

# Telomerase inhibition by retinoids precedes cytodifferentiation of leukemia cells and may contribute to terminal differentiation

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## Abstract

Human promyelocytic leukemia HL60 cells display high telomerase activity, a phenotype related to their immortal status. All-*trans* retinoic acid (ATRA) is a clinically effective cytodifferentiating agent. To understand the mechanism underlying ATRA-induced cytodifferentiation, we did a kinetic analysis of the role of ATRA in inhibiting telomerase in HL60 cells. Our studies indicate that telomerase inhibition by ATRA occurred relatively early after treatment of HL60 cells due to a rapid decrease in *hTERT* gene expression. More importantly, however, we found through monitoring the expression of CD11b, a marker for granulocytic differentiation of HL60 cells, that down-regulation of telomerase preceded the differentiation of HL60 cells. These observations suggest that the *hTERT* gene may be a primary target of ATRA regulation of cellular differentiation and the anti-leukemia activity of ATRA may be mediated by its ability to induce the differentiation of the promyelocytic leukemia cells through down-regulation of the *hTERT* gene. [Mol Cancer Ther 2004;3(8):1003–9]

## Introduction

Human promyelocytic leukemia HL60 cells are derived from peripheral blood leukocytes of a patient with acute promyelocytic leukemia, which is a subtype of myeloid leukemia characterized by the accumulation of cells blocked at the promyelocytic stage (1). Retinoids are natural compounds structurally related to vitamin A. They play essential roles in normal developmental processes such as cell

proliferation, differentiation, and apoptosis. Retinoids also possess pharmacologic properties as chemopreventive agents and are often used in clinical treatment of cancers including acute promyelocytic leukemia, laryngeal papilloma, and oral leukoplakia lesions (2, 3). The most extensively studied retinoids in cancer medicine include all-*trans* retinoic acid (ATRA), 9-*cis* retinoic acid, and 13-*cis* retinoic acid. ATRA is one of the most biologically active retinoids, and several clinical studies have established that ATRA can induce differentiation of leukemia cells and remission through an unknown mechanism in almost all patients (4). This has led to the development of ATRA therapies for the prevention and treatment of various human cancers such as renal cell carcinoma, breast cancer, prostate cancer, and other malignancies (3).

Recently, extensive interest has been focused on the aberrant regulation of telomerase activity in tumorigenesis. Telomerase is a reverse transcriptase that synthesizes telomeric repeats at the ends of chromosomes. The length of telomeric repeats shortens gradually with cell division, and this telomere shortening is associated with cellular senescence. Two major components of human telomerase have been identified: the RNA template and the reverse transcriptase (hTERT). The RNA template is expressed in both normal and cancerous tissue, whereas hTERT is often detectable in cancerous cells but not in normal somatic cells (5). The mRNA level of hTERT often correlates positively with telomerase activity and has thus been considered as the major determinant of telomerase activity (6). Telomerase activity is present in human stem cells, progenitor cells, and germ cells but is undetectable in the vast majority of adult somatic tissues (7). Although alternative mechanisms of maintaining telomere length have been reported (8), most tumors examined thus far seem to employ telomerase activation as a means to escape senescence and retain high proliferative potential (9).

High telomerase activity has been observed in HL60 cells, a phenotype related to their immortal status (9, 10). HL60 cells display distinct morphologic and histochemical commitment toward myeloid differentiation. Previous studies have established that HL60 cells respond well to cytodifferentiating reagents like DMSO and 1,25 $\alpha$ -dihydroxyvitamin D<sub>3</sub> and have the potential to give rise to monocytes and macrophage cells (11–13). Addition of ATRA to the growth medium often induces differentiation of these progenitors to neutrophils. The mechanism of this differentiating function by ATRA treatment is not yet understood, although existing evidence indicates that telomerase activity may be somehow linked with this cytodifferentiating activity (14). The current study addresses the molecular pathways involved in neutrophil differentiation of HL60 cells by ATRA treatment. Our studies indicate that ATRA down-regulates hTERT and thus

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telomerase activity in early differentiation, and this process precedes cellular differentiation of the promyelocytic cells, indicating that ATRA may exert its anticarcinogenic effects through down-regulation of the *hTERT* gene.

## Materials and Methods

### Cell Culture and Proliferation Assessment

HL60 cells (American Type Culture Collection, Manassas, VA) were grown in suspension culture as described previously (15). For all experiments, the HL60 cells were seeded at  $4 \times 10^5$  cells/mL in fresh medium and ATRA (Sigma Chemical Co., St. Louis, MO) was added to 1  $\mu$ mol/L final concentration. Control cells were grown in parallel under the same conditions as treated cells but without the addition of ATRA. Morphologic changes in the cells were recorded using a Nikon Coolpix990 digital camera (Nikon, Tokyo, Japan). The HL60 cells used for growth curve studies were grown for a total of 12 days. Cell counting was done in parallel cultures, and cells were counted on days 0, 3, 6, 9, and 12 following ATRA treatment using trypan blue (0.25%) to stain for monitoring the viability of the cells, which were counted on a Neubauer hemacytometer.

### Fluorescence-Activated Cell Sorting Analysis of CD11b Expression

Fluorochrome conjugates of monoclonal antibody against human CD11b antigen, the maturation marker for granulocytes, were used to detect CD11b expression in ATRA-treated and nontreated HL60 cells following the protocol from the manufacturer (Sigma Chemical). Briefly,  $\sim 0.5 \times 10^6$  cells were harvested, washed twice with cold PBS, and incubated with antibody (2  $\mu$ g) conjugated with FITC for 30 minutes at room temperature. After incubation, the cells were washed twice with diluent solution (PBS containing 1% bovine serum albumin and 0.1% NaN<sub>3</sub>), fixed with 2% paraformaldehyde, and analyzed on a Becton Dickinson FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). The fluorescence intensity of the viable cells (identified by their light scattering characteristics) was analyzed using CellQuest software. Nontreated HL60 cells incubated only with diluent solution were included as control to set the gating threshold value (0.9%).

### RNA Extraction, cDNA Synthesis, and PCR Amplification

Total cellular RNA was purified using the RNeasy Mini kit (Qiagen, Valencia, CA). Extracted RNA was digested with RQ1 RNase-free DNase (2 units, Promega, Madison, WI) to remove any DNA contamination. After digestion, DNase was removed by phenol-chloroform extraction. RT-PCR was done as described previously (16) with slight modifications. RNA ( $\sim 2$   $\mu$ g) was reverse transcribed in 20  $\mu$ L of final reaction volume using the SuperScript preamplification system for first-strand cDNA synthesis (Life Technologies, Rockville, MD). From this reaction, cDNA (1.5  $\mu$ L) was used as the template for PCR reactions with a primer set specific to a 219-bp fragment of the *hTERT* gene coding region: forward 5'-GACTCGACAC-

CGTGTTACCTAC-3' and reverse 5'-GACACACATTC-CACAGGTCG-3'. Amplification of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA was done as an internal control as described previously (16). After 32 cycles of amplification, PCR products were resolved on a 1.5% agarose gel and analyzed using the Kodak 1D 3.6.1 image software (Eastman Kodak Company, Rochester, NY).

Quantitative real-time PCR was done on cDNA from ATRA-treated and nontreated cells by use of the ABI Prism 7900 Sequence Detection System (PE Applied Biosystems, Foster City, CA) following the Assays-on-Demand protocol. *GAPDH* was included as a control reference gene to normalize the amount and the quality of cDNA. Primers for *hTERT* and *GAPDH* were preoptimized and mixed with TaqMan MGB probe (FAM dye labeled, PE Applied Biosystems). Quantitative data were analyzed using the Sequence Detection System software version 2.1 (PE Applied Biosystems). This relative quantification is given by the ratio of the mean value of the target gene (*hTERT*) to the mean value of the endogenous control (*GAPDH*) gene in each sample.

### Telomeric Repeat Amplification Protocol Assay

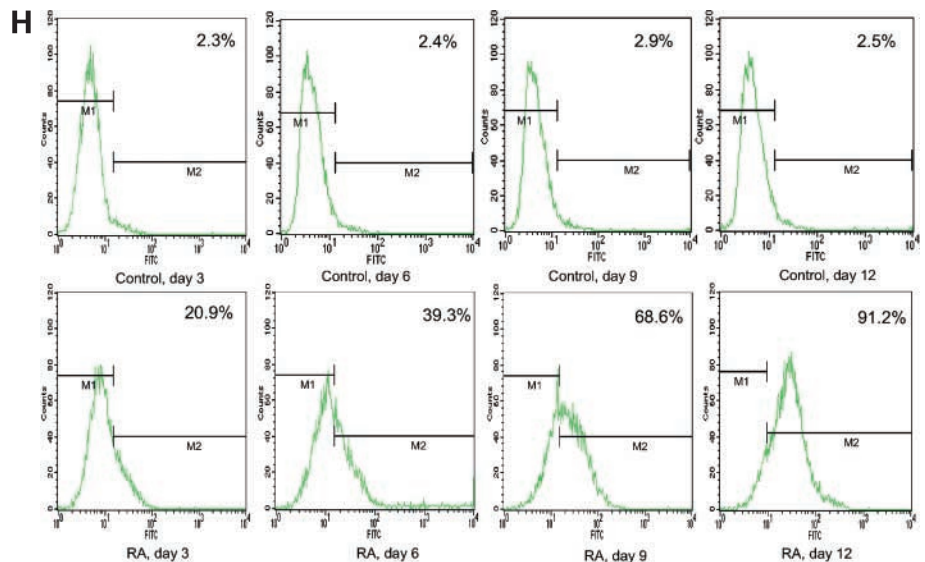
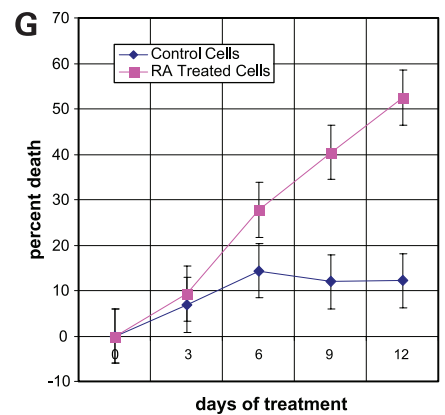
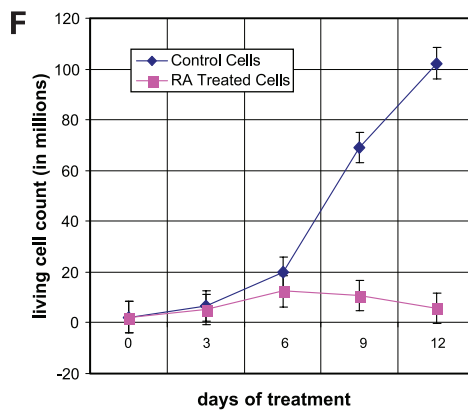
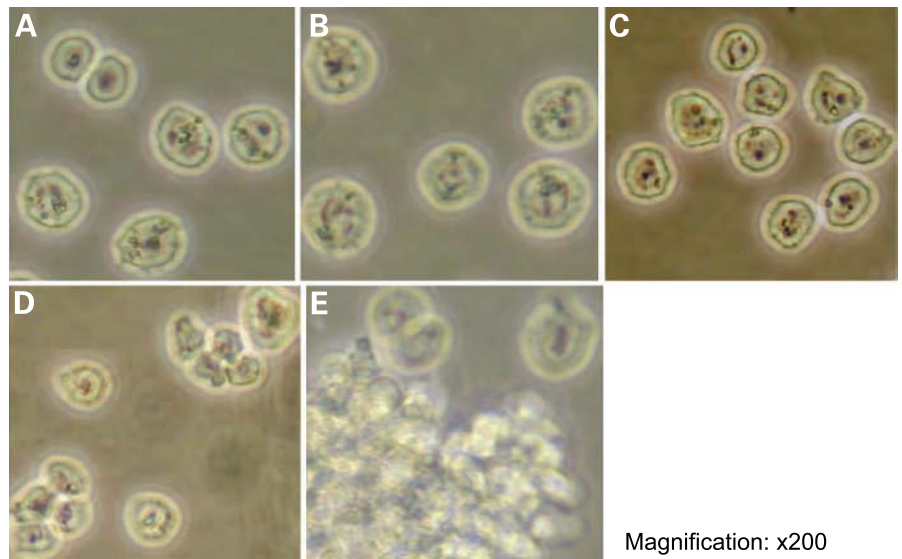
The telomeric repeat amplification protocol (TRAP) assay was done as described previously (17). Briefly, cells were harvested and lysed in CHAPS lysis buffer. The lysate was centrifuged for 20 minutes at  $12,000 \times g$  at 4°C, and the supernatant (2  $\mu$ L) was used in PCR amplification according to the manufacturer's protocol (Intergen, Burlington, MA). In addition, supernatant (10  $\mu$ L) from each sample was incubated at 94°C for 10 minutes to serve as a heat inactivation control. PCR was done at 30°C for 30 minutes followed by 38 cycles at 94°C for 30 seconds, 59°C for 30 seconds, and 72°C for 1 minute. The reaction is finished at 55°C for a 25-minute extension step. An internal control together with a specific primer was added to the reaction mixture to monitor the efficiency of PCR. To determine whether ATRA directly inhibits telomerase enzyme activity in cell-free system, protein extracts from control HL60 cells were incubated with various concentrations of ATRA (0.2, 1, and 5  $\mu$ mol/L) at 37°C for 10 minutes prior to the standard TRAP assay. The PCR products were electrophoresed on a 10% nondenaturing polyacrylamide gel and stained with SYBR Green (Molecular Probes, Eugene, OR).

## Results

### Changes in Morphology and Proliferation Rate of HL60 Cells in Response to ATRA Treatment

When suspension cultures of HL60 cells were treated with ATRA, the cells displayed gradual morphologic changes toward neutrophil differentiation as indicated by the segmented nucleus (Fig. 1A–C) within 12 days, whereas the nontreated control cells maintain stable morphology and proliferative rate throughout the experiments (data not shown). In addition to the changes in nuclear structure, a striking feature observed was that the treated cells began to cluster together to form cell clumps from the single cell suspension. The size of the cell clumps increased

**Figure 1.** **A to E**, morphologic changes following addition of retinoic acid in the growth medium. **A**, nontreated control cells (identical morphologies of nontreated cells were observed throughout the experiment; thus, only one control for day 12 was presented for all retinoic acid experiments). **B to E**, morphologies of the cells following 3, 6, 9, and 12 days of retinoic acid treatment, respectively. Magnification,  $\times 200$  (**A to E**). **F and G**, graphic presentations of growth kinetics of HL60 cells following addition of retinoic acid in the growth medium: total number of cells (**F**) and percentage of dead cells during retinoic acid treatment (**G**). Similar results were obtained from independent counting. *Bars*, SE. **H**, representative fluorescence-activated cell sorting results showing the gradual expression of the CD11b adhesive molecule during development of neutrophils. *Percentage values*, percentage of CD11b-positive HL60 cells determined by the fluorescence-activated cell sorting. FITC characteristics of nontreated control (*upper panel*) and retinoic acid-treated (*lower panel*) cells at different experimental days.



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with the number of treatment days. By day 12 in the presence of ATRA, most of the cells were growing in clusters (Fig. 1A–E). Following the maturation of HL60 cells, a decrease in the proliferation rate was clearly visible by day 6, which was accompanied by an increase in the number of dead cells (Fig. 1F and G). Spontaneous cell death of the untreated cells was also observed, but this process did not interfere with log-phase proliferation of the cells. Cell clumps were not observed in the control cells. These findings show that, after treatment with ATRA, promyelocytic leukemia cells slowed down in proliferation and gradually differentiated toward cells that have characteristics of neutrophils. ATRA-induced differentiation led to growth inhibition and eventual death of the differentiated cells.

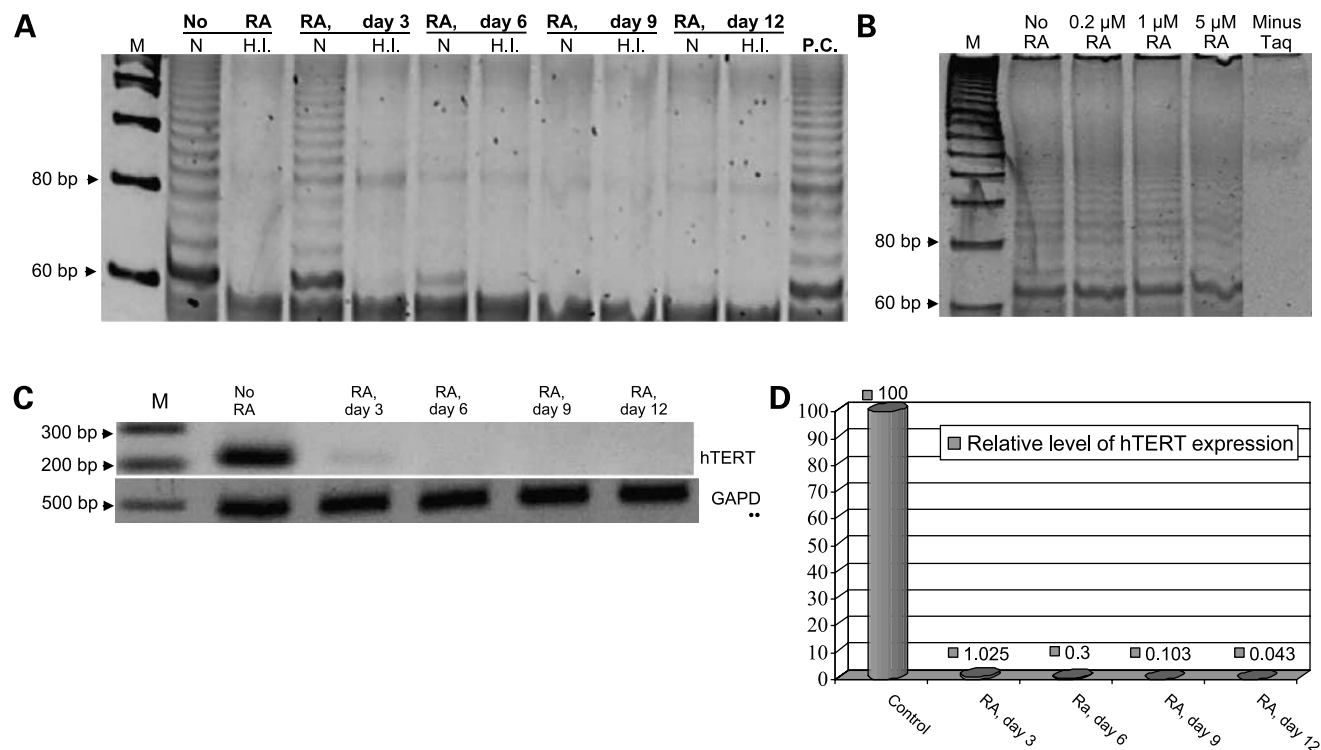
#### Gradual Increase in Expression of CD11b in ATRA-Treated HL60 Cells

Expression of the CD11b antigen was monitored every 3 days during ATRA-induced differentiation of the HL60 cells. Cells were stained with FITC-conjugated mouse monoclonal antibody against human CD11b after 3, 6, 9, and 12 days of ATRA treatment. Nontreated cells were grown in parallel and included as controls. A basal level of CD11b expression was present in nontreated control cells

(Fig. 1H, *upper panel*), reflecting the fact that these cells underwent spontaneous maturation that was followed by apoptosis as indicated from the growth kinetic analysis in Fig. 1F and G. Following ATRA treatment, a gradual increase in CD11b expression can clearly be seen from the fluorescence-activated cell sorting data depicted in Fig. 1H (*lower panel*). By day 12, >90% of the cells expressed CD11b, the adhesion molecule most likely responsible for the cell clumps in the culture as shown in Fig. 1E. These kinetics of CD11b expression were consistent with the observed gradual morphologic changes in the ATRA-treated cells, confirming that ATRA-induced differentiation tended to be a gradual process occurring in terms of days rather than hours.

#### Significant Loss of Telomerase Activity following ATRA Treatment of HL60 Cells

To address whether telomerase activity was involved in the differentiation process of HL60 cells, we did TRAP assays on nontreated control and ATRA-treated HL60 cells at the aforementioned days following addition of ATRA to the medium. As illustrated in Fig. 2A, the presence of telomerase activity was shown by the 6-bp ladder banding pattern that started at 61 bp. A 56-bp internal control band to monitor the PCR efficiency was visible in every sample.



**Figure 2.** **A** and **B**, TRAP assay showing changes in telomerase activity during differentiation of HL60 cells. The presence of telomerase activity was shown by the 6-bp ladders starting from 61 bp. **A**, days of exposure to retinoic acid (*top of each lane*). *M*, 20-bp ladder marker; *N*, normal cell extracts; *H.I.*, heat-inactivated cell extracts; *P.C.*, positive control sample for TRAP assay. **B**, incubation of cell extract directly with retinoic acid at different concentrations (*top of each lane*). Minus Taq is included as a negative control. **C** and **D**, changes in expression levels of *hTERT* mRNA during retinoic acid-induced differentiation of HL60 cells. **C**, RT-PCR product of the *hTERT* gene and the *GAPDH* gene amplified from control and retinoic acid-treated cells. *M*, 100-bp ladder DNA molecular weight marker. **D**, graphic presentation of relative levels of *hTERT* mRNA in control and retinoic acid-treated cells as determined by real-time PCR.

**Table 1. Representative average threshold cycle values derived from real-time PCR analysis of *hTERT* and *GAPDH* expression**

Samples	<i>hTERT</i>	<i>GAPDH</i>
No retinoic acid	26.87	18.44
Retinoic acid, day 3	36.18	21.14
Retinoic acid, day 6	36.33	19.52
Retinoic acid, day 9	38.00	19.65
Retinoic acid, day 12	40.0	20.38

NOTE: Average threshold cycle values were derived from duplicated real-time PCR amplifications of *hTERT* and *GAPDH* expression. Each value indicates the average number of threshold PCR cycles prior to reaching amplification plateau in that specific sample. The relative expression values of *hTERT* were determined through comparing threshold cycle values between *hTERT* and *GAPDH* by the Sequence Detection System software (PE Applied Biosystems).

Control cells (no retinoic acid treatment) clearly displayed the 56-bp control band and a ladder of PCR products with 6-bp increments (more than seven bands), which indicated a high level of telomerase activity in the undifferentiated HL60 cells. With the addition of ATRA in the growth medium, however, telomerase activity decreased rapidly and was almost nondetectable by day 9 (Fig. 2A-A). All samples that were subjected to heat inactivation displayed no telomerase activity, which confirmed that the observed telomerase activity was indeed of endogenous origin. When cellular protein extracts from telomerase-positive control HL60 cells were directly incubated with various concentrations of ATRA (0.2, 1, and 5  $\mu\text{mol/L}$ ), there was no obvious loss of telomerase activity observed (Fig. 2A-B), which indicates that ATRA does not directly inhibit telomerase protein activity.

#### Loss of Telomerase Activity Is Preceded by a Rapid Decrease in *hTERT* Expression

Telomerase activity is closely correlated with *hTERT* expression. To confirm that a decrease in telomerase activity was indeed due to down-regulation of *hTERT* mRNA expression, we did RT-PCR to assess the expression status of the *hTERT* gene during differentiation of HL60 cells in a similar manner as our analysis using the TRAP assay. As shown in Fig. 2B-A, the control cells possessed high levels of *hTERT* mRNA as indicated by the intense band at 219 bp. The mRNA level decreased rapidly by day 3 and was almost undetectable by day 6 and later. Amplification of the internal control *GAPDH* gene, however, showed no change among ATRA-treated and control cells (Fig. 2B-A). To quantify the relative levels of *hTERT* mRNA in each sample, we did real-time PCR using the *GAPDH* gene as an endogenous control to normalize the amount and quality of cDNA. Relative expression levels of *hTERT* in each sample were derived from normalizing the threshold cycle value of *hTERT* against that of *GAPDH* (Table 1). The results shown in Fig. 2B-B indicate that ATRA triggered an  $\sim 100$ -fold decrease of *hTERT* mRNA after only 3 days of treatment. Despite the dramatic decrease in the level of *hTERT* mRNA in the cells after 3 days, the telomerase activity was still readily detectable by

TRAP assay (Fig. 2A), suggesting that the *hTERT* protein remained stable and functional for a period of time after the rapid shutdown of the transcription of the *hTERT* gene, but that the enzymatic activity decreased within 3 days following down-regulation of the *hTERT* gene.

## Discussion

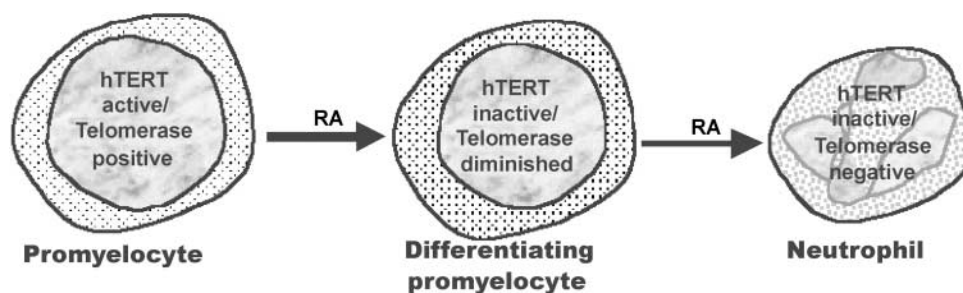
In this study, we showed that the differentiation process is more gradual than the down-regulation of telomerase activity in response to ATRA treatment, which has not been discussed before. The expression of the CD11b adhesion molecule has been frequently employed as a maturation marker for HL60 cells in response to drug-induced differentiation. Previous studies reported that  $\sim 90\%$  of cells became CD11b positive following 2 to 3 days of treatment with DMSO or 1,25 $\alpha$ -dihydroxyvitamin D<sub>3</sub>, which had a more acute and rapid differentiation effect than ATRA with maturation of HL60 cells toward monocytes and macrophages (12, 13). Our studies showed that differentiation of HL60 by ATRA took longer than that reported previously for DMSO and 1,25 $\alpha$ -dihydroxyvitamin D<sub>3</sub> as indicated by the gradually increased expression of CD11b. The lineage of differentiation may affect the pace of CD11b synthesis. Alternatively, cells may be relatively less sensitive in response to ATRA treatment because physiologic levels of retinoids are required during normal development and growth. Adaptive systems may have evolved to tolerate transient increases of retinoids at the pharmacologic level but will eventually succumb to an extended presence of increased levels of retinoids. This lower sensitivity to ATRA treatment is also consistent with a protracted change in morphology following the initial treatment of cells as we discussed above.

In contrast to the slow onset of synthesis of CD11b, telomerase activity is more rapidly decreased in differentiating

**Table 2. Differences between kinetic loss of telomerase activity and kinetic increase in terminal cellular differentiation in differentiating HL60 cells**

	% Loss of Telomerase Activity	% Terminal Cellular Differentiation	Differences between Columns 2 and 3
No retinoic acid	0	2.5	-2.5
Retinoic acid, day 3	26.7	20.9	5.8
Retinoic acid, day 6	68.5	39.3	19.2
Retinoic acid, day 9	100	68.6	31.4
Retinoic acid, day 12	100	91.2	8.8

NOTE: Percentage values in column 2 were derived from quantification of seven representative bands of the TRAP assay results for each retinoic acid-treated sample as shown in Fig. 2A. The relative telomerase activity in each sample was determined by comparing the respective total value for each sample with nontreated control (No retinoic acid). Percentage values in column 3 were derived from fluorescence-activated cell sorting analysis of CD11b expression as shown in Fig. 1C.



**Figure 3.** Schematic illustration of kinetic events of hTERT expression and differentiation of promyelocytes after retinoic acid treatment. Inactivation of *hTERT* gene led to decreased telomerase activity and was subsequently followed by differentiation of HL60 cells into neutrophils. RA, retinoic acid.

HL60 cells and is almost nondetectable at 6 days of ATRA treatment. Obviously, the differentiation status of HL60 cells followed the diminished activity of telomerase, which suggests that telomerase activity may be essential in maintaining the undifferentiated stem cell status of HL60 cells (Table 2). Following diminished activity of telomerase, most of the promyelocytic cells became terminally differentiated. Whether down-regulation of telomerase activity was the sole cause of differentiation of HL60 cells remains to be further addressed; however, our findings assessing the kinetics of telomerase down-regulation and HL60 cellular differentiation suggest that telomerase may be a key player during the differentiation process.

As indicated by CD11b expression, a small percentage of cells seemed to be resistant to ATRA induction. Maturation of the HL60 cells did not reach completion even by day 12, suggesting that a heterogeneity of cell response to ATRA treatment may interfere with its clinical efficacy under certain circumstances. This heterogeneity may explain the clinical failure of cytodifferentiation therapy by ATRA as observed in some relapsed acute promyelocytic leukemia patients that leads to the resistance of those patients to further ATRA treatment (18). A recent study compared the effects of ATRA with those of 11-*O*-hydrophenanthrene, a new derivative of retinoic acid, on differentiation and apoptosis of HL60 cells (19). The results suggest that 11-*O*-hydrophenanthrene has more antiproliferative activity than ATRA and holds promise for future clinical applications. Further characterization of the molecular processes modulated by these agents will serve to better define their role in the prevention and treatment of human cancer and to tailor specific targeted therapies in combination with other compounds.

The *hTERT* gene may be a prime target gene subject to ATRA regulation because its level of expression was rapidly affected by ATRA administration as we have shown in this study. Reduced expression of hTERT led to diminished telomerase activity followed by maturation of the promyelocytic cells toward neutrophils (Fig. 3). These observations are consistent with our previous studies on human teratocarcinoma cells (15, 16). Because ATRA has a large range of target genes involved in different biological processes (20), its therapeutic significance may pose potential harm to normal cells while being used as a general anticancer agent. Given the efficacy of diminished telomerase activity on growth inhibition of HL60 cells, drug

development aimed specifically at telomerase activity, or its hTERT subunit by the use of RNA interference technology, may prove to be highly effective in future cancer therapies. Taken together, our results show that ATRA treatment of leukemia cells seems to involve down-regulation of hTERT expression and telomerase activity, contributing to a decrease in the proliferative potential of these cells. Ablation of telomerase activity may also contribute to the terminal differentiation of the promyelocytic cells. Further efforts are being undertaken to elucidate the inactivation mechanism of the *hTERT* gene on ATRA treatment.

#### Acknowledgments

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