Inhibition of Human Telomerase Enhances the Effect of the Tyrosine Kinase Inhibitor, Imatinib, in BCR-ABL-positive Leukemia Cells

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Abstract

**Purpose:** Telomerase is a ribonucleoprotein enzyme that maintains protective structures at the ends of eukaryotic chromosomes. Earlier findings have supported an association between progressive telomere shortening in the chronic phase of chronic myelogenous leukemia and the up-regulation of telomerase activity occurring late in the evolution of the disease. We examined the impact of telomerase inhibition by dominant negative-human telomerase reverse transcriptase (DN-hTERT) on the biological features of BCR-ABL-transformed cells.

**Experimental Design:** We introduced vectors encoding DN-hTERT, wild-type (WT)-hTERT, or a control vector expressing only a drug-resistant marker into Philadelphia chromosome-positive K562 cells and OM9:22 cells and assessed the biological effect of telomerase inhibition on cellular immortality.

**Results:** Ectopic expression of DN-hTERT resulted in complete inhibition of telomerase activity and reduction of telomere length. The entire population of telomerase-inhibited K562 cells exhibited cytoplasmic blebbing and chromatin condensation, features of apoptosis. In contrast, K562 cells expressing WT-hTERT, which differ from the mutants by only two amino acids, exhibited normal morphology. The evidence of apoptosis in the telomerase-inhibited cells was determined by flow cytometric analysis with APO2.7 monoclonal antibody. We also observed enhanced induction of apoptosis by imatinib seen in DN-hTERT-expressing K562 cells, as compared with WT-hTERT-expressing cells.

**Conclusions:** These results demonstrate that disruption of telomere maintenance limits the cellular life span of leukemia cells and show that the combined use of imatinib and telomere maintenance inhibition may be effective in the treatment of BCR-ABL-positive leukemia.

Introduction

BCR-ABL is a chimeric oncoprotein generated by reciprocal translocation between chromosomes 9 and 22 and implicated in the pathogenesis of Ph+—positive leukemia (1). BCR-ABL fusion proteins exhibit elevated tyrosine kinase activity and transforming properties (2, 3). The 2-phenylaminopyrimidine derivative imatinib is a recently designed tyrosine kinase inhibitor that competitively inhibits ATP binding in the kinase domains of both c-ABL and BCR-ABL (4). Imatinib is specifically active both in vitro and in vivo against a variety of BCR-ABL-transformed cells (4–7). Imatinib has shown promise in human clinical trials of Ph-positive leukemia, but the emergence of imatinib resistance in patients with acute forms of Ph-positive leukemia highlights the need for combination chemotherapy to eradicate disease (6, 8).

Telomerase is a cellular RNA-dependent DNA polymerase that serves to maintain the tandem arrays of telomeric TTAGGG repeats at eukaryotic chromosome ends (9). Telomeres are highly conserved in organisms ranging from unicellular eukaryocytes to mammals, indicating a strong role of their protective mechanisms in preventing chromosomal ends from undergoing degradation and ligation with other chromosomes (9). Without telomeric caps, human chromosomes would undergo end-to-end fusions, with formation of dicentric and multicentric chromosomes (10). These abnormal chromosomes would break during mitosis, resulting in severe damage to the genome and activation of DNA damage checkpoints, leading to cell senescence or initiation of the apoptosis cell death pathway (11). Indeed, it has been proposed that telomere length specifies the number of cell divisions a cell can undergo before senescence (12). Telomerase activity is up-regulated in the vast majority of human tumors, as compared with normal somatic tissues. Ex-
pression of the catalytic subunit of telomerase, hTERT, in cultured human primary cells reconstitutes telomerase activity and allows immortal growth (13–16). TERT-mediated telomerase activation is able to cooperate with oncogenes in transforming cultured primary cells into neoplastic cells (17). In addition, it has been shown that TERT-derived cell proliferation results in activation of the c-myc oncogene (18). These findings in cultured cells have opened up the possibility that telomerase up-regulation may contribute actively to tumor growth (19). As a corollary to this hypothesis, the inhibition of telomerase in tumor cells should disrupt telomere maintenance and turn malignant cells toward proliferative crisis, followed by senescence or cell death. Genetic experiments using a DN-hTERT have demonstrated that telomerase inhibition can result in telomere shortening followed by proliferation arrest and cell death by apoptosis (20, 21). This makes telomerase a target not only for cancer diagnosis but also for the development of novel therapeutic agents. We examined the impact of telomerase inhibition by DN-hTERT on the biological features of BCR-ABL-transformed cells. We found that inhibition of telomerase activity by DN-hTERT induced sensitivity to imatinib.

Materials and Methods

Generation of Stable Clones Expressing WT-hTERT and DN-hTERT Mutants. K562 cell line was obtained from the American Type Culture Collection (Manassas, VA). OM9;22 cell line, a human leukemia cell line derived from a Ph-positive acute lymphoblastic leukemia patient, was derived as described previously (22). DN-hTERT was created by substituting the aspartic acid and valine residues at positions 710 and 711 with alanine and isoleucine residues by site-directed mutagenesis, as described previously (20). The resulting mutant was completely sequenced and subcloned into the vector pBABE-puro (20). K562 cells and OM9;22 cells were transfected with the expression vectors pBABE-puro, pBABE-puro-DN-hTERT, or pBABE-puro-DN-hTERT by electroporation (23). Beginning 48 h after electroporation, cells were selected with 2 μg/ml puromycin and cloned by limiting dilution. PD0 was defined as the time at which cultures reached confluence in 10-cm culture dishes.

Telomerase Assay and Measurement of TRF. Telomerase activity was examined by TRAP assay with a TRAP E-assay telomerase detection kit (Oncor, Gaithersburg, MD; Ref. 24). The PCR products were subjected to 12% acrylamide denaturing electrophoresis in an automated laser fluorescence DNA sequencer II (Pharmacia LKB Biotechnology, AB) and analyzed by the Fragment Manager program (Pharmacia LKB Biotechnology; Ref. 24). Activity in the extract-based PCR TRAP assay was detected as a periodic 6-bp peak of telomerase products and, in each sample, relative telomerase activity was calculated semi-quantitatively in comparison with a 36-bp internal standard (24). To measure TRF, genomic DNA was digested with the restriction enzymes, HinfI and Rsal, fractionated on 0.7% agarose gels, and transferred to nylon membranes. Hybridization was performed by using the Telo TTAGGG telomere length assay kit (Roche Molecular Biochemicals, Mannheim, Germany).

Apoptosis Assay. Imatinib was kindly provided by Novartis, Inc. (Basel, Switzerland). The incidence of apoptosis was determined by flow cytometric analysis with the FITC-conjugated APO2.7 mAb (clone 2.7), which was raised against the M, 38,000 mitochondrial membrane protein (7A6 antigen) and is expressed by cells undergoing apoptosis (25).

Statistical Analysis. Comparisons between groups were analyzed by the Mann-Whitney t test. Values of P < 0.05 were considered to show statistical significance. The statistical tests were performed with the Microsoft Word/Excel (Brain Power, Inc., Calabashes, CA) software package for the Macintosh personal computer.

Results

Effects of DN-hTERT on Telomerase Activity and Telomere Length. Previous reports have identified several point mutants in reverse transcriptase motifs of the telomerase catalytic subunit hTERT that exhibit dramatically reduced telomerase activity (20, 21). To study the effects of long-term expression of DN-hTERT on BCR-ABL transformed cells, we introduced DN-hTERT, WT-hTERT, or a control vector expressing only a puromycin-resistant marker into BCR-ABL-positive K562 and OM9;22 cells. After puromycin selection, successfully transfected cells were cloned and isolated. We selected clones expressing DN-hTERT (clones 3, 6, 7), WT-hTERT (clones 1, 2), or a control vector (clones 1, 2) for the TRAP assay (Fig. 1A). For the OM9;22 cells, we also selected clones expressing DN-hTERT (clones 1, 2) or a control vector (clones 1, 2; Fig. 1A). We investigated telomerase activity in these clones. Expression of DN-hTERT in K562 (clones 3, 6, 7) and OM9;22 (clones 1, 2) dramatically reduced telomerase activity (Fig. 1A). In contrast, expression of WT-hTERT (clones 1, 2) increased almost 2-fold the overall telomerase activity in K562 cells (Fig. 1A). Thus, the expression of a catalytically inactive hTERT mutant results in the disruption of telomerase activity. We next determined whether inhibition of telomerase activity influenced telomere length in K562 cell lines (Fig. 1B). We assessed peak telomere length in the K562 cell clones expressing either DN-hTERT (clones 3, 7), WT-hTERT (clone 1), or a control vector (clone 1; Fig. 1B). The telomerases in the parental K562 cells and K562 cells expressing control vector (clone 1) were maintained at 5.2 kb in length. As the K562 cell clones (clones 3, 7) expressing DN-hTERT were passaged, gradual telomere shortening was observed (Fig. 1B). Whereas all WT-hTERT- (clones 1, 2, 3) or control vector- (clones 1, 2, 3) expressing cells grew successfully in culture, DN-hTERT-expressing cells (clones 3, 6, 7) showed a loss of viability (see below). Therefore, we harvested DN-hTERT-expressing cells on PD22 (clone 3) and PD30 (clone 7). We estimated that DN-hTERT-expressing cells on PD22 (clone 3) and PD30 (clone 7) lost 1.7 and 2.3 kb in telomere length from the time of transfection (Fig. 1B). As the process of isolating clones expends ~20 population doublings, the loss of telomere sequence per population doubling in DN-hTERT-expressing clone 3 and clone 7 were 38 and 46 bp, respectively. In contrast, cells expressing WT-hTERT (clone 1) showed a telomere length of 8.0 kb (Fig. 1B).

Effects of DN-hTERT on Cell Proliferation and Apoptosis. We characterized the growth properties of cells expressing either DN-hTERT (clones 3, 6, 7) or WT-hTERT
or control vector (clone 1; Fig. 2). The growth kinetics of K562 cells expressing WT-hTERT (clone 1) did not differ substantially from those of cells carrying a control vector (clone 1; Fig. 2). K562 cells expressing DN-hTERT (clones 3, 6, 7) showed slow growth and eventually stopped proliferating on PD14 (clone 6), PD22 (clone 3), and PD30 (clone 7; Fig. 2). OM9;22 cells expressing DN-hTERT (clone 1, 2) stopped proliferating on PD20 (clone 1) and PD18 (clone 2; Fig. 2). DN-hTERT-expressing K562 clones (clone 3, 6, 7) resulted in the disruption of telomerase activity (Fig. 1A), however, clone 6 underwent a growth arrest more quickly than clone 3 and clone 7 (Fig. 2). The response to DNA damage induced by telomere dysfunction might reflect this variation between DN-hTERT-expressing clones. The telomerase-inhibited K562 cells (clone 3) exhibited morphological changes accompanied by apparent cell death over PD22 (data not shown). The entire population of DN-hTERT-expressing K562 cells (clone 3) exhibited cytoplasmic blebbing and chromatin condensation, morphological features of apoptosis (data not shown). In contrast, K562 cells expressing WT-hTERT (clone 1), which differed from the mutant by only two amino acids, exhibited morphology resembling that of original K562 cells (data not shown). To determine whether DN-hTERT-expressing K562 cells underwent apoptosis, we used flow cytometry analysis of living cells with a FITC-conjugated APO2.7 mAb (clone 2.7; Fig. 3; Ref. 23). After 40 days, 61.4 and 56.6% of DN-hTERT-transfected K562 cells (clone 3 and clone 6) were observed to undergo apoptosis (Fig. 3). In comparison, WT-hTERT- (clone 1) and control vector- (clone 1) transfected K562 cells underwent apoptosis at rates of 5–15% throughout the entire experimental period (Fig. 2).
3). These results demonstrate that the expression of DN-hTERT in K562 cells inhibits telomerase activity, resulting in additional telomere shortening, which may lead to chromatin damage and the subsequent induction of apoptosis.

**Enhancement of Sensitivity to Imatinib in DN-hTERT-expressing K562 Cells.** To assess the effects of telomerase inhibition in modulating responses to imatinib, which is a selective inhibitor of BCR-ABL tyrosine kinase, experiments have focused on early passaged K562 cells (PD10) expressing DN-hTERT (clone 3, 6, 7) and WT-hTERT (clones 1, 2, 3; Fig. 4A). DN-hTERT-expressing K562 cells at PD10 showed no difference in the morphology (data not shown). In a series of experiments, pools of K562 cells that expressed DN-hTERT (clone 3, 6, 7) or WT-hTERT (clones 1, 2, 3; Fig. 4A). The incidence of apoptosis was determined by flow cytometric analysis with APO2.7 mAb for 48 h to exclude the variation between each clones (Fig. 4A). The incidence of apoptosis was determined by flow cytometric analysis with APO2.7 mAb for 48 h to exclude the variation between each clones (Fig. 4A). The incidence of apoptosis was determined by flow cytometric analysis with APO2.7 mAb for 48 h to exclude the variation between each clones (Fig. 4A). The incidence of apoptosis was determined by flow cytometric analysis with APO2.7 mAb for 48 h to exclude the variation between each clones (Fig. 4A). The incidence of apoptosis was determined by flow cytometric analysis with APO2.7 mAb for 48 h to exclude the variation between each clones (Fig. 4A).

Although K562 cells expressing DN-hTERT at PD20 showed features of apoptosis spontaneously (Fig. 3), DN-hTERT-expressing K562 cells at PD10 showed no difference in the features of apoptosis without imatinib (Fig. 3). These results suggest that there is a cytotoxic synergy between telomerase inhibition and imatinib. To elucidate the mechanism of a cytotoxic synergy between telomerase inhibition and imatinib, we examined the effect of imatinib on telomerase activity and telomere length in K562 cells (Fig. 4B).
are able to maintain the length of their telomeres, indicating the existence of one or more nontelomerase mechanisms for telomere maintenance that have been termed ALT (36, 37). ALT has been detected in a variety of human tumors, including sarcomas, glioblastomas, and cancers of the lung, kidney, adrenal, breast, and ovary (38). An implication of the existence of ALT is that tumors using this telomere maintenance mechanism (including mixed telomerase-positive/ALT-positive tumors) will be resistant to telomerase inhibitors. Also, telomerase inhibitors will put tumors that are initially telomerase-positive under strong selection pressure for activation of ALT. Therefore, combination therapy using ALT and telomerase inhibitors may help prevent the emergence of drug resistance. In this context, a novel telomerase inhibitor, telomestatin, is a clinical candidate as a dual inhibitor of ALT and telomerase (39).

Lee et al. (40) reported that neoplastic cells from telomerase RNA null mice (mTERC−/−) showed enhanced chemosensitivity to doxorubicin or related double strand DNA break-inducing agents. Telomere dysfunction, rather than telomere inhibition, is proposed to be the principal determinant governing chemosensitivity specifically to double strand DNA break-inducing agents (40). We observed enhanced induction of apoptosis by imatinib in DN-hTERT-expressing K562 cells compared with WT-hTERT-expressing cells (Fig. 4A). DN-hTERT-expressing K562 cells also showed enhanced induction of apoptosis compared with WT-hTERT-expressing cells after exposure to daunorubicin and vincristine, whereas significant chemosensitivity was not observed in cells exposed to 6-mercaptopurine and methotrexate; 4-amino-10-methylpteroyl-glutamic acid (data not shown). We have not determined the telomere dysfunction in K562 cells in this study, however, DN-hTERT-expressing K562 cells at PD10 (TRF 4.8 kb) is enough to show the cytotoxic synergy with imatinib.

The enhanced sensitivity to imatinib may imply that there is cytotoxic synergy between telomerase inhibition and imatinib. Recently, Maida et al. (41) reported that epidermal growth factor activates telomerase through Ras/MEK/ERK pathways. In this study, we observed that imatinib suppressed telomerase activity and shortened TRF in K562 cells (Fig. 4, B and C). In this regard, imatinib may suppress telomerase activity via inhibition of Ras pathways. It has been shown that activation of the nuclear c-Abl protein can contribute to the induction of apoptosis (42). Actually, Kharbanda et al. (43) have reported that c-Abl protein is directly associated with hTERT and inhibits telomerase activity. Because imatinib stimulates nuclear import of BCR-ABL by combining with leptomycin B (44), imatinib may affect subcellular localization of BCR-ABL in the situation of telomere dysfunction. Although the exact mechanism of this apoptotic effect in DN-hTERT-expressing cells requires additional elucidation, our observation suggests that certain antineoplastic agents may enhance the telomere position effect in neoplastic cells (45). We observed only the enhanced induction of apoptosis by imatinib in K562 cells expressing DN-hTERT (Fig. 4), but inhibitors that are combined with the use of imatinib seem to be very useful for BCR-ABL-positive leukemia.

There have also been concerns that inhibiting telomerase might lead to an increase in malignancy by enhancing the genomic instability of cells (46). These concerns have arisen from observations of mTERC−/− knockout mice, which have an increased incidence of malignancies in both early and late

Discussion

Direct genetic manipulations have shown that telomerase-mediated telomere elongation plays a key role in determining cellular replicative capacity and senescence (9). The mechanisms regulating the production of active telomerase enzyme are still predominantly unknown, but roles for transcriptional control of hTERT or alternative-splicing of hTERT have been advocated (26–30). Several alternatively spliced variants of hTERT have been identified, including two deletions (α and β) and four insertions of intron sequences into hTERT mRNA (29, 30). Although all known splice variants of hTERT are inactive, the observation that hTERT-α is a dominant-negative inhibitor of telomerase indicates that splicing may indeed represent a means for cells to fine-tune their levels of active enzyme by differentially shifting the balance between the hTERT-α form and the full-length transcript (29, 30). Point mutations of hTERT protein have emerged as an additional mechanism of regulating telomerase activity (31). Telomerase activity can be abolished by replacement of any of the three conserved aspartic acid residues of amino acid motifs A and C to the hTERT subunits in budding yeast and in humans (31). Ectopic expression of these hTERT mutants in human cancer cells results in telomerase inhibition, telomere shortening, and apoptosis (20, 21). Therefore, these mutant hTERT subunits act as dominant negatives of wild-type hTERT and appear to be effective tools for studying the characteristics of telomerase.

In our study, we observed that inhibition of telomerase by DN-hTERT reproducibly results in not only telomere shortening but also induction of apoptosis in human leukemia cells (Figs. 1–3). These results suggest that telomerase activity is the dominant mechanism providing telomere maintenance and long-term viability to human leukemia cells. Earlier findings support an association between progressive telomere shortening in the chronic myelogenous leukemia-chronic phase and the up-regulation of telomerase activity occurring late in the evolution of the disease (32–34). Thus, specific inhibitors of the hTERT enzyme seem to be very effective in limiting the growth of leukemia cells. We have not identified cells that survive the period of crisis induced by DN-hTERT, however, Sachsinger et al. (35) demonstrated that telomerase activity was efficiently reactivated in murine RenCa cells expressing DN-mTERT and suggested the fundamental differences in telomere regulation between human and murine cells.

Some human tumor cells without any telomerase activity are able to maintain the length of their telomeres, indicating the existence of one or more nontelomerase mechanisms for telomere maintenance that have been termed ALT (36, 37). ALT has been detected in a variety of human tumors, including sarcomas, glioblastomas, and cancers of the lung, kidney, adrenal, breast, and ovary (38). An implication of the existence of ALT is that tumors using this telomere maintenance mechanism (including mixed telomerase-positive/ALT-positive tumors) will be resistant to telomerase inhibitors. Also, telomerase inhibitors will put tumors that are initially telomerase-positive under strong selection pressure for activation of ALT. Therefore, combination therapy using ALT and telomerase inhibitors may help prevent the emergence of drug resistance. In this context, a novel telomerase inhibitor, telomestatin, is a clinical candidate as a dual inhibitor of ALT and telomerase (39).

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There have also been concerns that inhibiting telomerase might lead to an increase in malignancy by enhancing the genomic instability of cells (46). These concerns have arisen from observations of mTERC−/− knockout mice, which have an increased incidence of malignancies in both early and late
generations, particularly in the setting of p53 mutant tumors (46). There is no evidence to suggest that this would be the case in humans (47). Mouse telomeres are much longer than even the longest human telomeres and do not appear to have a role in signaling senescence. Although a critical and careful evaluation of telomerase inhibitors in clinical development is certainly required, inhibition of telomerase activity appears to be a potential approach for the treatment of leukemia. This work suggests that telomerase inhibition may serve as an effective tool for eliminating leukemia cells. These experiments await the development of specific inhibitors of the components of the telomerase complex.

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References


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