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Evidence for a mitotic clock in human hematopoietic stem cells: Loss of telomeric DNA with age

HOMAYOUN VAZIRI*†, WIESLAWA DRAGOWSKA‡, RICHARD C. ALLSOPP*, TERRY E. THOMAS‡§, CALVIN B. HARLEY*, AND PETER M. LANSDORP‡§¶

*Geron Corporation, 200 Constitution Drive, Menlo Park, CA 94025; ‡Terry Fox Laboratory for Hematology/Oncology, British Columbia Cancer Agency, 601 West 10th Avenue, Vancouver, BC V5Z 1L3, Canada; and §Department of Medicine, University of British Columbia, Vancouver, BC, Canada

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ABSTRACT **The proliferative life-span of the stem cells that sustain hematopoiesis throughout life is not known. It has been proposed that the sequential loss of telomeric DNA from the ends of human chromosomes with each somatic cell division eventually reaches a critical point that triggers cellular senescence. We now show that candidate human stem cells with a CD34$^+$CD38^{lo} phenotype that were purified from adult bone marrow have shorter telomeres than cells from fetal liver or umbilical cord blood. We also found that cells produced in cytokine-supplemented cultures of purified precursor cells show a proliferation-associated loss of telomeric DNA. These findings strongly suggest that the proliferative potential of most, if not all, hematopoietic stem cells is limited and decreases with age, a concept that has widespread implications for models of normal and abnormal hematopoiesis as well as gene therapy.**

The requirement for primers and the undirectional 5′ → 3′ nature of DNA synthesis by DNA polymerases results in incomplete replication of the terminal 3′ strands of linear chromosomes (1–3). Eukaryotic chromosomes end in specialized nucleoprotein structures called telomeres (4) and in vertebrates, including humans, telomeres terminate in tandem repeats of $(TTAGGG)_n$ (5). Telomeres have been shown to be critical for chromosome stability and function (4) and telomere loss has been shown to signal cell cycle arrest and chromosomal instability in yeast (6, 7). Telomeric repeats are synthesized by telomerase, a ribonucleoprotein capable of elongating telomeres *de novo* (8, 9). In the absence of telomerase, human telomeres shorten with cell divisions and such telomere loss may act as a mitotic clock to eventually signal cell cycle exit and cellular senescence (10, 11). Loss of telomeric DNA upon proliferation *in vitro* has been observed in fibroblasts (10, 12) and lymphocytes (13) but not in immortalized tumor cells, which were found to express telomerase activity (9, 14, 15). Shortening of telomeres during aging *in vivo* has been observed with skin dermal and epidermal cells (12, 16), peripheral blood leukocytes (13, 17), and colon epithelia (17) but not in sperm DNA (12).

Most mature cells of the hematopoietic system are relatively short-lived cells that need to be replaced continuously throughout life. A relatively small population of hematopoietic stem cells is ultimately responsible for producing the staggering numbers of cells estimated to represent the daily output of the hematopoietic system (i.e., $>10^{11}$ cells per day in human adults). Stem cells are usually defined as multipotential cells with self-renewal capacity—i.e., the capacity to give rise to more cells with indistinguishable properties and developmental potential. The maintenance of these properties is believed to be an essential feature of both steady-state hematopoiesis and the regeneration of hematopoiesis from a fraction of the number of stem cells present in the adult as occurs following the administration of myeloblative chemoradiotherapy protocols and injection of a marrow transplant (18–22).

Primitive hematopoietic cells in human bone marrow are contained in a very small subpopulation of cells that is characterized by cell surface expression of high levels of CD34 (23), low levels of Thy-1 (24, 25), and an absence or low levels of CD38 (26), CD45RA, and CD71 (27). Recent studies have shown striking ontogeny-related changes in the functional properties of cells with this phenotype (28). In view of these findings and the age-related loss of telomeric DNA observed in fractionated peripheral blood leukocytes (13) and other somatic cells (12, 16, 17), we undertook experiments to analyze the length of telomeric DNA in hematopoietic cells in relation to their stage of development.

MATERIALS AND METHODS

Small-Scale Cell Purification. CD34$^+$ CD45RA^{lo} CD71^{lo} cells from fetal liver (13–18 weeks of gestation), umbilical cord blood, and bone marrow (organ donors, 19 and 58 years, respectively) were purified by flow cytometry and cell sorting as described (27, 28). Purified cells were cultured for up to 4 weeks in serum-free medium supplemented with Steel factor, interleukin 6 (IL-6), granulocyte/macrophage colony-stimulating factor (GM-CSF)/IL-3 fusion protein (PIXY), macrophage CSF, granulocyte CSF, and erythropoietin as described (29). At various time intervals, the cells in the cultures were harvested, counted, and used for analysis of mean terminal restriction fragment (TRF) length, calculation of population doublings, and continuation of the cultures. Human material used in this study was obtained following protocols approved by local Institutional Review Boards as well as the Ethical Screening Committee of the University of British Columbia.

Large-Scale Cell Purification. Previously frozen bone marrow cells were depleted from cells expressing CD45RA, glycophorin, CD3, CD67, and an unknown platelet antigen (recognized by antibody 3H2 developed in our laboratory) using density separation and high gradient magnetic separation procedures (30). The magnetically preenriched CD34$^+$ cells (70–80% CD34$^+$) were stained for CD34 and CD38 antigens and sorted by fluorescence-activated cell sorting. A total of 0.65×10^6 and 0.41×10^6 CD34$^+$CD38^{lo} cells and 4.2×10^6 and 2.3×10^6 CD34$^+$CD38$^+$ cells was recovered after sorting from 17×10^8 and 38×10^8 nucleated cells, respectively, from each organ donor prior to density separation and immunomagnetic selection.

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Abbreviation: TRF, terminal restriction fragment.

†Present address: Ontario Cancer Institute, Princess Margaret Hospital, 500 Sherbourne Street, Toronto, ON M4X 1K9, Canada.
¶To whom reprint requests should be addressed at the ‡ address.

Telomere Length Analysis. The methods for measurement of mean TRF length have been described elsewhere (12, 13). In brief, 10^6 cells at different time points were washed twice in phosphate-buffered saline and the pellet was lysed. Genomic DNA was extracted and 2–5 μg was digested with excess *Hin*fI and *Rsa* I (BRL, 10–20 units each), ethanol precipitated, and quantified by fluorometry. One microgram of digested DNA was resolved in 0.5% agarose gels. Gels were dried and subjected to hybridization with 5′ end-^{32}P-labeled $(C_3TA_2)_3$. Gels were washed and then exposed to a PhosphorImager screen (Molecular Dynamics).

RESULTS

Loss of Telomeric DNA in Hematopoietic Cells *in Vivo*. Analysis of telomere length in the total nucleated cell population obtained from different bone marrow donors and different fetal liver and cord blood samples demonstrated a loss of telomeric DNA in these tissues at progressive stages of development (Fig. 1). The observed loss of telomeric DNA from early development (fetal liver or cord blood) to adulthood was highly significant ($P < 0.0001$, t test). Telomere length also appeared to decrease with the age of the adult bone marrow donors (calculated loss of 9 bp per year), although considerable individual variation in the mean TRF length at any given age was observed. Most likely, these differences reflect inborn differences in TRF length between individuals. This is also suggested by the observed differences in mean TRF length between the various fetal liver and cord blood samples (Fig. 1). However, differences between individuals in the proliferative history of their hematopoietic cells is another explanation for the observed variations in mean TRF length. Findings in childhood leukemia that indicate that leukemic blast cells have shorter TRFs than normal hematopoietic cells from the same patient support this possibility (31).

Loss of Telomeric DNA in the Cultured Progeny of Purified Hematopoietic Precursor Cells. Primitive hematopoietic cells with a $CD34^+CD45RA^{lo}CD71^{lo}$ phenotype were purified from fetal liver, umbilical cord blood, and adult bone marrow by flow cytometry and cell sorting. The purified cells were cultured in serum-free medium supplemented with a mixture of cytokines to stimulate their proliferation and to obtain sufficient numbers of cells for telomere length analysis. Genomic DNA of the cultured cells was isolated and the mean length of TRFs [which contain the telomeric $(TTAGGG)_n$ repeats] was analyzed by Southern analysis. The results of these experiments are shown in Figs. 2 and 3. Cells produced in cultures of purified adult bone marrow precursors could readily be distinguished from those produced in cultures of purified fetal liver and cord blood cells by virtue of their shorter mean TRF length. Furthermore, the cells produced in the cultures showed a proliferation-associated loss of telomeric DNA. The calculated loss of telomeric DNA by the cultured cells was 45 and 35 bp per population doubling for bone marrow cells, 54 and 19 bp per population doubling for fetal liver cells, and 46 and 23 bp per population doubling for cord blood cells (calculated average loss for all cell types: 37 bp per population doubling). The cells produced in these cultures are known to be highly heterogeneous and contain a minority of primitive precursors amid an abundance of cells at various stages of erythroid and myeloid differentiation (27, 29). As a result, the calculated loss of telomeric DNA per population "doubling" is not indicative of the possible loss of telomeric DNA per cell division in the most primitive precursors.

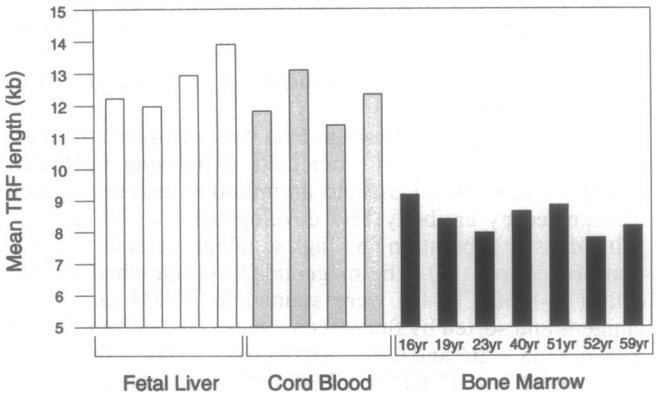

FIG. 1. Loss of telomeric DNA in hematopoietic cells with age. The mean TRF length in cells from the indicated tissue is shown. Fetal liver samples (13–18 weeks of gestation), umbilical cord blood (full-term pregnancies), and bone marrow (from donors of indicated ages) were separated by density centrifugation. DNA was extracted from low-density cells (>90% viable) for TRF analysis. The signal from the dried gels was detected and digitized with a PhosphorImager. The mean TRF length was calculated as described (12, 13).

Loss of Telomeric DNA in the Most Primitive Hematopoietic Cells. To exclude the possibility that the observed telomere loss in cells from adult bone marrow cultures was restricted to the progeny of a more mature subset of progenitors in the purified cell fraction we examined the mean TRF length of the purified cells themselves. This represented a considerable challenge as our current TRF analysis requires DNA from at least 0.5×10^6 cells. To purify sufficient candidate stem cells from $CD34^+$ bone marrow cells in these numbers, low-density cells from previously frozen organ donor bone marrow were first depleted from cells expressing lineage antigens using high gradient magnetic separation (30). $CD34^+CD38^{lo}$ cells (26) were then sorted from the magnetically preenriched cells by fluorescence-activated cell sorting.

The mean TRF length of purified $CD34^+CD38^{lo}$ cells (>99% pure) from the bone marrow of two different organ donors (aged 16 and 58 years) was compared to that of different cell fractions from the same donors and to that of (control) fetal liver cells (Fig. 4). As before (Figs. 1–3), bone marrow cells had a shorter mean TRF length than fetal liver cells and again an apparent decrease with the age of the marrow donor was observed. Small differences in mean telomere length between $CD34^+CD38^{lo}$, $CD34^+CD38^{hi}$, and total nucleated bone marrow cells were also found ($CD34^+CD38^{lo} > CD34^+CD38^+ >$ total nucleated bone marrow cells), in agreement with a higher proliferative potential

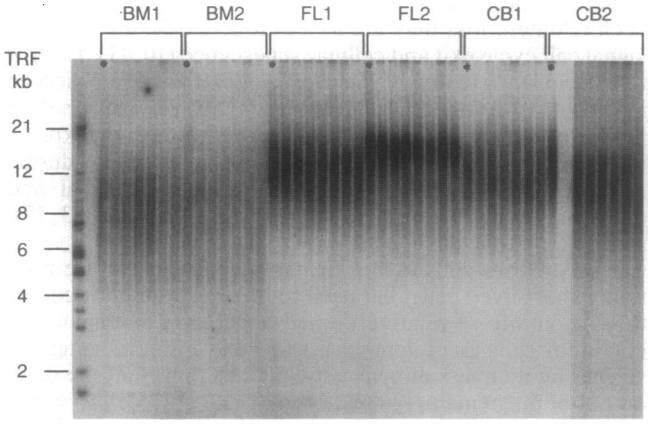

FIG. 2. Loss of telomeric DNA in human hematopoietic cells upon proliferation *in vivo* and *in vitro*. An autoradiogram showing the loss of telomeric DNA in the cultured progeny of primitive hematopoietic precursors purified from the indicated tissues is shown. BM, bone marrow; FL, fetal liver; CB, umbilical cord blood. DNA samples from total nucleated cells from each tissue before purification (indicated with an asterisk) are shown as a control. All subsequent lanes were loaded with digested DNA from cells present at increasing time intervals in cultures initiated with highly purified precursors from the indicated tissues (see also Fig. 3).

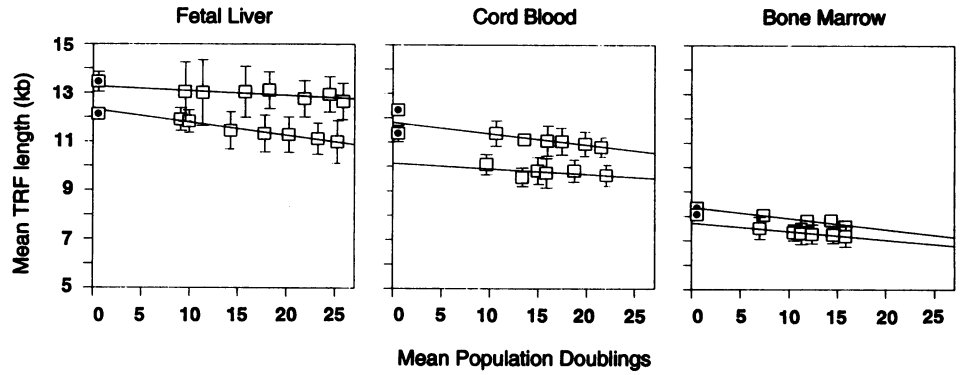

FIG. 3. Loss of telomeric DNA in cells from cultures of purified candidate hematopoietic stem cells. Quantitative analysis of experiments described in the legend to Fig. 2. □, TRF data obtained with cultured cells from two different donors; ⊡, total nucleated cells from the indicated tissues before purification and culture. The mean ± SE of two independent TRF measurements (different gels) for each DNA sample are shown.

of the $CD34^+CD38^{lo}$ cells. Although the presence of a minor subpopulation of cells within the population of $CD34^+CD38^-$ cells with long ("fetal length") telomeres or telomerase activity cannot be excluded by this experiment, the majority of adult bone marrow cells with this phenotype clearly appear to have shorter telomeres than unfractionated cells from fetal liver, presumably as a result of cell divisions *in vivo*.

DISCUSSION

The observed age- and proliferation-related loss of telomeric DNA in hematopoietic cells confirms previous observations with DNA from whole blood (17) and lymphocytes as well as neutrophils (13). Our data strongly suggest that fetal liver or cord blood cells have a significantly higher replicative potential than adult hematopoietic cells, a concept that would argue in favor of the use of such cells for transplantation purposes (32). However, the importance of telomere length relative to other intrinsic and extrinsic factors controlling the proliferative potential and behavior of hematopoietic cells is currently not known.

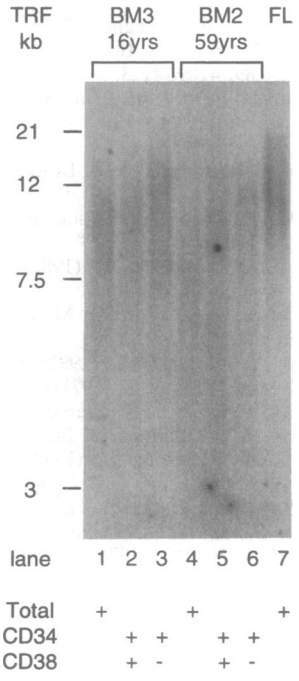

FIG. 4. Age-related loss of telomeric DNA in primitive hematopoietic adult bone marrow cells with a $CD34^+CD38^{lo}$ phenotype. DNA was extracted from $CD34^+CD38^{lo}$ cells (lanes 3 and 6), $CD34^+CD38^+$ cells (lanes 2 and 5), and total nucleated bone marrow (BM) cells (lanes 1 and 4) from two different organ donors (age 16, lanes 1–3; age 58, lanes 4–6) for measurement of mean TRF length. DNA from fetal liver (FL) cells (15–18 weeks of gestation) was used as a control (lane 7). The mean TRF lengths (in kb) of the samples in this gel were as follows: lane 1, 9.2; lane 2, 9.1; lane 3, 10.0; lane 4, 8.0; lane 5, 8.4; lane 6, 9.3; and lane 7, 12.5.

If the most primitive hematopoietic cells lose telomeric DNA at a rate that is roughly comparable to other somatic cells [50–100 bp per doubling (12, 13)], our observations are compatible with limited mitotic activity and a limited proliferative potential in such cells from adult bone marrow. More extensive TRF analysis of larger numbers of samples and the development of more refined techniques to allow telomere length measurements in smaller numbers of cells or, ideally, single cells are required to increase the accuracy of estimates of the turnover rate and proliferation potential in various hematopoietic cells.

In previous studies, we found that the fraction of cells responding to a mixture of hemopoietic cytokines, the production of $CD34^+$ cells, as well as the proliferation rate of $CD34^+$ cells all decrease during development (28). This study, showing a decrease in mean telomere length with cell and donor age, suggests that replicative senescence within the hemopoietic lineage may be causally linked to some of these functional differences. Developmental changes in the turnover rate and cytokine responsiveness of primitive human hematopoietic cells (28) are most likely not directly related to changes in the mean telomere length of such cells. However, limitations in the proliferative potential of purified precursors from adult bone marrow could be directly correlated to the observed decrease in mean telomere length. A major obstacle to direct experimental support for this hypothesis is that human telomerase genes have not yet been cloned (33). However, a large body of evidence on telomere length in somatic cells *in vitro* and *in vivo* indicates that telomere length serves as a biomarker of the replicative history of cells (10, 12, 13, 17, 34).

The findings reported here have several implications for models of both normal and deregulated hematopoiesis. A finite life-span of primitive hematopoietic cells is in agreement with the observed loss of repopulating ability of murine bone marrow in serial transplantation experiments (35–37) or repeated cycles of cytotoxic treatment (38, 39). Our findings are also compatible with the higher proliferative potential of fetal versus adult hematopoietic cells observed in various assays (35, 40, 41). However, data documenting hematopoietic reconstitution of multiple recipients with the progeny of a single marked precursor support the notion that at least some hematopoietic cells in this species are capable of self-renewal (20, 42, 43). Although telomerase expression in regenerating stem cells and/or possible differences between

species cannot be ruled out, another explanation is that in these experiments telomere-related limitations in the proliferative potential of primitive murine precursors were simply not yet reached. A similar situation may exist in various forms of leukemia. At initial stages of the disease, altered signal transduction pathways and/or altered probabilities of differentiation events in a single cell will result in numerical expansion of the leukemic cells. In our current model, this expansion should eventually be limited by critical shortening of telomeric DNA, resulting in signals that trigger cell cycle arrest but also chromosomal instability. Further progression of the disease may involve selection of cells that ignore or can bypass the cell cycle arrest signal and, eventually, selection of cells that express the enzyme telomerase (14, 15, 33). A thorough analysis of telomerase expression in cells from normal hematopoietic tissues at various stages of development as well as cells from patients with various hematological disorders will be required to fully understand the pathophysiology of telomere length dynamics in hematopoietic cells. Further studies are also needed to explore the possibility of extending or maintaining the proliferative potential of adult stem cells by forced expression of exogenous or endogenous telomerase activity. The information derived from such analysis should help to guide the development of novel therapeutic strategies involving transplantation and genetic manipulation of primitive hematopoietic cells for the treatment of a variety of disorders.

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