in up to 95% of breast tumors.[14,15] Yashima et al. and others have also shown that telomerase activity increases with tumor aggressiveness or histopathologic staging.[16,17] Recently, Poremba et al. reported that telomerase activity correlates with cell proliferation.[18] These articles gave rise to the importance of studying the effects of tamoxifen on telomerase activity.[1]

We examined the effects of tamoxifen on estrogen positive and estrogen negative breast carcinoma cell lines, focusing on telomerase activity and cell growth over time. We also characterized the effects of these drugs on the level of expression of the telomerase RNA component, the catalytic subunit of the enzyme, and the estrogen receptor RNA levels in both cell lines.

MATERIALS AND METHODS

Cell Cultures

The breast carcinoma cell lines MCF-7 and MDA-MB-231 were maintained in RPMI 1640–5% fetal bovine serum treated with dextran-coated charcoal, and supplemented with 100 U of penicillin per mL, 100 μg of streptomycin per mL, 2.5 μg of amphotericin B per mL, and estradiol 10^{-9} M at 37°C under 5% CO_2. Cultures were grown in 25 cm^2 flasks and seeded at approximately 3.5×10^5 cells per inoculum. Control flasks were trypsinized for total cell counts and harvesting of 100,000 cells to assay for telomerase.

Tamoxifen Treatment

Tamoxifen used to treat the breast cells was obtained from Sigma Chemicals (St. Louis, MO). Cells were cultured in the absence (media control) or presence of tamoxifen diluted in 100% ethanol at concentrations of 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} M for a period of 10 days. Each day a series of flasks was trypsinized for cell counting with a hemocytometer using trypan blue staining with 100,000 cells being processed for telomerase assays. Fresh media was added to remaining flasks daily with their appropriate drug concentration. The experiment was repeated five times.

Telomerase Assays

Telomerase activity was determined by the telomere amplification repeat protocol (TRAP assay) using the Telomerase PCR ELISA kit from Boehringer Mann-

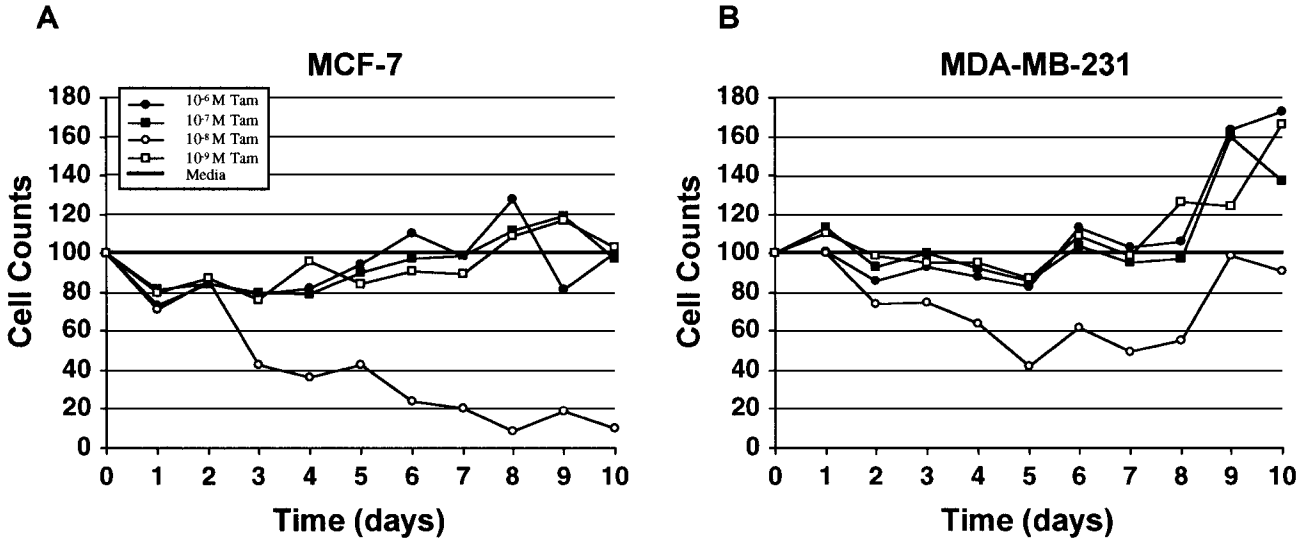

FIGURE 1. Effects of tamoxifen on cell counts (repetitions 1–4) are shown in (A) MCF-7 estrogen receptor positive cells and (B) MDA-MB-231 estrogen receptor negative cells. Media control is set at 100%. Tamoxifen treatment at a concentration of 10^{-8} M has a significantly different effect compared with other concentrations.

heim (Indianapolis, IN) according to manufacturer's instructions. TRAP assays were performed on both cell lines both at baseline before drug treatment and during drug treatment as a function of time. Ten thousand cell equivalents from the 100,000 cell lysate extracted above were assayed for each sample. Positive and negative controls were used from the kit. As a secondary test to determine telomerase activity, reverse transcriptase–telomerase chain reaction (RT-PCR) was used to amplify the RNA component (HTR) from the same protein extracts used for telomerase assays.

RT-PCR of Telomerase Genes and Estrogen Receptors

Total RNA was extracted from the cell lines treated and not treated with tamoxifen at all time points and concentrations from the third repetition. RT-PCR was performed on RNAs using primer sets (Table 1) for the human telomerase RNA component (HTR), reverse transcriptase catalytic subunit (RT), and estrogen receptor-α (ER). β_2 microglobulin was used as a PCR quantitation control. The RT-PCR conditions were 1 minute at 94°C, 30 seconds at 58°C, and 30 seconds at 72°C for 30 cycles. RT-PCR products were run on 1% agarose gels, stained with Sybr Green I, and then transferred by capillary action to Magnagraph nylon membranes. Standard procedures for DNA hybridization were performed using [γ-^{32}P] dCTP-labeled probes specific for either the human telomerase RNA (HTR) component, the reverse transcriptase-like catalytic subunit (RT), or the estrogen receptor. Images were detected using the Molecular Imager System from BioRad (Hercules, CA) and the corresponding Multi-Analyst software package.

Estrogen Receptor Assay

Estrogen receptor status was determined in both cell lines using the [^{3}H] estrogen receptor assay kit from Dupont (Billerica, MA) according to the manufacturer's instructions. In the assay, estrogen receptor binding sites were titrated to saturation with increasing concentrations of estradiol-17β under equilibrium conditions, whereby bound versus unbound substrate was measured by scintillation methods and analyzed by Scatchard plot. Assays were performed on cells before testing and again after the final repetition.

Statistical Analysis

Univariate pairwise regression analyses were performed to determine correlations among cell proliferation, telomerase activities, telomerase component levels ([RNA component [HTR] and catalytic subunit [RT]), and estrogen receptor levels. Analysis of variance was performed to determine the overall effect of all variables studied on both cell proliferation and telomerase activity.

RESULTS

Cell count studies and overall cell growth (n = 4) with tamoxifen treatment of both estrogen receptor positive (MCF-7) and estrogen receptor negative (MDA-MB-231) cells are shown in Figure 1. Cell count results

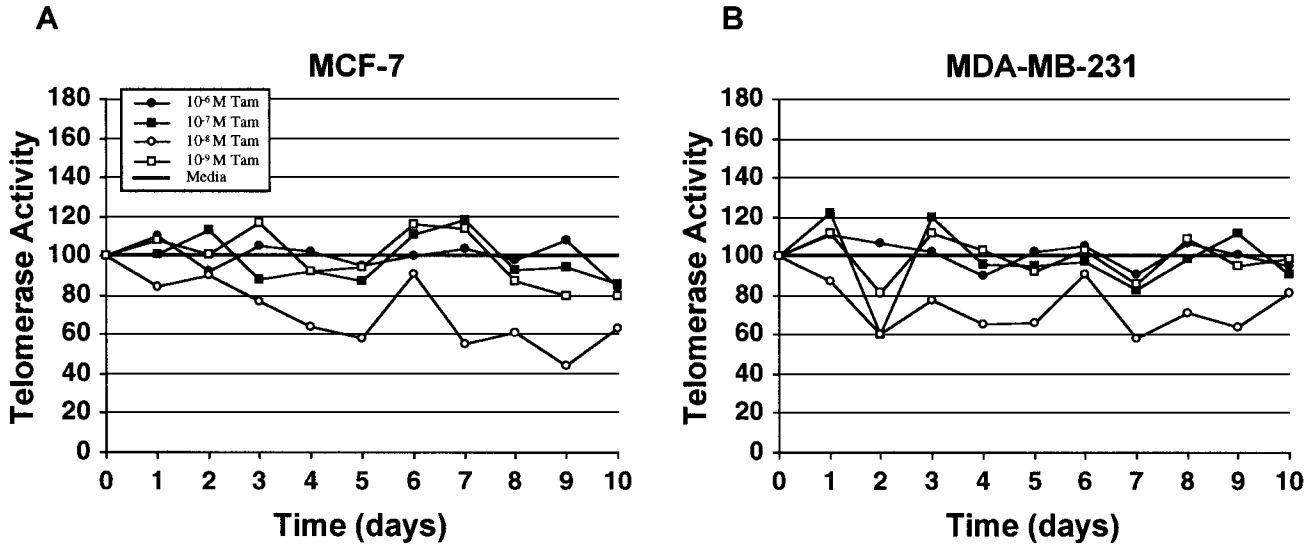

FIGURE 2. Effects of tamoxifen on telomerase activity (repetitions 1–4) are shown in (A) MCF-7 estrogen receptor positive cells and (B) MDA-MB-231 estrogen receptor negative cells. Media control is set at 100%. Tamoxifen treatment at a concentration of 10^{-8} M has a significantly different effect compared with other concentrations.

of tamoxifen-treated samples were averaged versus the media control (100%). Tamoxifen demonstrated a temporary suppressive effect versus controls in the MCF-7 cells at dosages of 10^{-6}, 10^{-7}, and 10^{-9} M, with recovery within 96–120 hours. Growth of the MCF-7 cells treated with 10^{-8} M remained depressed when compared with controls. The growth rate of the MDA-MB-231 cell line was relatively unaffected by tamoxifen treatment at 10^{-6}, 10^{-7}, and 10^{-9} M concentrations until 192 hours, when total cell counts appeared to be stimulated when compared with controls. Treatment of MDA-MB-231 cells with 10^{-8} M tamoxifen significantly affected cell growth until 192 hours, followed by an increase in cell counts. The effects of the 10^{-8} M concentration on cell growth in both cell lines were significantly different from the other concentrations used ($P = < 0.001$).

Telomerase activities of treated versus untreated cells (n = 4) are shown in Figure 2. Telomerase activities in both cell lines appear to be relatively unaffected by tamoxifen concentrations of 10^{-6}, 10^{-7}, and 10^{-9} M. Only the 10^{-8} M treated cells showed significantly lower activities than untreated control cells, although both cell lines demonstrated a rise in activity at 144 hours. The effects of the 10^{-8} M concentration on telomerase activity in both cell lines were significantly different from the other concentrations used ($P = < 0.001$). Regression analysis determined that cell counts correlated with telomerase activity regardless of cell line and that cell counts and telomerase activity both depended on drug concentration ($P = < 0.0001$).

To see whether tamoxifen might have a direct affect on telomerase activity, a telomerase repeat amplification protocol (TRAP) assay was performed using kit control samples spiked with the same concentrations of tamoxifen used in our study (Fig. 3). No significant changes in activity were detected between spiked samples and controls. We also ran an RT-PCR reaction in an attempt to amplify the RNA component with the same concentrations of tamoxifen in our reaction mixes, but we observed no effect on the PCR process (data not shown). Both experiments proved that, besides not having a direct effect on telomerase activity, the detected effects on telomerase activity in treated cells were actual effects and not simply artifacts of either the drug or the vehicle in which the drug was diluted.

RT-PCR was performed using total RNA to determine whether there were detectable changes in RNA expression of HTR or RT that might correlate with changes in enzyme activity. A sample of the RT-PCR results are shown in Figure 4. Multivariate analyses detected very little correlation between RT-PCR results and either telomerase activity or cell counts. Only RT levels significantly correlated with telomerase activity in MCF-7 cells ($P = 0.02$).

The estrogen receptor profile remained constant for both cell lines until the fifth repetition. In this repetition, the MCF-7 cells lacked detectable estrogen receptors and acted similarly to the MDA-MB-231 cells in both cell count and telomerase activity data (not shown). RT-PCR data showed no changes in expression of the estrogen receptor and thus no correlation with telomerase activity or cell counts.

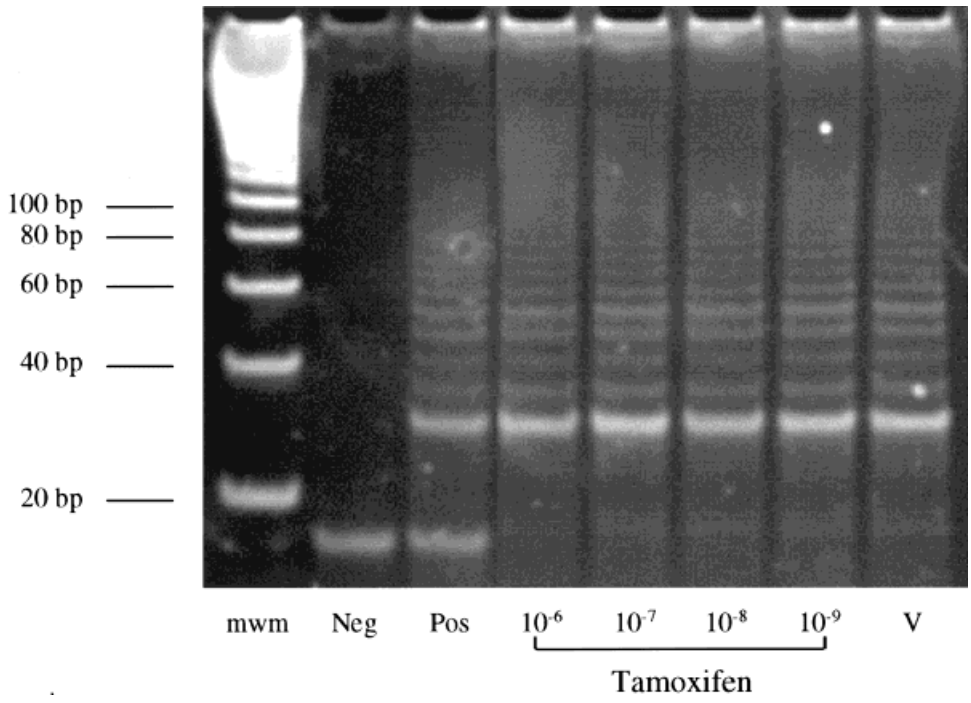

FIGURE 3. A telomerase assay of kit controls and controls spiked with tamoxifen or drug vehicle is shown. Neither tamoxifen nor the drug vehicle directly affected the assay for telomerase activity, as demonstrated by the equivalent levels of telomeric repeats in all lanes. mwm: size marker; V: drug vehicle.

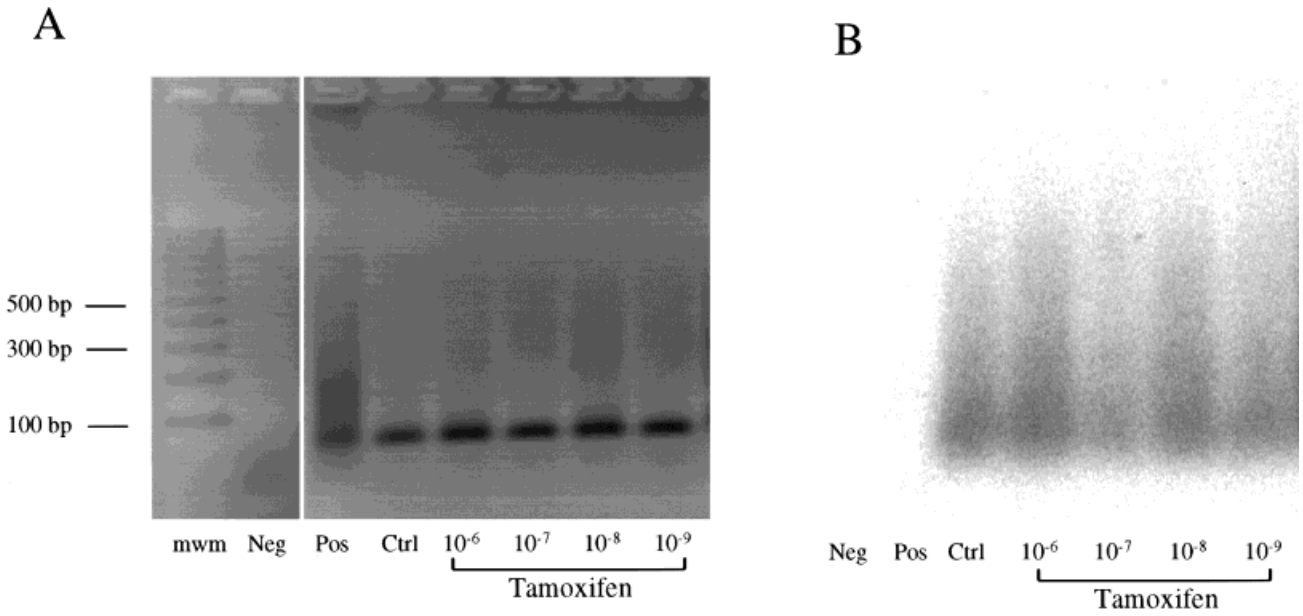

FIGURE 4. Reverse transcriptase–polymerase chain reaction is shown for (A) and Southern blot (B) results of human telomerase RNA component in tamoxifen treated cells. Predicted polymerase chain reaction product size is 89 bp.

DISCUSSION

Tamoxifen is a potent antiestrogenic agent indicated for treatment of breast carcinoma. It has been shown that positive estrogen and progesterone receptor values may help to predict the benefit of tamoxifen to the patient. In this study, tamoxifen affected both cell lines, causing short term effects on the MDA-MB-231 cell line and long term effects on the MCF-7 cell line. Cell count data correlated very strongly with telomerase activity, which corroborates the article published by Poremba et al.[18] Telomerase activity and cell counts also depended on the specific drug concentration used.

There was a narrow window of drug efficacy as

cells treated with 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} M of tamoxifen decreased cell counts and telomerase activity early, with recovery of cell counts to those seen in vehicle controls within 96–192 hours. The 10^{-8} M treatment was significantly different, with no recovery of MCF-7 cell numbers during the study period. One possible explanation for the rapid recovery of cells treated with other doses could be resistance to tamoxifen. There are several hypotheses for acquired tamoxifen resistance, including 1) loss or mutation of the estrogen receptor, 2) modification in estrogen receptor–associated parameters, 3) alteration in the estrogen response element, 4) high levels of antiestrogen binding sites, and 5) alteration of metabolism or availability of tamoxifen.[19] It is unknown whether similar mechanisms are responsible for the resistance of the MCF-7 cells to the higher doses (10^{-6} and 10^{-7}) and the lowest dose (10^{-9}) of tamoxifen used in this study. It does appear that both cell lines used did behave in a similar manner to the different doses of tamoxifen, with the 10^{-8} dose being the most effective in altering cell proliferation and telomerase activity.

MCF-7 cell lines with multiple passages have been known to lose their estrogen receptors. This appears to have happened in our study, as the fifth MCF-7 set appeared to have undetectable estrogen receptors over time as determined by ligand binding assay (data not shown). This cell line acted similarly to the MDA-MB-231 estrogen receptor-α negative cells with respect to cell counts and telomerase activity levels. However, MDA-MB-231 have been shown to contain estrogen receptor-β.[20] Although estrogen receptor-α and -β behave in a similar manner regarding the estrogen response element, they have differential activities in relation to AP1 elements.[21] These data affirm the importance of a positive estrogen receptor-α status for treatment with tamoxifen. It would be interesting to study further the role of estrogen receptor-β in how cells respond to tamoxifen and other antiestrogens.

Telomerase activity is associated with the cell cycle and proliferation.[22,23] Telomerase activity can be undetectable in cells collected from normal tissues; but when those cells are cultured and proliferate, telomerase becomes active. Many tumor cell lines, such as HL-60 and NB4 promyelocytic leukemia cells and NTERA-2 embryonal carcinoma cells, can be induced to differentiate in culture, with a concomitant decrease in telomerase activity.[24,25] Tamoxifen is known to arrest MCF-7 cell proliferation and increase the proportion of cells in the G0 or G1 phase of the cell cycle.[26,27] This study has shown that tamoxifen is not a direct inhibitor of telomerase, due to its lack of effect in inhibiting a positive sample in the TRAP assay. Therefore, the inhibition of telomerase by tamoxifen is due to an action of the hormone on the breast carcinoma cells. It remains to be determined whether tamoxifen directly affects the expression of the telomerase-related genes or acts through a secondary mechanism, such as p21 or other regulators of the cell cycle.

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