Tumor necrosis factor alpha inhibits hTERT gene expression in human myeloid normal and leukemic cells

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Specific contributions of all authors

N Prade-Houdellier, O Beyne-Rauzy, V Mansat-De Mas performed research and analyzed data; J Ayel provided discarded fragment from hip surgery; C Recher and C Demur provided fresh AML cells; G Laurent, O Beyne-Rauzy and V Mansat-De Mas designed research and wrote the paper.
Abstract

Telomerase catalytic subunit (hTERT) has been shown to play a critical role not only in telomere homeostasis but also in cellular survival, DNA repair, and genetic stability. In a previous study, we described that TNFα induced in the leukemic KG1 cells a senescence state characterized by decreased hTERT activity followed by prolonged growth arrest, increased β-galactosidase activity, telomere shortening and major chromosomal instability. Interestingly, GM-CSF abrogated all these events. In the present study, we show for the first time that TNFα acts by inhibiting hTERT gene in both normal CD34+ cells and fresh leukemic cells. Using KG1 cells as a representative cellular model, we show that TNFα induced sphingomyelin hydrolysis, ceramide production and JNK activation all of which are critical components of TNFα signaling resulting in hTERT gene inhibition. Moreover, we provide evidence that the protective effect of GM-CSF is related to its capacity to interfere with both ceramide generation and ceramide signaling. Negative regulation of the hTERT gene may represent one mechanism by which TNFα interferes with normal hemopoiesis.
Introduction

Telomerase is a large ribonucleoprotein complex containing two major subunits contributing to enzymatic activity: a RNA component (hTR) that serves as a template for the polymerase activity of the enzyme and a catalytic subunit with reverse transcriptase activity (hTERT). Previous studies have documented that hTERT represents the rate limiting step in telomerase function. For this reason, and because this enzyme has been found to play an essential role in the regulation of telomere elongation and cellular protection, hTERT regulation has been the subject of intense investigation in recent years. From these studies, it appears that hTERT is tightly regulated both at transcriptional and post-transcriptional levels. For example, the cloning and characterization of the hTERT 5’ gene regulatory elements have identified more than twenty transcription factor binding sites acting as activators or repressors (for a review, see 3).

In spite of increasing knowledge about hTERT transcription regulation, much less is known about which type of intra- or external stimuli could interfere with hTERT gene regulation. Recent studies have described that, in epithelial cells, oncogenes, such as Her2/Neu, growth factors, such as EGF, or steroids could activate hTERT at the transcriptional level. In contrast, TGFβ acts as a repressor of hTERT gene transcription in a variety of cellular models.

In a previous study, we described that, in leukemic myeloid KG1 cells, TNFα induced premature senescence characterized by cellular growth arrest, increased β-galactosidase activity, reduced hTERT activity, telomeric disturbances (shortening, losses, fusions), and intense chromosomal instability. Moreover, increased β-galactosidase activity was also found in normal CD34+ as well as in fresh acute
myeloid leukemia (AML) cells treated with TNFα. To the best of our knowledge, the role of TNFα in hTERT regulation in hematopoietic cells had not been previously documented. This question may be of great significance. Indeed, hTERT plays a critical role not only in telomere homeostasis but also in cellular survival, DNA repair, and genetic stability, whereas TNFα accumulates in a number of pathological situations, including not only chronic inflammation and neoplastic disorders, but also leukemia, myelodysplasia, and aplastic anemia.

The present study was aimed at evaluating whether TNFα could interfere with hTERT expression in normal progenitors cells and in leukemic cells derived from patients with acute myeloblastic leukemia (AML). In this study, we also investigated the signaling pathways by which TNFα influences hTERT gene regulation or which could interfere with TNFα.

Patients, materials and methods

Normal progenitors purification and culture

Normal bone marrow CD34+ hematopoietic progenitor cells (HPC) were obtained from discarded fragments of hematological healthy patients undergoing hip surgery after informed consent. Mononuclear cells from bone marrow were obtained by Ficoll-Hypaque density gradient centrifugation after which isolation of HPCs was performed by positive selection of CD34 expressing cells. Briefly, CD34+/HPCs cells were magnetically labelled using MACS CD34 microbeads then isolated by positive selection through MS separation column (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the CD34+ cells was evaluated by flow cytometry using anti-
CD34 and anti-CD45 monoclonal antibodies, and reached 85-98%. Human progenitors (5x10^5 cells/ml) were cultured in IMDM containing 10% of a commercial mixture of BSA, insulin and transferrin (BIT9500, StemCell Technologies, Grenoble, France) supplemented with 100 ng/ml stem cell factor, 1U/ml interleukin 3, 100 ng/ml fms-like tyrosine kinase type 3 ligand (FLT3-L), and 5 pg/ml thrombopoietin (R&D Systems, Oxon, UK). Human recombinant TNFα (PeproTech-TEBU, Rocky Hill, NJ, USA) was added to fresh medium containing cytokines every 2 days.

Patient samples culture

Fresh AML cells were obtained from patients diagnosed at the Hematology Department of Toulouse University Medical Center (France), after informed consent. AML cells were isolated from bone marrow by Ficoll-Hypaque density gradient centrifugation and were cryopreserved in IMDM medium with dimethyl sulfoxide (10% final concentration) and fetal calf serum (FCS, 50% final concentration) or immediately processed for assays. Leukemic cells (1x 10^6 cells/ml) were maintained in IMDM containing 10% 5637 conditioned medium (CM-5637) (5637 is a bladder carcinoma cell line). Human recombinant TNFα was added to fresh medium containing CM-5637 every 2 days.

Cell line

The human leukemic cell line KG1 was purchased from American type culture collection (ATCC, Rockville, MD, USA), cultured in IMDM medium supplemented with 20% heat-inactivated fetal calf serum (FCS) (Invitrogen Corporation, Gibco, Cergy Pontoise, France) and incubated in a humidified incubator containing 5% CO2 at 37°C. The cells were split with fresh medium every 2 or 3 days and maintained at
4x10^5 cells/ml. Cell viability was assessed by trypan blue exclusion. Cells were treated with TNFα (20 ng/ml), human recombinant GM-CSF (R&D Systems, Oxon, UK) or cell-permeant exogenous ceramide, C6-ceramide (10 µM) (Sigma Aldrich, Saint Quentin fallavier, France).

**Immunophenotypic analysis**

Anti-CD34 and anti-CD45 antibodies (Beckman Coulter, France) were used to examine surface antigen expression according to the manufacturer’s recommendations. Analysis was performed with an EPICS XL-MCL (Beckman Coulter, Villepinte, France).

**Quantitative RT-PCR**

Total RNA were isolated by using Trizol Reagent (Invitrogen, Cergy Pontoise, France). Quantitative RT-PCR was performed using the LightCycler TeloTAGGhTERT quantification kit (Roche Diagnostics, Mannheim, Germany) as indicated by the manufacturer. Briefly, hTERT mRNA was reverse transcribed and amplified with specific primers in a one-step RT-PCR reaction. The amplicon was detected by fluorescence, using a specific pair of probes that hybridize to an internal sequence of the amplified fragment during the annealing phase of the amplification cycle. The emitted fluorescence was measured by the LightCycler instrument. In a separate one-step RT-PCR, mRNA encoding for porphobilinogen deaminase (PBGD) was used as a house-keeping gene. Results were expressed as the ratio between hTERT and PBGD transcripts normalized to untreated cells as previously described^1^.

**Telomerase activity**
Quantitative determination of telomerase activity was performed using the Telomeric Repeat Amplification Protocol (TRAP) with teloTAGGG telomerase PCR ELISA PLUS kit (Roche Diagnostics, Mannheim, Germany) following the manufacturer’s instructions as previously described. The relative telomerase activity (RTA) was determined as absorbance of the sample compared to absorbance of the control template.

**Western Blot**

Cells were harvested and lysed with Laemmli buffer, sonicated, and boiled 5 min at 95°C. Samples were separated on 12.5 % SDS-PAGE gels, transferred onto nitrocellulose membrane, and immunostained. The following primary antibodies were used: anti-ERK, anti-JNK (Santa Cruz, Le Perray en Yvelines, France), anti-phospho-MAPK, anti-phospho-JNK, anti-phospho-p38, anti-p38, anti-phospho-MAPKAPK-2, anti-MAPKAPK-2 (Cell Signaling, Ozyme, Saint Quentin en Yvelines, France) and horseradish peroxidase labelled anti-mouse and anti-rabbit antibodies. Proteins were visualized using the ECL detection system (Pierce, Rockford, IL, USA).

**Metabolic cell labeling and ceramide quantitation**

Total cellular ceramide quantitation was performed by labeling cells to isotopic equilibrium with 1 µCi/ml of [9, 10-³H] palmitic acid (Amersham Biosciences) for 48 h in complete medium as previously described. Cells were then washed and resuspended in complete medium for time-course experiments. Lipids were extracted and resolved by thin-layer chromatography. Ceramide was scraped and quantified by liquid scintillation spectrometry.
Statistics

Results are expressed as mean values +/- standard deviation. Statistical analysis of the datas were performed by the Student t test. Differences were considered as significant for p values < 0.05.

Results

Effect of TNFα on hTERT gene expression in CD34+ normal cells

In a first set of experiments, we tested if TNFα altered hTERT expression in normal bone marrow progenitors. Normal marrow CD34+ cells were cultured in medium containing various cytokines (SCF, IL3, FLT3-L and TPO) in the presence or not of TNFα for 14 days. According to a previous study, we used the dose of 20ng/ml 15. In control cells, this mixture of cytokines induced 5.4-fold and 32-fold expansion at 7 and 14 days, respectively. As expected from previous studies using a similar mixture of cytokines 15, we found that TNFα reduced cellular expansion compared to controls. However, this difference became significant only after 14 days of culture (Fig. 1A). We did not observe apoptosis in TNFα-treated cells (data not shown). Moreover, TNFα was found to facilitate differentiation as reflected by reduced CD34 expression compared to control cells after 7 days culture (Fig. 1B), whereas CD34 became undetectable after 14 days of culture in both TNFα-treated cells and control cells (data not shown). In parallel, as shown in Fig. 1C, treatment with TNFα resulted in a dramatic decrease in hTERT gene expression compared to untreated cells as revealed by real time quantitative PCR (93.8 +/- 3.4 %) followed by a time-dependent
reduction in hTERT activity which became almost undetectable after 10 days culture (Fig. 1D).

*These results show that TNF$\alpha$ inhibits hTERT gene expression in normal myeloid lineage.*
Figure 1: Influence of TNFα on normal bone marrow progenitors

Fresh CD34+ were cultured in medium containing various cytokines as described in Material and Methods in the presence or not of TNFα (20 ng/ml) for 7 to 14 days. (a) Viable cells were counted by trypan blue dye exclusion. Results represent the mean +/- s.d. of five independent experiments. ***p=0.0002. (b) Differentiation was analysed by flow cytometry using anti-CD34 monoclonal antibody at 7 days of culture. Results are representative of 3 independent experiments (c) Transcripts hTERT were quantified using the LightCycler Telomerase hTERT quantification kit as described in Materials and Methods. HTERT/PBGD represents the ratio between hTERT and PBGD transcripts normalized to untreated control cells, at 7 days of culture. They are expressed as the mean hTERT/PBGD values from three independent experiments +/- s.d. ****p=0.0001 (d) Telomerase activity was measured by using the Telomerase PCR ELISA PLUS kit as described in Materials and Methods. Results are expressed as mean RTA values +/- s.d. of three independent experiments. *p=0.014; **p=0.002 and 0.004.

Effect of TNFα on hTERT gene expression in leukemic cells

We next investigated whether TNFα could also regulate hTERT gene expression in myeloid leukemic cells. These experiments were performed with both KG1 cells 1 and in fresh AML cells cultured in CM-5637 for 7 days. They revealed that, as for normal myeloid cells, 7 days exposure to TNFα resulted in a dramatic reduction in hTERT gene expression in KG1 cells 1. However, in these cells, hTERT inhibition was detectable as soon as 2 hours with a maximum at 6 hours and remained stable over 7 days 1. Dose-effect analysis revealed a maximum reduction of hTERT gene at 20
ng/ml (data not shown). In initial experiments, we obtained similar results with fresh AML cells but experimental conditions based on 7-days exposure were often questionable due to loss of viability in control cells. For this reason, further experiments were conducted with freshly thawed AML cells or with cells immediately harvested from AML patients and incubated in short-term liquid culture (2-6 hours) in the presence of CM-5637 with or without TNFα. As depicted in Table 1, treatment with TNFα resulted in rapid hTERT gene reduction in virtually all cases, hTERT transcripts becoming undetectable in 2/10 cases. These results show that, as in myeloid normal cells, TNFα negatively influences hTERT expression in myeloid leukemic cells.

<table>
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<tr>
<th>Sample number</th>
<th>hTert/PBGD untreated cells</th>
<th>hTert/PBGD TNFα 2 hours</th>
<th>hTert/PBGD TNFα 6 hours</th>
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<tr>
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<td>100</td>
<td>ND</td>
<td>60</td>
</tr>
<tr>
<td>2</td>
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<td>Undetectable</td>
</tr>
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<td>73.1</td>
<td>ND</td>
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<td>KG1</td>
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<td>49.4+/− 9.8**</td>
<td>27.2 +/-2.1****</td>
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</table>

ND = not done

Table 1: Influence of TNFα on hTERT gene expression in AML cells

Fresh AML cells and KG1 leukemia cell line were treated or not with TNFα (20 ng/ml) for 2 to 6 hours. hTERT transcripts were quantified as described in Materials and Methods. Results are expressed as undetectable or as the ratio between hTERT and PBGD transcripts normalized to untreated cells. For KG1, results are the mean of ten independents experiments. ****p=0.00001, **p=0.006.
Role of MAPK in TNF-α-induced inhibition of hTERT in KG1 cells

In order to investigate signaling pathway regulating hTERT, we used KG1 cell line as a representative model of myeloid cells. Previous studies have described that TNF-α activates a complex network of signaling pathways among which MAPK may represent an important functional component through regulation of a wide variety of genes. Therefore, we hypothesized that ERK, JNK or p38MAPK might play an important role in mediating the inhibitory effect of TNF-α on hTERT gene regulation.

As a matter of fact, we found that, in KG1 cells, treatment with TNF-α induced a rapid increase (as soon as 15 minutes) in JNK and p38MAPK, but not ERK phosphorylation as revealed by immunoblotting with antibodies directed against phosphorylated forms of p44/p42ERK, p56/p44JNK, and p38MAPK (Fig. 2 A, B, C respectively). Moreover, we found that JNK activation was abrogated by pre-treatment with the JNK inhibitor SP600125 (20 µM) whereas the p38MAPK inhibitor SB203580 (1 µM) abrogated the activation of MAPKAPK-2, a substrate of p38MAPK (Fig. 2 B, D). Then, we investigated whether the JNK or p38MAPK pathway inhibition may interfere with TNF-α-induced hTERT gene inhibition. As shown in Table 2, we found that pre-treatment with the JNK inhibitor SP600125 (20 µM) abrogated the inhibitory effect of TNF-α on hTERT whereas pre-treatment with the p38MAPK inhibitor SB203580 (1 µM) had no effect.

These results suggest that JNK is specifically involved in TNF-α-mediated hTERT gene regulation.

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A
Figure 2: Role of MAPK in TNFα-induced inhibition of hTERT in KG1 cells

KG1 cells were treated or not with TNFα for 15 to 120 minutes after overnight serum deprivation. MAPK expression was evaluated by western blot analysis as described in Material and Methods. (a) Phospho-p42/44 ERK and p42/44 ERK expression. FCS was used as positive control. (b) Phospho-p46/54 JNK and p46/54 JNK expression. (c) Phospho-p38 and p38 expression. (d) Phospho-MAPKAPK-2 and MAPKAPK-2 expression.

<table>
<thead>
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<th>Cells</th>
<th>hTert/PBGD</th>
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<tr>
<td>KG1</td>
<td>100</td>
</tr>
<tr>
<td>KG1 + TNFα 2 hours</td>
<td>49.9 +/- 9.8**</td>
</tr>
<tr>
<td>KG1 + SP600125</td>
<td>108.1 +/- 18.1</td>
</tr>
<tr>
<td>KG1 + TNFα 2 hours + SP600125</td>
<td>98.5 +/- 23.1*</td>
</tr>
<tr>
<td>KG1 + SB203580</td>
<td>33.1 +/- 4.5</td>
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<tr>
<td>KG1 + TNFα 2 hours + SB203580</td>
<td>34.2 +/- 11.6</td>
</tr>
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</table>
Table 2: Influence of MAPK inhibitors on TNFα induced inhibition of hTERT in KG1 cells

KG1 cells were pretreated or not with SP600125 (20 µM) or SB203580 (1 µM) for 1 hour, then treated with TNFα (20 ng/ml) for 2 to 6 hours. hTERT transcripts were quantified as described in Materials and Methods. hTERT/PBGD represents the ratio between hTERT and PBGD transcripts normalized to untreated control cells. Results are expressed as mean hTERT/PBGD values +/- s.d from 3 independent experiments. *p=0.014; **p=0.006.

Role of ceramide in TNFα-induced inhibition of hTERT in KG1 cells

Previous studies have documented that, in myeloid leukemic cells, TNFα stimulates the JNK pathway by activating the sphingomyelin cycle. This pathway consists in the stimulation of a neutral Mg²⁺-dependent sphingomyelinase responsible for sphingomyelin hydrolysis and subsequent generation of ceramide that activates the JNK cascade through a redox-dependent mechanism. Moreover, independent studies have shown that, at least in non-hematopoietic cellular models, ceramide interferes with hTERT gene expression. For all these reasons, we speculated that ceramide could mediate the effect of TNFα on hTERT expression in KG1 cells. As shown in Fig. 3A, labeling studies revealed that, in KG1 cells, intracellular ceramide concentration increased upon TNFα stimulation with a maximum of 145 % at 3-5 minutes, and then returned to basal line. These results suggest that TNFα may indeed activate the sphingomyelin cycle in KG1 cells. In order to evaluate the functional consequence of endogenous ceramide release, we treated KG1 cells with cell-permeant exogenous ceramide (C6-ceramide) (10 µM) for 2 hours and hTERT transcripts were measured using real-time RQ-PCR. As shown in Fig. 3B, treatment with C6-ceramide resulted in a 40% decrease in hTERT PCR products. Ceramide-mediated hTERT inhibition was therefore in a same range of that observed with TNFα. The fact that C6-ceramide mimicked the effect of TNFα strongly suggests that
TNFα-induced ceramide production is responsible for hTERT regulation. Moreover, the effect of C6-ceramide was inhibited by the JNK inhibitor SP600125, suggesting that, like for TNFα, JNK mediated the effect of C6-ceramide on hTERT (Fig. 3B). These results suggest that ceramide plays an important role in mediating the effect of TNFα on hTERT through a JNK-dependent mechanism.

Figure 3: Role of ceramide in TNFα-induced activation of JNK
(a) KG1 cells were pre-labeled with [3H]-palmitic acid for 48 hours, washed, and treated with TNFα (20 ng/ml) at the indicated time. Intracellular levels of ceramide were analyzed as described in Materials and Methods. Results are expressed as the mean values from three independent experiments +/- s.d. *p<0.05. (b) KG1 cells were preincubated or not one hour with JNK inhibitor SP600125.
SP600125 (20 µM) then treated or not with C6-ceramide (10µM) for 2 hours. Transcripts hTERT were quantified using the LightCycler TeloTAGGGhTERT quantification kit as previously described. ****p=0.00001; *p=0.03.

**Influence of GM-CSF on TNFα effects**

In further experiments, we considered the possibility that GM-CSF could interfere with the inhibitory effect of TNFα on hTERT gene. This hypothesis was supported by the fact that GM-CSF has been found to negatively regulate stress-induced ceramide production. For this reason, KG1 cells were co-treated with TNFα (20 ng/ml) and GM-CSF (13 ng/ml), and ceramide production was investigated. These experiments revealed that GM-CSF inhibited TNFα-induced ceramide generation as illustrated in Fig. 4A. Moreover, GM-CSF inhibited the effect of both TNFα and ceramide on hTERT gene (Fig. 4B), suggesting that GM-CSF acted both upstream and downstream ceramide production.
Figure 4: Influence of GM-CSF on ceramide pathway
(a) Intracellular ceramide variations measured at the peak of stimulation in KG1 cells treated with TNFα (20 ng/ml) and GM-CSF (13 ng/ml). Results are expressed as mean +/- s.d. from 5 independent experiments. **p=0.008. (b) KG1 cells were incubated with or without TNFα, C6-ceramide (10 µM) or GM-CSF for 2 hours. Transcripts hTERT were quantified using the LightCycler TeloTAGGGhTERT quantification kit as previously described. ***p=0.0002; **p=0.004.

Discussion

This study shows that TNFα regulates telomerase in both normal and leukemia cells. This regulation involves ceramide and JNK and is inhibited by GM-CSF.

Concerning CD34⁺ normal progenitors, previous studies have demonstrated that telomerase activity is detectable at low levels in hematopoietic progenitors cells and is up-regulated in response to cytokine stimulation²¹,²². Our study suggests that, in hematopoietic cells, regulation of hTERT enzymatic activity is under control of hTERT gene as described in other cellular models, although we cannot rule out
complementary post-transcriptional mechanisms. Our study shows for the first time that, in vitro, TNFα inhibited hTERT gene and enzymatic activity in normal myeloid progenitors. Although the functional consequence of this regulation remains uncertain, it is possible that hTERT inhibition mediates the negative effect of TNFα in cellular expansion 23, through two hypothetical mechanisms. First, based on the protective function of hTERT in many tissues 24, it is conceivable that TNFα-induced hTERT inhibition results in an increase in cell loss along myeloid differentiation even if we found no increase in apoptosis. Second, it is possible that hTERT plays by itself an important function in differentiation commitment as recently suggested 25.

In order to investigate signaling pathway regulating hTERT, we used the KG1 cell line as a representative model of myeloid cells. We show that TNFα induces early and transient ceramide generation in KG1 cells. Others and we have documented that, in other AML cell lines such as U937 cells, TNFα induced ceramide generation due to stimulation of a neutral sphingomyelinase and subsequent sphingomyelin (SM) hydrolysis (SM cycle) 26, 27. The kinetics and the magnitude of ceramide production observed in KG1 cells are very similar to that of TNFα-treated U937 cells. These results suggest that TNFα activated the SM cycle in KG1 cells. Previous studies showed that ceramide inhibited hTERT in epithelial tumor cells 18, 19. Therefore, we hypothesized that, in KG1 cells, ceramide could mediate the effect of TNFα. Actually, cell-permeant ceramide inhibited hTERT gene expression in KG1 cells. This result suggests that the SM cycle is an important component of TNFα signaling leading to hTERT inhibition. If this is the case, it can be assumed that any negative regulators of this pathway, including increased protein kinase C activity or enhanced anti-oxidative defenses 28 may confer a significant protection of AML cells towards TNFα.
In additional studies, we investigated the effect of TNFα on MAPK. Indeed, previous studies have documented that TNFα may activate the three MAPK modules i.e., ERK/MAPK, JNK/SAPK, and p38MAPK in various AML cellular models. Moreover, p38MAPK has been recently implicated in cellular senescence. For this reason, we used specific pharmacological inhibitors to investigate the respective contribution of these pathways in TNFα-induced hTERT regulation. We found that JNK, but not ERK or p38MAPK, is involved in TNFα signaling leading to hTERT gene inhibition. This result contrasts with other studies, which showed that, at least in epithelial cells, JNK is an activator of hTERT gene suggesting that the role of JNK in hTERT regulation is cell-specific. Further studies are needed to identify the regulatory mechanisms, which operate downstream JNK and interfere with hTERT regulation in myeloid cells.

GM-CSF was found to protect cells from hTERT inhibition by TNFα. GM-CSF is not a unique growth factor capable to positively regulate hTERT. Indeed, it has been reported that IGF-1 or IL6 abrogated dexamethasone-induced down-regulation of hTERT activity in multiple myeloma cells, and that PI3K and NF-κB pathways mediated protective effects of IGF-1 and IL6. In KG1 cells, we found that GM-CSF inhibited ceramide generation. Moreover, this growth factor inhibits C6-ceramide inhibition of hTERT. These results suggest that GM-CSF exerts a dual mechanism that involves both abrogation of ceramide generation induced by TNFα and interruption of ceramide signaling.

In summary, our study shows that TNFα down-regulates the hTERT gene in normal and leukemic cells through ceramide-JNK pathway. Hematopoietic growth factors efficiently counter-regulate this signaling. Based on the role of hTERT in cellular protection, chromosome stability, and perhaps differentiation, the negative
effect of TNFα may have important functional consequences, including abnormal regulation of hematopoietic cell differentiation, genetic instability and marrow insufficiency.

References


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