Advances in Brief

Telomerase Activity in Human Acute Myelogenous Leukemia: Inhibition of Telomerase Activity by Differentiation-inducing Agents[1]

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Abstract

The terminal regions of human chromosomes, the telomeres, shorten with each cell division in most normal somatic cells. Telomerase, a ribonucleoprotein that synthesizes telomeric DNA onto chromosomal ends, is activated in germline cells and almost all tumor cells. Telomerase activity maintains the stability of telomere length, resulting in indefinite cellular proliferation (immortality). In the present study, telomerase activity was analyzed in leukemic mononuclear blood cells obtained from 56 patients with acute myelogenous leukemia (AML) with known cytogenetic alterations. Heterogenous levels of telomerase activity were observed and generally correlated with cytogenetic status. Patients with 11q abnormalities and -5/-7 (unfavorable cytogenetics) tended to have high telomerase activity compared with cells obtained from AML patients with other types of cytogenetics. Additional studies with a larger cohort of patients will determine whether these differences are statistically significant. Chemotherapy agents that result in differentiation of leukemic cells also resulted in inhibition of telomerase activity. Knowledge of telomerase activity in patients with AML, before and throughout therapy, may have clinical utility for following disease progression and may predict early cancer relapse.

Introduction

The telomeres of human chromosomes consist of repeated TTAGGG hexamers (1, 2). Telomeres may protect the chromosomal ends from degradation and prevent them from fusing with each other and from recombining with internal DNA (1). Telomeric repeats are also lost as part of the end-replication problem as originally described by Watson (3) and Olovnikov (4). The end-replication problem arises from the inability of the lagging strand of DNA to replicate the very end of a linear chromosome. As a consequence, progressive shortening of the telomeres occurs with each round of DNA synthesis in somatic cells (5–8). However, in germline cells, the length of the telomeric repeat is maintained by the presence of telomerase, an enzyme that adds TTAGGG repeats to the chromosome ends. Telomerase is a ribonucleoprotein in which the RNA component contains a template region that is complementary to TTAGGG, permitting the binding to and synthesis of telomeric repeats (9–10). Although the RNA moiety of human telomerase has been recently cloned (11), the protein components of human telomerase are still not known. However, a sensitive TRAP[4] assay has been developed to detect telomerase activity in tissue extracts derived from a very small number of cells (12).

Telomerase activity is detectable in many primary human tumor specimens and tumor-derived cell lines (12–26). Elevation of telomerase activity was first identified in ovarian carcinoma (14) and has now been detected in more than a dozen different tumor types including hematological malignancies and neuroblastoma, gastric, lung, liver, colorectal, brain, prostate, and breast cancers (12–26). High levels of telomerase activity were found to correlate with an unfavorable prognosis in neuroblastomas and gastric cancer (18, 19). Telomerase activation in hematopoietic cells has also recently been studied (12, 15–17, 19). One study reported detectable telomerase activity in one patient with chronic lymphocytic leukemia but the absence of telomerase activity in five patients with ALL (15). Four AML patient samples in this study were also found to be negative for telomerase activity. However, this study did not use the more sensitive telomerase activity assay (12). In the study by Kim *et al.* (12), telomerase activity was detected in 16 of 18 samples of acute lymphocytic leukemia and chronic lymphocytic leukemia. Thus, it appears that telomerase activity is repressed in most somatic cells and is reactivated in most immortal cells and human cancers. The presence of telomerase in $CD34^+CD38^+$ stem cells (17) suggests the possibility that some leukemias may actually represent progressive selection of telomerase-expressing clonogenic stem cells in differentiation arrest. Because the presence of telomerase activity is such a widespread phenomenon in human tumors, telomerase may be a specific target for

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[4] The abbreviations used are: TRAP, telomeric repeat amplification protocol; AML, acute myelogenous leukemia; CHAPS, 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride; ATRA, all-*trans* retinoic acid; ITAS, internal telomerase assay standard.

cancer therapy. Therefore, it is important to understand the regulatory mechanisms which govern telomerase activity.

AML is characterized by an accumulation of immature leukemic blast cells in the marrow and peripheral blood of patients due to abnormal proliferation and blocked myeloid differentiation (27). The abnormal proliferation and/or blocked differentiation can occur at different stages of maturation along the myeloid differentiation pathway. Genetic alterations, including distinct cytogenetic rearrangements, are specific prognostic indicators. For example, -5, -7, and 11q are associated with an unfavorable prognosis, inversion 16 and t(8;21) are associated with a good prognosis, and other cytogenetic abnormalities and an apparently normal karyotype are associated with an intermediate prognosis (28, 29).

In the present study, we examined telomerase activity in 56 patients with AML with known cytogenetic alterations and observed that unfavorable cytogenetics generally correlated with the presence of high telomerase activity, although this correlation did not reach statistical significance in this small sample. We also observed that telomerase activity in HL-60 promyelogenous leukemia cells could be inhibited by differentiation-inducing agents.

Materials and Methods

Patient Samples. Leukemic cells were collected from patients with AML by continuous flow centrifugation and stored in liquid nitrogen. Samples for analysis were obtained during regularly scheduled diagnostic evaluations as part of protocols approved by the Institutional Review Board of the University of Texas M. D. Anderson Cancer Center. There were 29 men and 27 women in this study. The median age was 49.5 (range, 21–80) years. There were patients in all French-American-British classification (30) except M3. There were samples from 6 patients classified as M1, 22 classified as M2, 12 as M4, 3 as M5, 1 was unclassifiable, and 12 without French-American-British information.

Telomerase Assay. The frozen leukemic cells were thawed rapidly in a 37°C water bath. After washing with PBS, protein was extracted for 30 min with ice-cold lysis buffer (10^6 cells/1 ml: 0.5% CHAPS, 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 5 mM β-mercaptoethanol, 0.1 mM AEBSF) and centrifuged at 12,000 × *g* for 20 min at 4°C. Two μl of each extract (2000 cell-equivalents) were assayed for telomerase activity in 50 μl reaction mixture as described previously (12, 31, 32). The telomerase reaction products were amplified in a thermal cycler for 30 rounds at 94°C for 30 s, 50°C for 30 s, and 72°C for 45 s, in the presence of 2 μCi [α-^{32}P]dCTP. One half of the reaction was electrophoresed in 0.5× tris-borate EDTA on 10% polyacrylamide nondenaturing gels and analyzed in a PhosphorImager using the ImageQuant software (Molecular Dynamics, Sunnyvale, CA). To obtain semiquantitative information about levels of telomerase activity, an ITAS (33) was included that can be amplified by the same two primers that is used in the telomerase activity assay (12).

Differentiation Induction. HL-60 promyelocytic leukemic cells were maintained in RPMI 1640 medium supplemented with 10% FCS in a 37°C incubator containing 5% CO_2. For differentiation induction, HL-60 cells were incubated in the presence of 1.25% DMSO or 1 μM ATRA for 1 to 3 days. Under these conditions, HL-60 cells differentiate as indicated by the appearance of a specific differentiation marker CD11b (34, 35).

Table 1 Disease-free survival by cytogenetics in patients in complete remission since 1990[a]

Cytogenetics	No. of patients	Probability of disease-free survival at 1 yr (%)	*P*
-5/-7 or abnormal 11q	62	25	
inv16 or t(8;21)	49	80	
Other abnormalities	69	45	<0.001
Normal	107	60	

[a] Comparisons of disease-free survival among patients with different cytogenetics were based on log rank tests.

Statistical Analysis. Comparisons of disease free-survival among patients with different cytogenetics were based on log rank tests. Distributions of therapy response among telomerase activity groups and distributions of cytogenetics among telomerase activity groups were compared using the χ^2 test.

Results and Discussion

There is a strong correlation between the cytogenetics of patients with AML and response to current therapy. The disease-free survival of patients in complete remission at the M. D. Anderson Cancer Center since 1990 is shown in Table 1. Patients with inv16 and t(8;21) have the highest probability to be disease free at 1 year; patients with -5/-7 (monosomic deletion of chromosome 5 and/or 7) and 11q abnormalities have the least probability; patients with other abnormalities and normal karyotypes have an intermediate diagnosis at 1 year. Although the correlation between disease-free survival of patients with the above cytogenetics is highly statistically significant ($P < 0.001$, log rank test), the underlying mechanism is unknown.

We examined telomerase activity levels in the leukemic cells of 56 patients with AML with known cytogenetics. Heterogenous levels of telomerase activity were observed in different patient samples as manifested by the intensities of the telomerase reaction ladder (Fig. 1). There was detectable telomerase activity in 41 (73%) of 56 patients. To estimate the relative telomerase activity levels and to rule out potential false negatives, we used an internal control DNA template (ITAS), which is amplified by the same two PCR primers used in the telomerase activity assay protocol. To obtain normalized telomerase activity values, the radioactivity of the telomerase reaction ladders was divided by that of the amplified internal standard (ITAS). We then compared telomerase activity levels both with response to the therapy and cytogenetics of the patients. Since there is low but detectable telomerase activity in normal peripheral blood mononuclear cells, we defined 0.5 as the basal level of activity. If the value of a sample was higher than 0.5, it was considered to have telomerase activity higher than normal levels; if the sample had a value of above 10 it was considered to have very high telomerase activity. Analysis of response to therapy showed that very high telomerase activity was often associated with resistance to therapy (Table 2), although the differences were not statistically significant ($P = 0.26$). We then compared telomerase activity with the cytogenetics of the pa-

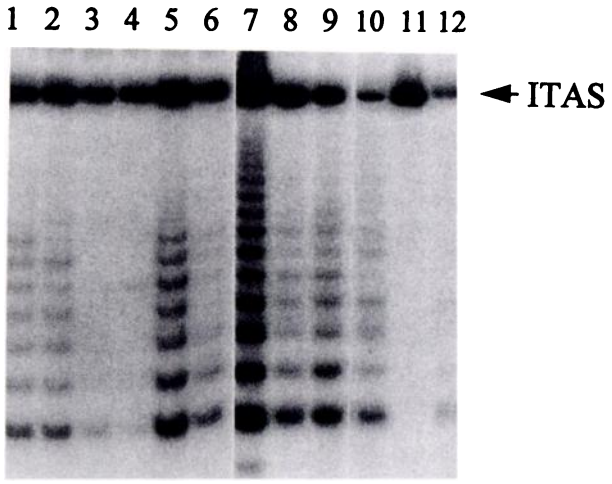

Fig. 1 The heterogenous levels of telomerase activities in AML samples. Protein extracts of 2000 cell equivalents were examined using the TRAP assay. ITAS was included in these assays. *Lanes 1–12*, 12 AML samples.

Table 2 Correlation of telomerase activity with response to therapy[a]

	Patients classified by telomerase activity levels			
Response	Negative	Low	High	Very high
Complete remission	11	14	11	3
Failure and resistance	4	3	6	4

[a] Distributions of therapy response among telomerase activity groups were compared using the χ^2 test. $P = 0.26$.

Table 3 Correlation of telomerase activity and cytogenetic status[a]

		Telomerase activity	
Cytogenetics	No. of patients	Low/negative	High/very high
Abnormal 11q	5	1 (20%)	4 (80%)
-5/-7	5	2 (40%)	3 (60%)
inv16 or t(8;21)	11	7 (63%)	4 (36%)
Other abnormalities	13	8 (62%)	5 (38%)
Normal	22	14 (64%)	8 (36%)
Total	56	32 (57%)	24 (43%)

[a] Distributions of cytogenetics among telomerase activity groups were compared using the χ^2 test. $P = 0.39$.

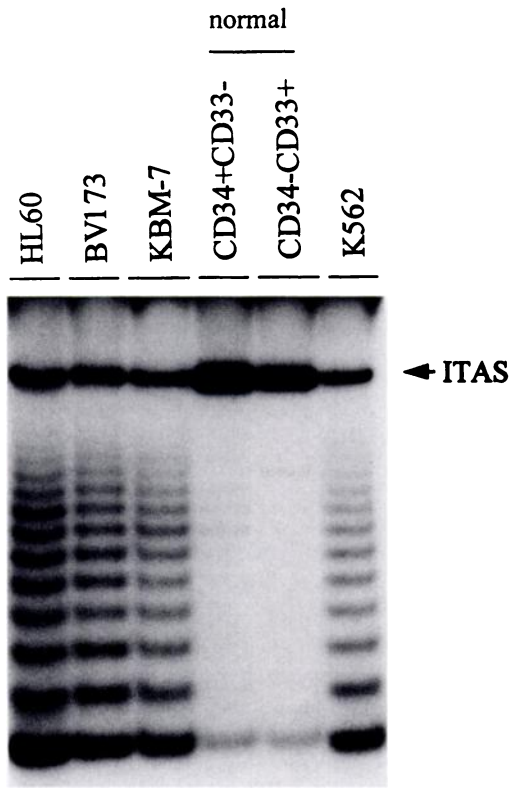

Fig. 2 Normal myeloid cells have low telomerase activity. Protein extracts of 2000 cell equivalents from four leukemia cell lines (HL-60, BV173, KBM-7, and K562) and two myeloid cell populations ($CD34^+CD33^-$ and $CD34^-CD33^+$) from a normal donor were analyzed for telomerase activity using the TRAP assay. ITAS was included in these assays.

tients and observed a general trend between high telomerase activity with unfavorable cytogenetics of -5/-7 and 11q abnormalities (Table 3). However, the sample size of certain groups was not large enough to be statistically significant. Although additional studies are required, high telomerase activity in AML may be a new prognostic indicator of chemoresistance associated with unfavorable cytogenetics.

Telomerase has been implicated in the maintenance of chromosome stability. A truncated human chromosome 16 associated with α-thalassemia was found to be stabilized by addition of telomeric repeats $(TTAGGG)_n$ (36), probably by the activity of telomerase (37). It has been proposed that telomerase may help rejoin the fragments of broken human chromosomes (38, 39). Therefore, high levels of telomerase in tumor cells may correlate with and possibly contribute to the resistance to the drugs that cause chromosome fragmentation. Future studies will be required to clarify whether this mechanism may account in part for the chemoresistance.

It has also been shown that telomerase activity in normal marrow and blood cells from adults is low compared to several tumor cell lines (16, 17). To test these findings, we isolated myeloid progenitor cells ($CD34^+CD33^-$) and mature myeloid cells ($CD34^-CD33^+CD13^+$) from normal marrow (40). Telomerase activities were measured from 2000 cell equivalents. As shown in Fig. 2, essentially no telomerase activity was detected from these two populations, whereas telomerase activity was readily detected in 2000 cells from four leukemia cell lines (HL-60, BV173, KBM-7, and K562). In a separate study, telomerase activity was detected in $CD34^+CD38^+$ myeloid progenitor cells but not in $CD34^+CD38^-$ cells, which are more primitive progenitor cells (17). This result indicates that activated telomerase is more easily detected in rapidly dividing cells. Thus, agents that decrease telomerase activity may largely affect a subpopulation of myeloid progenitor cells that are

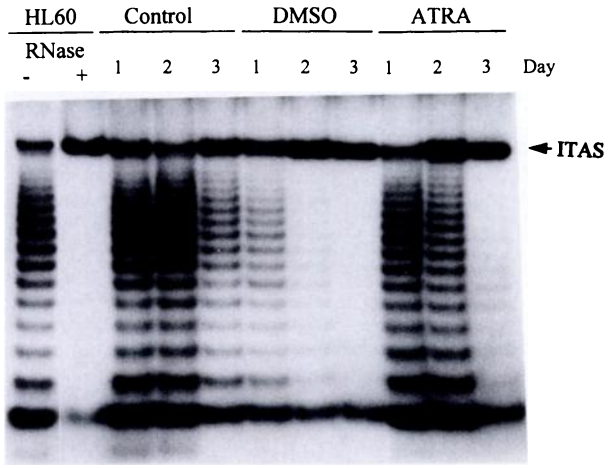

Fig. 3 Differentiation-inducing agents inhibit telomerase activity in HL-60 cells. HL-60 cells were treated with 1.25% DMSO or 1 μM ATRA for 1 to 3 days and at various time points. Cellular extracts of 2000 cell equivalents were analyzed for telomerase activity using the TRAP assay. ITAS was included in these assays.

rapidly proliferating. This suggests that therapeutic modalities targeting telomerase activity may have less toxicity on the less frequently dividing primitive stem cells.

To test whether telomerase activity can be modulated *in vitro*, we used a well-established model of cell differentiation. HL-60 cells were treated with 1.25% DMSO or 1 μM ATRA for 1 to 3 days to induce differentiation. The results in Fig. 3 show that telomerase activity, exemplified by the disappearance of the telomerase reaction ladder, is markedly decreased in DMSO- and ATRA-treated cells, starting as early as the second day. After this article was submitted, Sharma *et al.* (41) also reported that differentiation of HL-60 leukemia cells and epithelial and embryonal stem cells was accompanied by an inhibition of telomerase activity.

It is likely that this inhibition of telomerase reflects the nondividing (G_0) state that accompanies cell differentiation. We have shown (42) that telomerase activity decreases when cells become quiescent, regardless of the specific pathway. Contact inhibition, serum starvation, reexpression of the senescent phenotype in a reversibly immortalized cell line, and the induction of differentiation all lead to the disappearance of telomerase activity in a variety of model systems in which telomerase-expressing immortal cells are induced to enter G_0 (42). This notion is supported by the observation that telomerase activity in quiescent peripheral blood mononuclear cells is very low and that the activity increased dramatically after incubation with interleukin 2 and phytohemagglutinin (17). This repression is reversible and probably reflects an entirely different pathway than the one by which telomerase is silenced during the development of most somatic cells.

It is not yet known whether the telomerase activity in most AML cells reflects the reactivation of a previously silenced telomerase or, more likely, the persistence of telomerase in a hematopoietic stem cell derivative in which telomerase was never turned off (43, 44). We believe the levels of telomerase that we measured likely reflect the fraction of circulating cells in the proliferative compartment. This concept suggests that patients with AML with the highest levels of telomerase would be those exhibiting the combination of the highest production of proliferating cells and the greatest inhibition of the differentiation of those cells to a nonproliferating compartment. This combination is precisely that which would be expected to carry the poorest prognosis. However, larger patient populations will need to be examined to fully evaluate the prognostic value of telomerase measurements.

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