Telomerase Activity in Candidate Stem Cells From Fetal Liver and Adult Bone Marrow

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Telomerase is a ribonucleoprotein polymerase that synthesizes telomeric repeats onto the 3' ends of eukaryotic chromosomes. Activation of telomerase may prevent telomeric shortening and correlates with cell immortality in the germline and certain tumor cells. Candidate hematopoietic stem cells (HSC) from adult bone marrow express low levels of telomerase, which is upregulated with proliferation and/or differentiation. To address this issue, we stimulated purified candidate HSC from human adult bone marrow with stem cell factor (SCF), interleukin-3 (IL-3), and Flt3-ligand (FL). After 5 days in culture, activity was detected in total cell extracts from IL-3-, SCF + FL-, SCF + IL-3-, FL + IL-3-, and SCF + IL-3 + FL-stimulated cultures, but not from cells cultured in SCF or FL alone. Within the CD34+ fraction of the cultured cells, significant activity was found in the CD34+CD71+ subset of stem cells or, more likely, is not sufficient to prevent telomere shortening.

The hematopoietic system replenishes the loss of mature blood cells via the recruitment of stem cells that have been defined as pluripotential cells with self-renewal properties. We and others have shown low levels of telomerase activity in normal bone marrow and peripheral blood cells, both in progenitors of the myeloid and lymphoid lineages as well as in terminally differentiated cells such as T and B cells. Furthermore, we and others found that “candidate” hematopoietic stem cells (HSC) upregulate telomerase activity upon stimulation in vitro. However, as the vast majority of stem cells are quiescent during steady-state hematopoiesis, the status of telomerase expression in cycling HSC has not yet been elucidated. This is an important issue because the self-renewal and replicative potential of the most primitive hematopoietic cells may depend on telomerase to maintain stable telomeres.

In this report, we describe results of experiments designed to address the question of telomerase expression in cycling stem cells. For this purpose, we measured telomerase activity in extracts from purified candidate HSC from human adult bone marrow stimulated with different cytokines and in extracts from CD34+CD38- candidate HSC purified from human fetal liver. Our data indicated that telomerase activity is expressed in most if not all cycling stem cells but is repressed in quiescent stem cells.

MATERIALS AND METHODS

Purification of HSC from adult bone marrow and fetal liver. Candidate HSC with the phenotype CD34+CD45RA-CD71+ were obtained from previously frozen cadaver marrow as previously described. Briefly, mononuclear cells retrieved from the interface after density separation were stained with 8G12-Cy5 (anti-CD34), 8d2-PE (anti-CD45RA), and OKT9-FITC (anti-CD71) for 30 minutes at 4°C. The hematopoietic system replenishes the loss of mature blood cells via the recruitment of stem cells that have been defined as pluripotent cells with self-renewal properties.
Cells were washed twice in Hanks’ buffered saline with 0.2% BSA (HB) and stained with 2 µg/mL of propidium iodide (PI) before suspending in HB at a density of 5 × 10^6 cells/mL for sorting. Cells were sorted on a FACStar® plus (Becton Dickinson, San Jose, CA) equipped with argon (488 nm) and helium-neon (633 nm) lasers. CD34+CD45RA−CD71lowCD38− cells were obtained from adult marrow and fetal liver in the 17th and 18th week of gestation according to previously described protocols.33,34 Cells were stained with 8G12-Cy5, 8d2-FITC, OKT9-FITC, and anti-CD38-phycocerythrin (PE; Becton Dickinson) for 30 minutes at 4°C. The washing and sorting procedures were performed as above.

Cell culture. Sorted candidate HSC were cultured in serum-free medium consisting of Iscove’s modified Dulbecco’s medium (IMDM) supplemented with the following reagents: bovine serum albumin (BSA) at 2%, sodium bicarbonate at 0.1%, transferrin at 200 µg/mL, insulin at 10 µg/mL, 2-mercaptoethanol at 10^{-5} mol/L, low-density lipoprotein (Sigma, St Louis, MO) at 40 µg/mL, and penicillin-streptomycin at 100 U and 50 µg/mL, respectively. Both stem cell factor (SCF) and Fl3-ligand (FL) were used at a final concentration of 50 ng/mL, whereas interleukin-3 (IL-3) was used at 20 ng/mL. All growth factors were purchased from Pepro-tech (Rocky Hill, NJ).

Telomerase repeat amplification protocol (TRAP) assay. Telomerase activity was measured by TRAP assay using an end-labeled telomerase substrate (TS) primer as described.17,27,28 Briefly, cell extracts were prepared by lysing the cells in CHAPS extraction buffer at a concentration of 500 cells per µL of buffer, centrifuged at 1200 g at 4°C, and 2 µL of these extracts were used in the assay.

The telomerase reaction was performed in 50 µL of TRAP reaction buffer containing 20 mmol/L tris-HCl (pH 8.3), 1.5 mmol/L MgCl_2, 6°C, and 2 µL of these extracts were used in the assay.

Telomerase activity in candidate stem cells from adult bone marrow. Cell extracts from adult marrow candidate HSC with the phenotype CD34+CD45RA−CD71lowCD38− were assayed for telomerase activity by a modified version of the PCR-based TRAP assay. In addition to the typical ladder of 6 bp repeats that correspond to the amplified product of the TS primer, an internal control (TSU2) was coamplified that yields a single lower band of 35 bp (Fig 1). By normalizing the signal intensity of the telomerase ladder to that of the internal control, sample to sample variation due to PCR amplification efficiency was minimized, thus allowing for semiquantitative analysis. Treatment with RNase obliterated the 6-bp ladder, indicating that telomerase was responsible for this reaction (Fig 1). This modified TRAP assay allowed us to detect telomerase activity in cell extracts obtained from 10 to 100 cell equivalent of 293 cells, a telomerase-positive immortalized kidney cell line.

Low levels of telomerase activity were detected in candidate HSC from four different marrow samples (Fig 1). The telomerase levels in the purified candidate HSC varied from 3% to 20%
of that generated by 1 amol of M2R8, an oligonucleotide with 8 T2AG3 repeats used as a quantitation standard, which translated to an equivalent of 0.06% to 0.4% of the activity of 293 cells when normalized on a per-cell basis.

**Telomerase activity in candidate HSC after culture in cytokine combinations of SCF, FL, and IL-3.** Because telomerase activity was reported to increase upon cellular activation,29,33-37 candidate HSC were cultured in the presence of SCF, IL-3, and FL to examine potential upregulation of telomerase activity. When the purified cells were cultured in SCF or FL alone for 5 days, no upregulation of telomerase activity was observed, whereas IL-3 by itself enhanced telomerase activity (Fig 2). Among combinations of two cytokines, those containing IL-3 (SCF + IL-3 or FL + IL-3) were more effective in upregulating telomerase activity than the combination SCF + FL, giving rise to a twofold increase in telomerase activity (Fig 2). Total cell extracts derived from candidate HSC cultured in the presence of SCF + IL-3 + FL also showed enhanced telomerase activity (Fig 2). The relatively low levels of telomerase activity did not appear to result from inhibitory substances to the PCR reaction because the internal control was amplified as expected (Fig 2). Furthermore, mixing extracts from hematopoietic cells with those from 293 cells did not result in any significant decrease in 293 telomerase activity, further indicating that inhibitors of telomerase were unlikely to be present (data not shown).

**Telomerase activity in cultured candidate HSC is restricted to cycling cells.** Because telomerase activity was upregulated in cultures containing IL-3, SCF + IL-3, FL + IL-3, and SCF + FL + IL-3, we next investigated whether increased levels of telomerase activity were restricted to cells of a particular phenotype. Flow cytometric analysis of purified candidate HSC after 5 days of culture in the seven different cytokine combinations revealed that in cultures with SCF or FL alone, the majority of cells retained the CD34+CD45RAloCD71hi phenotype (Fig 3). On the other hand, in cultures containing IL-3, the percentage of CD34+CD45RAloCD71hi cells decreased to below 50% of total viable cells (Table 1), and increased numbers of CD34-, CD34+CD45RAloCD71hi, and CD34+CD45RAloCD71hi cells were observed (Fig 3, Table 1). Candidate HSC cultured for 5 days in SCF + FL + IL-3 were sorted into CD34-, CD34+, CD34+CD45RAloCD71hi, CD34+CD45RAloCD71hi, and CD34+CD45RAloCD71hi cells. Semi-quantitative analysis of telomerase activity indicated that CD34+ cells expressed fourfold higher levels than those in the CD34- cells (Fig 4). Among the CD34+ cells, most telomerase activity resided in the CD34+CD45RAloCD71hi and CD34+CD45RAloCD71hi populations, whereas CD34+CD45RAloCD71hi cells had negligible telomerase activity (Fig 4).

To further investigate the relationship between telomerase activity and cell proliferation in hematopoietic progenitors, we used PKH26 to track cell divisions at the level of single cells. Sorted candidate HSC were labeled with PKH26, a fluorescent dye that stably incorporates into the lipid bilayer and is diluted among the daughter cells with each successive cell division. After 8 days of culture in SCF + FL + IL-3, cells were sorted into PKHlo and PKHhi fractions (Fig 5A). Telomerase activity in PKHlo cells was 5 to 10 times higher than the ones in PKHhi cells (Fig 5B), indicating that viable cells that remained
Telomerase activity in fetal liver CD34⁺CD38⁻ cells. Although telomerase activity was upregulated in 5-day cultures of adult marrow candidate HSC stimulated by SCF, IL-3, and FL, the activity was found to reside predominantly in the proliferating cells containing mainly committed progenitors. To address the question of whether cycling stem cells express telomerase activity, we examined telomerase expression in CD34⁺CD38⁻ candidate HSC from fetal liver. Higher telomerase activity was detected in the fetal liver CD34⁺CD38⁻ cells than those from adult bone marrow (Fig 6). Pooled data from three different samples showed that the activity in fetal liver CD34⁺CD38⁻ cells ranged from 0.5% to 1.5% of that found in 293 cells, whereas in adult bone marrow the activity was at most 0.3% of that present in 293 cells. These results suggest that either all or a subfraction of cycling fetal liver stem cells express telomerase activity.

DISCUSSION

The ability to induce or enhance telomerase activity may be important in maintaining the replicative potential of normal stem cells found in self-renewing tissues such as those of the hematopoietic system. The studies reported here were aimed to investigate telomerase expression in the most primitive hematopoietic cells in humans. We confirmed our previous results regarding the low level of telomerase activity in freshly isolated “candidate” HSC with the CD34⁺CD45RA⁻CD71⁻ phenotype from adult marrow. These sorted cells are highly enriched (several hundred-fold when compared with unpurified cells) in long-term culture-initiating cells (LTC-IC), arguably the best in vitro assay for human stem cells. Unfortunately, most bone marrow cells with a CD34⁺CD45RA⁻CD71⁻ phenotype are unable to initiate long-term cultures, and rare committed progenitors from the adult bone marrow also share this phenotype. The latter, which are actively proliferating, could...
contribute partially or completely to the low but readily detectable levels of telomerase in purified candidate HSC before culture.

In the present study, we found that the level of telomerase activity in CD34\(^+\)CD45RA\(^-\)CD71\(^-\) cells sorted from cytokine-stimulated cultures was reduced as compared with that in freshly isolated cells with the same phenotype. On the other hand, telomerase activity was increased in cultured candidate HSC concomitantly with the upregulation of CD45RA and CD71 expression. By sorting various subpopulations after stimulation with SCF, FL, and IL-3 for 5 days, we found that telomerase activity was mainly confined to CD34\(^+\)CD45RA\(^-\)CD71\(^-\) and CD34\(^+\)CD45RA\(^-\)CD71\(^-\) cells, which are known to be enriched in cycling progenitors committed to differentiate into the erythroid and myeloid lineages, respectively.\(^{32}\) In addition, tracking cellular division by PKH fluorescence confirmed that telomerase activity was confined to cells that had proliferated in culture. Cells with a CD34\(^+\)CD45RA\(^-\)CD71\(^-\) phenotype present after 5 days in cytokine culture could represent a population that failed to respond to SCF, FL, and IL-3 stimulation and remained quiescent or exited from the cell cycle. In both cases, telomerase activity is expected to be low in view of the data describing upregulation of telomerase activity upon entry into the cell cycle.\(^{35-37,40}\)

Because cycling stem cells are extremely rare in the adult bone marrow,\(^{30,33}\) and because our culture conditions are unable to induce the selective self-renewal of adult bone marrow candidate stem cells, we next examined telomerase expression

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**Fig 4.** Telomerase activity in subpopulations of cells present after 5 days in cultures of purified candidate HSC from adult marrow. See also Fig 3G. (A) CHAPS extracts from BM4-derived cells (lanes 1 to 10): CD34\(^-\) fraction (lanes 1 and 2), CD34\(^+\) fraction (lanes 3 and 4), CD34\(^+\)CD45RA\(^-\)CD71\(^-\) fraction (lanes 5 and 6), CD34\(^+\)CD45RA\(^-\)CD71\(^+\) fraction (lanes 7 and 8), and CD34\(^+\)CD45RA\(^-\)CD71\(^+\) cells and (lanes 9 and 10); CHAPS extracts from BM1-derived cells (lanes 11 to 14): CD34\(^-\) fraction (lanes 11 and 12) and CD34\(^+\) fraction (lanes 13 and 14). All extracts were generated from 1,000 cells.

**Fig 5.** PKH26 staining of purified candidate HSC before and after culture in SCF + IL-3 + FL. (A) PKH26 was incorporated into the lipid bilayer of purified candidate HSC at day 0. On day 8, PKH26 intensity was analyzed again and cells were sorted into PKH\(^{hi}\) (gate1) and PKH\(^{lo}\) fractions (gate2) for TRAP. The cells in the PKH\(^{hi}\) fraction have undergone several rounds of division, resulting in diminished dye fluorescence. (B) Telomerase activity in PKH\(^{hi}\) and PKH\(^{lo}\) cells. PKH\(^{hi}\) and PKH\(^{lo}\) cells were sorted from BM1 after 8 days of culturing SCC in SCF + FL + IL-3. TRAP assay was performed on CHAPS extracts equivalent to 1,000 cells in the absence (−) and presence (+) of RNase A.
in CD34+CD38− cells from fetal liver. It has been shown that the proliferative potential of hematopoietic cells changes during ontogeny and that candidate HSC from fetal liver contain a very high proportion of cycling cells as compared with candidate HSC from bone marrow. Our finding that CD34+CD38− fetal liver cells express readily detectable levels of telomerase activity strongly suggests that such cycling candidate HSC express telomerase activity. In view of the loss of telomerase DNA in hematopoietic cells with age, this observation can be explained by assuming that the measurable telomerase activity is not preventing the overall telomere shortening in candidate HSC. Alternatively, telomerase could be expressed in only a proportion of the cells. If the latter hypothesis is correct, then identification and selective expansion of such telomerase positive clones could be useful in transplantation and gene transfer protocols because their progeny would possibly maintain a high proliferative potential. To address this issue, more information about telomerase expression in single CD34+CD38− cells from fetal liver and in vivo of the telomerase levels detected by telomerase assays is urgently needed. Antibodies to the telomerase reverse transcriptase protein could possibly be used to address this issue.

In our study we found that in day-5 cultures, CD34+ cells expressed higher telomerase activity than CD34− cells. One possible explanation for this observation is that as CD34+ cells differentiate, their telomerase activity is downregulated together with their proliferative potential. Similarly, freshly isolated CD34+ cells from adult marrow also have lower telomerase activity compared with CD34+71+ cells, and when the latter were placed in culture over a period of 10 days, their telomerase activity declined. One recent report also shows that leukemic cell lines lose telomerase activity when induced to differentiate.

In a couple of transgenic mouse models, the reactivation of telomerase activity was correlated with tumorigenesis. However, the role of telomerase in normal somatic cells is still largely unknown. Telomerase activity is expressed in germline cells and is required to maintain telomere length and preserve the unlimited proliferative potential of these cells. In the hematopoietic system and skin epidermis, two other examples of self-renewing tissues, low levels of telomerase activity have been found. Possibly this activity could extend the proliferative potential of the stem cells in these tissues to a certain extent but not sufficient to confer immortality as telomeric DNA is still lost upon replication. In a similar manner, peripheral blood lymphocytes express low telomerase activity that is upregulated upon activation. Although the levels of telomerase activity once again appear insufficient to override telomeric decline, the enzyme activity could reduce the loss of telomeric DNA to allow repeated clonal expansion of immune cells upon antigenic stimulation.

In a recent study, telomerase activity in murine hematopoietic “candidate” stem cells and various progenitors was assayed on a single-cell basis and found to be associated with “self-renewal” potential, with lower levels in committed progenitors than in their pluripotent precursors. In contrast, we and others have consistently detected higher telomerase levels in committed progenitors relative to those observed in “candidate” stem cells using human hematopoietic tissues and this study. This discrepancy suggests that telomerase expression is regulated differently in murine versus human hematopoietic cells, a phenomenon that was previously observed with other cell types from these two species.

Because to date telomere length measurements are typically based on bulk DNA analysis using Southern hybridization, subtle changes in telomeric length on individual chromosomes could escape detection. This notion was recently confirmed in studies of cells from telomerase RNA knockout mice. On the basis of the distribution of telomere length in individual chromosomes of cultured hematopoietic cells using fluorescent in situ hybridization analysis, we proposed that telomerase in these cells may preferentially act on short telomeres to maintain the proliferative potential of adult hematopoietic cells becomes more pertinent. Possibly the low levels of telomerase that we measured are able to maintain the length of some but not many short telomeres. In this model, telomerase allows for a limited extension of the replicative lifespan of HSC. Studies in this general area using in situ hybridization to measure telomere length on individual chromosomes of clonally propagated hematopoietic cells in combination with assays of telomerase activity and protein expression should further clarify the role of telomerase in hematopoietic cells.
TELOMERASE EXPRESSION IN HUMAN STEM CELLS

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