HEMATOPOIESIS AND STEM CELLS

Prolonged self-renewal activity unMASKS telomerase control of telomere homeostasis and function of mouse hematopoietic stem cells

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Methods for expanding hematopoietic stem cells (HSCs) could have significant utility for transplantation-based therapies. However, deleterious consequences of such manipulations remain unknown. Here we examined the impact of HSC self-renewal divisions in vitro and in vivo on their subsequent regenerative and continuing ability to sustain blood cell production in the absence of telomerase. HSC expansion in vitro was obtained using a NUP98-HOXA10hd transduction protocol and, in vivo, using a serial transplant protocol. We observed ~10 kb telomere loss in leukocytes produced in secondary mice transplanted with HSCs regenerated in primary recipients of NUP98-HOXA10hd-transduced and in vitro-expanded Tert−/− HSCs 6 months before. The second generation leukocytes also showed elevated expression of γH2AX (relative to control) indicative of greater accumulating DNA damage. In contrast, significant telomere shortening was not detected in leukocytes produced from freshly isolated, serially transplanted wild-type (WT) or Tert−/− HSCs, suggesting that HSC replication posttransplant is not limited by telomere shortening in the mouse. These findings document a role of telomerase in telomere homeostasis, and in preserving HSC functional integrity on prolonged self-renewal stimulation. (Blood. 2011;118(7):1766–1773)

Introduction

The existence of HSCs with a capacity for sustained self-renewal is essential for lifelong blood cell production. Expansion of the stem cell pool requires the stimulation of symmetric self-renewal divisions and is critical both during early development1 and in later life,2,3 as well as after transplantation or after hematopoietic injury. When small numbers of HSCs are transplanted into myeloablated or pre-immune hosts, the increases in HSC numbers that follow may be even larger than those seen during development.4,6 These findings document the high replicative potential of HSCs.7 In mice, retroviral marking studies and, more recently, reconstitution studies starting from a single transplanted cell have shown that a single HSC can reestablish the hematopoiesis of a mouse, by continual creation of new HSCs capable of regenerating the system.8,9

The self-renewal function of HSCs and their ability to reestablish hematopoiesis permanently in a myelosuppressed host is the basis of an increasing range of therapies for (BM) failure, malignant and genetic disorders.10,11 Broader use (eg, from cord blood sources) and improved safety (eg, by accelerating recovery) of such transplant therapies would be greatly facilitated by the development of methods for achieving significant prior expansion of HSC numbers ex vivo.12-14 However, it remains unclear, whether HSC self-renewal activity, provoked by either extrinsically or intrinsically induced mechanisms would at some point have deleterious consequences (eg, by inducing HSC senescence or an impairment of some critical aspect of HSC function). Studies reported to date indicate that despite detectable levels of telomerase expression,15 the telomeres of leukocytes present in the blood of increasingly older people are increasingly shorter. This finding suggests that some HSC proliferation is constantly occurring in humans.16 Similar results have been reported for hematopoietic cells produced in murine recipients of serially transplanted hematopoietic cells.17-19

The severe consequences of genetically determined telomerase deficiencies provide additional compelling evidence of the importance of telomerase in both humans and mice. Patients suffering from dyskeratosis congenita (DKC) or acquired aplastic anemia with loss of function mutations in telomerase complex genes20,21 have short telomeres, frequently associated with a decreased proliferative capacity of their hematopoietic progenitors, BM failure and sometimes evidence of malignant progression because of genomic instability.22,23 Similar to DKC patients, late generation telomerase-deficient mice (as mice possess significantly longer telomeres than humans) provide compelling evidence of the importance of telomerase in telomere homeostasis, and in preserving HSC functional integrity on prolonged self-renewal stimulation.24

The purpose of this study was to examine the extent of HSC telomere loss and possible impairment of HSC function under conditions of HSC self-renewal stimulation in vitro and in vivo and}


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the effect of absent telomerase (Tert) on the responses obtained. The average length of telomere repeats in HSCs progeny was measured by a highly sensitive flow-fluorescent in situ hybridization (FISH) method, here adapted for application to murine cells. This method uses labeled peptide nucleic acid probes specific for telomere repeats in combination with fluorescence measurements by flow cytometry thereby enabling the rapid analysis of large numbers of different cell types, discriminated by differences in light scattering and immunophenotypic properties. The conditions used to stimulate HSC self-renewal involved serially transplanting freshly isolated BM samples (self-renewal stimulation in vivo) or, after their transduction with a NUP98-HOXA10hd (NA10hd) transgene and expansion in vitro before transplant (self-renewal stimulation in vitro and in vivo). This in vitro treatment was based on our previous finding that expression of this variant Hox transcription factor (NUP98-HOXA10hd = NUP98 fused to the homeodomain of HOXA10) reproducibly expands HSCs >1000-fold in 10-day cultures with retention of normal HSC differentiation and increased self-renewal activity posttransplant.14 The results of the present studies confirm the importance of intact telomerase activity in maintaining the integrity of telomere length in HSCs that have undergone many self-renewal divisions and demonstrate its necessity in preserving the genomic integrity of their progeny.

Methods

Mice

Mice were bred and maintained at the Biomedical Research Center (BRC) and the British Columbia Cancer Research Center animal facilities according to the guidelines of the Canadian Council on Animal Care. Transplant donor and recipient pairs were the following: C57Bl6 that express CD45.2 and either C57Bl6/JSJL or C57Bl/6Ly-Pep3b that express CD45.1. The recipient mice for telomere length measurements. Donor-derived BM cells were isolated on a FACSVantage (Becton Dickinson), 3 months posttransplant from the 3 primary recipients showing the highest level of reconstitution within each group. Secondary recipients were lethally irradiated (1.1 Gy of X-rays) and transplanted with 5 × 10^6 donor-derived BM cells from the primary mice by intravenous injection. Tertiary transplantations were performed in a similar fashion with 5 × 10^6 donor-derived cells isolated from secondary recipients 3 months posttransplantation. PB was analyzed for donor-derived granulocytes and the reconstitution level evaluated one week before each transplantation.

Assessment of HSC frequencies

HSCs were detected and quantified using the limiting dilution competitive repopulating unit (CRU) assay, as previously described. Briefly, lethally irradiated C57Bl6/6Ly-Pep3b mice (0.81 Gy X-rays) were transplanted by intravenous injection with variable numbers of fresh or NUP98-HOXA10hd-transduced and in vitro expanded cells from WT or TERT-KO mice, along with a life-sparing dose of 10^5 normal C57Bl/6Ly-Pep3b BM cells. The proportion of mice in each group that showed multilineage reconstitution with donor-derived (CD45.2+ or GFP+) cells was determined by flow cytometric analysis of PB WBCs a minimum of 16 weeks posttransplantation. Only mice whose PB contained >1% donor-derived myeloid cells (Ly6G+ and Mac-1+), B cells (B220+), and T cells (CD4+ and CD8+) were considered to be positive. CRU frequencies were calculated using L-Calc software (StemCell Technologies).

Peripheral blood analysis

Blood samples (100 μL) were obtained from the tail vain, erythrocytes lysed with ammonium chloride (StemCell Technologies) and the leukocytes suspended in BSS with 2% FBS (StemCell Technologies). Thereafter, the remaining leukocytes where incubated on ice for 20 minutes with the following antibodies: CD45.1-FITC, CD45.2-APC and Gr1-PE for analysis of recipients transplanted with unmanipulated BM cells. For the analysis of recipients transplanted with NA10-hd-transduced bm cells, all lysed blood samples were split into 3 fractions, each incubated on ice for 20 minutes with following antibody combinations: biotinylated anti-Ly5.2 in combination with either PE-labeled antibody to B220; or a combination of PE-labeled antibodies to Ly6G and Mac-1; or a combination of PE-labeled antibodies to CD4 and CD8, followed by a 20-minute incubation with APC-labeled streptavidin. Finally, all samples were washed with HBSS with 2% FBS and 1 μg/mL propidium iodide (PI, Sigma Chemicals) before analysis on a FACS caliber (Becton Dickinson).

Colony forming cell assays

Clonogenic hematopoietic progenitor cells were assayed by plating 1500 cells per mL of methyl-cellulose culture medium (Methocult M3234; StemCell Technologies), containing 3 U/mL human erythropoietin, 10 ng/mL mouse IL-3, 10 ng/mL human IL-6, and 50 ng/mL mouse stem cell factor (SCF; all recombinant from StemCell Technologies). After 7 days of culture, colonies...
were counted using standard scoring criteria, the cells pooled and the viable cells counted also. Equal cell numbers were replated in duplicates for a total of 3 times at weekly intervals to assess serial replating capacity.

**Flow-FISH**

After lysis of the red blood cells with ammonium chloride, FACS-isolated donor granulocytes (CD45.2<sup>+</sup>) from unmanipulated or donor (GFP<sup>+</sup>) WBCs from transduced BM samples were frozen and stored either in liquid nitrogen or at −120°C until analysis. The majority of transduced BM samples analyzed were > 90% GFP+ and analyzed without further enrichment. Samples < 90% GFP+ (lowest positive being 30%) were FACS-sorted for the transduced population before telomere length measurements. To measure telomere lengths with flow-FISH, each sample (2 × 10<sup>5</sup>-1 × 10<sup>6</sup> cells) was split in half and mixed with 2 × 10<sup>5</sup> fixed cow thymocytes of known telomere length. One sample was stained, leaving the second unmarked to account for auto-fluorescence. DNA was then denatured for 15 minutes in 75% formamide at 87°C, followed by hybridization for 90 minutes at room temperature with a 0.3 μg/mL FITC-labeled (CCCTAA) <sub>3</sub> PNA specific for the telomere sequence. Excess probe was removed by several washes in a Hydra robotic washing station. The first lysis of the red blood cells with ammonium chloride, FACS-isolated donor granulocytes (CD45.2<sup>+</sup>) from unmanipulated or donor (GFP<sup>+</sup>) WBCs from transduced BM samples were frozen and stored either in liquid nitrogen or at −120°C until analysis. The majority of transduced BM samples analyzed were > 90% GFP+ and analyzed without further enrichment. Samples < 90% GFP+ (lowest positive being 30%) were FACS-sorted for the transduced population before telomere length measurements. To measure telomere lengths with flow-FISH, each sample (2 × 10<sup>5</sup>-1 × 10<sup>6</sup> cells) was split in half and mixed with 2 × 10<sup>5</sup> fixed cow thymocytes of known telomere length. One sample was stained, leaving the second unmarked to account for auto-fluorescence. DNA was then denatured for 15 minutes in 75% formamide at 87°C, followed by hybridization for 90 minutes at room temperature with a 0.3 μg/mL FITC-labeled (CCCTAA) <sub>3</sub> PNA specific for the telomere sequence. Excess probe was removed by several washes in a Hydra robotic washing station. The first

**Results**

**Telomerase deficiency affects telomere homeostasis under conditions of prolonged self-renewal stimulation**

In an initial series of experiments, primary recipients were transplanted with BM cells from either WT or Tert<sup>−/−</sup> mice which were not found to be different based on flow cytometric analyses of cells with a Lin<sup>−</sup> Sca-1<sup>+</sup> c-kit<sup>+</sup> (LSK) phenotype and assessment of HSC frequency by limiting dilution transplant assays of cells with long-term lympho-myeloid repopulating activity (data not shown). Six weeks and 3 and 9 months after transplantation of primary mice the donor-derived BM cells regenerated were sampled by BM aspiration and their telomere lengths measured using flow-FISH (Figure 1A). To examine the effects of telomerase deficiency on HSC function, the cells were subjected to a serial transplant protocol. Secondary and tertiary transplants were performed by transplanting donor-derived BM cells from the primary and the secondary recipients, respectively, and at the time of each transplantation, telomere length measurements were again performed on donor-derived bm cells (Figure 1C). BM cells from early generation Tert<sup>−/−</sup> mice had significantly shorter telomere repeats (∼24kb) than those of WT mice (∼34kb; Figures 1B,D and 2B,E), most likely as a consequence of the telomerase deficiency in the parental germ line combined with the proliferative stress that occurs during early development. Surprisingly, evidence of further erosion of telomeres related to the serial transplantation protocol, even in cells unable to produce telomerase, was not observed over 9 months post transplantation within individual mice (Figure 1B,D). Consistent with this, both WT and Tert<sup>−/−</sup> HSCs showed a similar reconstitution capacity in primary, secondary and tertiary transplants (Figure 1E). However, in the third round of transplantation, we observed a decrease in PB chimerism in recipients of either WT or Tert<sup>−/−</sup> HSCs (Figure 1E), that was not correlated with evident telomere attrition.

Subsequent experiments were designed to determine whether stimulating even higher levels of HSC expansion in vivo would lead to telomere attrition and/or further effect on HSC function. Therefore, in addition to proliferative stress provoked by serial transplantation, we used forced expression of NAI10hd to enhance the self-renewal of WT and Tert<sup>−/−</sup> BM cells regenerating in vivo. Accordingly, we transduced BM cells with GFP (control) or NAI10hd retroviral vector and then transplanted them into primary recipients either immediately after infection or after an additional 6-day period in culture sufficient to achieve >1000-fold expansion of the input HSCs. Expansion cultures were initiated with either large or reduced numbers of BM cells, estimated to contain ∼30 or 1-2 HSCs, respectively. The latter approach allowed monitoring the possibility of clone to clone variation. Secondary transplants were performed 6 months later and telomere length measurements of donor-derived (NAI10hd-transduced) BM cells were performed after 3 and 6 months in primary hosts and 3 months post transplantation of secondary recipients (Figure 2A,D). The growth factor cocktail used for gene transduction and in vitro expansion was chosen to induce HSC cycling required for high level gene transfer, rather than to promote HSC self-renewal and maintain HSC numbers in culture. Consequently, recipients of control GFP-transduced bm cells showed low chimerism for transduced cells (for recipients of WT, > 20%, or for Tert<sup>−/−</sup>, > 4%) or essentially no chimerism for transduced cells (for recipients of WT or Tert<sup>−/−</sup> transduced and additionally cultured cells), thus imped-
These results indicate that NA10hd-stimulated symmetric HSC self-renewal divisions required to explain the expansion measured in vivo and/or in vitro does not affect telomere homeostasis. A similar result was seen for the progeny of NA10hd-transduced Tert<sup>−/−</sup> BM cells initially transplanted immediately after transduction into primary and then secondary recipients which showed only a slight but still nonsignificant decrease in telomere length (Figure 2B).

In sharp contrast, the progeny of NA10hd-transduced Tert<sup>−/−</sup> HSCs and/or clones that had undergone the 6-day in vitro expansion exhibited a significant (~5kb) telomere loss during the 6 months of regeneration in primary transplant recipients and an additional ~5kb telomere loss after another 3 months of regeneration in the secondary transplant recipients (Figure 2E). This result highlights the significance of telomerase in maintaining HSC telomere homeostasis when sufficient symmetric self-renewal divisions are stimulated to occur. Importantly, the level of chimerism obtained in recipients of WT or Tert<sup>−/−</sup> HSCs, stimulated to self-renew by NA10hd either in vivo (Figure 2A) or in vitro (Figure 2D) also revealed a significantly decreased reconstituting ability of Tert<sup>−/−</sup> BM cells in primary and secondary transplants (Figure 2C,F). Interestingly, such a deficit in reconstituting ability of Tert<sup>−/−</sup> BM cells (Figure 2F) was accompanied by skew to myeloid cells (data not shown) and a significant telomere loss (Figure 2E) only after NA10hd-enhanced HSC expansion in vitro (Figure 2D). However, the phase of NA10hd-enhanced HSC expansion in vivo (Figure 2A,C), appears to be independent of detectable telomere attrition (Figure 2B). We also performed a limiting dilution analysis of the HSC frequency in the in vitro expanded cell suspensions initiated with WT and Tert<sup>−/−</sup> BM cells (Figure 3B). As expected, results revealed very significant levels of net HSC expansion in both WT and Tert<sup>−/−</sup> cultures (~1800- and ~260-fold, respectively) although the net expansion was ~7-fold less for NA10hd-transduced Tert<sup>−/−</sup> HSCs relative to WT HSCs (Figure 3B).

To determine whether a more rapid indicator of the role of telomerase in maintaining the proliferative potential of primitive hematopoietic cells could be developed, we examined the response of NA10hd-transduced clonogenic bm cells to being serially replated in methylcellulose medium. NA10hd-transduced WT progenitors consistently formed large granulocyte-macrophage colonies over at least 4 passages. In contrast, NA10hd-transduced Tert<sup>−/−</sup> progenitors were essentially exhausted by a third replating (Figure 3A). Taken together, these results demonstrate that the
absence of telomerase activity blunts the ability of NA10hd to maintain the self-renewal capability of primitive hematopoietic cells, including HSCs, thereby implicating telomerase in the regulation of this property.

**Primitive hematopoietic cells lacking telomerase activity exhibit signs of enhanced DNA damage**

Recent studies performed in mouse models of accelerated aging, including mice lacking telomerase activity, and/or defective in DNA repair pathways have shown that accumulation of DNA damage can deleteriously impact HSC function.25,35 In fact, competitive transplantation assays have revealed reduced long-term reconstituting and self-renewal activity of HSCs from aged mutants relative to WT HSCs, ultimately leading to premature exhaustion of their numbers. These studies thus suggest that impaired HSC function may be a general consequence of DNA damage accumulation.

Because our findings demonstrated a decreased ability of primitive Tert−/− hematopoietic cells to respond to prolonged NA10hd-enhanced self-renewal stimulation in vivo and/or in vitro (Figures 2C,F and 3), we designed experiments to investigate whether this might be associated with a corrupted genomic integrity of these cells. To investigate this possibility, we used flow cytometry to analyze the LSK subset of unmanipulated, second generation Tert−/− BM cells for expression of phosphorylated histone H2AX (γ-H2AX), an indicator of DNA damage. This
analysis showed an average of a 4-fold increase in γ-H2AX expression within the telomerase deficient LSK subset compared with their WT counterparts (Figure 4A). A similar trend was observed when donor-derived BM cells were obtained from recipients reconstituted with unmanipulated WT and Tert⁻/⁻ cells (Figure 4B), as well as donor-derived bm cells in recipients reconstituted with NA10hd-transduced and in vitro expanded WT or Tert⁻/⁻ BM cells (Figure 4C). This trend was further reinforced by direct comparison of γ-H2AX expression in recipient- and donor-derived LSK compartment within each analyzed reconstituted recipient shown in 4B and C. Taken together, these results show that γ-H2AX expression was not increased simply as a result of the stimulus for HSC regeneration in transplanted mice and/or NA10hd-enhanced expansion in vitro or in vivo, and that the evidence of DNA damage accumulation seen in primitive (LSK) Tert⁻/⁻ hematopoietic cells may represent an early indicator of the reduced functional integrity of these cells when stimulated to proliferate.

**Discussion**

Our findings confirm and extend accumulating evidence for 2 functions of telomerase in HSC regulation. First, is a postulated role of telomerase in telomere maintenance. Second, is a role of telomerase in preserving HSC function. These activities were revealed in experiments in which Tert⁻/⁻ HSCs were stimulated to execute increased self-renewal divisions by serial transplantation, as previously examined in studies by others, combining with forced expression of NA10hd and exposure to growth factors in vitro. Importantly, under the same conditions, telomere homeostasis was not affected when NA10hd was used to induce symmetric self-renewal divisions of WT HSCs either in vivo or in vitro. Contrary to previous findings, we did not detect significant telomere shortening in the progeny of serially transplanted WT or...
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**Authorship**

Contribution: S.S. and V.G. designed and performed the research and analyzed the data; I.V. performed and analyzed flow-FISH
data; M.G. and C.S. helped perform and analyze flow cytometry for γH2AX; Y.E. and C.B. helped perform mouse experiments; C.J.E., P.M.L. and F.M.R. designed the research and edited the manuscript; R.K.H designed the research; and S.S. and R.K.H. wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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