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Biochemical and Biophysical Research Communications 305 (2003) 150–154
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Telomerase upregulates expression levels of interleukin (IL)-1α, IL-1β, IL-6, IL-8, and granulocyte–macrophage colony-stimulating factor in normal human fibroblasts

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Received 4 April 2003

Abstract

Expression of human telomerase reverse transcriptase (hTERT) in normal human fibroblast cell strain, TIG-3, extends their replicative life span. We found that expression levels of interleukin (IL)-1α, IL-1β, IL-6, IL-8, and granulocyte–macrophage colony-stimulating factor (GM-CSF) mRNA were up-regulated in hTERT-induced fibroblasts irrespective of population doubling level (PDL). Expression levels of these cytokines were low in growing young TIG-3 cells and in control vector-transfected TIG-3 cells but were up-regulated in growth-arrested young cells maintained at high cell density. In senescent TIG-3 cells, expression of IL-1β, IL-6, and GM-CSF was moderately increased. These results indicate that the introduction of hTERT into normal fibroblasts up-regulates the expression of some inflammatory cytokines, and caution should be paid when introducing the hTERT gene to establish cell lines with normal phenotype.

Keywords: Telomerase; IL-1α; IL-1β; IL-6; IL-8; GM-CSF; Fibroblast

Telomeres are specialized structures at the end of eukaryotic chromosomes that are involved in chromosome stability, positioning, replication, and meiosis [1]. Normal human diploid fibroblasts have a limited proliferative lifespan [2] and telomere length has been proposed as a counting mechanism for the number of cell divisions (mitotic clock) that controls cellular senescence [3–5]. A causal relation between telomere shortening and cellular senescence has been established by showing that the ectopic expression of human telomerase reverse transcriptase (hTERT) gene in human mortal somatic cells results in telomere length elongation and extension of the in vitro replicative life span [6–8]. It may be reasonable to expect (1) that the expression of genes that halt the cell cycle such as p16 and p21 increases, while expression of genes that promote the cell cycle such as *c-fos* and cyclin CDK decrease according to the progression of in vitro replicative senescence [9–13], and (2) that the changes in expression of these genes are reversed by the introduction of the hTERT gene followed by an extended proliferative lifespan. We recently reported that the down-regulation of keratinocyte growth factor and insulin-like growth factor-II expression with cellular senescence was restored by telomere elongation after introduction of hTERT cDNA [14]. In addition to telomere-dependent replicative senescence, several types of apparently unrelated stimuli are reported to induce cellular phenotypes that are indistinguishable from those observed in cells undergoing replicative senescence. These stimuli include oxidative stress [15,16], DNA damage [17], so-called culture shock [18], and forced expression of activated Ras oncogene in NHF cells [19]. Cellular senescence is now understood as a cellular response against stresses, including telomere shortening. The increased expression levels of other

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doi:10.1016/S0006-291X(03)00717-4

genes, including such inflammatory cytokines as interleukin (IL)-1 and IL-6, have also been reported [20–23], although the biological significance of these changes in cytokines is unclear during cellular senescence. If the increased expression of inflammatory cytokines were a kind of cellular response against stresses that lead to telomere shortening and replicative senescence, these consequences may be reduced by telomere elongation following introduction of hTERT. We report here that the expression of IL-1β, IL-6, and granulocyte-macrophage colony-stimulating factor (GM-CSF) was increased with cellular senescence and, unexpectedly, the expression of IL-1α, IL-1β, IL-6, IL-8, and GM-CSF was highly increased by the introduction of hTERT cDNA.

Materials and methods

Cell culture. A normal human fetal fibroblast strain, TIG-3, was obtained from the Japanese Cancer Research Resources Bank (JCRB) [24]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Nissui, Tokyo) supplemented with 10% fetal calf serum (FCS) (Invitrogen, Carlsbad, CA), glucose (3.5 mg/ml), penicillin (100 U/ml) and streptomycin sulfate (100 μg/ml). Cells were cultured at 37 °C in a 5% CO_2 humidified atmosphere. To obtain growth-arrested cells, TIG-3 cells at 37 population doubling level (PDL) were maintained in the confluent state for 10 days, and the medium was changed every other day.

Transfection of hTERT and cloning of cells. Transfection of hTERT and cloning of cells were done as reported previously [14]. Here we used the hTERT-introduced clones TT1 and TT2 in addition to the previously isolated clone Tel-5. N5 and N25 were used as control clones, ones that were infected with empty vector as reported [14].

Southern blot analysis, TRAP assay and RT-PCR. Southern blot analysis, TRAP assay and RT-PCR were done as reported previously [14]. RT-PCR analysis was performed using 0.1 μg of total RNA, 1 U of *rTth Taq* polymerase and reaction buffer (Perkin–Elmer, Branchburg, NJ) in a final volume of 20 μl. Each PCR cycle included 30 s of denaturation at 94 °C, and 1 min of primer annealing and extension/synthesis. The specific primer sequence pairs, annealing temperature, number of PCR cycles, and predicted size of PCR products are shown in Table 1. All primer sequence pairs were derived from separate exons of the gene sequence, thus spanning one or more introns so that the DNA products from genomic DNA could be distinguished from those of reverse-transcribed mRNA. The number of PCR cycles was selected to yield linear signal intensity. The amplified products were electrophoresed on a 2% agarose gel, visualized by staining with SYBRR Gold (Molecular probes, Eugene, OR) staining, and detected by a Bio Imaging Analyzer (BAS-2000, Fuji).

Results and discussion

TIG-3, a human normal fetal fibroblast cell strain, has a limited proliferative lifespan of around 70–80 PDL. The expression of cytokines by this cell line was examined using RT-PCR within a linear range. The expression level of IL-1α, IL-1β, IL-6, IL-8, and GM-CSF was found to increase by growth-arrest of young

Table 1
PCR primer sequences

	Sequence (5′–3′)	RT temperature (°C)	Annealing temperature (°C)	Cycle	PCR product size (bp)
Interleukins					
IL-1α	GTC TCT GAA TCA GAA ATC CTT CTA TCA CAT GTC AAA TTT CAC TGC TTC ATC	62	62	28	421
IL-1β	AAA CAG ATG AAG TGC TCC TTC CA GAG AAC ACC ACT TGT TGC TCC A	60	65	24	389
IL-3	GTC CTG CTC CTG CTC CAA CTC AAG ATC GCG AGG CTC AAA GTC	55	58	30	440
IL-6	CCA GCT ATG AAC TCC TTC TCC A GTG GGG CGG CTA CAT CTT T	60	64	20	133
IL-7	TGT TCC ATG TTT CTT TTA GGT ATA TCT T TTC TCA AAT GCC CTA ATC CGT T	55	55	30	675
IL-8	ATG ACT TCC AAG CTG GCC GTG GCT TCT CAG CCC TCT TCA AAA ACT TCT	55	60	23	292
Others					
G-CSF	TGG CAC AGT GCA CTC TGG ACA GTG GAG GAA AAG GCC GCT ATG GAG TTG	64	64	30	294
GM-CSF	TGT GAA TGC CAT CCA GGA GG TGG CTC CCA GCA GTC AAA GG	64	64	26	328
M-CSF	CAG GAA CAG TTG AAA GAT CCA CTC ATA GAA AGT TCG GAC GC	60	58	30	207
Housekeeping gene					
GAPDH	CGT ATT GGG CGC CTG GTC AC ACG TAC TCA GCG CCA GCA TCG	65	65	15	248

(37 PDL) cells as compared with those observed in growing cells at 32 PDL and 50 PDL (Fig. 1A). When the cells were plated by 1:2 split ratio at 72 PDL, they did not form confluent monolayer even after 2 weeks, and we defined the cell population as senescent cells. When the cells reached the senescent state, the expression level of IL-1β, IL-6, and GM-CSF was increased, while the extent of increase was smaller than that observed in growth-arrested young cells. The expression level of IL-1α and IL-8 in senescent cells was the same as that of young growing cells. We could not explain why the expression of these cytokines in senescent cells was not increased to the level observed in growth-arrested young cells at 37 PDL. Although the growth-arrested cell populations of young and senescent cells contained different numbers of proliferating cells, this may not be the reason for the difference in expression in these cytokines because the proliferating cell fraction determined by BrdU incorporation for 24 h was very small in both growth-arrested young cells (1%) and senescent cells (3–5%). Maintenance of normal fibroblasts at very high density, rather than the resulting growth-arrest, may be a stressful condition that may lead to induction of cytokines.

Whatever the mechanism was for the increase in expression level of these cytokines in growth-arrested young and senescent cells, we expected that the expression level of these cytokines in cells transfected with hTERT would be the same as for young growing cells. Human TERT-transfected TIG-3 clones (Tel-5, TT1, and TT2) expressed hTERT mRNA and telomerase activity (Fig. 2), possessed unshortened telomeres (Fig. 3) and continued proliferation over 200 PDL, whereas control vector-transfected clones (N5 and N25) did not express hTERT mRNA, did not show telomerase activity (Fig. 2) with short telomeres (Fig. 3) and ceased proliferation around 72–73 PDL similar to the original TIG-3 cells. The expression levels of IL-1α, IL-1β, IL-6, IL-8, and GM-CSF were all high in growing hTERT-transfected cells, similar to growth-arrested cells at 37 PDL (Fig. 1). The expression level did not change with passage (Fig. 1B). The expression level of these cytokines was low in control vector-transfected cells, similar to young growing cells. Not all cytokines were expressed at high levels in hTERT-transfected cells. For example,

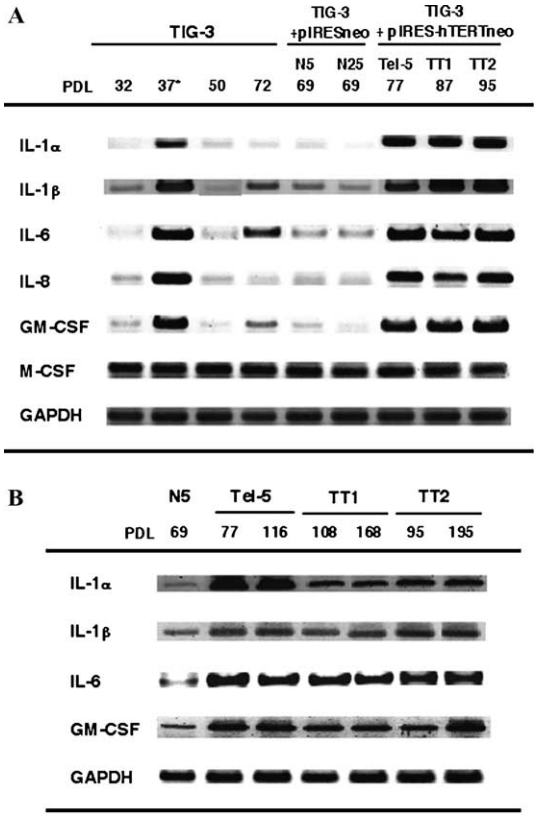

Fig. 1. Expression of cytokines and GM-CSF mRNA in TIG-3 and hTERT-transfected TIG-3 cells. The expression level of mRNA was determined by RT-PCR. (A) TIG-3 cells are normal human fetal fibroblasts. N5 and N25 are clones of TIG-3 cells transfected with a control vector (pIRESneo). Tel-5, TT1, and TT2 are three independent clones of TIG-3 transfected with hTERT expression vector. PDL: population doubling level. The asterisk indicates the growth-arrested state due to contact inhibition. GAPDH: glyceraldehyde-3-phosphate dehydrogenase. (B) Expression of cytokines and growth factor in hTERT-transfected clones at different PDLs.

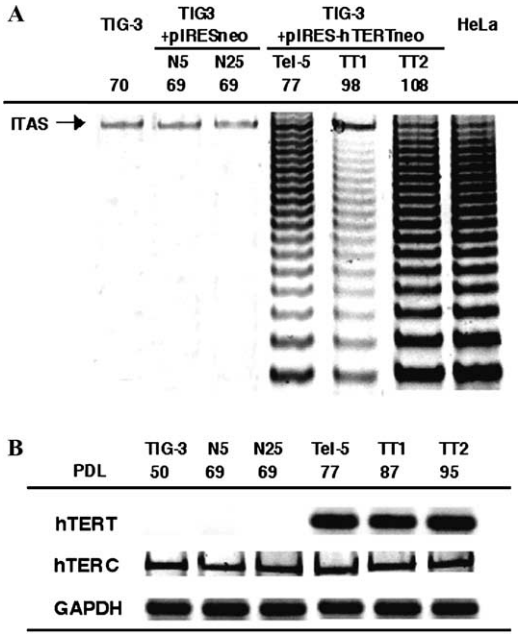

Fig. 2. Telomerase activity and hTERT mRNA expression in hTERT-transfected normal human fibroblasts. (A) Telomerase activity was measured by TRAP assay. TIG-3 cells are parental human fibroblasts. N5 and N25 are clones of TIG-3 cells transfected with a control vector (pIRESneo). Tel-5, TT1, and TT2 are clones of TIG-3 cells transfected with hTERT expression vector (pIRES-hTERTneo). HeLa is a telomerase-positive cervical cancer cell line used as a control. ITAS: internal telomerase assay standard. (B) Expression of hTERT and hTERC mRNAs by RT-PCR in TIG-3, control vector-transfected and hTERT-transfected TIG-3 cells.

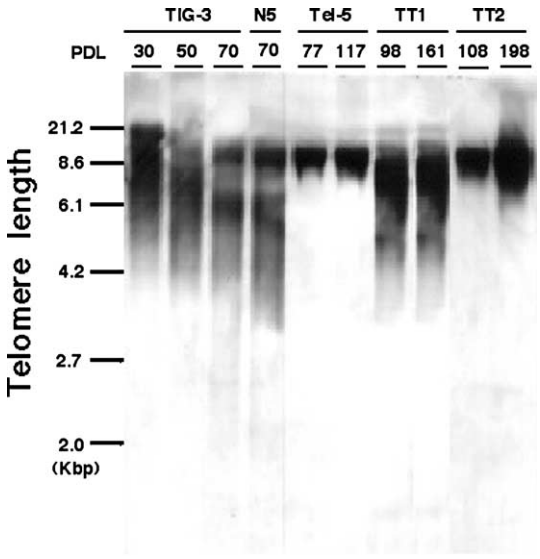

Fig. 3. Telomere length analysis in hTERT-transfected fibroblasts. A Southern blot analysis for terminal restriction fragments (TRFs) was performed on TIG-3 cells, the control vector-transfected clone (N5) and the three hTERT-transfected clones (Tel-5, TT1, and TT2) at different PDLs.

expression of macrophage colony-stimulating factor (M-CSF) was at the same level among different cell lines and strains under different conditions (Fig. 1A). Expression of IL-3 and IL-7 was not detected in the cell lines and strains that we used (data not shown).

Introduction of hTERT into normal human somatic cells elongates telomere length and restores reduced functions such as proliferative capability, and expression level of some genes in senescent cells. We have no reasonable answer to the question, however, why the expression level of IL-1α, IL-1β, IL-6, IL-8, and GM-CSF was high in both growth-arrested young cells and hTERT-transfected proliferating cells, whereas it was low in young growing cells. We also do not have an answer to the question: what is the common condition between growth-arrested young cells and hTERT-transfected proliferating cells that leads to increased expression of common cytokines. We do not know whether the hTERT gene product has functions to modify expression levels of cellular genes other than telomere elongation or whether change in expression in cellular genes occurs via telomere elongation.

Recently, it has been reported that hTERT interacts with NF-κB p65 directly and hTERT locates in the nucleolus via binding with NF-κB p65 protein [25]. It is well known that the transcriptional activator, NF-κB, regulates the transcription of IL-1, IL-6, and IL-8 genes. Therefore we examined whether NF-κB is activated in hTERT-transfected fibroblasts. When Tel-5 cells were stimulated with TNF-α, NF-κB activation was shown in terms of gel shift assays using an NF-κB binding site probe and a western blotting assay using antibodies against NF-κB p65 and Ser-536 of NF-κB p65. However, the activation of NF-κB was not observed at all in hTERT-transfected cells by any of the above parameters (data not shown).

Introduction of hTERT into normal human somatic cells elongates telomere length and restores functions of young cells such as proliferative capability, expression level of some genes [26–28] and tissue formation in transplanted animals [29]. Introduction of hTERT gene into normal human somatic cells results in an extension of proliferative lifespan without any cancer-associated changes [30,31]. Therefore, telomerase expression would be applicable as a useful strategy for regeneration medicine or cell therapy of age associated diseases. However, it was reported that the introduction of hTERT creates some risk for tumorigenesis [32–37]. In addition, in the study presented herein we found that hTERT-transfected human fibroblasts showed unexpectedly high expression of some inflammatory cytokines compared with parental fibroblasts, and raises a caution against application of hTERT to establish lifespan-extended cells with normal phenotype.

Acknowledgments

This work was supported, in part, by a Grant-in-Aid for Scientific Research and a Grant-in-Aid for Cancer Research from The Ministry of Education, Culture, Sports, Science and Technology of Japan.

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