

The Biology Behind

Telomerase Therapeutics: Telomeres Recognized as a DNA Damage Signal¹

Commentary re: K. Kraemer *et al.*, Antisense-mediated hTERT inhibition specifically reduces the growth of human bladder cancer cells. *Clin. Cancer Res.*, 9: 3794–3800, 2003.

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Introduction

On the basis of a consolidation of a number of principles that underpin our current understanding of the transformation of normal human cells to malignant tumors, more mechanistic and rationale approaches to cancer therapeutics are emerging. Cancer cell immortality (limitless replicative potential) is one of the hallmarks of cancer (1). The topic of telomeres and telomerase as it relates to cell immortalization and advances in targeting this almost universal characteristic of cancer for drug development forms the central theme for this commentary. The ability of cancer cells to divide indefinitely is attributable to the activation of the ribonucleoprotein enzyme, telomerase. The most well-understood function of telomerase is to act as a cellular reverse transcriptase to synthesize telomeric (TTAGGG) repeats on the end of chromosomes to compensate for the progressive telomeric shortening that occurs each time a cell divides. All replicating cells lose some telomeric repeats with each division attributable to both the end replication problem as well as processing events that are attributable to yet unidentified nucleases. Before 1994, most research efforts on telomeres and telomerase were conducted by biochemists and molecular biologists using model organisms such as yeast and protozoa. With the discovery that telomerase is repressed in most normal human tissues but expressed in >90% of human tumors (2, 3), cancer biologists have initiated experiments to determine whether inhibition of telomerase has use in cancer therapeutics. The logic for pharmacological inhibitors of telomerase is that this approach would have the potential to be more cancer specific and perhaps with fewer cytotoxic side effects compared with currently approved therapies. Telomerase inhibitors might not only directly stop the growth and kill cancer cells but also might work effectively in combination with conventional cancer treatments such as surgery, chemotherapy, and radiation therapy to delay or prevent tumor regrowth (Fig. 1).

Many novel approaches targeting telomeres and telomerase have been described (Table 1). Those agents that target only telomeres are likely to have nonspecific toxicities on normal

cells, whereas those that target telomeres through inhibition of telomerase would be predicted to have fewer side effects (Refs. 4–10 for reviews). The current study by Kraemer *et al.* (11) tested AS-ODNs³ complementary to the catalytic subunit of telomerase (hTERT) for their effectiveness in influencing cell growth in a bladder cancer cell line, EJ28. Although the mechanism of action of these AS-ODNs would have been predicted to inhibit telomerase activity, it would also have been expected they would have taken time to progressively shorten the telomeres of EJ28 cells before causing a growth arrest of the cells. Interestingly, the present study showed dramatic short-term effects of 5 of the 23 AS-ODN tested on the EJ28 cells leading to rapid cell death of the tumor cells. Certainly, one possibility to explain this is that the inhibition of hTERT mRNA was causing an effect on telomere state as opposed to telomere loss. Alternatively, because telomere dynamics were not examined in the present study, it is conceivable that the EJ28 cells have at least some critically short telomeres, and upon hTERT inhibition, a rapid DNA damage-signaling pathway is activated leading to a rapid cell death. This raises a central issue in the telomerase therapeutic field: the amount of time or divisions required to observe efficacy of telomerase inhibitors.

Telomerase Therapeutics: Telomeres Recognized as a DNA Damage Signal

Recent results suggest that there may be several options available for targeting telomeres and telomerase in cancer therapeutics (Fig. 2). Mammalian telomeres are distinctive DNA-protein structures consisting of repetitive hexameric sequences (TTAGGG) that cap the ends of linear chromosomes (Fig. 2). Telomeres end in a large-tailed loop resembling a lasso, termed the T-loop. The formation of T-loops is dependent on TRF2, one of the telomere-associated proteins that bind double-stranded telomeric DNA (12). Indirect evidence suggests the single-stranded 3' overhang strand invades the telomeric duplex DNA (Fig. 2). Thus, the T-loop may provide a structure that hides the chromosomal ends, preventing them from resembling DNA double-strand breaks. Disruption of even a single T-loop via chemotherapy or telomerase inhibitors may potentially signal a cellular response that resembles a double-strand break. It is generally thought that uncapped chromosome ends are at great risk for degradation, recombination, or fusion by cellular DNA repair systems, leading to the loss of genetic information, rearrangement of chromosomes, and increased genomic instability. In normal cells without other alterations, this leads to replicative senescence that may have evolved as an anticancer protection

Received 7/15/03; accepted 7/16/03.

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³ The abbreviation used is: AS-ODN, antisense oligonucleotide.

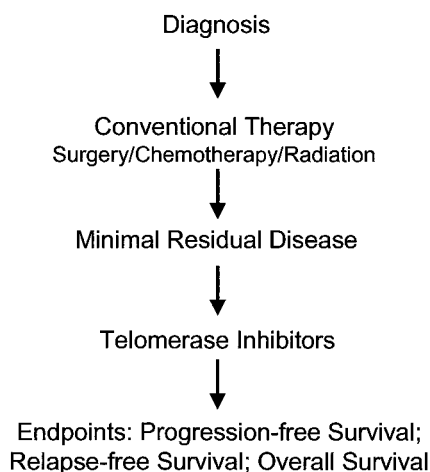


Fig. 1 Telomerase repression in a clinical setting for cancer therapy

mechanism acting as a failsafe mechanism to prevent the proliferation of cells at risk for neoplastic transformation (13, 14). In the presence of other cancer predisposing alterations, uncapped telomeres could lead to increased genomic instability and an increased probability of cancer formation, including telomerase reactivation. Thus, telomeres can be lost or rendered dysfunctional by DNA damage, repeated cell divisions in the absence of telomerase, or changes in telomere-associated proteins. In response to dysfunctional or damaged telomeres, cells can undergo apoptosis and die, continue to divide until a replicative senescence-induced growth arrest occurs, or develop genomic instability, leading to a mutant phenotype (Fig. 2). These cellular phenotypes can have profound consequences for cancer cells. On the basis of the recent work of Kraemer *et al.* (11) and another recent study (15), the telomerase therapeutics field has to now consider that there may also be pathways that target the rapid degradation of telomeres, resulting in terminal growth arrest leading to cell death (15–17).

Senescence (Stasis) May Be Induced by Nontelomere-based Mechanisms or Changes in Telomere State not Telomere Loss

Although cell senescence in the strictest sense is defined as telomere-based replicative growth arrest that occurs in normal cells after a limited number of divisions, there has also evolved a more broadly defined field of research that is similar in many regards to telomere-based replicative senescence. It has been documented that cells can be triggered to undergo an exogenously induced rapid G₁ growth arrest. This growth arrest is similar to replicative senescence because the cell cannot divide even if stimulated by mitogens. However, these cells remain metabolically and synthetically active and show characteristic changes in morphology (13, 14). It is believed that somatic cells have innate defense mechanisms that guard against unrestrained proliferation, irrespective of telomere status. Both the telomere-based and nontelomere-based growth arrest may be due, in part, to repression of genes required for cell cycle progression (*e.g.*, *myc* and *c-fos*)

Table 1 Telomerase/telomere inhibitor approaches

Targeting the telomerase (template) RNA component (hTR)
ODNs: hTR template antagonist
Hammerhead ribozymes
2-5A-anti-telomerase RNA oligonucleotides
hTR mutants/gene therapy
Targeting the catalytic protein component of telomerase (hTERT)
Dominant negative hTERT/gene therapy
hTERT promoter: oncolytic virus
Immunotherapy
ODNs/siRNA hTERT mRNA
Ribozyme cleavage of hTERT mRNA
Molecular chaperone inhibitors affecting hTERT assembly
Nucleoside analogues, reverse transcriptase inhibitors
Targeting telomeres
G-quadruplex interacting compounds
Telomere-associated and binding proteins
Targeting interactions of hTR, hTERT, and telomeres and associated signaling pathways

and up-regulation of growth inhibitory genes (*e.g.*, p21 and p16). It has been demonstrated that these growth inhibitory genes can also be activated in cell culture because of a variety of environmental stresses that have been termed, premature senescence, culture shock, stasis, and stress-induced senescence (18–20). For example, the growth arrest elicited in primary fibroblasts in response to oncogenic Ras or Raf, oligonucleotides, inadequate culture conditions, or the limited proliferation of mouse embryonic fibroblast with very long telomeres operate just as effectively in human cells expressing telomerase. This demonstrates that this type of growth arrest does not involve counting cell replications (*e.g.*, telomere-based replicative senescence). This type of growth arrest has received a variety names that are easily confused with replicative senescence so the term “stasis” (stress or aberrant signaling-induced senescence) has been suggested to convey the notion that cells engage a common senescence-like arrest mechanism in response to diverse stresses (20). Recently, the concept of tumor cell senescence (stasis) in cancer treatment was reviewed (21). Prospective drugs targeting the induction of senescence-associated regulatory pathways include conventional chemotherapy, radiation, and hormone ablation. These can work via affecting central cell cycle checkpoint pathways involving p21 and p16, as well as by forcing expression of secreted and/or intracellular growth inhibitors. Thus, in some instances, stasis could be thought of as an evolutionarily conserved defense mechanism through which cells *in vivo* are guarded against potentially oncogenic insults and *in vitro* as a response to inadequate culture conditions. In some instances, stasis could be attributable to a change in telomere state and in other instances attributable to alternative telomere-independent mechanisms. Importantly, these mechanisms may operate by independent pathways in different cell types (*e.g.*, fibroblast *versus* epithelial) or in different species (*e.g.*, man *versus* mouse; Ref. 22).

Rapid Telomere Loss Mechanisms

The studies by Kraemer *et al.* (11) demonstrating a rapid loss of cell viability after treatment with hTERT oligonucleo-

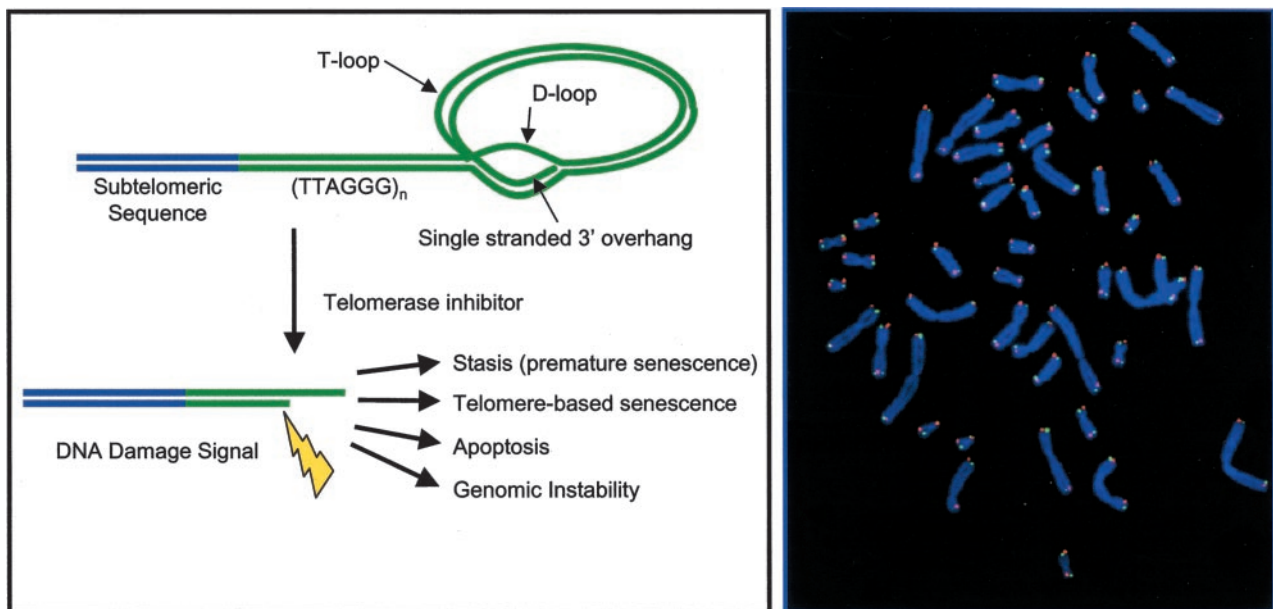


Fig. 2 Potential cellular outcomes after treating cancer cells with telomerase inhibitors (left). Dual color telomeric chromosome orientation-fluorescence *in situ* hybridization using digital fluorescence microscopy to visualize both human telomere leading strand (green) and lagging strand (pink) in a metaphase spread (right).

tides could be attributable to the cells having at least some very short telomeres, oligonucleotide-induced stasis, or to a rapid telomere loss mechanism. One example of the rapid telomere loss mechanism has recently been reported (15). RB94 lacks the NH₂-terminal 112 amino acid residues of the full-length retinoblastoma protein (RB110) and is a potent tumor and growth suppressor. In this study (15), RB94, but not wild-type RB110, produced marked growth inhibition and caspase-dependent apoptosis in human telomerase-positive bladder cancer cell lines. Importantly, RB94 produced rapid telomere length shortening (48 h) and loss of telomere signal (Fig. 3) as demonstrated by both terminal restriction fragment and fluorescence *in situ* hybridization analysis. However, the underlying mechanism causing this rapid telomere loss is not presently known. Interestingly RB94 showed no cytotoxicity to telomerase negative human normal urothelial cells or to cells that maintain their telomere length through an alternative lengthening of telomeres recombination mechanism (2, 23). These results suggest that the induction of rapid telomere erosion and chromosomal crisis by RB94 occurs via a telomerase-specific pathway and may provide a high therapeutic index when used in gene therapy protocols for the treatment of bladder and other cancers. Other approaches such as altering telomere-binding proteins that also induce a rapid loss of telomeres (24) are much less likely to have a cancer-specific therapeutic index because the telomere shortening is not mediated through telomerase. Finally, telomeres, with many kilobases of the triplet G repetitive hexameric sequence (TTAGGG), provide an ideal target for oxidative damage (16, 17). Inducing oxidative damage would accelerate the rate of telomere shortening, but this would not be predicted to be cancer specific.

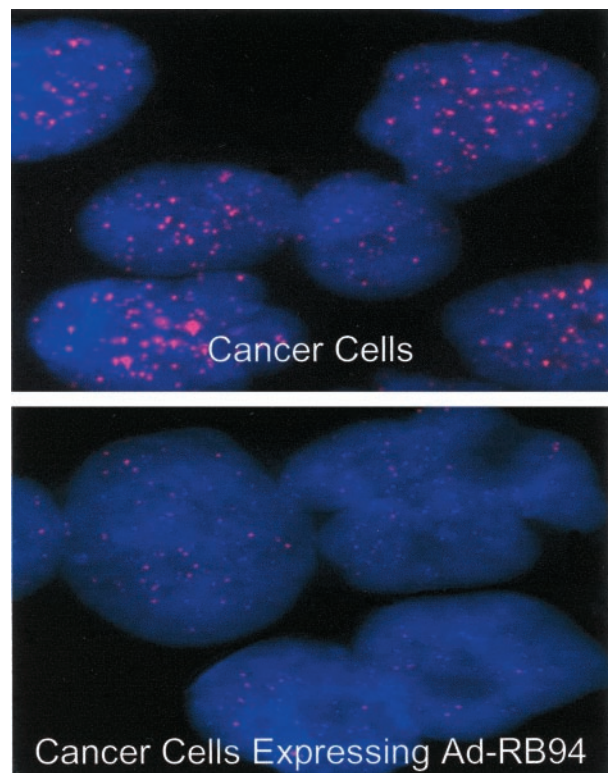


Fig. 3 Rapid loss of telomere signal after 48-h treatment of bladder cancer cells (UN-UC14) with Ad-RB94 (15). This effect is not seen in telomerase-negative cells or cells that use telomerase-independent mechanisms for telomere length maintenance. The RB94-induced cytotoxicity is not attributable to direct telomerase inhibition but is mediated through a telomerase-associated effect on the telomere complex.

Senescence Induced by Progressive Telomere Loss through Telomerase Inhibition

Both telomere shortening and telomerase activation may have what appear to be paradoxical roles in human tumorigenesis. Telomere shortening may be a protective anticancer mechanism limiting the maximal number of cell divisions, whereas successful cancer cells up-regulate or reactivate telomerase, resulting in the unlimited ability of cells to divide. This has led to the general idea that telomerase activity is necessary for the proliferation of most (but not necessarily all) advanced cancers (25, 26) and that telomerase inhibitors may be a powerful and novel cancer therapy strategy (4–10). Normal cell types either fully maintain (germ-line cells, embryonic stem cells) or partially maintain (pluripotent stem-like cells) their telomeres via activation or up-regulation of telomerase. Successful tumor cells almost universally maintain their telomeres by the activity of telomerase, albeit at a short length. Because telomeres are generally shorter in human tumors compared with adjacent cleared margins, this suggests that there may be a therapeutic window to achieve a reduction of telomeres to a critical length without irreversibly affecting normal cells.

Although functional telomeres may be essential for the many cell proliferations a cancer cell must undergo, it is still unclear if telomerase activation in tumors actually facilitates tumor growth. Telomerase overcomes growth limitations because of telomere dysfunction, but it does not cause growth deregulation and hence is not a typical oncogene. There are many normal cell types that have been immortalized by over-expressing the catalytic subunit of telomerase (hTERT) without signs of cancerous changes (27–30). However, in precancerous or weakly cancerous cells with many genetic alterations, telomerase may facilitate cell (and thus tumor) growth (31), perhaps by enhancing genomic stability and DNA repair (32).

There have already been many antitelomerase approaches tested in cell culture and xenograft models (Ref. 10, for recent review). The results to date are highly encouraging, and there is room for optimism that similar results will be observed in clinical trials. Although there is a possibility that telomerase inhibitors could select for more malignant subclones from the tumors by enhancing genomic instability (Fig. 2), it may be that the risk of outgrowth of resistant subclones could be minimized by combination therapy. Thus, after initial dose escalation and toxicity trials, it is likely that telomerase inhibitors will be administered as an adjuvant therapy in combination with standard therapeutic regimens. Although conventional therapies result in a reduction of tumor mass, these may not by themselves or in combination affect telomere length or telomerase activity. Telomerase inhibitors by themselves will result in telomere shortening, but it may take many cell replications to achieve the benefit of such therapies. Thus, combining conventional therapies to produce a setting of minimal residual disease and combining this approach with a telomerase inhibitor may improve overall survival or at least delay the time to tumor regrowth (Fig. 1). Telomere length may be a critical consideration in the design of future clinical trials, and it may be important to screen for patients whose tumors are characterized by relatively short telomeres. Antitelomerase therapies would greatly benefit from shortening the time between initiation of treatment and obser-

vation of positive effects. Thus, an important challenge for basic and preclinical work will be to determine combination therapies to best enhance the erosion of telomeres to cause a more rapid decrease in cancer cell proliferation without affecting normal cell telomeres.

Future Directions and Final Comments

Targeting tumor cells in patients with telomerase-based strategies has never before looked so promising. Despite the complexities of telomere dynamics on cancer initiation and progression in mice and unresolved questions in humans, the evidence is persuasive that telomerase inhibitors may lead to effective interventions for the treatment of patients with cancer. Although advances continue to be made in the discovery of telomerase inhibitors, other approaches to specifically kill telomerase-positive tumor cells have achieved robust experimental support (10). Telomerase (hTERT) promoter-driven oncolytic viruses have proven effective *in vitro* and in animal models, and therapeutic hTERT immunotherapy strategies are now in human clinical studies (10). Although one can always make hypothetical arguments for and against any novel cancer therapeutic, the preclinical experimental evidence for telomerase as a universal target for cancer therapy is encouraging. Several approaches are currently being translated into hopefully well-designed clinical trials, and this will determine the ultimate use of this novel approach. Clearly, targeting telomere maintenance mechanisms will be important in our repertoire of future cancer strategies.

Acknowledgments

I thank Ying Zou, Sergei Grayznov, and William F. Benedict for technical assistance, and CA70907.

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