Interferon α down-regulates telomerase reverse transcriptase and telomerase activity in human malignant and nonmalignant hematopoietic cells

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Recently, the derepressed expression of the catalytic subunit of telomerase, human telomerase reverse transcriptase (hTERT), the enzyme that elongates telomeres, has been implicated as an important step in the immortalization process. The exact regulation of hTERT expression, which is the rate-limiting factor for telomerase activity, is at present unclear. As transformed cells seem to be dependent on a constitutive telomerase activity, the availability of inhibitors would potentially be of great value in antineoplastic therapy. Interferons (IFNs) have been successfully used in the treatment of several forms of malignancies, but the underlying molecular mechanisms responsible for the antitumor activity are poorly defined. In this study we have investigated the effects of IFNs on hTERT expression and telomerase activity. We found that IFN-α rapidly (commonly within 4 hours) and significantly down-regulates the expression of hTERT and telomerase activity in a number of human malignant hematopoietic cell lines, primary leukemic cells from patients with acute leukemia as well as T-lymphocytes from healthy donors. This effect of IFN-α did not seem to depend on IFN-α–mediated cell growth arrest or alterations in c-myc expression. The finding that IFN induces a repression of hTERT and a decrease in telomerase activity suggests a novel mechanism that may play a significant role in the antitumor action of IFN. (Blood. 2000;96:4313-4318)

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Introduction

The acquisition of replicative immortality is a critical step in malignant transformation. One mechanism limiting the number of cell divisions in nontransformed cells is through the loss of telomeric sequences at each cell division, a process thought to eventually trigger the cellular senescence program.[1] The complete replication of telomeric sequences at the termini of eukaryotic chromosomes requires a special enzyme complex, telomerase, an RNA-dependent DNA polymerase,[1,2] which is not present in most somatic cells. The cloning and characterization of the catalytic subunit of the telomerase enzyme, human telomerase reverse transcriptase (hTERT), has allowed direct testing of the telomerase hypothesis, showing that derepression of hTERT is clearly implicated in immortalization.[3] Consistent with these observations, telomerase activity is found in most malignant cells,[3] and therefore finding substances that can inhibit telomerase activity could thus be of great therapeutic value in malignant disease.

Interferons (IFNs) are highly pleiotropic cytokines produced by eukaryotic cells when encountering viruses and other infectious agents and have potent antiviral, immunoregulatory, and antiproliferative properties.[4] IFNs have also been found to exert antitumor activity in a variety of malignant diseases.[5] Despite the demonstrated therapeutic effectiveness of IFNs, and a substantial progress in understanding their biochemical and biologic functions, elucidating molecular mechanisms underlying the antitumor action of IFNs has remained a critical challenge. This study was undertaken to explore the possibility of a role for IFNs in regulating the expression of hTERT and telomerase activity.

We demonstrate that type I IFNs trigger a rapid down-regulation of hTERT expression, followed by suppression of telomerase activity in susceptible immortal hematopoietic cell lines, primary leukemic cells, and normal T-lymphocytes. The data also indicate that this effect occurs through a direct effect of IFN on hTERT transcription, independently of IFN-induced cell cycle arrest. This effect suggests a novel mechanism for the antitumor action of IFN, and may provide a basis for future antitelomerase-based therapies, as well as providing a tool for better understanding of the regulation of telomerase.

Materials and methods

Cell lines, culture conditions, and IFN preparations

The cell lines used in this study were the Burkitt lymphoma cell lines Daudi and P3HR-1 (kindly provided by Dr K. Wiman, Karolinska Institute, Stockholm, Sweden), the myeloma cell line U266, and the H9 cell line derived from a cutaneous T-cell lymphoma.[6,7] All cells were grown in complete medium (RPMI 1640 medium [GIBCO, Berlin, Germany]) supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, 50 μg/mL penicillin, and 50 μg/mL streptomycin) in a humid atmosphere at 37°C. Exponentially growing cells were cultured in the presence and absence of IFNs for up to 96 hours. At harvest, 2×10^6 cells were centrifuged at +4°C, washed with ice-cold phosphate-buffered saline (PBS), and frozen as a dry cell pellet in liquid nitrogen.

To synchronize exponentially growing cells at the G1/S boundary, the

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cells were preincubated with aphidicholine (Sigma Chemical Co, Stockholm, Sweden) for 24 hours and then further cultured with the addition of aphidicholine alone or in combination with IFN-α.

To determine the role of de novo protein synthesis, cells were cultured in the absence or presence of 50 μg/mL of cycloheximide (CHX) (Sigma Chemical Co).

Recombinant human IFN-α$_2$b (Introna, Schering-Plough, Kenilworth, NJ), as well as recombinant IFN-β (Betaferon, Schering Nordiska, Stockholm, Sweden) and IFN-γ (Imukin, Boeringer Ingelheim, Stockholm, Sweden) were used for the in vitro experiments.

Primary leukemic cell separation and culture

Peripheral blood obtained from 7 newly diagnosed acute myeloid leukemia (AML) patients and 2 patients with T-cell acute lymphoblastic leukemia (T-ALL) was drawn into heparinized glass tubes. Leukemic cells were isolated by Lymphoprep gradient centrifugation (Nycomed, Oslo, Norway), where after the cells were washed in PBS and subsequently incubated in complete medium in the absence or presence of IFN-α$_2$b for up to 96 hours at a concentration of 10^6 cells per milliliter.

Normal T-lymphocyte preparation and culture

Mononuclear cells from heparinized buffy coats from healthy blood donors were isolated by Lymphoprep gradient centrifugation and T-lymphocytes isolated using nylon wool columns, were stimulated, and were cultured as previously described.[8]

RNA extraction and reverse transcription

Total RNA was extracted using the Ultraspec-II RNA kit (Biotecx Laboratories, Houston, TX). RNA yield and purity were determined spectrophotometrically at 260 to 280 nm, and the integrity of RNA verified by electrophoretic size separation in 1% agarose gels stained with ethidium bromide (EB). Complementary DNA (cDNA) was synthesized using random primers (N6) (Pharmacia, Uppsala, Sweden) and MMLV reverse transcriptase as described.[9]

Quantitative determination of hTERT messenger RNA expression by competitive reverse transcriptase-polymerase chain reaction

The construction of the hTERT competitive template and the quantification of hTERT messenger RNA (mRNA) levels were described elsewhere.[9] Briefly, cDNA corresponding to 50 ng of RNA was coamplified with 5000 competitive molecules using 32 cycles for cell lines and with 1000 competitive molecules using 34 cycles for primary cells, respectively. Polymerase chain reaction(PCR) products were resolved in 4% Metaphor agarose gels stained with EB, visualized in ultraviolet light, and photographed. Volumetric integration of signal intensities was performed by using NIH Image software (Version 1.58; http://rsb.info.nih.gov/nih-image). The relative levels of hTERT expression were calculated from the ratio of hTERT and competitor signal density normalized to the loaded amount of total RNA.

Telomerase activity assay

Protein extraction and measurement was performed as described.[9] A commercial telomerase PCR enzyme-linked immunosorbent assay (ELISA) kit (Roche, Scandinavia AB, Stockholm, Sweden), based on the telomeric repeats amplification protocol (TRAP) was used to determine telomerase activity in all samples according to the manufacturer's protocol. In each assay, 0.5 μg of protein was added and subject to PCR amplification with 20 cycles for cell lines and 28 cycles for primary human cells.

Determination of cell cycle distribution by flow cytometry and determination of ³H-thymidine incorporation

DNA histograms, fixation of cells, DNA labeling, and measurements of cellular protein content were performed and analyzed as previously described.[7] For determination of ³H-thymidine incorporation, 2×10^5 cells (in triplicate) were cultured in U-shaped 96-well microtiter plates in the presence or absence of 1 μmol/L of aphidicholine. One hour before harvesting, 0.037 MBq (1.0 μCi) of ³H-thymidine (Amesrsham, Uppsala, Sweden) was added to each well. Harvesting was performed with a Tomtec harvesting machine (Wallach Sverige, Upplands Vasby, Sweden), and the radioactivity was measured in a 1450 Microbeta Trilux (Wallach).

Northern blotting

Preparation of total RNA and Northern blotting was performed as previously described.[8] The filters were hybridized with a 800-base pairs (bp) c-myc cDNA probe (kindly provided by Dr M. Henriksson, Karolinska Institute, Stockholm, Sweden).

Results

Down-regulation of hTERT expression and telomerase activity in malignant lymphoid cell lines

We assessed the effect of IFNs on basal hTERT mRNA levels and telomerase activity in a panel of 4 susceptible malignant hematopoetic cell lines. These cell lines have previously been investigated for the integrity of the IFN signal transduction pathway, demonstrating that they are all highly sensitive to IFN-α as measured by the induction of the IFN-stimulated gene 2′ 5′ oligoadenylate synthetase (25 AS). An 11- to 40-fold induction of 25 AS activity was detected in the cell lines after incubation with 5000 U/mL of IFN-α for 24 hours.[6]

All 4 cell lines under study exhibit high levels of hTERT mRNA and telomerase activity as determined by competitive RT-PCR and TRAP assay, respectively. After the exposure of all of these cell lines to 5000 U/mL IFN-α, a rapid decline in hTERT mRNA level was observed by 4 hours. Daudi and P3HR-1 cells exhibited less than 10% to 15% of the original amount of hTERT mRNA after 24 to 96 hours of treatment, whereas in H9 and U266 cells, there was an equally rapid decrease at early time points, after which the levels reached a plateau of about 40% to 50% of the levels in the untreated cells (Figure 1A). U266 and H9 cells, in contrast to Daudi and P3HR-1 cells, are sensitive to IFN-induced apoptosis,[6] and our experiments were therefore terminated at 48 hours in these cell lines, the time at which many cells displayed signs of apoptosis. The effect of IFN-α on hTERT down-regulation in Daudi and U266 cells was dose-dependent from 5 to 500 U/mL (Figure 1B and data not shown). As expected, a suppression of telomerase activity followed the decrease in hTERT mRNA expression, but occurred after a lag period (Figure 1A), presumably because of the long half-life of the hTERT protein.[10] To evaluate whether the IFN-induced reduction of hTERT levels and telomerase activity is a reversible event, Daudi cells were incubated with IFN-α for 48 hours and subsequently washed and incubated in fresh medium in the presence or absence of IFN-α. We found that the repression of hTERT and telomerase activity was maintained throughout the 7-day culture period in the presence of IFN-α, whereas withdrawal of IFN resulted in the reappearance of hTERT and telomerase activity within 24 hours (data not shown). This also indicates that the effect of IFN-α on hTERT is not secondary to the possible effects of IFN on irreversible cellular programs such as differentiation.

To evaluate the impact of other IFN species, Daudi cells were exposed to IFN-β and γ. An effect similar to that observed with IFN-α treatment, with respect to hTERT levels and telomerase activity, was caused by IFN-β, whereas, in contrast, IFN-γ
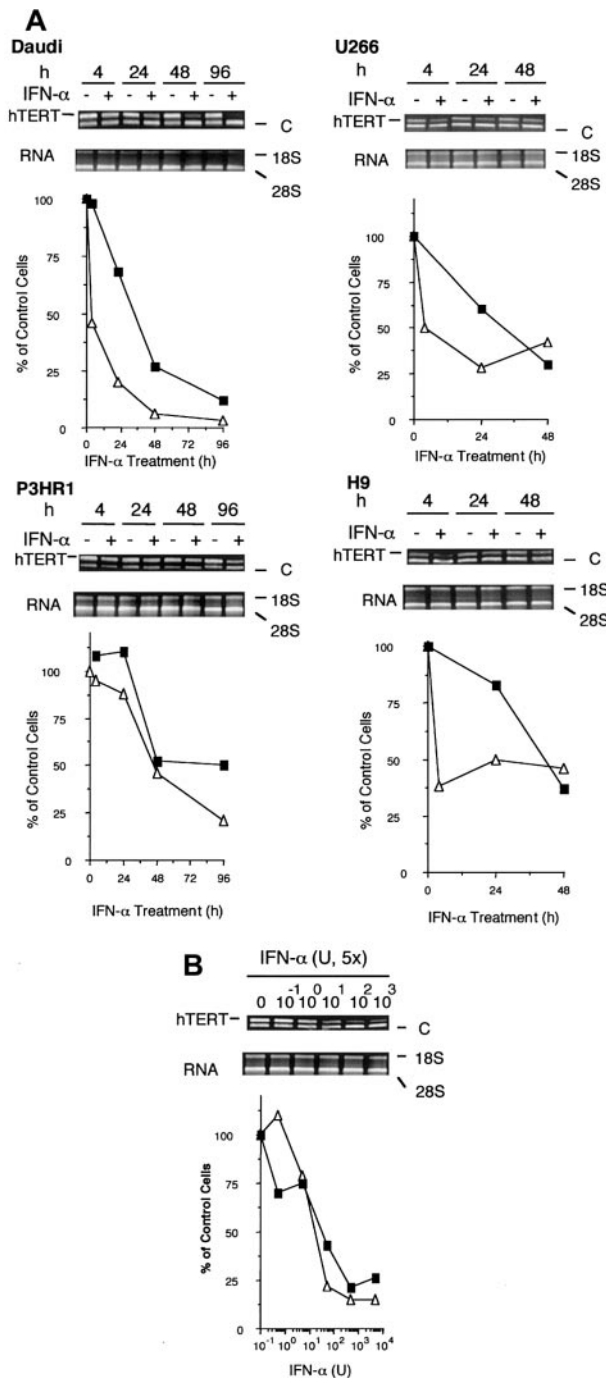
Figure 1. Effects of IFN-α on hTERT levels and telomerase activity in Daudi, P3HR-1, U266 and H9 cells. (A) Exponentially growing cells were cultured in the presence or absence of IFN-α (5000 U/mL) for the indicated times and hTERT levels (△) and telomerase activity (■) were determined using the competitive RT-PCR and TRAP assays respectively, as described in "Materials, and methods." C denotes the competitor band. Data shown are representative of 3 independent experiments. (B) Influence of various concentrations of IFN-α on hTERT levels (△) and telomerase activity (■) in Daudi cells. Exponentially growing cells were cultured in the presence or absence of IFN-α for 48 hours and hTERT levels and telomerase activity were determined.

treatment transiently up-regulated the levels of hTERT mRNA and telomerase activity in Daudi cells, as well as in P3HR-1 cells (data not shown). Although type I and type II IFNs have many similar effects on the cellular and molecular level, there are also a number of differences; they use different receptors and partly different signaling molecules, which is also reflected in differential transcrip-

tional regulation, resulting in overlapping but often distinct cellular effects.[4] Furthermore, few malignancies have been shown to respond favorably to IFN-γ treatment.[5]

IFN-induced changes in telomerase activity occur independently of cell cycle effects

IFN-α is a well-known inhibitor of cellular proliferation in susceptible cells. Because some previous studies have shown that the levels of telomerase activity may be proliferation-related,[11] we wanted to investigate whether the effects on hTERT mRNA expression may be dependent on an IFN-α–induced G1 arrest. We have previously shown that IFN-α inhibits proliferation in Daudi, P3HR-1, and U-266 cells by arresting them in G1-phase of the cell cycle.[7,12] H9 cells are, on the other hand, completely resistant to this effect of IFN-α.[6,12] To further demonstrate whether the effect of IFN-α on hTERT levels may occur independently of IFN-induced cell cycle arrest, Daudi cells were pretreated for 24 hours with aphidicholine, which is known to arrest cells in early S-phase because of the efficient blockage of DNA-pol-α. Treatment of Daudi cells with aphidicholine for 24 hours resulted in a near complete cell cycle arrest in early S-phase, as shown by a greater than 90% reduction in ³H-thymidine incorporation, as well as by the analysis of cellular DNA content by flow cytometry (data not shown). The cell cycle blockage caused by aphidicholine treatment alone for 24 to 72 hours did not result in any change in telomerase levels. However, pretreatment for 24 hours with aphidicholine before the addition of IFN-α did not abrogate the IFN-α–induced down-regulation of hTERT mRNA levels (Figure 2A). This indicates that cell cycle arrest in early S-phase does not in itself lead to telomerase down-regulation, and that IFN-α–induced G1 arrest is not a prerequisite for its ability to regulate hTERT levels.

The effects of interferon on hTERT levels are independent of de novo protein synthesis

IFNs bind to specific cellular surface receptors and induce/repress transcription of specific genes. IFN receptors lack intrinsic kinase activity but rely on associated Janus family kinases, in turn activating different Stat molecules. Stimulation with IFN-α leads to phosphorylation of Stat1 and Stat2, which form an oligomeric complex called ISGF3. Activated (phosphorylated) Stats translocate to the nucleus and induce transcription of IFN-stimulated genes (ISGs) through their binding to so-called IFN-stimulated responsive elements (ISREs), thereby eliciting a number of biologic effects.[4] To show whether IFN-α–induced down-regulation of hTERT levels is a consequence of direct signaling, or is secondary to other IFN-induced phenotypic changes, we investigated whether the repression of hTERT expression was dependent on de novo protein synthesis. This was carried out by pretreatment of U266 cells with CHX and subsequent incubation with IFN. We found that CHX treatment by itself caused a significant induction of hTERT mRNA levels (Figure 2B). However, addition of IFN-α to CHX-treated cells caused a significant reduction of hTERT mRNA levels. Although not entirely conclusive due to the prominent effect of CHX alone, the relative reduction in hTERT mRNA levels by IFN was comparable between CHX-treated and CHX-untreated cells (Figure 2B). These data strongly indicate that the hTERT gene is a direct transcriptional target for the IFN-α signaling pathway.
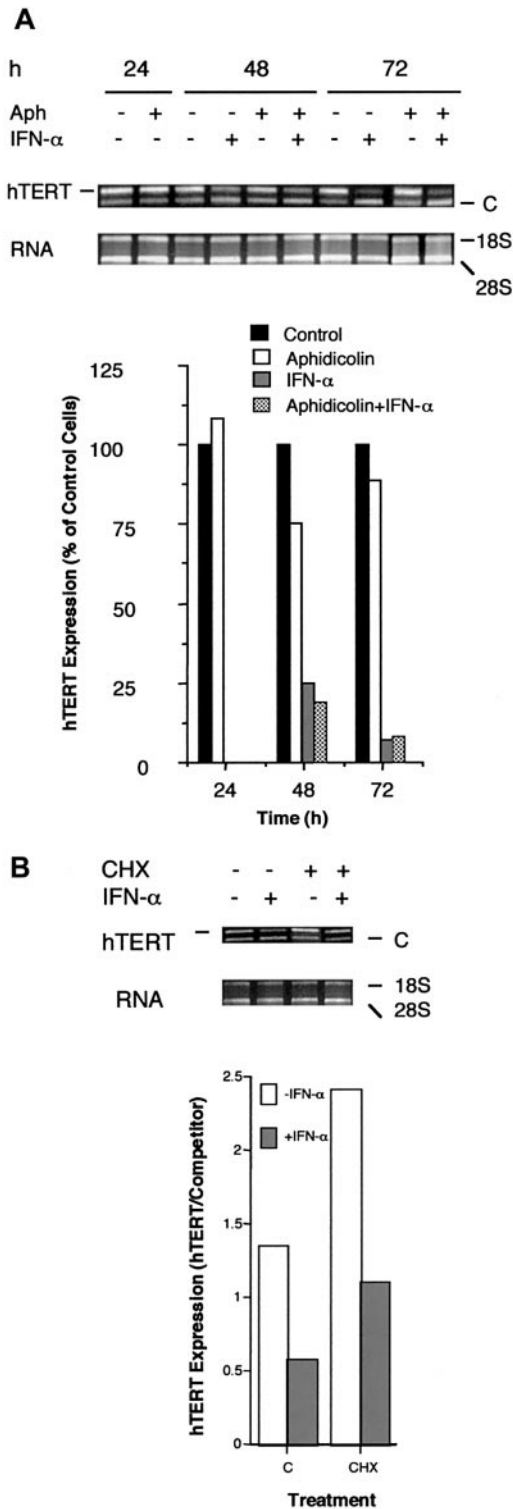
Effects of interferon on telomerase in primary leukemic cells and T lymphocytes from healthy donors

As primary malignant cells generally demonstrate derepressed hTERT expression,[13] we decided to investigate whether IFN-α can exert a similar effect on telomerase levels and activity in primary leukemia cells. Leukemic cells from 2 patients with acute lympho-cytic leukemia (ALL) and 7 patients with acute myeloid leukemia (AML) were treated with IFN-α at 5000 U/mL. In the leukemic cells from both ALL patients, IFN-α caused a 70% to 99% decrease in steady state hTERT levels (Figure 3A and data not shown). In primary AML cells, IFN-α caused a significant decrease in hTERT levels (67%-99%) and telomerase activity in 2 of the cases, whereas no effect was observed in 2 additional patients (Figure 3A
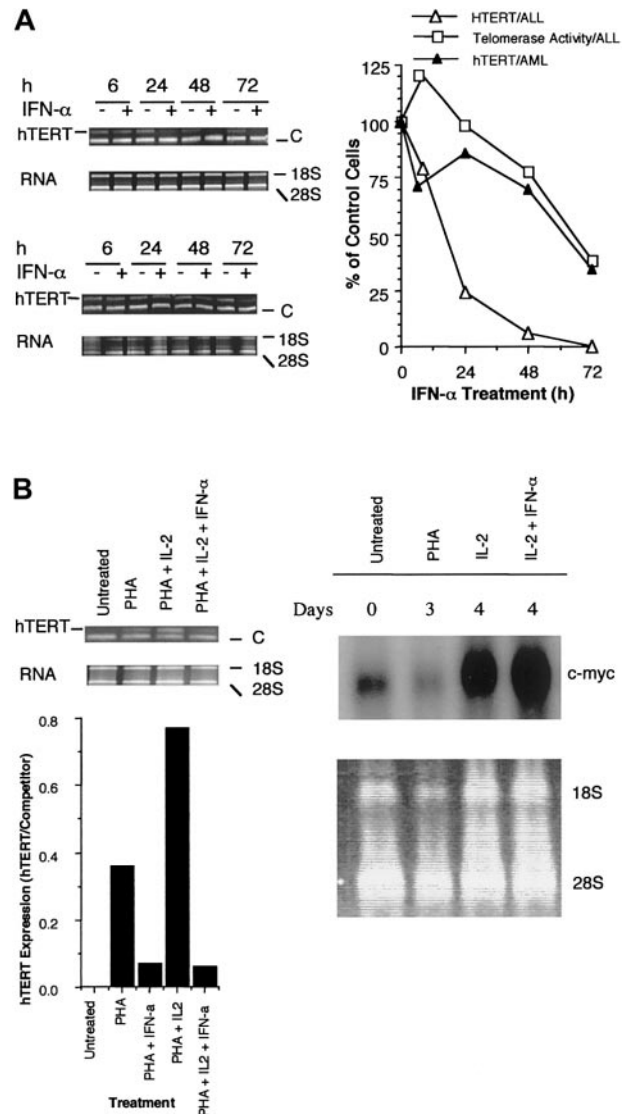

Figure 2. Effects of aphidicholine and CHX on IFN-α–mediated down-regulation of hTERT levels. (A) Exponentially growing Daudi cells were cultured in the presence or absence of aphidicholine (1 μmol/L) for 24 hours, and the cells were then further treated with either aphidicholine alone or aphidicholine plus IFN-α for an additional 24 and 48 hours. (B) Exponentially growing U266 cells were cultured in the presence or absence of CHX (50 μg/mL) for 15 minutes, after which the cells were treated with either CHX alone or CHX plus IFN-α for an additional 4 hours. Data shown are representative of 3 independent experiments. C denotes the competitor band.

Figure 3. Effects of IFN on telomerase in primary leukemic blasts and telomerase and c-myc in normal T-lymphocytes. (A)Effects of IFN-α on hTERT levels and telomerase activity in leukemic blast from patients with ALL (left, upper panel) and AML (left, lower panel). Leukemic blasts were separated using lym-phoprep separation, cultured in the presence or absence of IFN-α (5000 U/mL) for the indicated times, where after hTERT levels and telomerase activity were determined. (B) Influence of IFN-α on hTERT levels and telomerase activity and c-myc expression in T-lymphocytes during stimulation with PHA and IL-2. c-myc expression was analyzed by Northern blotting in quiescent (day 0), PHA (day 3), PHA/IFN-α (day 3), IL-2 (24 hours), and IL-2/IFN-α (24 hours)–treated cells. 18S and 28S rRNA serves as a control for equal loading. Data shown are representative of 3 independent experiments. C denotes the competitor band.

and data not shown). In leukemic cells from the 3 remaining AML patients, data were not evaluable because hTERT mRNA levels in these samples were barely detectable both in the absence and presence of IFN. The cell cycle status of the leukemic cells in the absence and presence of IFN-α was also investigated using flow cytometry. We found that the absolute majority of cells (more than 95%) in all patients at all time points were located in the G1 phase of the cell cycle, irrespective of treatment with IFN-α. This further strengthens the notion that the effect of IFN-α on telomerase levels is independent of the ability of IFN to induce cell cycle arrest.

Although most human somatic cells lack telomerase activity, some exceptions exist, such as stem cells and activated T-lymphocytes. To analyze whether IFN-α is able to regulate hTERT expression also in untransformed cells, we investigated the effects of IFN-α on hTERT levels during T-cell activation. As found by others,[14] stimulation of T-lymphocytes from normal donors with PHA leads to the appearance of a low level of hTERT mRNA. We have found that further stimulation of these cells with interleukin 2 (IL-2) leads to an additional up-regulation of the hTERT transcript (Figure 3B). In line with the effects of IFN on malignant cell lines and primary leukemia cells, IFN-α was found to significantly inhibit both PHA and IL-2–induced up-regulation of hTERT expression (Figure 3B).

It has recently been suggested that the c-myc protein may play an important role in telomerase regulation. For example, inhibition of c-myc expression leads to suppression of telomerase activity in HL60 cells,[15] whereas c-myc overexpression induces hTERT mRNA and activates telomerase in normal human fibroblasts lacking telomerase activity.[16] The recently cloned hTERT promoter has been found to contain binding sites for c-myc,[17] compatible with a direct induction of hTERT expression by c-myc. As IFNs have been shown to decrease c-myc levels under some circumstances, this could be one plausible mechanism for the IFN-induced reduction in hTERT levels. Indeed, IFN-α rapidly reduces c-myc mRNA levels in Daudi cells.[6] Even so, c-myc is unlikely to be the only downstream player that modulates hTERT expression in IFN-α–treated cells. First, despite that IFN-α has no effect on myc expression in U266 and H9 cells,[6,12] a clear inhibition of hTERT expression still occurred in these cells following their exposure to IFN-α. Furthermore, an equal increase in c-myc expression was observed between PHA/IL-2 and PHA/IL-2 plus IFN-α–treated T-lymphocytes (Figure 3C), in contrast to the effects of IFN-α on the PHA- and IL-2–induced up-regulation of hTERT mRNA levels, which were almost completely abrogated by IFN. We noted that the down-regulation of hTERT mRNA in U266 and H9 cells was only partial in comparison to the near complete disappearance of hTERT levels in Daudi and P3HR-1 cells. Whether such a difference reflects different capacities of IFN-α to regulate myc expression in these cells remains to be determined. Another possibility could be that the ability of IFN to induce apoptosis in H9 and U266 cells may keep telomerase levels up, as the induction of apoptosis has recently been suggested to increase telomerase levels.[18]

Discussion

The activation of telomerase activity seems to be one crucial step for cells to acquire indefinite proliferative potential and to transform, a notion that is supported by the finding of telomerase activity in a variety of immortal tumor cell lines and primary malignant tissues. IFNs have, in a large number of studies, been found to exert an antitumor effect in certain malignancies. The exact mechanism responsible for the clinically beneficial effects of

IFN treatment are not known, but IFN-α–mediated hTERT/telomerase suppression may play a significant role in its antitumor action. It is conceivable that IFN-α, by inhibiting hTERT/telomerase expression, would abrogate the telomere compensation mechanism in malignant cells, which may resume the mitotic clock function of telomeres, and eventually direct the cells to replicative senescence or crisis. This may explain the need for the prolonged treatment with IFN commonly required for a detectable response to occur, as observed in several malignancies.[5] It would thus be of major interest to investigate whether IFN-α treatment may have an impact on tumor telomere length and senescence in vivo, and whether such an effect correlates with the clinical outcome of IFN therapy.

In several systems, telomerase activity is clearly linked to proliferation.[11] As IFN-α is one of the few antiproliferative cytokines defined to date, one possibility could be that the effects of IFN on telomerase levels could be secondary to IFN-induced cell growth inhibition. Several pieces of data, however, argue against this notion. First, blocking of cells at the G1/S boundary by aphidicholine did not abrogate the IFN-induced reduction of hTERT levels. Furthermore, one of the cell lines under study, H9, is not cell cycle arrested by IFN,[12] but still shows a clear repression of hTERT expression. Finally, cells with a very small S-phase fraction (less than 5%), such as primary leukemic cells, still down-regulated the telomerase activity in response to IFN-α.

The mechanism by which IFNs exert this activity is not clarified in the present study. However, our data gives some indications on where to focus further studies. The fact that the IFN induced repression of hTERT levels is independent of de novo proteins synthesis, strongly indicates that IFN signaling may directly repress the hTERT promoter. To date little is known about how hTERT transcription is regulated. Searching with the published hTERT promoter sequence using the MatInspector V2.2 software [http://transfac.gbf-braunschweig.de/cgi-bin/matSearch/mat-search.pl0] reveals no classical ISRE sites. It is thus possible that transcription factor complexes other than ISGF3 may mediate this effect of IFN-α, and studies are ongoing, using hTERT promoter/reporter constructs, to identify such putative transcription factors. One factor that has been shown to positively regulate hTERT levels in other studies is the c-myc protein, through its binding to E-boxes in the hTERT promoter. IFN can strongly reduce c-myc levels in some systems. However, our data indicate that there are other factors acting as downstream players modulating hTERT expression in IFN-α–treated cells. In several instances we observe IFN-α–induced reduction in hTERT levels in the absence of c-myc down-regulation as in the case of the cell lines U266, H9, and in normal T-lymphocytes.

The unique effect of IFN-α on telomerase may reflect a novel and important mechanism by which this cytokine can exert its antitumor action, and may also help to clarify the molecular regulation of hTERT levels. The present finding that IFN-α potently down-regulates hTERT/telomerase expression not only in immortal cell lines but also in primary leukemia cells may thus have important clinical implications.

Acknowledgments

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