Cancer Therapy: Preclinical

Neural Tumor-Initiating Cells Have Distinct Telomere Maintenance and Can be Safely Targeted for Telomerase Inhibition

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Abstract

Purpose: Cancer recurrence is one of the major setbacks in oncology. Maintaining telomeres is essential for sustaining the limitless replicative potential of such cancers. Because telomerase is thought to be active in all tumor cells and normal stem cells, telomerase inhibition may be nonspecific and have detrimental effects on tissue maintenance and development by affecting normal stem cell self-renewal.

Methods: We examined telomerase activity, telomere maintenance, and stem cell maturation in tumor subpopulations from freshly resected gliomas, long-term, primary, neural tumor-initiating cells (TIC) and corresponding normal stem cell lines. We then tested the efficacy of the telomerase inhibitor Imetelstat on propagation and self-renewal capacity of TIC and normal stem cells in vitro and in vivo.

Results: Telomerase was undetectable in the majority of tumor cells and specific to the TIC subpopulation that possessed critically short telomeres. In contrast, normal tissue stem cells had longer telomeres and undetectable telomerase activity and were insensitive to telomerase inhibition, which results in proliferation arrest, cell maturation, and DNA damage in neural TIC. Significant survival benefit and late tumor growth arrest of neuroblastoma TIC were observed in a xenograft model (P = 0.02). Furthermore, neural TIC exhibited irreversible loss of self-renewal and stem cell capabilities even after cessation of treatment in vitro and in vivo.

Conclusions: TIC exhaustion with telomerase inhibition and lack of telomerase dependency in normal stem cells add new dimensions to the telomere hypothesis and suggest that targeting TIC with telomerase inhibitors may represent a specific and safe therapeutic approach for tumors of neural origin. Clin Cancer Res; 17(1); 111–21. ©2011 AACR.

Introduction

Pediatric central and peripheral nervous system tumors comprise the largest group of childhood solid tumors and are responsible for the most morbidity and mortality from childhood cancer (1). These tumors, specifically gliomas and neuroblastomas, share unique extremes of clinicobiological behavior: spontaneous growth arrest, on the one hand, and relentless progression even with maximal therapy, on the other (2, 3). Previous work from our group and others has shown that the lack of telomere maintenance is responsible for growth arrest and predicts the risk of recurrence in pediatric low-grade gliomas (2), ependymomas (4, 5), and stage IV-S neuroblastomas (6), suggesting that telomerase inhibition could reverse their malignant phenotype by altering their self-renewal capabilities.

The ability of cancer to recur, even after minimal undetected disease is achieved with aggressive therapy, is one of the major setbacks in oncology. Limitless self-renewal is necessary for these tumors for continuous recurrence. This can be explained by telomere maintenance on the molecular level and existence of tumor-initiating cells (TIC) on the cellular level. Telomeres are repetitive sequences of DNA present at the ends of chromosomes (7) that protect them from degradation (8). Because of the end-replication problem, telomeres become shorter, with each cellular division resulting in cellular senescence. Immortal cells such as germ cells, stem cells, and memory lymphocytes are thought to maintain telomeres for self-renewal through reactivation of the enzyme telomerase. Telomerase is a complex ribonucleoprotein composed in part of telomerase RNA (TR) and the telomerase reverse transcriptase.
We hypothesized that telomerase-dependent telomere maintenance might be critical only to the survival of the self-renewing TIC tumor subpopulation and not to the bulk of tumor cells or the corresponding tissue stem cells. We have established long-term primary normal stem cell lines from the human fetal brain (31) and pediatric dermis (32), and malignant stem cell lines from both freshly resected adult and pediatric gliomas and neuroblastomas (17, 31). We chose to interrogate these tumors as model systems to examine the importance of telomere maintenance in TICs relative to the bulk of tumor cells and normal tissue stem cells.

Here we report that telomerase activity is not a property of all tumor cells but rather confined to the TIC subpopulation and is not detectable in the majority of tumor cells. Furthermore, normal tissue stem cells lack telomerase activation and are insensitive to telomerase inhibition, which is a highly specific and effective anti-TIC therapy. Furthermore, as opposed to conventional chemotherapies, telomerase inhibition resulted in TIC exhaustion by irreversibly altering their self-renewal capacity.

Because maintaining normal stem cells is critically important for children undergoing cancer therapy, our findings present a rationale for using telomerase inhibition in the treatment of these devastating tumors.

Materials and Methods

Primary glioma cell culture and FACS sorting

High-grade glioma samples (N = 14) were obtained with patients’ consent according to the Research Ethics Boards at The Hospital for Sick Children and Toronto Western Hospital. Tumors were washed and dissociated as previously described (16) and cells were stained with anti-CD15/SSEA-1-FITC (BD Biosciences). FACS sorting was conducted on a MoFlo (Beckman Coulter) and cells were collected into DMEM/F12 growth factor–free media. Sorted cells (5 × 10^4/po) were lysed with 50 μL of NP-40 buffer (33) and the 2 subpopulations were assessed for telomerase activity by using TRAP assays. Primary spheres (nonadherent) and adherent cells were obtained as described earlier and cultured for 48 hours in tumor sphere medium (34). Both cell populations (5 × 10^4 cells/po) were then isolated, lysed with 50 μL of NP-40 buffer, and telomerase activity was assessed by TRAP assays.

Cell lines

Fetal neural stem HF240 cells (31), human mesenchymal progenitor (hMP) cells, human umbilical cord perivascular cells (35), and human skin-derived precursors (hSKP; ref. 32) were previously described and used as normal tissue stem cell lines. Glioma TIC lines G179 and GLINS1 (described as G144ED in Pollard et al.) were derived from patients with a diagnosis of classic glioblastoma multiforme and maintained as we previously described (31). Neuroblastoma NB12, NB88R2, and NB122R TICs were isolated from bone marrow aspirates and maintained as described previously (17).
Telomeric repeat amplification protocol assay

Telomeric repeat amplification protocol (TRAP) assays were conducted using a TRAPEze kit (Millipore) according to the manufacturer’s instructions. In brief, $5 \times 10^4$ cells were lysed with 50 µL of NP-40 buffer (33), placed on ice for 30 minutes, and spun, and 2 µL of supernatant was used for the PCR reaction. HeLa cell lysates were used as a positive control and lysis buffer as a negative control. Approximately 30 µL of each PCR reaction was loaded onto a 10% nondenaturing acrylamide gel and run for 3 hours at 180 V. Gels were visualized using the Odyssey infrared imaging system (LI-COR Biosciences).

Terminal restriction fragment assay

Cell pellets were digested overnight in TAE buffer (10 mmol/L Tris, 1 mmol/L EDTA; Invitrogen) and 0.5% SDS. Total DNA was isolated through phenolic extraction and ethanol precipitation. Telomere length was determined using the nonradioactive chemiluminescent TeloTAGGG assay (Roche) following manufacturer’s instructions and autoradiographs were developed using a Kodak X-OMAT 2000A film processor.

In vitro telomerase inhibition

Cells were seeded at $3 \times 10^3$ (G179) or $4 \times 10^3$ (HF240) in poly-L-ornithine/laminin-coated P100 dishes or $3 \times 10^5$ cells/75-cm$^2$ flask (NB12, NB88R2, and NB122R) and treated twice a week with 5 µmol/L of either Imetelstat sodium or mismatch control. Population doublings were assessed every week by using the formula log(number of cells collected/the number of cells plated)/log 2 and cells replated with fresh media and corresponding drug or control. All experiments were done in triplicate.

In vivo telomerase inhibition

Fifty thousand neuroblastoma TICs (NB12) were resuspended in 50 µL of proliferation media (17). Cells were mixed 1:1 with Matrigel basement membrane matrix (BD Biosciences) and the cell suspension was immediately injected into the inguinal fat pad of 6- to 8-week-old non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice ($n = 5$/group; The Jackson Laboratory). Intraperitoneal treatment with Imetelstat (30 mg/kg thrice a week, Geron Corporation), mismatch control oligonucleotide (5’-palm-TAGGTGTAAGCAA-NH2-3’, 30 mg/kg thrice a week, Geron Corporation) or PBS was initiated immediately or when the tumor was established (50–100 mm$^3$; approximately 3 weeks postimplantation). Tumor volumes were monitored using calipers and calculated with the formula $V = (length \times width^2)/2$. Once tumors reached 1,000 mm$^3$, animals were sacrificed and tumor cells were isolated. Xenograft tumors were cut into 2- to 3-mm$^3$ pieces, triturated in DMEM:F12 media, and cells were filtered through a 40-µm cell strainer. Approximately $5 \times 10^5$ cells/tumor were lysed with 50 µL of NP-40 buffer and telomerase activity was assessed by TRAP assays. Animals were housed in a facility certified by the Canadian Council of Animal Care, and all in vivo procedures were approved by the Hospital for Sick Children’s Animal Care Committee.

Statistical analysis

The analysis of tumor volumes, γH2AX quantification (Students’ $t$-test), and survival (log-rank test) were done with GraphPad Prism, Version 4.

Results

Telomerase activity is specific for the TIC subpopulation

To assess whether telomerase activity is specific to a certain tumor cell subpopulation, we separated freshly resected gliomas into TIC and the bulk of tumor cells employing different techniques. First, we assessed telomerase activity in 2 subpopulations of cells from the same tumor sorted by CD15/SSEA1 expression, a known marker for TICs (refs. 36–38; Supplementary Fig. S1A). To show that CD15$^+$ and CD15$^-$ cell subpopulations are both glioma tumor cells, we assessed copy number alterations in each subpopulation by using high-throughput Affymetrix SNP6.0 microarrays. We observed similar copy number alterations, including amplification or deletion of large chromosomal regions, in the whole tumor and each subpopulation as commonly observed in high-grade gliomas (Supplementary Fig. S1B).

The whole tumor cell population and the CD15$^+$ subpopulation (referred to in this study as TICs; ref. 38) revealed high telomerase activity. Strikingly, this property was limited to the CD15$^+$ population; the CD15$^-$ cells, which comprise the majority of tumor cells, exhibited undetectable telomerase activity (Fig. 1A). We then repeated the same experiments with cell subpopulations from high-grade gliomas that were separated according to morphologic differences after 48 hours of culture in neural stem cell media. Two cell subpopulations were observed in this assay: spheroid aggregates of nestin-positive floating cells, which again revealed high telomerase activity (Fig. 1B; ref. 34) and adherent mature GFAP (marker of glial differentiation)-positive and nestin-negative glioma cells (Fig. 1B), which exhibited undetectable telomerase activity. They were also unable to proliferate in vitro (34) and were positive for senescence-associated (SA)-β-galactosidase activity (Fig. 1B). In total 14 tumors were analyzed using TRAP assays, of which we show 3 examples (G291, G296, and G308). In all cases, only the sphere cell subpopulation displayed significant telomerase activity (Fig. 1C) and, as previously reported by our group, only this subpopulation could recapitulate tumors after serial transplantation (16).

Normal tissue stem cells have undetectable telomerase activity in contrast to TICs

We next explored the differences in telomere maintenance between TICs and the putative normal tissue stem cells. We previously established normal neural stem cell and high-grade glioma (glioblastoma) precursor cultures in serum-free media on laminin (ref. 31; referred to as glioma...
TIC lines) and grew neuroblastoma precursors in sphere conditions (ref. 17; referred to as neuroblastoma TIC lines) for a prolonged period of time. Although both normal neural stem cell lines and glioma TIC lines grown in identical culture conditions express high levels of nestin, a marker for neuronal precursor cells (Fig. 2A), we observed undetectable telomerase activity in the normal neural cells and extremely high telomerase activity in the glioma TIC lines (Fig. 2B). These findings were recapitulated in normal pediatric neural crest–like sphere lines (skin-derived precursor cells; ref. 32), mesenchymal stem cells (human umbilical cord perivascular cells; ref. 35), and the corresponding neuroblastoma TIC lines that arise from the neural crest (Fig. 2B). We then carried out quantitative PCR analysis of hTERT expression (the catalytic subunit of the telomerase complex) in the same populations (Fig. 2C). TICs had significantly higher levels of hTERT expression than normal tissue stem cells. Finally, Terminal restriction fragment (TRF) Southern blot analysis revealed that normal tissue stem cells have very long telomeres whereas TICs had extremely short telomeres (Fig. 2D). Taken together, the long telomeres and undetectable telomerase activity observed in normal stem cells suggest that these cells may be less sensitive to telomerase inhibition.

Telomerase inhibition targets TICs but not normal tissue stem cells

To determine whether normal tissue stem cells or TICs are dependent upon telomerase for cell proliferation, we evaluated the effects of the RNA TR oligonucleotide antagonist Imetelstat (GRN163L; Geron Corporation) on these cells. Consistent with our previous findings, the treatment of normal neural stem cells with Imetelstat did not affect their replicative ability even after prolonged exposure (Fig. 3A). Conversely, telomerase inhibition led to changes in stem cell properties in 2 glioma TIC lines (G179 and
Telomerase inhibition of G179 glioma TIC line resulted in profound growth arrest (Fig. 3B) as well as a pronounced loss of telomerase activity that persisted throughout the period of treatment. Telomerase inhibition (Fig. 3C) was accompanied by a reduction in TIC telomere length (Fig. 3D) and increased SA-β-galactosidase activity, differentiation as determined by immunostaining with GFAP for glial differentiation and DNA damage as measured by immunostaining for γH2AX (Fig. 3E and Supplementary Fig. S2). Moreover, telomerase inhibition of the GLINS1 glioma TIC line also revealed similar evidence of senescence, DNA damage, and loss of self-renewal capability as shown by sphere-forming assays (Supplementary Fig. S3).

Telomerase inhibition promotes loss of self-renewal capacity and cell growth arrest in a neuroblastoma TIC model

To broaden our analysis of telomerase-dependent TIC self-renewal, we investigated the effects of telomerase inhibition on 3 neuroblastoma TIC lines. Imetelstat treatment of the neuroblastoma TIC line NB12 resulted in a faster and more dramatic loss of replicative potential after only 3 to 5 weeks of treatment (Fig. 4) than glioma TIC lines. It also led to the inhibition of telomerase activity throughout the treatment period (Fig. 4B), loss of the spheroid morphology (Fig. 4C), and rapid telomere attrition (Fig. 4D). Furthermore, the treated NB12 cells lost their sphere-forming ability, indicating a loss of self-renewal capability (Fig. 4E). These results were repeated and confirmed in 2 other neuroblastoma TIC lines (NB88R2 and NB122R) freshly isolated from bone marrow metastases from different pediatric patients (Supplementary Fig. S4).

In vivo treatment with telomerase inhibitor leads to tumor growth arrest and increased survival

To study the effects of telomerase inhibition on TICs in vivo, we implanted human NB12 neuroblastoma TICs into NOD/SCID mice (17). Cells that were pretreated with Imetelstat over a period of 4 weeks failed to form tumors.

Figure 2. TICs have substantially higher telomerase activity than normal tissue stem cells. A, immunofluorescence studies show that both normal neural stem cells (HF240) and glioma TICs (G179) express high levels of nestin (neuronal precursor cells marker) and do not express the glial differentiation marker GFAP. Scale bar, 63 μm. B, TRAP assay of tissue stem cell lines HF240 (neural), hMP (mesenchymal), and hSKP (neural crest-like) showing undetectable telomerase activity when compared with the neuroblastoma TIC line NB12 and the glioma TIC lines G179, G144, and GLINS1. C, comparative quantitative PCR analysis of hTERT expression shows very low levels of hTERT expression in the tissue stem cell lines when compared with TIC lines. Column bars, mean of 3 measurements. D, TRF analysis showing that normal tissue stem cells have longer telomeres than TICs.
Figure 3. Telomerase inhibitor, Imetelstat, inhibits growth of glioma TICs, but not normal neural stem cells. Cell population doubling analysis of the HF240 normal neural stem cell line (A) and the TIC line G179 (B) after treatment with Imetelstat. The blue lines indicate untreated cells, black lines the mismatch drug control, green line the recovered cells whose treatment with Imetelstat was stopped at week 8, and the red line indicates Imetelstat treatment. Only TICs show loss of replicative potential after telomerase inhibition. Data points represent the mean of triplicate cell counts ± SD. C, TRAP assay of the G179 TIC cell line after 3, 6, 9, or 12 weeks of Imetelstat treatment. Inhibition of telomerase activity was observed only in treated cells. U, untreated; M, mismatch control; I, Imetelstat treated. D, TRF assay of G179 TIC line throughout 15 weeks of Imetelstat treatment. Cells treated with the telomerase inhibitor show progressive shortening of telomeres when compared with negative controls. E, cell characterization after 12 weeks of Imetelstat therapy. Treated TICs exhibit senescent profile as shown by positive β-galactosidase (β-Gal) activity, positive immunostaining for GFAP (astrocyte marker indicating differentiation), and increased γH2AX foci (DNA damage marker). Nestin is a neuronal precursor cell marker. Quantifications are shown under each respective panel.
even though no therapy was given to these animals (Fig. 5A). We then compared tumor formation and growth in mice treated either immediately after implantation or after tumor establishment (Fig. 5B). Animals whose treatment started immediately after implantation showed slower tumor progression relative to all other groups and by the end of the experiment their tumor volumes were significantly smaller than those in the PBS (vehicle) control group ($P = 0.02$) as well as established treated and mismatch (control oligonucleotide) groups ($P = 0.02$; Fig. 5B). Interestingly, a striking decrease in tumor growth rate was observed late in the treatment phase, suggesting a loss of replicative capacity due to telomere dysfunction. The early treatment group also showed a significant increase in survival when compared with PBS controls ($P = 0.03$), mismatch controls (mice were treated with the mismatch control immediately after implantation), ($P = 0.02$) and mice treated after tumor establishment ($P = 0.009$; Fig. 5C).

In an effort to address whether in vivo Imetelstat treatment affects self-renewal capability of TICs, we harvested tumors from the different groups of mice and carried out sphere-forming assays (Fig. 5D). TICs from both of the treated groups showed a significant loss of sphere-forming ability when compared with any of the controls, suggesting that telomerase inhibition impairs stem cell ability to self-renew in vivo in addition to causing inhibition of tumor growth.

**Telomerase inhibition results in irreversible loss of TIC self-renewal capabilities**

Because many brain tumors or advanced neuroblastomas recur after the cessation of therapy, we next assessed whether the growth inhibition and loss of stem cell
self-renewal ability displayed by Imetelstat-treated TICs are reversible. Glioma TICs grown in adherent conditions were treated with Imetelstat for a total of 8 weeks and then therapy was discontinued. Although telomerase activity was rapidly restored, these cells did not resume growth as the rate of control cells 7 weeks after cessation of treatment (Fig. 3B). We then carried out long-term colony forming assays for a period of 7 weeks by using neuroblastoma TICs pretreated with Imetelstat. While control TICs continued to form larger spheres, indicating replication ability, the pretreated TICs formed only small spheres (Supplementary Fig. S4G), without evidence of new sphere formation or growth. These results suggest that the pretreated neuroblastoma TICs have lost the self-renewal capacity of untreated TICs. In addition, the inability of the pretreated TICs to form tumors when implanted into mice in vivo (Fig. 5B) and the loss of clonogenic ability of TICs after in vivo treatment with Imetelstat (Fig. 5D) both suggest an irreversible loss of TIC self-renewal capability after a period of telomerase inhibition.

**Discussion**

In this study, we show that TICs are a subpopulation of tumor cells that are highly dependent on telomerase activity for their self-renewal. This property is not observed in the bulk of tumor cells or corresponding normal human tissue stem cells (Figs. 1A and C and 2B). Taken together, these 2 observations may have significant clinical implications for the treatment of patients with cancer.

The cancer stem cell hypothesis is still controversial (39, 40) and this is beyond the scope of this study. We used
previously published robust platforms (17, 31, 32, 38) to reassess the role of telomere maintenance in cell subpopulations.

Specifically, our data support the concept that tumors are composed of a heterogeneous group of cells with different telomere maintenance (13). The current telomere hypothesis states that telomerase is activated late in tumorigenesis by all tumor cells that can escape crisis (41). This concept was supported by experiments in cell line models in which hTERT activation was not sufficient for oncogenic transformation unless accompanied by the introduction of oncogenes and tumor suppressors (42–44). Because ex vivo separation of tumor cells into subpopulations according to stem cell markers has only recently been introduced, this concept was never challenged. Furthermore, because TRAP is a PCR-based assay, it is very sensitive and can produce positive results from tumors in which only a small cell component can activate the enzyme. Here we show that in freshly resected glioma tumor samples, telomerase is active only in TICs that represent 6% to 13% of the whole tumor cell population (data not shown and Supplementary Fig. S1; ref. 36). Phenotypically, these cells are precursors that show no evidence of cell senescence or differentiation (Fig. 1B). As the majority of glioma cells do not have telomerase activity and reveal a mature and senescent phenotype, they may be merely "passengers" in the ability of the tumor for continuous proliferation and recurrence. TICs, which are the "drivers" of these processes, require telomerase for long-term survival and ability to cause tumor recurrence.

Prior studies have failed to conduct a thorough comparative analysis of telomere maintenance in normal and tumor stem cells, primarily due to a lack of primary stem cells for use in long-term experiments. Although TICs mimic normal tissue stem cells in many characteristics, including the expression of progenitor markers (Fig. 2B), the ability to differentiate under certain conditions, and their replicative potential, our observations reveal striking differences in their telomere maintenance. TICs possess high levels of hTERT (Fig. 2C) and telomerase activity, accompanied by very short telomeres (Fig. 2D) and high sensitivity to telomerase inhibition (Figs. 3B and 4A and Supplementary Fig. S4A and B), suggesting telomerase "addiction" (45) and that this mechanism is regulated by hTERT. This is very different from normal neural stem cells and neural crest–like stem cells, both of which have very long telomeres (Fig. 2D), undetectable telomerase activity (probably due to low hTERT expression; Fig. 2C), and are resistant to long-term telomerase inhibition (Fig. 3A). These observations have major biological and clinical implications and should be confirmed in stem cells from other origins such as endodermal and hematopoietic. Although TICs were reported to have high telomerase activity in several tumor types (22), this was thought to be a part of their "stem cell" abilities and not a feature acquired during their transition to cancer. Concern about "exhausting" normal stem cells is the major reason for the hesitation in treating children with telomerase inhibitors. Our findings suggest that normal tissue stem cells lack telomerase "addiction" and therefore telomerase inhibition will not prevent them from continuing to replenish tissues throughout life. Because normal tissue stem cells may divide very slowly with prolonged periods of quiescence (46, 47), to avoid errors during DNA replication and maintain their long-term proliferation ability (48), it may be possible that these cells reactivate telomerase only rarely and, given their long telomeres, may have enough telomere length to survive throughout human life without consistently requiring telomerase.

Imetelstat has proven effective in inhibiting telomerase activity in multiple cancer cell lines and preclinical models (22, 33, 49, 50) and recent publications have confirmed inhibition of TIC growth by Imetelstat in different glioma and prostate cancer models (22, 23), our findings add the necessary translational safety and specificity aspect to enable the use of telomerase inhibition in children with cancer.

Interestingly, our studies revealed that telomerase inhibition resulted in late tumor growth arrest and therefore failed to have a significant survival benefit on established tumors. These observations are within the telomere hypothesis, in which time is required for telomeres to become dysfunctional in order for tumor cells to reach crisis. If confirmed, the clinical implications of this finding is that telomerase inhibition must be given in conjunction with other cytotoxic therapies or in the phase of minimal residual disease to prevent tumor recurrence by reducing TIC self-renewal.

Finally, our data suggest that telomerase inhibition leads to TIC "exhaustion" due to loss of self-renewal capability as shown by the inability of treated cells to resume cell growth (Fig. 3B) to generate new clones after cessation of telomerase inhibition (Figs. 4E and 5D and Supplementary Figs. S3D and S4G) and the inability of pretreated cells to form tumors in vivo (Fig. 5B). Although these findings are intriguing, they should be interpreted with care and should serve as a proof of principle. It would be important to follow TIC-treated cells for a longer period of time in order to further support this concept. Because most cancers tend to recur after cessation of therapy, cancer stem cell exhaustion can add a new dimension to the current cytotoxic approach to cancer therapy.

In summary, our data indicate that telomerase inhibitors can act specifically on TICs, causing irreversible loss of their self-renewal capacities and sparing normal tissue stem cells. Cancer stem cell exhaustion can serve as a novel therapeutic approach for cancer and be both effective and safe for children with neural tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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