Selective Inhibition of Telomerase Activity during Terminal Differentiation of Immortal Cell Lines

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ABSTRACT

Telomerase, the enzyme that maintains the ends of linear eukaryotic chromosomes, is active in human germ cells and in a majority of tumor tissues and immortalized cell lines. In contrast, most mature somatic cells and tissues contain low or undetectable telomerase activity, implying a stringent negative regulatory control mechanism. We report here that telomerase activity is dramatically inhibited during the terminal differentiation of HL-60 human promyelocytic leukemia cells to monocytic and granulocytic lineages. A loss of telomerase activity was seen in response to three different inducers of differentiation, was independent of differentiation-induced apoptosis, and occurred in the presence of unaltered expression of the RNA component of telomerase. Reduction in telomerase activity was also observed during the differentiation of murine F9 teratocarcinoma and C2C12 myoblast cells. In contrast, induced differentiation of murine p19 embryonal carcinoma and Neuro 2a neuroblastoma cells did not result in a loss of telomerase activity. These results are therefore consistent with the absence of telomerase activity in human somatic cells and the presence of telomerase activity in many somatic murine cells and tissues.

INTRODUCTION

Telomeres, the specialized structures present at the ends of eukaryotic chromosomes, consist of tandem arrays of highly conserved hexameric (TTAGGG) repeats in vertebrates, and telomeres in humans are an average of 10–15 kbp in length (1, 2). Telomeres have been implicated in stabilizing chromosomes from exonucleolytic degradation and chromosome-to-chromosome fusions, preventing other forms of aberrant recombination, the attachment of chromosomes to the nuclear matrix (reviewed in Ref. 3), and as a "mitotic clock" in determining the maximum replicative capacity of human somatic cells (4–6).

Normal diploid somatic cells lose approximately 50–200 bp of telomeric DNA/mean population doubling due to the inability of DNA polymerase to completely replicate the ends of linear chromosomes (5, 7). In contrast, germ line and most immortal cell lines maintain their telomeres at a constant length irrespective of the number of divisions they undergo due to the enzyme activity of telomerase, a ribonucleoprotein that is able to synthesize and add telomeric repeats onto chromosomal termini. Because telomerase activity is absent in most normal human somatic cells and tissues that have limited replicative life spans (8) but is activated during cellular immortalization (9), the telomere hypothesis of cell aging and immortalization has been proposed, in which the attrition of telomeric sequences ultimately interferes with the expression of genes required for continued cell growth (4–6, 10). Strong correlative support for this hypothesis was provided by the observation of telomerase activity in 98% of established (immortal) cell lines and 90% of tumors tested and the absence of telomerase activity in over 50 normal human somatic tissues (8).

In contrast to human somatic cells, many rodent cell types display telomerase activity (11, 12), and the length of telomeres in mouse cells derived from different normal somatic tissues has been reported to be hypervariable (13, 14). These results, combined with the observation that rodent cells undergo spontaneous immortalization at a frequency that is approximately 1,000,000-fold higher than that in human cells (15), have led to the suggestion that the expression of telomerase activity is a requisite for immortal human cancer cell formation (8). Therefore, the absence of telomerase activity in normal human somatic cells seems to reflect stringent controls that act developmentally to repress enzyme activity, perhaps as a mechanism for limiting cell growth. Because reacquisition of telomerase activity seems to be related to the emergence of immortal human cancer cells, this enzyme may offer a unique target for cancer therapies. To better understand the conditions leading to the suppression of telomerase activity, we used five independent model systems of differentiation. The HL-60 human promyelocytic leukemia cell line can be chemically induced to differentiate into a variety of cell types that functionally and morphologically resemble normal monocytes/macrophages and granulocytes (reviewed in Ref. 16). We found that differentiation of human HL-60 cells by several different agents is associated with the rapid inhibition of telomerase activity. The effect is also seen in the murine F9 (teratocarcinoma) and C2C12 (myoblast) cell lines; however, two of four murine cell lines, P19 (embryonal carcinoma) and Neuro 2a (neuroblastoma), did not display decreased telomerase activity when induced to differentiate.

MATERIALS AND METHODS

Cells and Cell Culture. HL-60 cells were maintained as a suspension in RPMI 1640 supplemented with 10% FBS (Life Technologies, Inc.) at 37°C, 5% CO2,95% air. Cells for differentiation studies were seeded at 2 × 106 cells/ml with or without 1 μM RA and 5 × 104 cells/ml with or without 160 nM TPA (a gift from D. Edwards, University of Calgary, Calgary, Alberta, Canada) or 1.25% v/v DMSO on 15-cm tissue culture plates (Corning Glass). Cells were monitored for viability by trypan blue exclusion and were counted with a hematocytometer. Morphological assessment of differentiated HL-60 cells was made with a hemocytometer. Morphological assessment of differentiated HL-60 cells was made with a LeukoStat stain kit (Fisher Scientific). Murine F9 teratocarcinoma, P19 embryonal carcinoma, and Neuro 2a neuroblastoma cells were maintained and differentiated as described (17–19). C2C12 myoblast cells were maintained and differentiated in DMEM supplemented with 10% FBS and were differentiated as described (20). Differentiated C2C12 cells were processed for telomerase activity assays after 10–14 days in differentiation medium. Differentiated P19 cells were incubated in the absence of FBS for up to 60 days, with media changes every 3–4 days to ensure complete differentiation as well as the death of undifferentiated P19 cells before analysis of telomerase activity.

NBT Reduction and CD11b Determination. Biochemical maturation of RA- and DMSO-treated HL-60 cells was assessed by NBT reduction as a measure of macrophage differentiation.

Received 1/31/96; accepted 6/1/96.

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1Supported by grants to K. T. R. from the Medical Research Council of Canada and the National Cancer Institute of Canada. L. J. B. received support from the Leukemia Research Fund of Canada and is the recipient of a studentship from the Alberta Heritage Foundation for Medical Research.

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3The abbreviations used are: FBS, fetal bovine serum; RA, retinoic acid; TPA, 12-O-tetradecanoylphorbol-13-acetate; NBT, nitro blue tetrazolium; TRAP, telomere repeat amplification protocol; GADD45, gadd45, a growth arrest and DNA damage-inducible protein; RTPCR, reverse transcription-PCR.
Fig. 1. Morphological maturation of terminally differentiating HL-60 cells. A, HL-60 cells were treated with the phorbol ester TPA (160 ng) for 4 days to induce features of differentiated monocytes/macrophages or with RA (1 μM) for 7 days or DMSO (1.25% v/v) for 8 days to induce granulocytic characteristics as described. Panels 1 and 3, untreated control cells. Arrow, a prominent nucleolus; Panel 2, TPA-treated for 48 h; Panel 4, RA-treated for 6 days. The efficiency of differentiation with TPA, RA, and DMSO was determined by CD11b expression [TPA (D)-treated cells] or NET staining [RA (O)- and DMSO (O)-treated cells]. B, the percentage of total CD11b-positive and NET-positive cells at different times after addition of the inducing agent.

described previously (21). Briefly, 2 × 10⁶ cells/ml were incubated for 25 min at 37°C with an equal volume of 0.2% NBT dissolved in PBS containing TPA (200 ng/ml). Cytospin preparations were made and were stained with Leukostat, and the percentage of cells that contained reduced blue-black formazan deposits was determined. Expression of the CD11b surface marker was measured using a commercially available antibody (Becton Dickinson, San Jose, CA) to stain cells followed by fluorescence-activated cell sorting analysis using standard parameters.

Telomerase Assays. Cells from each time point after induction of differentiation with TPA, RA, or DMSO were collected and counted, and viability was assessed as described above. In the case of TPA-treated HL-60 cells at later time points, only the most adherent cells were assessed. Pellets of 3–5 × 10⁶ cells were collected, and S100 extracts were prepared (8). In every case, unless otherwise indicated, cell extracts from the different cell types represented 1 × 10⁶ cells/20 μl of lysis buffer. To assess telomerase activity, the TRAP assay was used (8) with minor modifications. Aliquots containing 2 μl of S100 extract were added to tubes containing 20 mM Tris-HCl (pH 8.3), 63 mM KCl, 0.005% Tween-20, 1 mM EGTA (pH 8.0), 50 μM deoxynucleoside triphosphates, 0.1 μg of telomerase substrate oligonucleotide, 1 μg of T4g32 protein (Boehringer Mannheim), 0.1 mg/ml BSA, 2 units of Taq DNA polymerase (Pharmacia Biotech), and 0.4 μM [α-³²P]dCTP (10 μCi/μl, 3000 Ci/mmol) in a total volume of 50 μl. Reactions were incubated for 20 min at 23°C. Control reactions were performed with cell extract incubated with 1 mg/ml RNase A or with TPA, RA, or DMSO at concentrations used to induce differentiation for 20 min at room temperature. Reactions were placed in a thermal cycler for 27 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 90 s. Reverse primer (CX, complementary to telomeric repeats) was added manually to each reaction tube when the temperature reached 92°C during the first cycle. After amplification, 10 μl of stop buffer (100 mM EDTA, 50% glycerol, 0.02% xylene cyanol, 0.02% bromphenol blue, and 0.02% SDS) was added to each tube, and 30 μl of the contents were size-fractionated on a 15% nondenaturing polyacrylamide gel. All TRAP assays on differentiating cell systems were performed at least twice, with comparable results between trials.

Quantitation of TRAP Assays. TRAP assays were performed on HL-60 cell extracts representing various numbers of viable cells to determine the optimal cell number for linear PCR amplification, and these studies indicated that 1 × 10⁶ cells/20 μl of lysis buffer gave accurate, reproducible results. Individual bands of TRAP assays were quantitated by computerized scanning
Differential Loss of Telomerase Activity with Differentiation

Cont. + TPA

Fig. 2. Loss of telomerase activity in differentiating HL-60 cells. A, HL-60 cells were induced to differentiate along the monocytic pathway with TPA, and telomerase activity was assessed at various time points with the TRAP assay, as described in "Materials and Methods." Lane 1, HS68 normal diploid fibroblast cell extract; Lanes 2-5, uninduced HL-60 cell extracts incubated with increasing concentrations of TPA; Lane 6, uninduced cell extract; Lane 7, uninduced extract treated with 1 mg/ml RNase; Lanes 8-11, extracts from TPA-treated cells. HL-60 cells were also induced to differentiate toward the granulocytic lineage with RA (B) or DMSO (C), and telomerase activity was assessed as described. In each case: Lane 1, uninduced cell extract incubated with RA or DMSO; Lane 2, uninduced (control) extract; Lane 3, uninduced extract incubated with RNase. Days of drug treatment are indicated. Drug-free cells were allowed to grow in culture for 5 days without subcultivation to ensure loss of activity was not related to excessive cell density or poor culture conditions (C, Lane 12). Relative telomerase activity during differentiation was determined and was expressed as a fraction of the undifferentiated HL-60 cell signal. Activity in each lane was normalized to the standard and were expressed as a fraction of the maximum value of 100.

PCR Analysis of RNA. Total RNA was obtained by the acid guanidinium method (22), and PCR was performed as described (23). Aliquots of each reaction were equalized for the internal control GAPDH and were run on 2% agarose gels containing ethidium bromide (0.2 mg/ml). Primers for hTR were synthesized using the TRC 3 sequence (24). Amplification was carried out for 28–32 cycles for hTR and 19–22 cycles for GAPDH and c-myc. Gels were scanned by computerized densitometry, and relative concentrations of hTR and c-myc were determined by comparing the ratio of hTR or c-myc to GAPDH in each lane. An arbitrary value of 100 was given to the untreated HL-60 hTR and c-myc levels. Experiments were repeated in two to five independent trials, and values were either averaged or the SE was determined.

Quantitation of Apoptosis by Flow Cytometry. Cells were assessed for apoptosis by the ethanol-fixation method, as described (25). Briefly, 3 × 10^6 HL-60 cells were collected at various stages of TPA-, RA-, or DMSO-induced differentiation and were centrifuged at 200 × g, and the cell pellet was fixed in 2 ml of ice-cold 70% ethanol for 1 h. After centrifugation, cells were washed in 1 ml of PBS and were resuspended in 0.5 ml of PBS. A volume of 0.5 ml of RNase A (Sigma Chemical Co.; 1 mg/ml in PBS) was added, and the cell suspension was mixed gently, followed by the addition of 1 ml of propidium iodide (Sigma; 1 mg/ml in PBS). Cells were incubated in the dark for 15 min at room temperature and were stored in the dark for 24 h at 4°C before flow cytometry was performed.

Photomicroscopy. All cells were photographed using phase contrast or differential interference optics and Kodak Technical Pan film set at 160 DIN.

RESULTS AND DISCUSSION

HL-60 cells differentiating into mature monocytes/macrophages upon treatment with TPA increased in size, became adherent, and displayed prominent pseudopodia within 24 h, whereas cells stimulated with RA or DMSO decreased in size and exhibited less prominent nucleoli along with segmented or lobulated nuclei (Fig. 1A, Panels 1–4). Cells treated with TPA exhibited maximal expression of CD11b (a surface marker of differentiation) 36 h after stimulation, at which point 91% of the cells scored as positive (Fig. 1B). The majority of cells treated with RA remained functionally undifferentiated until 4 days after the addition of RA, when their ability to produce superoxide anion and subsequently reduce the water-soluble dye NBT increased from 30% to 70%. Maximal NBT reduction was seen after 7 days of RA treatment, when the majority (~85%) of cells stained, indicating that the cells had acquired biochemical characteristics of granulocytic maturation. Cells treated with DMSO displayed morphological and functional characteristics similar to those of RA-
Fig. 3. hTR and c-myc expression in differentiating HL-60 cells by RT-PCR. Total RNA was prepared, and "primer-dropping" RT-PCR was performed as described (23). Aliquots of each reaction were equalized for the internal control GAPDH and were run on 2% agarose gels containing ethidium bromide (0.2 mg/ml). A-C, times of treatment with different drugs are as indicated. Graphs show the relative concentrations of hTR (white bars) and c-myc (solid bars) mRNA as determined by computerized densitometry by comparing the ratio of hTR or c-myc to GAPDH in each lane. An arbitrary value of 100 was given to untreated HL-60 hTR and c-myc levels, and changes in response to differentiating agents were plotted as a function of time.

To determine whether the loss of telomerase activity was specific to a particular pathway of differentiation, HL-60 cells were induced to differentiate along the granulocytic lineage with RA (Fig. 2B) or with DMSO (Fig. 2C), followed by assessment of telomerase activity. In both cases, telomerase activity was seen to decline rapidly over the course of treatment, similar to results seen with TPA treatment (Fig. 2D), indicating that the mechanism(s) responsible for suppressing telomerase activity is activated by differentiation along both granulocytic and monocytic pathways. The loss of telomerase activity in DMSO-treated cells occurred more rapidly and more completely than in RA-treated cells. Cells treated with DMSO exhibited a total loss of detectable telomerase activity after 3 days, whereas after 7 days, RA-treated cells still expressed very low levels of residual telomerase activity (as indicated by the faint low molecular weight bands) that were approximately equal to 3.5% that of uninduced HL-60 but still higher than that of normal diploid cells, in which activity was undetectable. This residual activity may be due to a small proportion of the HL-60 population that failed to respond to RA. Telomerase activity in the uninduced HL-60 cell extract was not inhibited by adding RA and DMSO at concentrations that induced cell differentiation, again indi-
DIFFERENTIAL LOSS OF TELOMERASE ACTIVITY WITH DIFFERENTIATION

Fig. 4. Levels of apoptosis in differentiating HL-60 cells. DNA content of HL-60 cells induced to differentiate with TPA, RA, and DMSO was assessed by flow cytometry before addition of TPA (Control), or 1 (TPA 1D), 2 (TPA 2D), 3 (TPA 3D), or 4 (TPA 4D) days after TPA addition. M1, the DNA fluorescence of cells with a subdiploid DNA content that constitute the apoptotic cell population (33). Values derived from fluorescence-activated cell sorting analysis for TPA, DMSO, and RA were plotted in Table 1.

Telomerase is a complex enzyme containing both protein and an RNA component that acts as a template for telomere elongation. Although the cloning of genes encoding the protein components of telomerase has not yet been reported for any vertebrate (perhaps due to the rarity of the enzyme), the RNA component has been isolated and cloned from Tetrahymena, Oxytricha, Euplotes, mouse, and human sources (24, 26–30). To assess the potential role of the RNA component of human telomerase (hTR) in the loss of telomerase activity, the levels of hTR RNA were determined in differentiating cells by RT-PCR, as shown in Fig. 3. Cells differentiating toward monocytes/macrophages in response to TPA (Fig. 3A) and toward granulocytes in response to RA (Fig. 3B) or DMSO (Fig. 3C) showed no significant differences in the amount of hTR expressed when compared with the internal control “housekeeping” gene GAPDH. In contrast to the unaltered levels of hTR and in agreement with other reports (16), the levels of the c-myc gene transcript decreased very rapidly in response to all three chemical inducers (Fig. 3A–C), corroborating the previous observations of morphology, surface antigen expression, and dye reduction that differentiation was being induced efficiently.

Terminal differentiation of HL-60 cells is associated with a loss of proliferative capacity and increased programmed cell death or apoptosis (31, 32). To investigate whether the decreased telomerase activity observed during treatment with TPA, RA, or DMSO might result from increased apoptosis, HL-60 cells induced to differentiate with TPA, RA, and DMSO were analyzed for DNA content by flow cytometry. Apoptotic cells show decreased DNA staining with a variety of fluorochromes as a result of the activation of an endogenous endonuclease and the subsequent diffusion of low molecular weight DNA from the cells. As a result, cells undergoing apoptosis frequently display a subdiploid DNA content on a DNA-content frequency histogram (33). Fig. 4 shows the DNA content of HL-60 cells undergoing TPA-induced differentiation. Inhibition of cell growth is reflected by the accumulation of the vast majority of cells in the G0/G1 phase of the cell cycle and the loss of cells in the S phase and G2-M phase after 2 days of treatment. Also evident is a small apoptotic cell population that displays a sub-G0/G1 DNA content that increases with time of exposure to TPA. The percentages of apoptotic cells accumulating in response to TPA or RA, although increasing over the course of differentiation, did not display levels that could adequately account for the loss of telomerase activity seen upon differentiation (Table 1). In addition, DMSO-treated cells, which do not exhibit morphological characteristics of apoptosis such as membrane blebbing, cell shrinkage, or nuclear fragmentation (32), also did not display a subdiploid DNA content that was significantly different from that of uninduced cells, demonstrating that the loss of telomerase activity was not due to apoptosis in this model system.

These data demonstrate that the terminal differentiation of HL-60 human promyelocytic leukemia cells to monocytic and granulocytic lineages is accompanied by a loss of telomerase activity. This is most likely the result of regulatory changes incurred as a result of differentiation rather than drug-induced apoptosis or changes in the levels of telomerase activity.
Fig. 5. Differential loss of telomerase activity during differentiation of murine cell lines. F9 teratocarcinoma, C2C12 myoblast, P19 embryonal carcinoma, and Neuro 2a neuroblastoma cells were induced to differentiate, and telomerase activity was assessed with the TRAP assay. A-D contain photomicrographs of undifferentiated (PRE) and differentiated (POST) cells as well as the TRAP assay results of undifferentiated (−) and differentiated (+) cell extracts and RNase controls (R). A, photomicrographs of uninduced and RA (1 µM)-treated F9 cells at 4 days. Lane 1 of the TRAP assay, uninduced cell extract control incubated with 1 µM RA. Lane 2, RNase control. Lane 3, uninduced F9 cells. Lanes 4–6, F9 cells treated with RA for 4, 8, and 12 days, respectively. All extracts represent 1 µg of protein. B, photomicrographs of uninduced C2C12 and differentiated myoblasts after 1 week in differentiation medium. Lane 1 of the TRAP assay, RNase control. Lanes 2–4, extracts representing 1 × 10³, 5 × 10³, and 10³ untreated cells. Lanes 5–7, extracts representing 1 × 10³, 5 × 10³, and 10³ cells grown for 10 days in differentiation medium. C, photomicrographs of undifferentiated P19 and cells treated with RA for 4 days and allowed to form neurite outgrowths. Lane 1, RNase control. Lanes 2 and 3, untreated P19 cells at 1 × 10⁶ and 5 × 10⁶ cells/20 µl of lysis buffer. Lane 4, differentiated P19 cells at 1 × 10⁶ cells/20 µl of lysis buffer. Cells were maintained under serum-free conditions for 60 days before assessment of telomerase activity to ensure complete differentiation. D, photomicrographs of uninduced and 3-day serum-starved Neuro 2a cells. Lane 1 of the TRAP assay, RNase control. Lane 2, uninduced cells. Lanes 3 and 4, cells treated with 20 µM of RA for 3 and 4 days. Lane 5, serum-starved cells at 4 days.

of the RNA component of telomerase, although alterations in the RNA secondary structure cannot be ruled out as a factor in blocking telomerase activity.

In addition to HL-60, a variety of human and mouse cell lines can be induced to differentiate, including the mouse F9 teratocarcinoma, C2C12 myoblast, P19 embryonal carcinoma, and Neuro 2a neuroblastoma cell lines. To determine if the loss of telomerase activity observed in HL-60 cells was specific to this cell type, we determined the effect of differentiation on telomerase activity in these murine cell lines. Fig. 5A–D shows the changes in phenotype associated with differentiation of the murine cell lines and the TRAP assays performed on cell extracts both pre- and postdifferentiation. Treatment of F9 teratocarcinoma cells with RA and growth of C2C12 myoblast cells in low-serum medium induces their differentiation to cells with properties of parietal endoderm (17) and skeletal muscle (20), respectively. Both differentiated cell types exhibited a dramatic down-regulation of telomerase activity (Figs. 5A and B). In contrast, telomerase activity was retained in RA-treated P19 cells that were induced to differentiate to neurons and glial cells by RA treatment.
ACKNOWLEDGMENTS

We wish to thank Dr. R. Woodman and F. Johnston for helpful advice on NBT staining. Drs. T. Tamaoki and G. Schulz for the gifts of P19, C2C12, and F9 cells, and Shari Berridge for typing the manuscript.

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