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TELOMERASE ACTIVITY IN HUMAN HEMATOPOIETIC PROGENITOR CELLS

STEFAN HOHAUS,° MARIA TERESA VOSO,* ELETTRA ORTU-LA BARBERA,* SALVATORE CAVALLO,° ALFONSO BELLACOSA,° SERGIO RUTELLA,* CARLO RUMI,* MAURIZIO GENUARDI,° GIOVANNI NERI,° GIUSEPPE LEONE*

*°Departments of Medical Genetics and *Semeiotica Medica, Università Cattolica S. Cuore, Rome, Italy*

ABSTRACT

Background and Objective. Telomerase is the enzyme that stabilizes and elongates the telomeric ends of chromosomes. It is expressed in germline and malignant cells and absent in most human somatic cells. The selective expression of telomerase has thus been proposed to be a basis for the immortality of germline and malignant cells. Recently, telomerase activity has been observed in human bone marrow (BM) and peripheral blood (PB) samples. The objective of our study was to further characterize the telomerase expressing population in BM and PB.

Methods. $CD34^+$ cells were isloated from BM and PB, cultured *in vitro* and telomerase activity was assessed by the PCR-based TRAP assay.

Results. Telomerase activity in human BM and PB could be almost exclusively assigned to the hematopoietic progenitor cell fraction expressing the CD34 antigen. We observed telomerase activity in $CD34^+$ cells from BM and cytokine-mobilized PB. $CD34^+$ cells lacking co-expression of CD33 demonstrated higher levels of telomerase than myeloid committed $CD34^+/CD33^+$ cells. *In vitro* culture of $CD34^+$ cells in the presence of a cocktail of growth factors inducing differentiation resulted in a decrease of telomerase activity. Telomerase activity increased in peripheral blood during cytokine-induced mobilization of hematopoietic progenitor cells.

Interpretation and Conclusions. Our data demonstrate that at least a subfraction of the hematopoietic stem/progenitor cell fraction expresses telomerase and downregulates its expression with differentiation.

Key words: telomerase, CD34+ cells, PBSCT

Telomeres, the genetic elements at the end of linear chromosomes, consist of multiple (TTAGGG) repeats of 10-15 kilobases and are essential for proper chromosome structure and function.[1,2] Telomeres are thought to protect the chromosomal ends from breaking and reannealing events leading to genomic instability.[3,4] DNA polymerase cannot replicate the very ends of linear chromosomes, with progressive shortening of the telomeres at each cell cycle. Therefore, the length of the telomere can serve as a mitotic clock, and a sufficiently short telomere may be the signal for replicative senescence in normal cells.[5-10] A variety of eukaryotic cells overcomes this *end-replication* problem using a different DNA polymerase, telomerase.[1,11] Telomerase is a ribonucleoprotein enzyme capable of extending chromosome ends with telomere repeat sequences, using a portion of its integral RNA component as a template and therefore functioning as a reverse transcriptase.[11]

The recent development of a highly sensitive and reproducible PCR-based assay for telomerase activity (TRAP-assay, for telomeric repeat amplification protocol)[12] has made it possible to examine small cell populations. In the TRAP-assay, telomerase synthesizes telomeric repeats onto a nontelomeric oligonucleotide. These extension products serve as templates for the following PCR amplification. The products are separated on a polyacrylamide gel, and telomerase activity is shown by a ladder of bands, which differ by 6 base pairs (bp). Telomerase is reported to be substantially repressed in human fibroblasts, resting lymphocytes and epithelial cells, whereas it is present in reproductive tissues and a broad range of tumor cell lines and tissues.[12-22] This is consistent with the apparent immortality of at least a subset of cells in these populations.

A small number of hematopoietic stem cells sustains a continous production of mature blood cells, and it has therefore been postulated that these stem cells possess self-renewal capacity, suggesting that they are immortal. A recent study however reported that the candidate human BM $CD34^+/CD38^{lo}$ stem cells have shorter telomeres than progenitor cells enriched from fetal liver or

Correspondence: Dr. Stefan Hohaus, Department of Internal Medicine V, University of Heidelberg, Hospitalstr.3, 69115 Heidelberg, Germany. Tel. international +49.6221.568008; Fax: international +49.6221.565813. E-mail: stefan_hohaus@krzmail.krz.uni-heidelberg.de.

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umbilical cord blood.[23] Furthermore, purified precursor cells cultured in the presence of cytokines show loss of telomeric DNA. These data suggested that most, if not all, hematopoietic stem cells undergo replicative senescence. These findings are challenged by recent results using the sensitive TRAP-assay to detect telomerase activity[24,25] that showed low telomerase levels in BM and PB cells.

We attempted to further define the hematopoietic subpopulation in which telomerase is expressed and analyse the pattern of expression in PB during mobilization for hematopoietic stem cell harvesting. We therefore enriched hematopoietic progenitors from BM and PB and analyzed the cell fractions during enrichment. Telomerase activity was almost exclusively restricted to the $CD34^+$ cell population suggesting that at least a subfraction of these cells expresses telomerase. *In vitro* differentiation of $CD34^+$ cells in the presence of cytokines resulted in a gradual loss of telomerase activity. Finally, we observed an increase of telomerase positive cells during cytokine-mobilization for PB stem cell harvests.

Materials and Methods

Purification of $CD34^+$ cells

We obtained BM samples from a normal donor undergoing bone marrow harvesting for allogeneic transplantation and from a patient with B-lymphoproliferative disorder without bone marrow involvement. PB samples were obtained from 7 patients undergoing PB stem cell mobilization by chemotherapy and G-CSF. All patients gave informed consent, and studies were performed according to our Institutional guidelines.

The absence of tumor cell contamination in patients' samples was documented using a sensitive PCR technique for Ig heavy chain gene rearrangement.[26] We could not detect a monoclonal population in any of the cell fractions studied (data not shown).

$CD34^+$ cells were separated from bone marrow according to the previously described immunomagnetic technique.[27] Briefly, after Ficoll gradient centrifugation, the mononuclear cells (MNC) were allowed to adhere to a plastic surface for 16 hours at 37°C in a 5% CO_2 humidified atmosphere. The non-adherent cell fraction was further separated by a Percoll density gradient centrifugation and MNC with a density < 1.066 g/mL were further incubated in the presence of an anti-CD34 antibody (HPCA-2, Becton Dickinson, BD, Mountain View, CA) and separated by magnetic beads (Dynabeads, Dynal, Oslo, Norway) into $CD34^+$ and $CD34^-$ cells.

PB MNC were obtained from 5 patients with lymphoma in complete remission undergoing leukapheresis during G-CSF supported post-chemotherapy mobilization. MNC were incubated in the presence of a biotin-conjugated anti-CD34 monoclonal antibody (clone 12.8), washed with PBS and absorbed on an avidin-conjugated column, with an acquisition capacity of 10^{11} mononuclear cells (CEPRATE SC System, Cellpro, Bothell, WA, USA). $CD34^+$ cells were then removed from the column, washed with PBS and resuspended in a final volume of 10-15 ml. FACS staining of an aliquot with a PE-conjugated anti-CD34 antibody (HPCA-2, BD) demonstrated an over 90% purity of this cell fraction.

Further separation of $CD34^+$ cells from mobilized peripheral blood of a patient with lymphoma into $CD33^+$ and $CD33^-$ cells was achieved by FACS-sorting. Immediately after staining with PE- and FITC-conjugated CD34 and CD33 monoclonal antibodies (BD), cells were resuspended in PBS supplemented with 1% human serum albumin and sorted on a FACStarplus flow cytometer (BD). Sorting windows were set to clearly separate $CD33^+$ and $CD33^-$ cells. Purity of each cell fraction exceeded 98%.

Clonogenic assay

Mononuclear cells from PB (10^5/mL) were plated in IMDM, supplemented with 2 mM glutamine, 100 U/mL penicillin, 100 mg/ml streptomycin (GIBCO-BRL, Gaithersburg, MD, USA), 0.9% methylcellulose, 40% FCS, 10% phytohemoagglutinin-leukocyte conditioned medium (PHA-LCM) and 3 U/mL erythropoietin. Plates were incubated for 15 days at 37°C, in a 5% CO_2 humidified atmosphere. Colonies (more than 50 cells) were counted after 14 days in phase-contrast microscopy.

Culture of $CD34^+$ cells

$CD34^+$ cells from mobilized PB of 2 patients with lymphoma were incubated in a liquid culture system (10,000 cells/mL), containing RPMI 1640 (GIBCO), 10% FCS, a low dose of human recombinant IL-3 (0.01 U/mL), and a cocktail of growth factors: G-CSF (10 ng/mL), erythropoietin (3 U/mL), SCF (10 ng/mL), IL-6 (10 ng/mL)(Amgen Corp., Thousands Oaks, CA, USA). Cells were cultured at 37°C, in a 5 % CO_2 humidified atmosphere.

Telomerase assay

Cells were washed once in a hypotonic buffer (10 mM Hepes-KOH, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol) and then lysed in ice-cold CHAPS lysis buffer (10 mM Tris-HCl, pH 7.9, 1 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 5 mM ß-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, PMSF, 0.5% CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate). The suspension was incubated 30 min. on ice and then centrifuged 15 min. in a microcentrifuge at 4° C and 17,000 g. The supernatant was removed and quickly frozen at –80°C. The protein concentration was analyzed by the Bradford assay (Biorad, Hercules, CA, USA).

Aliquots of the CHAPS-extracts were used for the TRAP-assay as described,[12] with minor modifications. For RNAse treatment, extracts were incubated with RNAse at a final concentration of 1 µg/µL for 15 min at 23°C. The TRAP reaction was performed in 50 µl mixture containing 20 mM Tris-HCl (pH 7.9), 1.5 mM $MgCl_2$, 63 mM KCl, 0.005% Tween 20, 1 mM EGTA, 50 µM of each deoxynucleoside triphosphate, 0.1 µg/ µl bovine serum albumin, 1 µg T4g32 protein (Boehringer Mannheim, Germany) and 0.1 µg of the TS oligonucleotide (5'-AATCCGTCGAGCAGAGTT-3') which had been radioactively labelled with γ^{32}P-ATP using T4 polynucleotide kinase. The reaction was incubated 20 min. at room temperature to allow the elongation of the TS primer by telomerase. After inactivation of telomerase by heating of the reaction mixture to 94° C for 5 min 0.1 µg of the second primer CX (5'-CCCTTACCCT-TACCCTTACCCTAA-3') and 2 U of Taq polymerase were added. The products were amplified by Polymerase Chain Reaction (PCR) (94°C for 45 seconds, 50°C for 30 seconds and 72°C for 90 seconds), using a number of cycles in the linear range of amplification. PCR products were separated by electrophoresis on a 15 % non denaturing polycrylamide gel and exposed to X-ray films. A sample was classified as positive when a typical RNAse sensitive 6 bp ladder could been seen after an overnight exposure. The signal was quantitated by densitometric analysis (Pharmacia LKB) according to the manufacturer's instructions, compared to a positive standard (as % of the myelomonocytic cell line U937), and normalized to the amount of protein. Each experiment contained U937 as positive and extracts from fibroblasts as negative controls. Experiments were repeated once.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from 10000 cells according to the single step isolation method.[28] First strand cDNA was synthesized in a reaction mixture containing total RNA, 2 mM dNTPs, 100 pmoles of random hexamers, 6 U RNAse inhibitor, 50 U reverse transcriptase (Perkin Elmer, Norwalk, CT, USA) and 5 mM $MgCl_2$. The PCR mixture contained one tenth of the RT-reaction, 0.8 mM dNTPs, 1.5 mM $MgCl_2$, 40 pmoles of 5' and

3' oligodeoxynucleotides and 1.5 U of Taq polymerase (Pharmacia Biotech, Uppsala, Sweden). Oligodeoxynucleotide sequences and annealing temperatures were as follows: CD11b: 5'-GCCATTGTCTGCTTTCG-3' [bp 2304 to 2320 of the human cDNA[29]]) and 5'-GCCATTGTCTGCGCTTTCG-3' (bp 2755 to 2739), 50°C; GAPDH: 5'-CCATGGAGAAG-GCTGGGG-3' [bp 388 to 405 of the GAPDH cDNA[30]], and 5'-CAAGTTGTCATGGATGACC-3' (bp 582 to 563), 50°C. A negative control reaction containing water instead of cDNA was included in each PCR amplification in order to exclude the presence of possible cDNA contamination.

The PCR reaction was performed using a number of cycles in the linear range for each primer pair (20 cycles for GAPDH and 30 cycles for CD11b) and for 70 ng RNA input, as previously shown.[27] PCR products were separated in a 1.5% agarose gel, stained with ethidium bromide and transferred to a nylon membrane (Biotrans, ICN, Costa Mesa, CA, USA). Messages were hybridized to the corresponding cDNA (generous gifts of Dr Daniel G. Tenen), labelled with a-^{32}P dCTP by the hexanucleotide random priming method.[31] Bands were quantified using an Instant Imager software (Packard, Meriden, CT, USA).

Results

Linear amplification of telomerase activity by the TRAP-assay

Telomerase activity is assayed by the ability of cell extracts to elongate an oligonucleotide with telomeric 6 bp repeats. The elongated oligonucleotides are subsequently amplified by PCR. The PCR amplification is linear so long as the reaction is performed in the exponential range of amplification, at a constant efficiency. For these reasons, we first established PCR conditions for the TRAP-assay in which we could measure differences in telomerase activity, in analogy to what we described before for RT-PCR.[27] A low protein input (0.5 µg) of the myelomonocytic cell line U937 was assayed and an aliquot of the PCR reaction was harvested after every 2 to 8 cycles and separated on a 15% PAGE (Figure 1a). The 6 bp ladder characteristic for telomerase activity could be first visualized with 28 cycles. Thirty-two PCR cycles proved to be in the linear range. In a second step, increasing amounts of protein (from 0.01 µg to 10 µg) were amplified with 32 cycles, and the reaction was linear between 0.05 and 5 µg protein input (Figure 1b).

Enrichment of telomerase activity during purification of hematopoietic progenitors from bone marrow

Hematopoietic progenitors were enriched from 2 BM samples [one normal donor (Figure 2), one

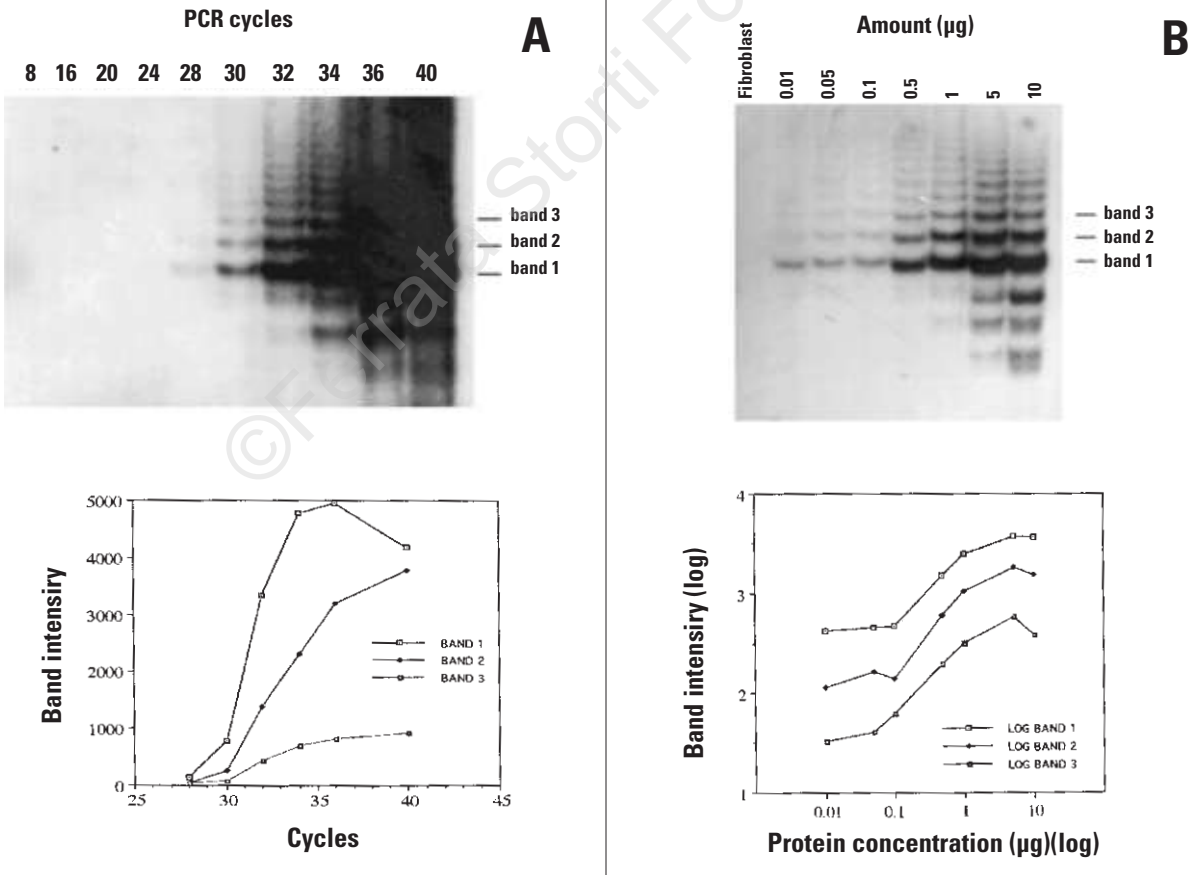


Figure 1. Quantification of the TRAP-assay. (A) The intensity of the 6 bp ladder characteristic for telomerase activity increases with the number of PCR cycles. Thirty-two PCR cycles are in the linear range of amplification. (B) Increasing amounts of protein from U937 cell extracts were assayed for telomerase activity. The reaction was linear between 0.05 µg and 5 µg protein input.

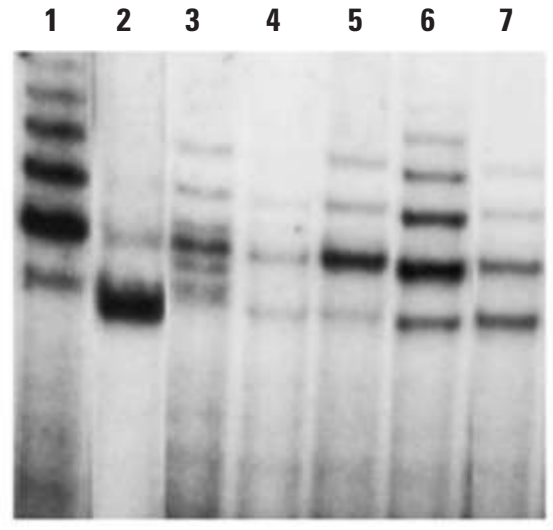
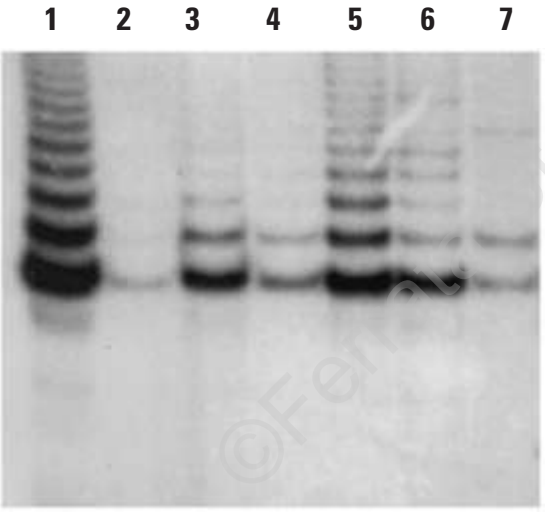


Figure 2. Telomerase activity in hematopoietic progenitor cells from bone marrow. Lane 1: U937 (0.5 µg); lane 2: total bone marrow (10 µg); lane 3: non-adherent MNC (10 µg); lane 4: pellet of Percoll gradient (10 µg); lane 5: Percoll fraction < 1.066 g/mL (10 µg); lane 6: $CD34^+$ cells (2 µg); lane 7: $CD34^-$ cells (10 µg).



Lanes	*Telomerase activity (%)*
1: U937 cells	100
2: fibroblasts	—
3: peripheral blood	2.0
4: PB $CD34^-$	0.3
5: PB $CD34^+$ cells	62.9
6: PB $CD34^+$ cells after 14h of culture	28.0
7: PB $CD34^+$ cells after 6 days of culture	0.6

Figure 3. Telomerase activity in hematopoietic progenitors from peripheral blood and during in vitro culture. Lane 1: U937 (0.5 µg); lane 2: fibroblasts (10 µg); lane 3: PB MNC (10 µg); lane 4: PB CD34- cells (10 µg); lane 5: PB $CD34^+$ cells (2 µg); lane 6 and 7: $CD34^+$ cells of the same patient cultured for 14 hours (2 µg) and 6 days (10 µg). Telomerase activity was standardized to the amount of protein input and the U937 signal.

patient with lymphoproliferative disorder] by a multistep procedure including adherence, density gradient centrifugation and positive selection of CD34+ cells by immunomagnetic beads. Cell fractions were harvested for telomerase activity. The starting BM cell population showed a low activity, which could be enhanced by collecting the non-adherent MNC (Figure 2, lanes 2 and 3). Separation of the non-adherent MNC by Percoll density centrifugation resulted in a recovery of almost all activity in the MNC fraction with a density < 1.066 (compare lane 4, the pellet of the Percoll centrifugation vs lane 5, the MNC). $CD34^+$ hematopoietic progenitors were further enriched by immunomagnetic selection to a purity of 91%. These cells showed a strong telomerase signal (lane 6, note that the reaction for $CD34^+$ cells contained a lower amount of protein).

$CD34^+$ cells from peripheral blood show telomerase activity

In a next step, we addressed the question of whether hematopoietic progenitor cells circulating in PB also expressed telomerase. Chemotherapy in combination with hematopoietic growth factors can increase the number of circulating progenitors several fold during the phase of leukocyte recovery and therefore facilitates the enrichment of this cell population.[32] We analyzed PB from 5 patients with lymphoma undergoing mobilization with chemotherapy and G-CSF. The PB sample of a representative patient showed a significant amount of telomerase activity (2.2% of U937 control, Figure 3, lane 3). We enriched $CD34^+$ cells by a biotin-avidin affinity column to 94% purity and could obtain 62.9% of the U937 telomerase activity (Figure 3, lane 5). The $CD34^-$ population showed only a very weak activity (Figure 3, lane 4).

Telomerase activity in $CD34^+/CD33^-$ cells

After having defined the hematopoietic progenitor cell fraction as the major telomerase expressing population in PB we attempted to further localize the activity to a subfraction according to the differentiation stage. We separated the peripheral blood $CD34^+$ population of a patient with lymphoma into $CD33^-$ and myeloid committed $CD33^+$ cells using FACS-sorting. The $CD34^+/CD33^-$ population showed a higher activity than the $CD33^+$ cells, although this cell population also demonstrated a considerable activity (Figure 4).

Telomerase activity during in vitro culture

The analysis of cell fractions from BM and PB suggested an expression pattern of telomerase during hematopoiesis depending on the differentiation stage with the highest activity in the most immature cell fraction. To study this phenomenon *in vitro*, $CD34^+$ cells had been enriched from mobilized PB of 2 patients with lymphoma as described above and were induced to differentiate during cultivation

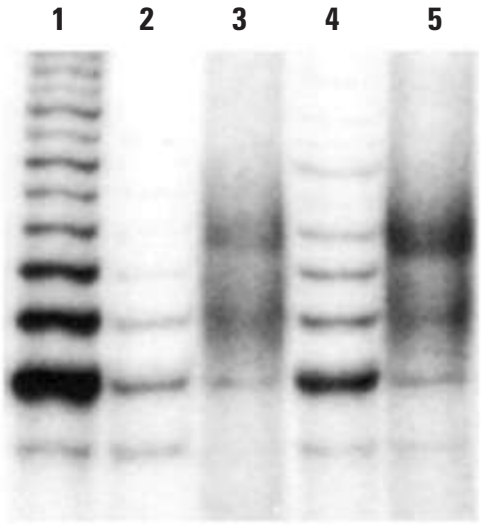
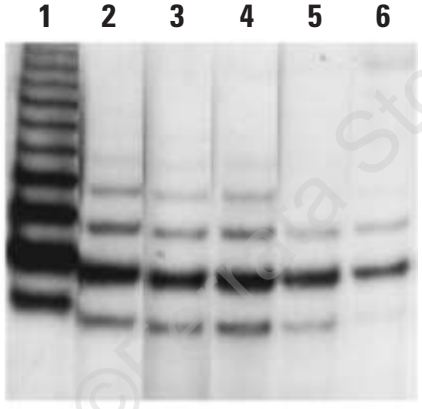


Figure 4. Telomerase activity in $CD34^+/CD33^-$ cells. Lane 1: U937 cells; lane 2: $CD34^+/CD33^+$ cells; lane 3: RNAse treated $CD34^+/CD33^+$ cell extract; lane 4: $CD34^+/CD33^-$ cells; lane 5: RNAse treated $CD34^+/CD33^-$ cell extract. 0.5 µg of protein was assayed in all lanes. The telomerase activity in PB $CD34^+/CD33^-$ and $CD34^+/CD33^+$ cells was 45.2% and 11.5% of the U937 control, respectively.



Telomerase act. (%) 100	2.1	0.8	0.9	0.2	0
$CD34^+$ cells (%)	2	4.2	3.9	2.5	1.7
CFU-GM (10^5 MNC)	ND	24	124	62	36
WBC (10^9/L)	1.5	4.1	8.4	24	30

Figure 5. Telomerase activity in peripheral blood during mobilization. The following amounts of protein extracts were assayed: lane 1: 0.5 mg U937 cell; lane 2-6: 10 µg $CD34^+$ cells at days 11-15 of G-CSF administration. The $CD34^+$ cell counts and CFU-GM colony data on the respective days are shown in the lower part of the figure. WBC: white blood cells. MNC: mononuclear cells plated.

in the presence of a growth factor cocktail. Cells were harvested after a short-term (14 hrs) and long-term (6 days) cultivation. Clonogenic capacity of the cell population decreased and differentiation occurred during this period. RT-PCR for the differentiation antigen CD11b showed strong upregulation of CD11b message at day 6 (data not shown). Concomitantly, telomerase activity decreased to less than 1% of the starting activity (Figure 3, lane 7).

Telomerase activity in peripheral blood during mobilization

Mobilization of hematopoietic progenitor cells into peripheral blood can be assessed by the formation of hematopoietic colonies in semisolid medium, CFU-GM, and the number of $CD34^+$ cells. We reasoned that telomerase activity might increase indicating the presence of hematopoietic progenitor and stem cells. We studied PB samples of 3 patients with lymphoma and observed an increased telomerase activity during the mobilization period defined by increased $CD34^+$ cell counts and CFU-GM colony formation. Figure 5 shows the results of the progenitor and telomerase assays of one patient.

Discussion

Telomerase expression has been proposed as a basis for immortality, and in humans it appeared to be restricted to germline and malignant cells.[12-22] Bone marrow and peripheral blood are among the few somatic tissues expressing telomerase.[24,25] We localized the major telomerase activity of bone marrow and peripheral blood to the $CD34^+$ cell population. We observed only weak activity in the $CD34^-$ cell fraction, what might be due to contamination by $CD34^+$ cells. We further characterized telomerase expression within the $CD34^+$ population by separating $CD34^+/CD33^+$ myeloid committed progenitor cells, and found a higher activity in the more immature $CD33^-$ subfraction. In the same line, Morrison *et al*. examined hematopoietic stem and progenitor cells at 4 different stages of differentiation and observed telomerase expression in 70% of murine hematopoietic stem cells.[33] In this study, the frequency of telomerase-expressing cells was proportional to the frequency of cells thought to have self-renewal capacity.

Telomerase expression decreased during *in vitro* culture of $CD34^+$ cells, paralleling the myeloid differentiation of these progenitor cells. This finding is in agreement with recent data on myeloid and other cell lines demonstrating a decrease of telomerase activity with differentiation.[34-38] The mechanism of down-regulation of telomerase with differentiation is unclear. A link between telomerase activity and progression through the cell cycle has been proposed.[39,40] Highest expression was found in S-phase cells and almost no activity in cells arrested at G_2/M phase.[40] However, peripheral blood $CD34^+$ cells, which we found to be telomerase positive, are almost exclusively in G_0/G_1.[41, 42] This indicates that telomerase expression in primary hematopoietic

cells is not restricted to actively cycling cells.

Telomerase activity increased in peripheral blood during mobilization of hematopoietic progenitors by G-CSF-supported chemotherapy. It will be intersting to extend this study to a larger group of patients to better define the kinetics of telomerase activity compared to the progenitor cell assays. Analysis of telomerase activity might add information on the quality of the mobilized stem cells.

Hematopoietic progenitor cells are not the only human somatic cells which express telomerase. Telomerase activity has been demonstrated in the proliferative basal cells of the epidermis providing another example of telomerase expression in a permanently regenerating human tissue.[43] Activated T and B lymphocytes express telomerase *in vitro* and *in vivo*.[25,44,45] Its expression appears to be developmentally regulated being high in thymocytes and absent in peripheral blood T cells.[45]

The detection of telomerase activity in these somatic tissues has raised the question of its functional role. Lymphocytes as well as even the most immature hematopoietic progenitor cells loose telomeric length *in vivo* and *in vitro*.[9,10,23] Telomerase activity in these cells might not be sufficient to sustain telomere length. Alternatively, telomerase activity might be restricted to a minor subpopulation of $CD34^+$ cells retaining the telomere length. This could be masked by the overall loss of telomere DNA in the whole population. Telomerase expression in somatic cells does probably not reflect immortality. The pattern of telomerase activity suggests that its expression is associated with a self-renewal potential and production of long-lived cells. Activated T cells generate in part long-lived memory cells which again enter into a quiescent state. Hematopoietic stem cells are thought to produce both long-lived progenitor cells exiting again from the cell cycle and differentiating progeny.[46,47]

The crucial role of telomerase expression for the continuous growth of immortal cells has recently been demonstrated by antisense experiments[48] and led to speculations on future anti-telomerase strategies in cancer treatments. The demonstration of telomerase activity in hematopoietic progenitor cells might point to some potential side effects of this therapeutic approach.

The restriction of telomerase expression during hematopoiesis might reduce the risk of cell transformation which is associated with cellular immortality. Studies on the mechanisms of the tight regulation of telomerase activity during hematopoiesis will result in greater insight in the process of self-renewal, lineage-commitment and differentiation on one hand and leukemic transformation of hematopoietic progenitor cells on the other.

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