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Role of Human Telomerase Reverse Transcriptase and Telomeric-repeat Binding Factor Proteins 1 and 2 in Human Hematopoietic Cells

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Telomerase, an enzyme that adds hexameric repeats of 5′-TTAGGG-3′, termed telomeres, to the ends of chromosomal DNA, has been implicated in cellular immortalization and cellular senescence. Recently several relevant genes have been cloned, including those encoding three major components of human telomerase: human telomerase RNA component (hTR), human telomerase reverse transcriptase (hTERT), and telomerase-associated protein-1 (TEP1). Also important are genes encoding human telomeric-repeat binding factor proteins (TRF) 1 and 2. We compared 10 human malignant hematopoietic cell lines, 19 samples from patients with acute leukemia and normal granulocytes and monocytes to study telomerase activity and expression of these various genes using a reverse transcription-polymerase chain reaction (RT-PCR). In all 10 malignant cell lines with telomerase activity, hTR, hTERT mRNA, and TEP1 mRNA were expressed, while in normal monocytes and granulocytes without telomerase activity, expression of hTR, but not hTERT mRNA was detected. TEP1 mRNA was expressed in normal monocytes, but not granulocytes. Expression of TRF1 and TRF2 mRNAs was greater in the normal cells than in human malignant hematopoietic cell lines and in 16 samples of patients with acute leukemia. When differentiation of the malignant hematopoietic cell line HL-60 was induced using tumor-necrosis-factor 471 and all-*trans* retinoic acid (ATRA), telomerase activity decreased gradually during differentiation. Of the three telomerase components, only hTERT mRNA expression showed changes paralleling telomerase activity, becoming undetectable with differentiation. In contrast, initially low expression of TRF1 and TRF2 mRNAs increased during differentiation. Not only hTERT, but also TRF1 and TRF2 are important regulators of telomerase activity that represent potential targets for gene therapy against cancer.

Key words: Telomerase — hTERT — TRF1 — TRF2 — Differentiation-inducing

Telomerase is an enzyme that replaces repetitive $(TTAGGG)_n$ sequences on ends of chromosomes that otherwise would be lost with successive cell divisions.[1)] Accordingly, telomerase activity is linked closely to attainment of cellular immortality, a step in carcinogenesis, while lack of such activity contributes to cellular senescence.[2, 3)] Telomerase apparently is activated in more than 85% of a variety of malignant tumors. In contrast, telomerase activity usually is repressed in normal somatic tissues, except in some self-renewing tissues with high regenerative potential. Manipulation of telomerase has generated considerable excitement as an anticancer strategy. Recently genes encoding three major components of human telomerase have been cloned, specifically those for human telomerase RNA component (hTR),[4)] human telomerase reverse transcriptase (hTERT),[5, 6)] and telomerase-associated protein-1 (TEP1).[7, 8)] Expression of hTR and TEP1 mRNAs has been detected not only in cancer cells with high levels of telomerase activity, but also in nonneoplastic cells.[9–14)] In contrast, many studies have found expression of hTERT mRNA to be limited to cancer cells.[5, 6, 11–14)] More recently, two human telomeric-repeat binding factor proteins (TRFs) have been cloned: TRF 1, considered to inhibit the action of telomerase at the telomeric region[15, 16)]; and TRF2, believed to prevent fusion of chromosome ends and, *in vitro*, to remodel linear telomeric DNA into large duplex loops.[17, 18)] However, details of the mechanisms regulating telomerase activity still are poorly understood, and specific components or binding proteins that might represent suitable targets for cancer gene therapy have not been identified. Expression levels of these genes have been studied by various methods, either northern blotting or reverse transcription-polymerase chain reaction (RT-PCR), which makes comparisons difficult.[4–14, 17, 19)] We therefore performed quantitative assays using a TaqMan RT-PCR for mRNAs encoding the telomerase components hTR, hTERT, and TEP1, as well as for those encoding TRF1 and TRF2.[20, 21)] To clarify the mechanisms regulating telomerase activity in human hematopoietic cells, we studied telomerase activity and

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gene expression of these telomerase components and TRFs in 10 malignant hematopoietic cell lines, in 16 samples of patients with acute leukemia and in normal granulocytes and monocytes. We performed similar studies in HL-60 cells as they differentiated under the influence of tumor necrosis factor (TNF) 471 and all-*trans* retinoic acid (ATRA).

MATERIALS AND METHODS

Cells The human malignant hematopoietic cell lines K-562 (erythrocytic leukemia), HL-60 (myelocytic leukemia), U-937 (monocytic leukemia), U266, RPMI 8226 (plasma cell), Ramos, Daudi, BALL-1 (B lymphocytic leukemia), MOLT-4, and Jurkat (T lymphocytic leukemia) cell lines were obtained from the Department of Pathology at Sapporo Medical University. All cells were cultured in RPMI 1640 medium (Nipro, Osaka) supplemented with 10% heat-inactivated fetal calf serum (FCS; Biological Industries, Kibbutzbeit Haemek, Israel).

Human leukocyte fractions Granulocytes were obtained from a Ficoll-Paque (Pharmacia, AB Uppsala, Sweden) pellet, separated from other leukocytes, from 3 different healthy donors, as previously reported.[22] Monocytes were isolated from whole blood by using anti-CD14 monoclonal antibody-conjugated Dynabeads (Dynal A.S., Oslo, Norway).

Patients and isolation of leukemic cells Leukemic cells were obtained freshly from the bone marrow (BM) or peripheral blood (PB) of 16 patients with acute leukemia. The characteristics of patients are shown in Table I. Isolation of leukemia cells was described previously.[23] PB (30 to 50 ml) or BM (5 to 10 ml) was withdrawn and heparinized, and 6% dextran T500 (Pharmacia Biotech)-0.9% NaCl was added for sedimentation of red blood cells. The leukocyte-rich supernatant was aspirated, and layered on Ficoll-Isopaque (Pharmacia Biotech). After density gradient centrifugation, the mononuclear cells (MNC) interface and bottom pellet were hemolyzed with lysis reagent (Ortho Diagnostic Systems, Tokyo), then the total pellet was washed twice, adjusted to 2×10^7 cells/ml with phosphate-buffered saline (PBS), and layered on top of a discontinuous Percoll (Pharmacia P-L Biochemicals, Milwaukee, WI) gradient as described earlier.[23] The top layer contained 40% Percoll (density of 1.052), and in the underlying layers the concentration of Percoll was increased by 2.5%, with the bottom layer containing 50% Percoll. One milliliter of the cells from the interface of the Ficoll-Paque gradient centrifugation was then layered on the Percoll gradient, and centrifuged at 600*g* for 30 min. The bands of each interface were aspirated and washed twice with RPMI-1640 medium (Nipro) and cultured in a tissue culture flask for 18 h at 37°C. Then, adherent cells were removed and free leukemic cells were cultured in a new flask in RPMI-1640 medium, supplemented with 20% heat-inactivated FCS (Biological Industries). Isolated samples from the proper layer of Percoll gradients contained more than 95% leukemic cells as determined on the basis of morphology.

Telomerase assay Cells were collected as pellets after centrifugation at 1000*g* for 5 min at 4°C. The pellets then were washed and lysed as described previously.[2] After

Table I. Expression of TRF1 mRNA and TRF2 mRNA in Leukemic Cells Obtained from Patients

No.	FAB type	Sex	Age	PB/BM	%blasts	TRF1 mRNA	TRF2 mRNA
1	ALL (L1)	M	14	BM	78.0	16.9	3.6
2	ALL (L2)	M	59	PB	80.0	4.4	1.8
3	ALL (L2)	M	48	BM	95.0	7.9	2.6
4	AML (M0)	M	14	PB	94.0	12.8	4.3
5	AML (M1)	M	56	BM	75.8	23.2	8.3
6	AML (M1)	M	45	BM	59.8	19.2	7.2
7	AML (M1)	M	52	PB	78.0	11.3	3.8
8	AML (M1)	M	54	PB	91.0	11.9	4.0
9	AML (M2)	M	75	BM	70.0	16.4	2.9
10	AML (M2)	M	51	BM	88.0	17.2	5.1
11	AML (M2)	F	78	PB	99.0	7.4	2.2
12	AML (M3)	M	41	BM	40.0	13.2	3.5
13	AML (M3)	M	69	PB	57.0	13.0	4.7
14	AML (M4)	M	43	PB	21.0	20.2	6.0
15	AML (M4)	M	43	PB	80.0	12.8	3.9
16	AML (M7)	M	35	BM	28.0	8.7	2.9

Abbreviations: FAB, French-American-British; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; M, male; F, female; PB, peripheral blood; BM, bone marrow; TRF, telomeric repeat binding factor protein.

incubation on ice for 30 min, lysates were centrifuged at 16 000*g* for 30 min at 4°C. Supernatants were collected and their protein concentrations were measured by a Gene Quant DNA/RNA Calculator (Pharmacia, Cambridge, UK); lysates contained 0.06 μg of cellular protein. Telomerase activity was measured using a PCR-based Telomeric Repeat Amplification Protocol (TRAP)-eze Telomerase Detection Kit (Oncor, Gaithersburg, MD) according to the manufacturer's instructions. Briefly, for end-labeling of TS primer, 10 μl of TS primer (5′-AATCCGTCGAGCAGAGTT-3′) was added to 10 μl of reaction mixture including 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; Amersham, Cambridge, UK), 2 μl of 10× kinase buffer (TaKaRa, Kyoto), 5 U of T4 polynucleotide kinase, and 5 μl of distilled (d) H_2O. The mixture was incubated for 20 min at 37°C andhen for 5 min at 85°C. One microliter of this primer mixture was added to a reaction mixture containing 5 μl of 10× TRAP buffer, 1 μl of 50× dNTPs, 2 μl of TS end-labeling primer, 1 μl of TRAP primer mix, 2 U of *Taq* DNA polymerase (TaKaRa), 38.6 μl of dH_2O, and 2 μl of CHAPS extract (total volume, 50 μl). Each TRAP reaction mixture was incubated at 30°C for 30 min followed by 27 cycles of 94°C for 30 s and 60°C for 30 s in a thermal cycler (model 9600; Perkin-Elmer, Foster City, CA). Fifteen microliters of the PCR product was electrophoresed in 0.5× Tris-borate EDTA buffer on 12.5% polyacrylamide nondenaturing gels. Gels were dried and processed for autoradiography with sensitive New A film (Konica, Tokyo) at −80°C for 3 h. Signal intensity on exposed films was measured using Personal Densitometer model SI (Molecular Dynamics, Sunnyvale, CA). The experimental sample was incubated at 85°C for 10 min prior to the TRAP assay to inactivate telomerase and serve as a negative control, and a cell extract of known telomerase content provided in the kit served as a positive control. Semiquantitative analysis to estimate relative telomerase activity was accomplished by performing the TRAP assay with a TSR8 control template provided in the kit in place of sample extract. Telomerase activity was calculated as units of total product generated (TPG) using a formula described previously[24]: $TPG=\{[(x-x_0)/c]/[(r-r_0)/c_R]\}\times 100$, where telomerase products from non-heat-treated sample extract were x, telomerase products from a heat-treated sample extract were x_0, a non-heat-treated sample extract as an internal control was c, telomerase product TSR8 quantification control was r, telomerase products from the lysis buffer only were r_0, and the internal control for TSR8 quantification was c_R.

Quantitative RT-PCR assays for hTR, hTERT mRNA, TEP1 mRNA, TRF1 mRNA, and TRF2 mRNA The cell pellets were collected by centrifugation at 1000*g* for 5 min at 4°C. SepaGene RV-R (Sanko Pure Chemical, Tokyo) was used to extract total RNA from cells, and this extract was assayed for RNA with a Gene Quant DNA/RNA Calculator (Pharmacia). For quantitative RT-PCR, fluorescent hybridization probes, TaqMan PCR Core Reagents Kit with AmpliTaq Gold (Perkin-Elmer) were used with the ABI Prism 7700 Sequence Detection System (Perkin-Elmer). Expression of hTR, hTERT mRNA and TEP1 mRNAs was quantified by methods previously reported.[20, 21] Expression of TRF1 and TRF2 mRNAs was quantified using a method similar to that for hTR, hTERT mRNA and TEP1 mRNAs.[20, 21] Primers and TaqMan probes for TRF1 and TRF2 mRNAs were as follows. Sequences of the forward primer for TRF1 mRNA were 5′-GCAACAGCGCAGAGGCTATTATT-3′ and the reverse primer, 5′-AGGGCTGATTCCAAGGGTGTAA-3′; the sequence of the TaqMan probe was 5′-TCCAGTCTAACAGCTTGCCAGTTGAGAACG-3′. Sequences of the forward primer for TRF2 mRNA were 5′-AAACGAAAGTTCAGCCCCG-3′ and the reverse primer, 5′-TCCTCCAAGACCAATCTGCTTA-3′; the sequence of the TaqMan probe was 5′-CAGCCCAAGAACAAGCGCATGACA-3′. Conditions of one-step RT-PCR were as follows: 30 min at 48°C (stage 1, reverse transcription), 10 min at 95°C (stage 2, RT inactivation and AmpliTaq Gold activation), and then 40 cycles of amplification for 15 s at 95°C and 1 min at 60°C (stage 3, PCR). Data for hTR, hTERT mRNA, TEP1 mRNA, TRF1 mRNA, and TRF2 mRNA were normalized to data for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

HL-60 cell differentiation We previously reported that the combination of TNF 471 and ATRA had greater differentiation-inducing activity than did either native TNF or ATRA (Sigma, St. Louis, MO).[25] So, 2×10^6 HL-60 cells were seeded into tissue culture dishes 100 mm in diameter (Falcon, Oxnard, CA) containing 10 ml of culture medium, then 100 ng/ml of TNF 471 and 1000 n*M* ATRA were added. Cells were harvested for assays for quantitative RT-PCR and telomerase activity pre and 1, 2, 3, 4, or 5 days after induction of differentiation. Differentiation of HL-60 cells was evaluated by using NBT (nitroblue tetrazolium) reducing activity, morphologic differentiation and analysis of monocytic antigen expression (CD11b, CD36, CD68), as we reported previously.[25]

Statistical analysis Analysis of variance (ANOVA) was used for comparison of TRF1 and TRF2 expression between normal leukocytes, clinical samples and malignant hematopoietic cells.

RESULTS

Expression of three genes for major components of human telomerase in malignant hematopoietic cell lines, normal monocytes and granulocytes During the logarithmic phase of tumor cell growth, hTR, hTERT mRNA, and TEP1 mRNA were expressed in all cell lines with telomerase activity examined (Fig. 1). In normal

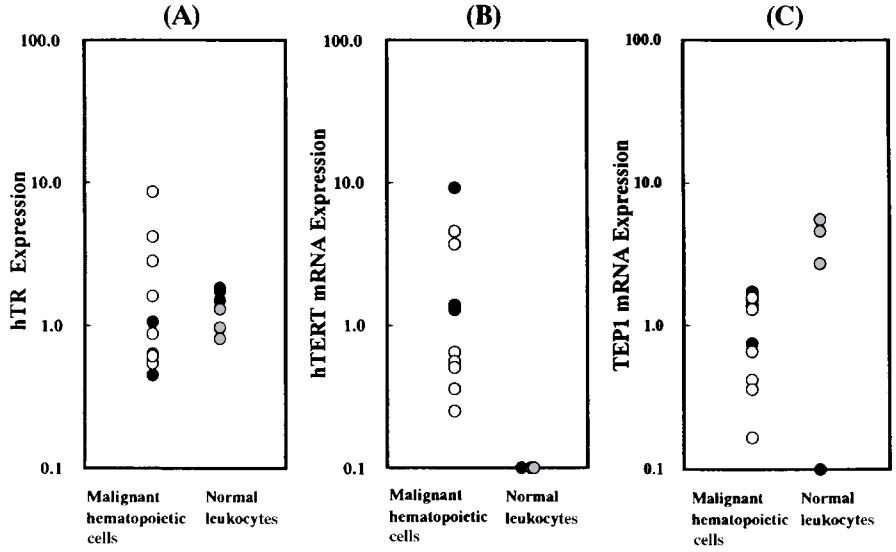

Fig. 1. Gene expression for telomerase components in human granulocytes (●) (n=3) and monocytes (○) (n=3), in 7 lymphoblastic cell lines (BALL-1, Daudi, RAMOS, MOLT-4, Jurkat, RPMI-8226, U266) (○), and 3 myeloblastic cell lines (HL-60, U937, K562) (●). Levels of hTR (A), hTERT mRNA (B), and TEP1 mRNA (C) expression were measured by a quantitative TaqMan reverse transcription-polymerase chain reaction (RT-PCR) assay. Expression is stated relative to that of glyceraldehyde-3-phosphate dehydrogenase. hTR, human telomerase RNA component; hTERT, human telomerase reverse transcriptase; TEP1, telomerase-associated protein-1.

monocytes and granulocytes without telomerase activity, hTERT mRNA were not detected, but hTR was expressed at the same level as in malignant hematopoietic cell lines (Fig. 1). Expression of TEP1 mRNA was detected only in normal monocytes, not granulocytes (Fig. 1).

Expression of genes encoding TRFs in malignant hematopoietic cell lines, patients' samples, normal monocytes and granulocytes In observations from 10 malignant hematopoietic cell lines, 16 samples from patients with acute leukemia, and normal monocytes and granulocytes from 3 different healthy donors, mean levels of TRF1 mRNA and TRF2 mRNA expression were highest in normal monocytes and granulocytes, followed by the patients' samples and the malignant hematopoietic cell lines (Fig. 2).

Telomerase activity and gene expression for telomerase components and TRFs after induction of differentiation in HL-60 cells To clarify the relationship between telomerase activity and gene expression for telomerase components and for TRFs, differentiation induced with TNF 471 and ATRA in HL-60 cells, was observed over time. A significant decrease in telomerase activity was observed within 2 days after induction of differentiation. After 5 days, telomerase activity had decreased to 1.8% of that in untreated cells. The level of hTERT mRNA expression sharply decreased to a nearly undetectable level within 1 day after induction of differentiation, a sequence

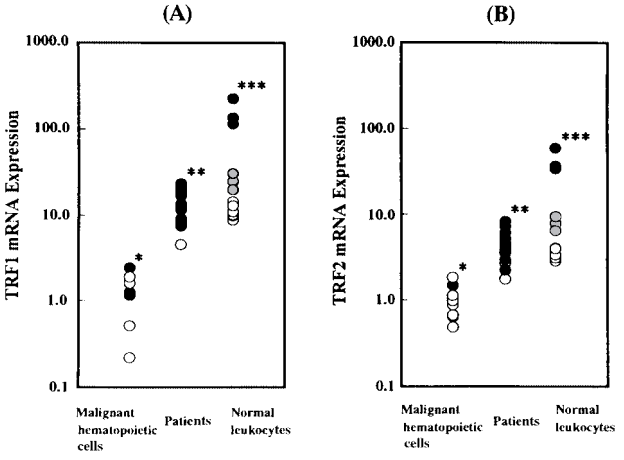

Fig. 2. Gene expression for telomeric-repeat binding factor proteins in human granulocytes (●) (n=3), monocytes (○) (n=3) and lymphocytes (○) (n=3), in 7 lymphoblastic cell lines (BALL-1, Daudi, RAMOS, MOLT-4, Jurkat, RPMI-8226, U266) (○), and 3 myeloblastic cell lines (HL-60, U937, K562) (●), and in 3 acute lymphoblastic leukemia (○) and 13 acute myeloid leukemia (●). Levels of TRF1 mRNA (A) and TRF2 mRNA (B) expression were measured by a quantitative TaqMan reverse transcription-polymerase chain reaction (RT-PCR) assay. TRF, human telomeric-repeat binding factor protein. (A) * vs. ** P<0.001, *** P<0.05, and ** vs. *** P<0.05. (B) * vs. ** P<0.001, *** P<0.05, and ** vs. *** P<0.05.

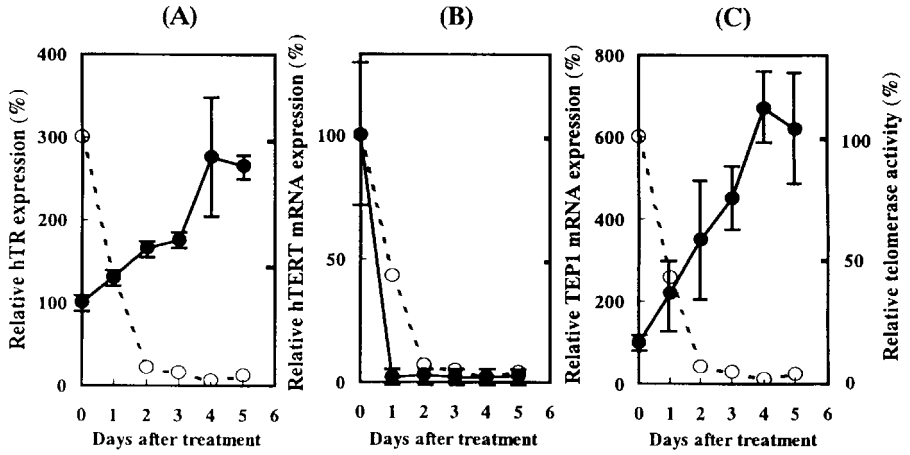

Fig. 3. Gene expression of telomerase components (●) and telomerase activity (○) in HL-60 cells during differentiation. Gene expression of telomerase components was measured by a quantitative TaqMan reverse transcription-polymerase chain reaction (RT-PCR) assay. Expression is stated relative to that of glyceraldehyde-3-phosphate dehydrogenase. Relative hTERT mRNA expression is calculated as a percentage of that shown by HL-60 cells just before differentiation. Telomerase activity was quantified as units representing total product generated (TPG), and is expressed relatively as a percentage of TPG measured in cells just before differentiation. hTR, human telomerase RNA component; hTERT, human telomerase reverse transcriptase; TEP1, telomerase-associated protein-1. The determinations for hTR, hTERT and TEP1 mRNA were made in triplicate, and are represented as mean±SE.

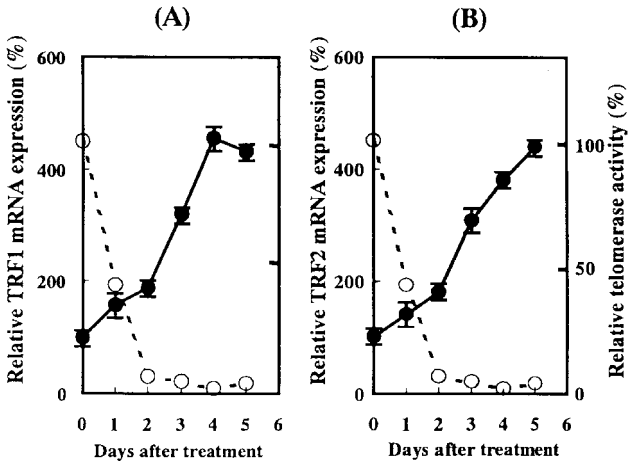

Fig. 4. Gene expression for telomeric-repeat binding factor proteins (TRF) (●) and telomerase activity (○) in HL-60 cells during differentiation. Gene expression of TRFs was measured by a quantitative TaqMan reverse transcription-polymerase chain reaction (RT-PCR) assay, and is stated relative to that of glyceraldehyde-3-phosphate dehydrogenase. TRF1 mRNA and TRF2 mRNA expression are expressed relatively, calculated as a percentage of the expression levels in cells just before differentiation. Telomerase activity was quantified in terms of units of total product generated (TPG), and is expressed relatively as a percentage of TPG measured in cells just before differentiation. The determinations of TRF1 and 2 mRNAs were made in triplicate, and are represented as mean±SE.

of change similar to that for telomerase activity. In contrast, hTR and TEP1 mRNA expression increased gradually after induction of differentiation (Fig. 3). As for gene expression for TRFs, expression of TRF1 mRNA and TRF2 mRNA increased gradually after induction of differentiation (Fig. 4), as telomerase activity declined.

DISCUSSION

This study attempted to investigate the regulatory mechanisms of telomerase activity in human hematopoietic cells. First, we examined and compared gene expression for telomerase components in malignant hematopoietic cells and normal granulocytes and monocytes. We found that hTERT mRNA was detected only in malignant hematopoietic cells. In addition, hTR in lymphoblastic cells and TEP1 mRNA in myeloblastic cells were higher than in myeloblastic cells and in lymphoblastic cells, respectively. Moreover, hTR in granulocytes and TEP1 mRNA in monocytes were higher than in monocytes and in granulocytes, respectively. Second, we examined the time course of changes in telomerase activity and gene expression for human telomerase components by means of a quantitative RT-PCR assay with a TaqMan fluorogenic detection system in HL-60 cells during differentiation, finding only hTERT mRNA to parallel changes in telomerase activity. Loss of telomerase activity has been reported with differentiation of HL-60 cells provoked by

two different inducers, ATRA, or 12-O-tetradecanoyl phorbol-13-acetate, associated with loss of hTERT mRNA expression.[5, 12] Our results using a quantitative RT-PCR assay and employing ATRA and TNF 471 as differentiation inducers, are consistent with these previous findings, indicating that HL-60 cells with initially high expression of hTERT mRNA differentiated into granulocytic and monocytic cells with almost undetectable expression. This tendency was reflected in the normal hematopoietic counterparts of these differentiated cells. These results suggest that telomerase activity is down-regulated via decreases in hTERT mRNA during the differentiation of HL-60 cells.

More recently, genes encoding TRF1 and TRF2 have been cloned.[15, 17] Transfecting a telomerase-positive tumor cell line (HT1080) that had maintained stable telomeres over 124 population doublings with a vector containing TRF1 resulted in gradual, progressive telomere shortening when cells were grown under conditions inducing vector expression.[16] In contrast, HTC75 cells expressing a dominant-negative mutant, $TRF1^{66-385}$, showed a gradual increase in telomere length.[16] On the basis of these findings, TRF1 is believed to inhibit the action of telomerase at the telomeric region.[16] A dominant-negative allele of TRF2 induced end-to-end chromosome fusion detectable in metaphase and anaphase cells.[26] TRF2, then, is considered to prevent such fusion of chromosome ends.[26]

Nonetheless, the functional relationship between telomerase activity and gene expression for TRFs in maintenance of telomere length in hematopoietic cells has not been clarified. We therefore examined expression of genes for TRFs in 10 human malignant hematopoietic cell lines and in 16 samples of patients with acute leukemia, finding that in normal monocytes and granulocytes without telomerase activity, TRF1 mRNA and TRF2 mRNA were expressed at a higher level than in human malignant hematopoietic cell lines and in 16 samples from patients with acute leukemia. Although the cellularity of purified leukemic cells was more than 95%, the contaminating normal cells might influence the results of TRFs mRNAs in clinical samples. In addition, we found that the levels of TRFs mRNAs in myeloblastic cells were higher than in lymphoblastic cells. This tendency was reflected in the normal hematopoietic counterparts of these leukemic cells. These results raise the possibility that high expression of TRF1 in normal monocytes and granulocytes prevents elongation of the telomeric region due to telomerase activity, while high expression of TRF2 in normal monocytes and granulocytes prevents fusion of chromosome ends and stabilizes the chromosome.

In addition, there is no report about the time course of changes of telomerase activity and gene expression for TRFs in hematopoietic cells. We therefore examined the time course of gene expression for TRFs during differentiation of HL-60 cells. Expression of TRF1 mRNA and TRF2 mRNA increased gradually during differentiation of HL-60 cells, which initially showed low expression of TRF1 and TRF2 mRNAs, but differentiated into granulocytic and monocytic cells that showed high expression levels of these genes. This result was reflected in the normal counterparts of the differentiated cells.

Our results suggested that not only hTERT, but also TRF1 and TRF2 play important roles in HL-60 cell differentiation induced by TNF 471 and ATRA. These results are consistent with previous reports that TRF1 inhibits the action of telomerase at the telomeric region while TRF2 prevents fusion of chromosome ends. In addition, expression of TRFs mRNAs in our clinical samples was lower than in normal leukocytes and hTERT mRNA was detected in all samples (data not shown). Although the number of clinical samples was too small to allow valid statistical analysis, the TRFs mRNAs in the cells from acute lymphoblastic leukemia (ALL) patients tended to be higher than in the cells from acute myeloid leukemia (AML) patients.

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