RAPID COMMUNICATION

Telomerase Activity in Normal Leukocytes and in Hematologic Malignancies

By Christopher M. Counter, Jyothi Gupta, Calvin B. Harley, Brian Leber, and Silvia Bacchetti

Telomeres are essential for function and stability of eukaryotic chromosomes. In the absence of telomerase, the enzyme that synthesizes telomeric DNA, telomeres shorten with cell division, a process thought to contribute to cell senescence and the proliferative crisis of transformed cells. We reported telomere stabilization concomitant with detection of telomerase activity in cells immortalized in vitro and in ovarian carcinoma cells, and suggested that telomerase is essential for unlimited cell proliferation. We have now examined the temporal pattern of telomerase expression in selected hematologic malignancies. We found that, unlike other somatic tissues, peripheral, cord blood, and bone marrow leukocytes from normal donors expressed low levels of telomerase activity. In leukocytes from chronic lymphocytic leukemia (CLL) patients, activity was lower than in controls in early disease, and comparable with controls in late disease. Relative to bone marrow, telomerase activity was enhanced in myelodysplastic syndrome (MDS) and more significantly so in acute myeloid leukemia (AML). Regardless of telomerase levels, telomeres shortened with progression of the diseases. Our results suggest that early CLL and MDS cells lack an efficient mechanism of telomere maintenance and that telomerase is activated late in the progression of these cancers, presumably when critical telomere loss generates selective pressure for cell immortality.

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MATERIALS AND METHODS

Isolation and culturing of cells. Normal human skin fibroblasts were cultured in α-minimal essential medium (α-MEM), supplemented with 10% fetal calf serum (FCS). 293 CSH cells were cultured in Joklik medium supplemented with 5% FCS. B4 cells, an immortal line derived from Epstein-Barr virus (EBV)-infected human B lymphocytes, were grown in RPMI with 10% FCS serum.

From the Departments of Pathology and Medicine, McMaster University, Hamilton, Ontario, Canada; and Geron Corp, Menlo Park, CA.

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Address reprint requests to Silvia Bacchetti, ScD, Department of Pathology, McMaster University, 1200 Main St W, Hamilton, Ontario, L8N 3Z5 Canada.

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Fig 1. Telomerase activity in normal and leukemic cells. Cell extracts were incubated with the TS oligonucleotide in the presence of dNTPs and α-PdCTP and, after addition of the CX primer, elongated TS oligonucleotides were amplified by PCR. As a control, RNase, which abolishes telomerase activity, was added to duplicate reaction before addition of α-PdCTP. Reaction products were resolved on acrylamide gels and visualized after exposure to PhosphorImager screens. (A) Telomerase activity in representative samples of leukocytes from patients with early (CLL2-1) or late (CLL14) CLL (10 μg protein per assay). (B) Activity in extracts from patients with MDS or AML (5 μg protein per assay). (C) Activity in peripheral blood (PBL5) and BM (BM4) leukocytes from normal individuals, and in control 293 and B4 cell lines, assayed at the indicated amounts of protein.

Samples were obtained with informed consent from normal donors, or from cancer patients at diagnosis or during follow-up. The age of normal adult donors ranged from 25 to 55 years, and that of patients from 40 to 80 years, without significant differences in the average age between groups. CLL was staged according to Rai and samples were obtained from blood when white blood cell (WBC) counts exceeded 15 × 10⁹/L. MDS samples were isolated from BM whereas normal and AML samples came from both sources. All samples were processed immediately after collection without expansion in culture. Following two washes in phosphate-buffered saline (PBS), low-density mononuclear cells were isolated by Ficoll-Hypaque (Pharmacia Biotech AB, Uppsala, Sweden) density gradient centrifugation and assayed.

Analysis of DNA. Genomic DNA was isolated and digested to liberate the terminal restriction fragments (TRFs), as previously described. Digested DNA was resolved in 0.5% agarose gels, which were dried, hybridized with the telomeric probe 32P(C3TA2)₅, stringently washed, and exposed to PhosphorImager screens (Molecular Dynamics, Sunnyvale, CA). TRFs are comprised of telomeric and subtelomeric DNA, and variability in the lengths of both components gives rise to their heterogeneous size. A mean TRF length was calculated using the total counts between 21 and 2 kbp, determined using ImageQuant software (Molecular Dynamics), and recorded for simplicity as TRF length.

Telomerase assay. Most S100 extracts were prepared from 10⁷ cells using hypotonic-detergent lysis as previously described, except that the lysis buffer contained 0.5% CHAPSO (Sigma, St Louis, MO) instead of 0.5% Nonidet P-40 (Sigma). A few extracts were prepared from 10⁶ cells using a hypotonic-Dounce homogenization method. Extracts were treated with RNase to a final concentration of...
of 0.1 μg/μL (negative control) or left untreated for 10 minutes at 21°C.

Telomerase was initially assayed by incubating cell extracts for 10 minutes at 21°C with 0.1 μg of TS oligonucleotide (5'-AATCCGAGCTT-3') in 20 mmol/L Tris-HCl pH 8.3, 1.5 mmol/L MgCl₂, 50 μmol/L each dNTP, 0.5 mmol/L T4-gene 32 protein, 2 μCi α²PdCTP (3,000 Ci/mmol), and 2 U Taq polymerase in a total volume of 50 μL in a tube containing 0.1 μg of CX primer (5'-CCCTTACCCTTACCTTACCTAA-3') separated from the reaction by a wax barrier. After elongation of the TS oligonucleotide by telomerase, products were polymerase chain reaction (PCR)-amplified by 27 cycles at 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 90 seconds. The first denaturation step inactivates telomerase and melts the wax barrier, releasing the CX primer for first-strand synthesis. This protocol results in a 10⁴-fold increase in sensitivity compared with previous methods. In later experiments, the CX primer and Taq polymerase were added to reactions prewarmed to 92°C to reduce background. Lastly, kinase-labeling of the TS oligo, substitution of the second primer with one (ACT) unable to dimerize with TS, and the use of a standard for measuring PCR amplification were used to increase sensitivity and to provide better quantitation and comparison between assays (Kim N.W., Prowse K.R., Chiu C.P., Harley C.B., in preparation). Results from all three assays were qualitatively consistent. Serial dilutions of S100 extracts were assayed in triplicate to establish the linear range of enzyme activity for the purpose of quantitation. Reaction products were resolved in 15% nondenaturing polyacrylamide gels and exposed to PhosphorImager screens. Extracts were considered negative if no products were detected on a 7-day exposure. Enzyme activity was expressed in arbitrary units as total counts in the RNase-sensitive products detected on a 7-day exposure. Enzyme activity was expressed in arbitrary units as total counts in the RNase-sensitive products detected on a 7-day exposure.

RESULTS AND DISCUSSION

To establish the temporal pattern of telomerase expression during leukemogenesis, leukocytes from patients with early...
(0-II) and late (III-IV) stage CLL, with MDS, and with AML were assayed for enzyme activity. As controls, leukocytes from cord, peripheral blood and BM of normal donors were used. A telomerase-positive extract elongates a single-stranded primer by addition of T2AG3 repeats and the elongated products are amplified in the PCR step. Pretreatment of the extract with RNase will abolish telomerase activity by degrading the templating RNA of the enzyme.

Assaying of samples from normal individuals showed that telomerase was present in leukocytes from cord (3/3), peripheral blood (6/6), and BM (3/3) (Fig 1 and Table 1). Activity was low in all three tissues (on average ≈0.8% of that in the 293 cell line, and ≈2% of that of the B4 lymphocyte cell line), suggesting that enzyme expression may be limited to a small subset of normal leukocytes or may be insufficient for telomere maintenance. In agreement with these possibilities, telomeres were significantly shorter in adult versus newborn leukocytes (Table 1), as reported by others.24-25 Leukocytes from early stage CLL patients (n = 14) expressed on average lower telomerase activity than control samples (Fig 1 and Table 1). In the vast majority (12/14 or 85%) activity was reduced on average by 70% (P = .03), and only two cases exceeded control values. Of interest, the latter samples (CLL19 and 20) were from patients with significant increase in WBC count and lymph nodes in the month before sampling. Conversely, in late-stage CLL (n = 7), four of the seven samples assayed (or 57%) had elevated enzyme levels compared to early stage samples (P = .016), although cases with no or negligible activity still persisted (Fig 1 and Table 1). The average value for late CLL patients was comparable with that of normal blood. In the myeloid diseases (Fig 1 and Table 1), MDS (n = 6) and AML (n = 7) samples on average had higher levels of telomerase than normal BM (=twofold for MDS and ≈fourfold for AML). The significance of these differences was borderline except for the comparison between AML and controls (P = .029). However, there was substantial variability in enzyme levels among samples and a subgroup of MDS patients (4/6 or 67%) with significantly higher activity than controls.
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(≈threefold on average) could be identified. Similarly, the majority (67% or 86%) of AML samples expressed on average ≈fivefold more telomerase than normal BM.

As previous studies in different tumors reported decreasing TRF length with increasing tumor grade or disease stage, we measured TRFs during leukemogenesis. In early stage CLL, TRF length varied considerably among samples but on average was 7.9 kbp, (Fig 2 and Table 1), a value consistent with those of leukocytes from age-matched normal donors. Furthermore, there was no detectable loss of telomeric DNA in samples taken 2 years apart from patients CLL1 and CLL4, as expected if telomere shortening occurred at the same rate as in normal leukocytes (≈40 bp/yr). These observations are in agreement with a normal turnover of CLL leukocytes. TRF lengths in late stage CLL were more homogeneous and much reduced, with an average size of 4.4 kbp, comparable with that detected for late-stage ovarian carcinoma. A similar trend was observed for the myeloid diseases (Fig 2 and Table 1), with TRFs being longer and extremely variable in size in MDS patients (average = 11.1 kbp), and consistently and significantly shorter in AML samples (average = 4.7 kbp), in agreement with previous studies.

The present study shows that telomerase activity is present in leukocytes from BM and peripheral blood from normal donors. Activity in blood has also been detected by others (Kim N.W., Chiu C.-P., Vaziri H., Weinrich S.L., Harley C.B., in preparation). To date, these are the only adult somatic tissues in which telomerase has been detected. The level of activity and the age-related shortening of telomeres in both tissues are compatible with enzyme expression being restricted to a subset of cells. The biologic significance of these findings and the identity of the positive cells is unclear at present. However, loss of telomeric DNA during in vitro culturing has been reported for the most primitive BM stem cells (CD34+CD38−), suggesting that telomerase-positive cells may belong to a more differentiated compartment. Despite the constitutive activity of the normal tissues, we detected distinct patterns of telomerase expression in samples from lymphoid and myeloid leukemias. In both diseases leukocytes from early stage patients generally have less telomerase activity and longer TRFs than those from late-stage patients. However, we also found samples not fitting either pattern, with short TRFs but low or no telomerase activity (CLL 3, 6, 10, and possibly AML 6). These may represent populations in transition, although inability to detect enzyme activity for technical reasons cannot be excluded. Kim et al have also detected telomerase activity in 2/2 late-stage CLL and in 14/16 AML samples. By contrast, Nilsson et al reported no activity in four AML samples, possibly because of the lower sensitivity of their assay that did not include an amplification step. Indeed, we were unable to detect activity in peripheral blood lymphocytes without amplification of products.

In the majority of early CLL, telomerase activity was undetectable or substantially reduced compared with control leukocytes. The most likely explanation is that activity present in a subset of normal cells is diluted by the more numerous telomerase-negative CLL cells, resulting in reduced levels in the whole population. Although normal cells may also contribute to telomerase levels in MDS samples, in 4/6 of these cases enzyme levels were significantly elevated over control. Our data suggest an inverse correlation between telomere length and telomerase activity that may reflect the proliferative history of the leukemic clone. Although our sample size is small, this pattern is consistent with recent observations on TRF length by Ohyashiki et al, who identified three classes of MDS patients with: (1) short TRFs at diagnosis and no change during disease evolution; (2) large TRFs decreasing in length as the disease evolved; or (3) large and stable TRFs. These data and ours suggest that telomerase is variably activated in MDS. Similar to our observations on late CLL, AML was associated with elevated telomerase activity relative to MDS. However, in the late stage of both leukemias activity was substantially lower (at most 16%) than that in a clonal population of immortalized B cells (Table 1), suggesting a preponderance of negative cells in the leukemic samples. Lastly, CLL and AML differed with respect to levels of telomerase, with CLL samples being generally less active. We speculate that this difference
may reflect the lower number of proliferating cells and/or the higher degree of cell differentiation characteristic of CLL.

Overall, our data are compatible with both CLL and AML arising in a progenitor cell that lacks, or has insufficient, telomerase activity to maintain telomere length during clonal expansion and differentiation. The presence of elevated telomerase levels in advanced leukemias suggests two alternative models for disease evolution (Fig 3). A "reactivation" model postulates that despite the capacity for self renewal, the leukemic stem cells arises from a precursor that is telomerase negative and will ultimately senesce. Thus, cellular expansion results in shortening of telomeres. During disease progression, telomerase becomes illegitimately activated, as proposed for other malignancies and a telomerase-positive subclone will ultimately predominate. Reactivation presumably occurs late, in terms of cell proliferation, because telomeres are short. An "expansion and retention" model, on the other hand, invokes the existence of a telomerase positive leukemic stem cell ab initio. This cell is capable of cell renewal but gives rise to telomerase-negative, possibly more differentiated, progeny, thus accounting for the observed shortening of telomeres. A prediction of this model is that telomere length in the leukemic population will ultimately increase as a result of replacing telomerase-negative cells having short telomeres and finite lifespan with positive cells that have not undergone telomere erosion.

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