Telomerase inhibition enhances the response to anticancer drug treatment in human breast cancer cells

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Abstract
Breast cancer is the most common malignancy among women. Current therapies for breast tumors are based on the use of chemotherapeutic drugs that are quite toxic for the patients and often result in resistance. Telomerase is up-regulated in 95% of breast carcinomas but not in adjacent normal tissues. Therefore, it represents a very promising target for anticancer therapies. Unfortunately, the antiproliferative effects of telomerase inhibition require extensive telomere shortening before they are fully present. Combining telomerase inhibition with common chemotherapeutic drugs can be used to reduce this lag phase and induce tumor cell death more effectively. Few studies have analyzed the effects of telomerase inhibition in combination with anticancer drugs in breast cancer cells. In this study, we inhibited telomerase activity in two breast cancer cell lines using a dominant-negative human telomerase reverse transcriptase and analyzed cell viability after treatment with different anticancer compounds. We found that dominant-negative human telomerase reverse transcriptase efficiently inhibits telomerase activity and causes telomere shortening over time. Moreover, cells in which telomerase was suppressed were more sensitive to anticancer agents independently of their mechanism of action and this sensitization was dependent on the presence of shorter telomeres. Altogether, our data show that blocking telomere length maintenance in combination with anticancer drugs can be used as an effective way to induce death of breast cancer cells. [Mol Cancer Ther 2006;5(7):1669–75]

Introduction
A hallmark of cancer cells is unlimited cell proliferation, which requires the ability to maintain telomere length during cell division. Telomeres are essential structures that cap the ends of eukaryotic chromosomes and are made of short G-rich DNA repeats associated with specific proteins (1). Their main function is to protect the chromosome ends from being recognized as DNA breaks by the DNA repair machinery (2). Another important function of the telomeres is to buffer the loss of terminal sequences due to the end replication problem, which results in DNA shortening at each round of cell replication (3, 4). When telomeres become critically short, cells stop dividing and enter replicative senescence (5). This irreversible growth arrest acts as a powerful tumor suppressor mechanism to block proliferation of cells in which dysfunctional telomeres may induce genome instability and therefore malignant transformation (6). Cells with unlimited proliferative potential, such as germ line, stem cells, and cancer cells, activate mechanisms to ensure the maintenance of telomere length. Most human cells engage telomerase, an RNA-dependent DNA polymerase with a reverse transcriptase subunit [called human telomerase reverse transcriptase (hTERT)], which uses its intrinsic RNA subunit (called hTR) as a template for synthesis of new telomeric repeats at the chromosome termini. Telomerase is mostly inactive in normal adult human cells, with the exception of germ line and stem cells, whereas it is reactivated in over 90% of human cancers (7–9). The almost universal presence of telomerase in human tumors suggests that targeting telomerase may represent an efficient way to specifically block tumor cell growth with minor effects on normal cells. Several approaches have been developed to block the activity of the telomerase holoenzyme, such as antisense oligonucleotides against either hTERT or hTR (10–12), inactive variants of hTERT that act as dominant negatives (13, 14), small chemical compounds against hTERT (15–18), and G-quadruplex-stabilizing agents that bind the telomeric ends and block telomerase access and elongation (19–21). In all cases, direct or indirect telomerase inhibition resulted in the inability of the cells to maintain telomere length and ultimately cell growth arrest or cell death. However, these effects were not immediately observed due to the requirement for extensive telomere shortening.
Combinations of telomerase inhibition and anticancer drugs have been used to reduce this lag phase and induce cell death more rapidly. Indeed, several reports have shown that these approaches may be more effective in killing tumor cells than telomerase inhibition alone, although in most cases they still depend on telomere shortening (11, 15, 22–27). Moreover, the effects of combination approaches were often cell line and drug type specific (11, 23, 25, 26).

Breast cancer is one of the most common malignancies among women. Typically after surgical removal of the tumor mass, breast cancer patients are treated with chemotherapeutic drugs that are quite toxic, lack selectivity, and often result in resistance. Estrogen-responsive tumors that initially respond to tamoxifen often develop resistance to this agent and progress to metastatic disease. The new generations of aromatase inhibitors, which have been shown to be highly effective in early breast cancer settings, also eventually induce resistance (28). Therefore, more effective treatments are needed to treat breast cancer.

Telomerase is up-regulated in 95% of breast carcinoma, but not in adjacent normal tissues, and its activity increases with tumor aggressiveness (29–33). Few studies have analyzed the effects of telomerase inhibition in combination with anticancer drugs in breast cancer cells (15, 25, 27). In this study, we inhibited telomerase activity in two telomerase-positive breast cancer cell lines through the introduction of a dominant-negative hTERT (dn-hTERT) variant and analyzed the effects on cell viability. We also measured cell survival and proliferative ability in the presence of anticancer agents commonly used for chemotherapy. We found that telomerase inhibition induces telomere shortening over time and affects cell viability in a telomere length–dependent manner. Moreover, cells in which telomerase was suppressed were more sensitive than the controls to a variety of anticancer drugs. Altogether, our data confirm that a combination approach based on telomerase inhibition and anticancer drugs could be used to effectively induce death of human breast cancer cells.

Materials and Methods

Cell Culture and Plasmids

YCC-B2 breast cancer cells (provided by Dr. Sun Young Rha, Cancer Metastasis Center, Yonsei, Korea; ref. 34) were grown in MEM with 10% heat inactivated fetal bovine serum (Wisent, St. Bruno, Quebec, Canada). MCF-7 cells (obtained from Dr. Pollack, Lady Davis Institute, Montreal, Canada) were grown in RPMI with 10% heat inactivated fetal bovine serum. phTERT containing wild-type hTERT and the puromycin resistance gene was obtained from Dr. Silvia Bacchetti (Regina Elena Cancer Institute, Rome, Italy); pdnhTERT, encoding for a dn-hTERT (D868N), was generated by site-specific mutagenesis (QuickChange Site-Directed Mutagenesis kit, Stratagene, La Jolla, CA) using phTERT as template and the following primers: 5'-CTTCGCGTTTGGTAACTGATTTCATTGTTG-3' and 5'-CAACAGAAATCTGTAACCAACCGCAGGAG-3' (35). The mutation was confirmed by sequence analysis and by digestion with HincII, a restriction site created by the mutation.

Telomerase Activity (Telomeric Repeat Amplification Protocol) Assay and Western Blot

Whole cells extracts were prepared by detergent lysis and assayed by the PCR-based telomeric repeat amplification protocol (36) using 50 and 100 ng extracts. Telomerase activity in the dn-hTERT derivatives was quantified relative to the internal PCR control and expressed as a percentage of the activity measured in the vector cells. Western blot analyses were done using 50 μg protein extracts. The following antibodies were used: antimouse estrogen receptor-α [MAB461, Chemicon International (Tomecula, CA), kindly provided by Dr. Miller, Lady Davis Institute, Montreal, Canada] and antimouse β-actin (MA1501, Chemicon International). Secondary antibodies were purchased from Sigma (Oakville, Ontario, Canada).

Telomere Length Analysis

For telomere restriction fragment analysis, DNA was extracted using standard procedures, digested with HinfI/RsaI and separated by pulse field gel electrophoresis (37). The gel was denatured, neutralized, partially dried, and hybridized with a [γ-32P]dATP 5' end-labeled telomeric probe (C3TA2)3 (38). After hybridization, gels were exposed to Phosphorimager, hybridization signals were quantified relative to the internal PCR control and expressed as a percentage of the activity measured in the vector cells. Western blot analyses were done using 50 μg protein extracts. The following antibodies were used: antimouse estrogen receptor-α [MAB461, Chemicon International] and antimouse β-actin (MA1501, Chemicon International). Secondary antibodies were purchased from Sigma (Oakville, Ontario, Canada).

Quantitative Fluorescence In situ Hybridization Analysis

Quantitative fluorescence in situ hybridization was done as previously described (38). Briefly, metaphase chromosome spreads were prepared from vector- and dn-hTERT–expressing YCC-B2 cells, fixed, hybridized with a telomeric (C3TA2)-Cy3 PNA probe, and counterstained with 4',6-diamidino-2-phenylindole. Fluorescent signals were captured using a charge coupled device camera (Photometrics-Sensys, Tucson, AZ) and quantified using the ImageQuant (Molecular Dynamics), and telomere length was calculated according to the formula $\frac{\Sigma (OD_i)}{\Sigma (OD/L_i)}$, where OD indicates the absorbance at the position $i$, and $L_i$ is the molecular weight marker at the same position (37, 38).

Reverse Transcription-PCR

RNA was isolated using TRIzol (Invitrogen, Burlington, Ontario, Canada) and the expression of dn-hTERT was analyzed by PCR with the following primers: dn2600, 5'-GGTTTGGTTAAGGATTTC-3', and hTERT3141, 5'-TGGATGAGTAGCAG-3'. As a control for RNA integrity, human glyceraldehyde-3-phosphate dehydrogenase was amplified using RT11,
showed that the introduction of dn-hTERT resulted in telomeric repeat amplification protocol assay analyses. Multiple regression test and significance of the correlation between proliferative ability and number of PDs expressed as *P < 0.05, **P < 0.01, and ***P < 0.001. The percentage of the vector clones (IC, internal control used for quantification. B, blank).

### Results

**dn-hTERT Inhibits Telomerase Activity and Induces Telomere Shortening**

To analyze the effects of telomerase inhibition in breast cancer cells, we used two telomerase-positive cell lines, YCC-B2 and MCF-7. MCF-7 cells maintain stable telomeres of ~7 kb in length, express wild-type p53, and have a functional estrogen receptor-α; YCC-B2 cells have an average telomere length of 10 kb, contain an inactive p53, and do not express the estrogen receptor-α (data not shown; Supplementary Fig. S1). 4 We inhibited telomerase activity in these cells through the introduction of a dn-hTERT variant (35) and selected clonal populations in puromycin for 1 to 2 weeks. The presence of dn-hTERT was detected by reverse transcription-PCR (Fig. 1A and B, top). Telomeric repeat amplification protocol assay analyses showed that the introduction of dn-hTERT resulted in significant inhibition of telomerase activity in both YCC-B2 and MCF-7 derivatives (2–29% of the activity in the vector clones; Fig. 1A and B, bottom). The biological effects of dn-hTERT expression and telomerase inhibition were evaluated by monitoring telomere length over time. YCC-B2 and MCF-7 vector clones maintained telomere lengths, whereas dn-hTERT–expressing clones showed a marked decrease in telomere length with increasing PDs (Fig. 2). Telomere shortening was more evident in the dn-hTERT clones derived from MCF-7 cells, which after 90 PDs harbored telomeres of 2.6 kb in length (Fig. 2B). Even at the latest passages analyzed (PD 80–94), the presence of dn-hTERT did not have any effect on cell viability and proliferative ability in mass cultures compared with controls in both cell lines (data not shown), suggesting that, although short, telomeres were still relatively functional and able to protect the chromosome ends. However, colony-forming assays of MCF-7 cells expressing dn-hTERT revealed a progressive reduction of proliferative ability compared with control cells, which correlated with cell division and presence of very short telomeres (Figs. 2 and 3). We did not observe a...
similar effect in dn-hTERT clones obtained from YCC-B2 cells, most likely due to the more limited telomere shortening compared with MCF-7 derivatives (Supplementary Fig. S2).4

Increased Sensitivity to Drugs in dn-hTERT–Expressing Clones Requires Telomere Shortening

Previous studies have shown that telomerase inhibition increases the sensitivity of several tumor cell lines to anticancer drugs (11, 15, 22–26). To investigate whether this held true also for breast cancer cells, we treated vector and dn-hTERT–expressing derivatives from MCF-7 and YCC-B2 cells with two DNA-damaging agents used for chemotherapy, etoposide and doxorubicin, and analyzed their ability to survive and proliferate using colony-forming assays. Cells were treated for 24 hours with either doxorubicin or etoposide at concentrations ranging between the IC15 and IC25 of the parental cells, and subsequently plated at low density and allowed to proliferate for 10 to 14 days until colonies were visible.

Figure 2. Expression of dn-hTERT induces telomere shortening. Telomere restriction fragment length was analyzed by pulse field gel electrophoresis in YCC-B2 (A) and MCF-7 (B) vector and dn-hTERT clones at the indicated PDs. Representative molecular weight markers are indicated. The average telomere length was calculated by densitometric scanning of each lane and is shown at the bottom.

Figure 3. Introduction of dn-hTERT in MCF-7 cells induces a progressive reduction of their proliferative ability. Colony-forming assays of MCF-7 dn-hTERT clones 7 and 8 at increasing PDs. The relative numbers of colonies were obtained as a ratio of the colonies in the dn-hTERT clones over the colonies in the vector clones. Columns, values of at least three independent experiments; bars, SE. r² values indicate the correlation between cell proliferative ability and PDs and are both significant with P < 0.01.

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Introduction of dn-hTERT in MCF-7 cells induced a significant reduction of the number of colonies compared with the vector cells, indicating that clones expressing dn-hTERT are more sensitive to both doxorubicin and etoposide (Fig. 4A). Similarly, YCC-B2 cells expressing dn-hTERT showed an increased sensitivity to drug treatment compared with the controls (Fig. 4B). Interestingly, these effects were present at early time points after the isolation of the clones (PD 6 and PD 4, in MCF-7 dn-hTERT clones 7 and 8; PD 8 and PD 6 in YCC-B2 dn-hTERT clones 10, 17, and 26). Telomere shortening likely occurred during the selection and the growth of the clones; however, it is technically unfeasible to measure telomere length at the onset or during clone selection. Therefore, we used quantitative fluorescence in situ hybridization to analyze the telomere profile in dn-hTERT clones at early and late PDs and compared them with the telomere profile in the controls. We found that the telomere profiles in YCC-B2 dn-hTERT clones 10 and 17 at early passages were broader with a higher frequency of short telomeres when compared with the vector cells, but became narrower at later passages, most likely due to overall telomere shortening (Fig. 5). In addition, although we detected a few chromosome ends without telomeric signals, no obvious increase in telomeric fusions or chromosome instability was seen (Fig. 5; Supplementary Fig. S3), which is in agreement with our data showing that the presence of the dn-hTERT did not significantly affect proliferative ability in YCC-B2 derivatives at the late PDs analyzed (Supplementary Fig. S2). Recent studies have indicated that telomerase elongates preferentially the shortest telomeres, which are more likely to be involved in chromosome aberrations and cause genome instability (40, 41). Therefore, it is possible that in the dn-hTERT–expressing cells, the loss of telomerase affected the shortest telomeres to a greater extent, and this could account for their higher sensitivity to the drugs at early time points compared with control cells.

Another chemotherapeutic drug that is largely used for breast cancer chemotherapy belongs to the group of the taxanes, which bind to the microtubules of the mitotic spindle and inhibit segregation of the sister chromatids (42, 43). A recent report showed that telomerase inhibition could sensitize HeLa cells specifically to DNA-damaging agents but not other drugs with different mechanisms of action (11). We therefore analyzed whether suppression of telomerase in YCC-B2 breast cancer cells increased their sensitivity also to this class of anticancer drugs. As shown in Fig. 4B, the number of colonies obtained with two YCC-B2 dn-hTERT–expressing clones (dn-hTERT clones 10 and 17) was significantly reduced compared with the controls after treatment with paclitaxel, excluding a DNA damage–specific response. Our results differ from those obtained in the above study; however, the use of different cell types may account for the discrepancy.

**Discussion**

Telomerase reactivation is a necessary requirement for the unlimited ability of cancer cells to proliferate. Most human breast tumors analyzed thus far express active telomerase, whereas telomerase cannot be detected in normal adjacent tissues (30, 31). Therefore, it may represent a useful and effective target to induce breast cancer cell death. However, the requirement for telomere shortening before the anti-proliferative effects of telomerase inhibition are observed implies that only cells with short telomeres would respond rapidly to such treatments, thus limiting their applicability. More recent reports have indicated that telomerase inhibition could be used to sensitize tumor cells to anticancer drugs (11, 15, 16, 22–26). These combination approaches have the advantage of killing tumor cells more rapidly than either treatment alone and allow the use of lower drug concentrations, thereby reducing cytotoxicity for patients. Although the feasibility of combination approaches has been tested in several tumor cell types,
with different degrees of success dependent on the cell type and the drug analyzed, very few studies have analyzed their effects on breast cancer cells (15, 25).

In this study, we analyzed the effects of telomerase inhibition in combination with anticancer drugs in two breast cancer cell lines with different p53 and estrogen receptor status. We report that breast cancer cells in which telomerase activity was suppressed by dn-hTERT undergo telomere attrition over time with a limited effect on proliferative ability depending on the initial telomere length. More importantly, these cells showed increased sensitivity to different chemotherapeutic drugs compared with control cells, and the sensitization was dependent on the presence of short telomeres. Indeed, telomere restriction fragment and fluorescence in situ hybridization analyses revealed higher frequencies of shorter telomeres at early PDs in dn-hTERT clones compared with control cells and marked telomere shortening with successive cell divisions.

Interestingly, telomerase inhibition sensitized breast cancer cells to various drugs with different mechanisms of action. Indeed, YCC-B2 cells in which telomerase was inhibited by dn-hTERT were more sensitive to both DNA-damaging agents, such as etoposide and doxorubicin, and to the microtubule-targeting compound paclitaxel, excluding a DNA damage–specific response. Our results differ from those of a recent study that reported a specific increase in sensitivity to DNA-damaging agents, but not to other classes of drugs upon telomerase inhibition in HeLa cells, and suggested a specific interaction between telomerase and the DNA repair process in human cells (11). However, this discrepancy may depend on the different cell lines used. Finally, we observed a similar behavior in response to drug treatment in MCF-7 and YCC-B2 cells with different p53 and estrogen receptor status, indicating that the dn-hTERT–dependent antiproliferative effects do not require functional p53-dependent checkpoints and could be achieved in both estrogen receptor–positive and estrogen receptor–negative breast tumor cells.

Previous studies have reported that in mouse cells lacking functional telomerase, treatment with anticancer drugs causes an increase in multichromosomal fusions and subsequently cell death, suggesting that telomere dysfunction due to telomerase inhibition, and not telomerase inhibition per se, is the most likely cause of increased drug sensitivity in those cells (44, 45). Although we did not detect any obvious increase in telomeric fusions or chromosome aberrations in cells expressing dn-hTERT without drug treatment, we have not characterized the cytogenetic profile of these cells after drug treatment. However, based on our data and the data reported in the literature, we speculate that shortened telomeres may be contributing to drug sensitization in the dn-hTERT derivatives. For instance, telomerase preferentially elongates the shortest telomeres (40, 41); thus, this group of telomeres may be more affected by the lack of telomerase activity upon dn-hTERT expression, and this might account at least in part for the higher drug sensitivity at early time points when most telomeres are still long.

In conclusion, our data show that telomerase inhibition in breast cancer cells cooperates with anticancer drugs to induce cell death and this effect depends on telomere shortening, although it does not require complete loss of telomeric sequences or functions. Rather, the presence of shorter telomeres may impair the ability of the cells to recover from drug treatment. The dependence on telomere shortening is supported by a recent observation that inhibition of telomerase by the chemical compound BIBR1532 (18) results in increased drug sensitivity only in...
cells whose telomeres shortened in response to BIBR1532 and removal of this compound reverses both telomere shortening and the increased drug sensitivity (15).

Our results, together with those of other laboratories using other cell types, confirm that a combination strategy based on telomerase inhibition and anticancer drugs may be effective in inducing cell death of breast cancer cells.

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References

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