Expression of telomerase inhibits hydroxyl radical-induced apoptosis in normal telomerase negative human lung fibroblasts

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Abstract In tumor cells telomerase activity is associated with resistance to apoptosis and the introduction of the human telomerase reverse transcriptase (hTERT) subunit into normal human cells is associated with life span extension of the cells. To determine the role of telomerase in regulating apoptosis, telomerase negative human embryo lung fibroblasts were transfected with the hTERT gene. Unlike the control fibroblasts, the telomerase-expressing cells had elongated telomeres and were resistant to apoptosis induced by hydroxyl radicals. The results indicate that expression of telomerase and, thus, the maintenance of telomere length in normal human somatic cells caused resistance to not only cellular senescence but also apoptosis. Moreover, we found that hydroxyl radical-induced apoptosis in telomerase-expressing and control fibroblasts was caspase-3 independent. These findings have revealed a new type of interrelation between telomerase and caspase-3, which may indicate that in this case the expressed telomerase may inhibit apoptosis at a site not related to the caspase-3 cascade.

Key words: Telomerase; Telomere; Apoptosis; Hydroxyl radical; Human telomerase reverse transcriptase subunit

1. Introduction

Telomerase, which maintains the telomere length by adding the DNA sequence TTAGGG to the termini of chromosomes, has been related to replicative senescence and malignancy. The life span of normal telomerase negative human fibroblasts is significantly extended and the cells are maintained in a phenotypically youthful state if these cells are transfected with the human telomerase reverse transcriptase (hTERT) gene [1,2]. On the other hand, it is well established that telomerase activity strongly correlates with cell immortality [3–6]. In tumor cells, inhibition of telomerase usually leads to telomere shortening and apoptosis while maintenance of telomerase activity is associated with an increased resistance to apoptosis [7–9]. However, there is no report thus far regarding the effect of the forced expression of telomerase on apoptosis in normal human somatic cells such as fibroblasts. To study this, we transfected human embryo lung fibroblasts with the hTERT gene to obtain telomerase-expressing cell clones.

We report here that telomerase introduction and expression in normal human lung fibroblasts (hLF) markedly increased the resistance to hydroxyl radical-induced apoptosis. Thus our study provides evidence for a role of telomerase in the regulation of apoptosis and life span extension in normal somatic cells. To our knowledge, this is the first report about the regulation of apoptosis by telomerase using hTERT-transfected cells.

2. Materials and methods

2.1. Cell culture

hLF obtained from the Beijing Institute of Biological Products (Beijing, PR China) were seeded at 1×10^5 cells per culture flask in 10 ml of MEM. The complete culture media contained 10% fetal calf serum, 100 μg/ml streptomycin, and 100 U/ml penicillin. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Exponentially growing cells were used for experiments.

2.2. Vector construction and stable expression of telomerase

The hTERT cDNA was in a pGRN251 plasmid, kindly provided by Professor Kaster (Geron Co.). It was cut out as a *Kpn*I–*Bgl*II fragment. An *Xho*I–*Bgl*II fragment, containing a *Xenopus* elongation factor 1a minimal promoter and a green fluorescence protein gene, was cut out from the plasmid pXGM2 [10]. These two fragments were cloned downstream of the CMV promoter in pcDNA3.1 (Invitrogen) to generate a construct of pCMV-hTERT-XGM2.

For transfection, cells at a density of 10^6 cells per 100 mm diameter plate were transfected with plasmid pCMV-hTERT-XGM2 DNA containing the full length hTERT and a selectable marker, a neomycin phosphotransferase gene, by calcium phosphate precipitation procedures as described [11–13]. The expression of telomerase in individually isolated clones was measured using a PCR-based telomeric repeat amplification protocol (TRAP) enzyme-linked immunosorbent assay (ELISA) kit. Once a stable cell line from each clone had been established, the drug was removed from the culture medium. The clonal lines were then maintained in a drug-free medium, and the telomerase-expressing was examined periodically. As a control, cells were transfected with the pCDNA3.1 plasmid containing a neomycin marker.

2.3. Induction of apoptosis and experimental treatments

Hydroxyl radicals generated through the reaction of 0.1 mM $FeSO_4$ and 0.3, 0.6, or 0.9 mM H_2O_2 were used for apoptosis induction for the indicated length of time (from 0 to 16 h). The continuous generation of hydroxyl radicals for more than 30 min was confirmed using the ESR spin trapping technique (data not shown).

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Abbreviations: hTERT, human telomerase reverse transcriptase; hLF, human lung fibroblast; •OH, hydroxyl radical; DAPI, 4′,6-diamidino-2-phenylindole; TUNEL, terminal deoxynucleotidyltransferase (TdT)-mediated dUTP-digoxigenin nick end labeling; PI, propidium iodide; TRAP, telomeric repeat amplification protocol

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2.4. Cell morphology

For phase-contrast microscopy, cells cultured on glass coverslips were washed with D-Hanks and then observed and photographed under a phase-contrast microscope (Nikon Diaphot).

2.5. 4′,6-Diamidino-2-phenylindole (DAPI) staining

24 h before the $FeSO_4/H_2O_2$ treatment the cells were seeded at a density of 3×10^4 cells/cm^2 onto glass coverslips, that precoated with 1 mg/ml of poly-L-lysine (Sigma, M_r 3.7×10^4). The cells were rinsed in D-Hanks and fixed with methanol. After another rinse, cells were incubated with 1 μg/ml DAPI (Sigma) in phosphate-buffered saline (PBS) for 30 min and counted. Apoptotic cells were determined by evaluating the nuclear morphology using a fluorescence microscope (Nikon Fluophot).

2.6. In situ detection of DNA cleavage by the terminal deoxynucleotidyltransferase (TdT)-mediated dUTP-digoxigenin nick end labeling (TUNEL) procedure

Apoptotic cells were also identified using a TUNEL kit (Boehringer Mannheim) as described by Gavrieli et al. [14]. Briefly, cells were plated on glass coverslips and incubated for different periods of time in medium with or without $FeSO_4/H_2O_2$. Cells were then fixed in freshly prepared paraformaldehyde solution (4% in D-Hanks, pH 7.4) for 30 min at room temperature. After rinsing with D-Hanks, the cells were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate buffer and incubated for 1 h at 37°C with TdT and fluorescein isothiocyanate-dUTP to label the cleaved DNA. After that, the coverslips were mounted in anti-fade mounting solution and observed under a fluorescence microscope (Nikon Fluophot). Cells showing a positive reaction were counted.

2.7. Annexin V assays

For quantitative analysis of the apoptosis, the living cells were trypsinized, washed once in ice-cold D-Hanks, and incubated with an annexin V fluorescence/propidium iodide (PI) kit (Clontech) in calcium-containing HEPES buffer. The cells were then immediately analyzed with a FACScan machine (Coulter).

2.8. Flow cytometry

Flow cytometry measurements were carried out according to the methods described by Ishibashi et al. [15]. About 1×10^6 cells were fixed in 70% ethanol and then incubated in 1 ml PBS containing 50 μg/ml PI and 250 μg/ml RNase A (Boehringer Mannheim) at 37°C for 30 min. The fluorescence intensity was measured using a Coulter Elite Flow Cytometer. For each sample, 20 000 cells were analyzed using the Coulter Elite workstation 4.0 software (Coulter Corp.).

2.9. Detection of telomerase activity

The telomerase activity was measured using a PCR-based TRAP ELISA kit (Boehringer Mannheim) according to the manufacturer's description with some modifications. In brief, approximately 1×10^6 cells were lysed in 200 μl lysis reagent and incubated on ice for 30 min. For the TRAP reaction, 2 μl of cell extract (containing 2 μg protein) was added to 25 μl of reaction mixture with the appropriate amount of sterile water to make a final volume of 50 μl. PCR was performed in a PTC-100™ Programmable Thermal Controller (MJ Research, Inc.) as follows: primer elongation (30 min, 25°C), telomerase inactivation (5 min, 94°C), product amplification by the repeat of 30 cycles (94°C for 30 s, 50°C for 30 s, 72°C for 90 s). Hybridization and the ELISA reaction were carried out following the manufacturer's instructions.

The extract of HeLa cells was used as positive controls. 10 μl of HeLa cell extract was incubated with DNase-free RNase (1 μg/μl) for 20 min at 37°C, and 2 μl of the RNase-treated extract was used as negative controls.

2.10. Analysis of terminal restriction fragment (TRF) length using Southern blot

To measure the TRF, genomic DNA was isolated from the cell pellets and digested with restriction enzymes *Hin*fI and *Rsa*I (Promega), and fractionated on 0.7% agarose gel. The separated DNA fragments were depurinated in 0.25 N HCl for 15 min, denatured in alkali and transferred to nylon membranes (Hybond-N; Amersham Life Science Ltd., Aylesbury, UK) using 20×SSC. The oligonucleotide probe $(TTAGGG)_4$ (synthesized by Sangon Co., Shanghai, PR China) was 5′-end-labeled with [γ-^{32}P]ATP using a DNA 5′-end-labeling kit (Megalabel™, Takara shuzo Co.). Prehybridization and hybridization were performed at 48°C using a thermostat-controlled hybridization incubator by the standard protocol. Filters were autoradiographed with an intensifying screen at −80°C for 12–48 h.

2.11. Measurement of telomere length using fluorescence in situ hybridization

Fluorescence in situ hybridization was carried out following the procedure described by Hultdin et al. [16] and Rufer et al. [17] with some modifications. Briefly, cells were suspended in a hybridization mixture containing 70% formamide (Fluka BioChemika, Buchs, Switzerland), 1% Blocking Reagent (Boehringer Mannheim GmbH, Mannheim, Germany) and fluorescein-$(CCCTAA)_3$-fluorescein PNA probe synthesized using the Expedite 8909 Nucleic Acid Synthesis System (PerSeptive Biosystems, Framingham, MA, USA) in 10 mM Tris pH 7.2. Samples were heated for DNA denaturation for 10 min at 82°C followed by hybridization in the dark at room temperature overnight, the cells were centrifuged and washed twice in PBS at 40°C. Cells were then resuspended in PBS containing RNase A (10 μg/ml) and PI (0.1 μg/ml), vortexed and incubated for 2–4 h at room temperature and analyzed immediately with a Coulter Elite Flow Cytometer. For the flow cytometric analysis, the FL_1 channel was used for detection of the fluorescein signal and the FL_3 channel was for PI. The list mode data from 1×10^4 cells in each experiment were collected and analyzed using Coulter Elite workstation 4.0 software (Coulter Corp.). The telomere fluorescence signal was defined as the mean fluorescence signal in cells after subtraction of the background fluorescence.

2.12. Assay of caspase-3 activity

HeLa cells were harvested and washed once with ice-cold D-Hanks and then resuspended in hypotonic cell lysis buffer (25 mM HEPES pH 7.5, 5 mM $MgCl_2$, 5 mM EDTA, 5 mM dithiothreitol, 2 mM PMSF, 10 μg/ml pepstatin A, 10 μg/ml leupeptin) at a concentration of 10^8 cells/ml. The cells were lysed for four cycles of freezing and thawing, the cell lysates were then centrifuged at $16\,000\times g$ for 20 min at 4°C and the supernatant fraction was collected. The caspase-3 activity was measured using a caspaACE™ Assay system, Fluorometric kit (Promega), following the manufacturer's description. The fluorescence intensity was measured with a fluorescence spectrophotometer at an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

2.13. Statistical analysis

Data are expressed as means ± S.D. The significance was assessed using the two-tailed Student's *t*-test or the one-way analysis of variance (ANOVA). All data represent at least three independent experiments performed in triplicates. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

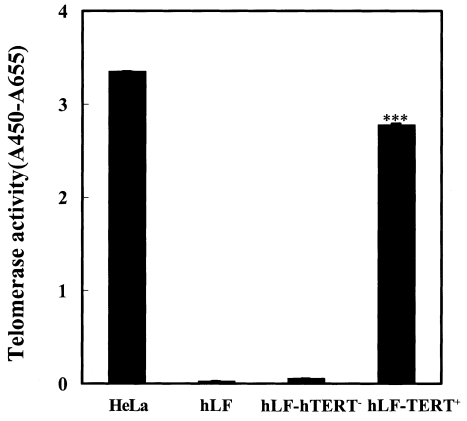

Fig. 1. Telomerase activity in a stable hLF-hTERT$^+$ clone. hLF transfected with a control vector (hLF-hTERT$^-$) or with a vector expressing the hTERT cDNA (hLF-hTERT$^+$) were analyzed for telomerase activity using TRAP ELISA assay. The positive control was the telomerase activity extracted from HeLa cells.

3. Results

3.1. Introduction of telomerase into normal human fibroblasts

hTERT$^-$ normal hLF were transfected with pCMV-hTERT-XGM2 vector at population doubling 28. Five of 12 resultant stable clones were identified to express telomerase activity ranging from 72% to 83% of that in the reference HeLa cells. One of the stable telomerase-expressing clones named hLF-hTERT$^+$ was used in the apoptosis study.

3.2. Expression of telomerase in hLF-hTERT$^+$ clone and control cells

As illustrated in Fig. 1, normal hLF and fibroblasts transfected with control vector (hLF-hTERT$^-$) were telomerase negative, whereas cells of the selected hLF-hTERT$^+$ clone showed a high telomerase activity which was 83% of that in the HeLa cells used as a positive control as detected by the TRAP assay. The results indicated that telomerase was successfully introduced and expressed in the selected hLF-hTERT$^+$ clone.

3.3. Telomere length in cells of the hLF-hTERT$^+$ clone and control cells

The telomere length was measured to determine if hTERT-reconstituted telomerase acts on a normal chromosomal sub-

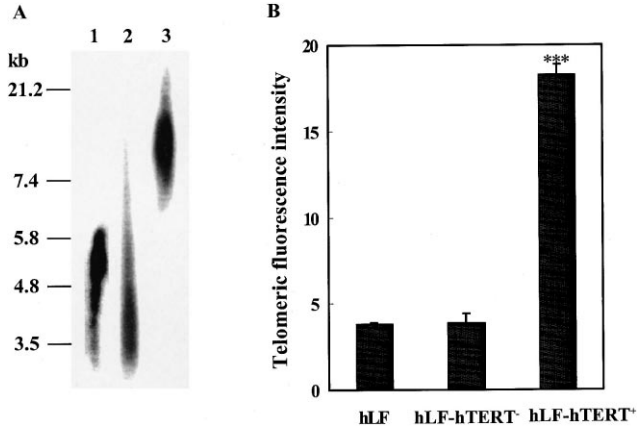

Fig. 2. Telomere length in the hLF-hTERT$^+$ clone. A: TRF length of DNA from hLF, hLF-hTERT$^-$ and hLF-hTERT$^+$ cells measured by Southern blot. Lanes: 1, DNA from hLF cells; 2, DNA from hLF-hTERT$^-$ cells; and 3, DNA from hLF-hTERT$^+$ cells. B: Quantitative analysis of telomere length by the method of fluorescence in situ hybridization.

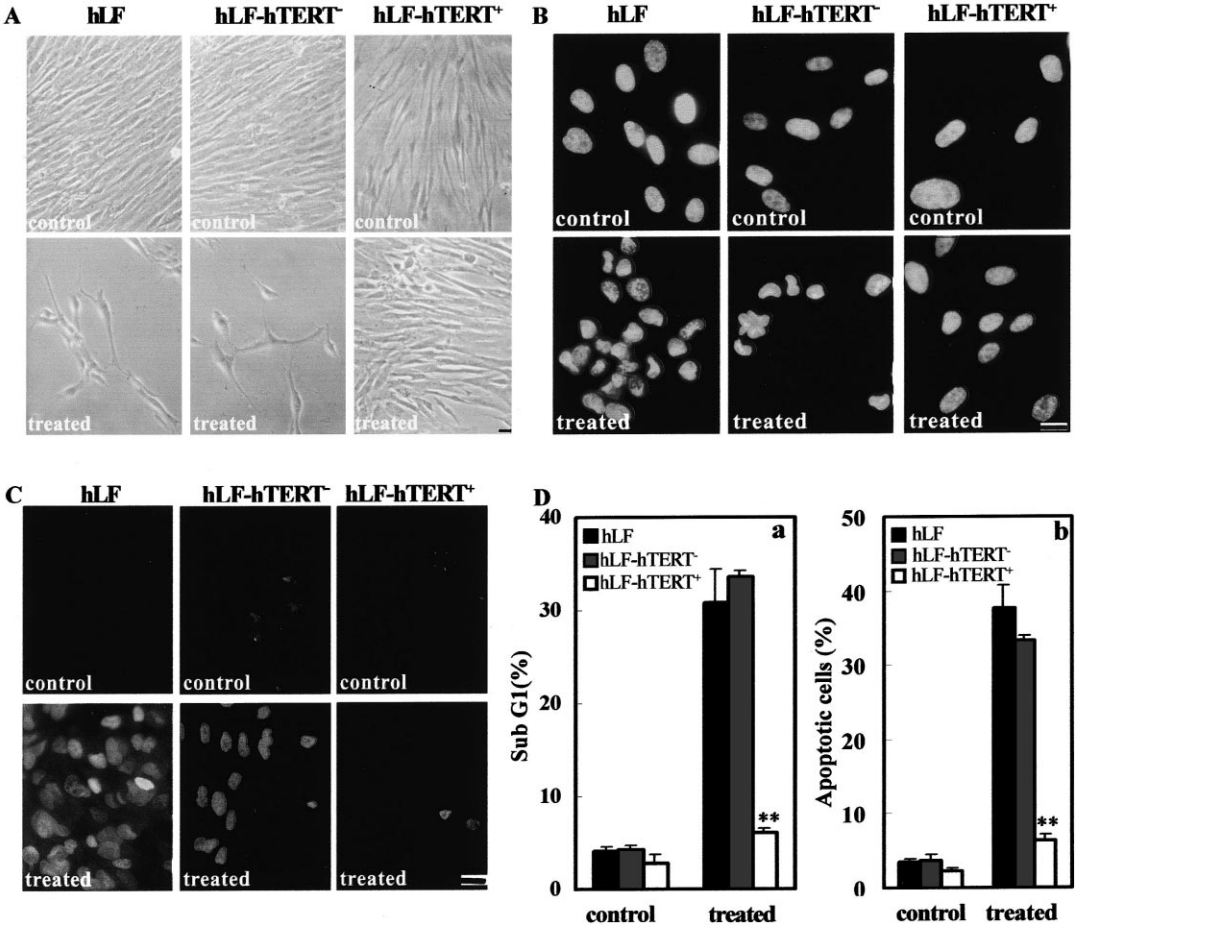

Fig. 3. Resistance to •OH-induced apoptosis in telomerase-expressed cells. Cells were treated with 0.1 mM $FeSO_4$/0.6 mM H_2O_2 for 3 h. A: Phase-contrast morphology of apoptotic cells. Bar, 15 μm. B: Detection of apoptosis with DAPI staining. Bar, 10 μm. C: Apoptosis detected by the TUNEL procedure. Bar, 10 μm. D: Quantitative analysis of •OH-induced apoptosis by annexin V (a) and flow cytometric (b) analysis. The values are the means of three independent experiments done in triplicate. Error bars indicate standard deviations.

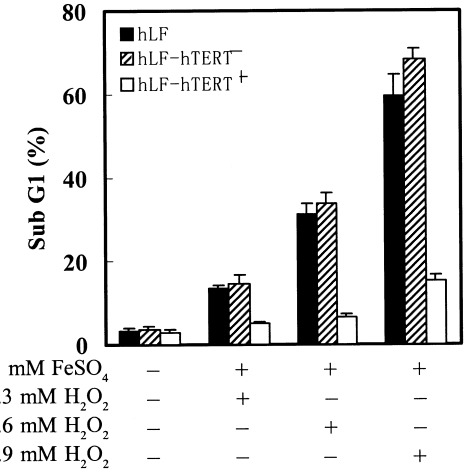

Fig. 4. Dose–response of hLF-hTERT$^+$ cells resistant to $^\bullet$OH-induced apoptosis as measured by flow cytometry.

strate. Fig. 2A displays the telomeric signals of hLF, hLF-hTERT$^-$ and hLF-hTERT$^+$ cells as measured by Southern blot. Quantitative analysis showed a 4.8-fold increase in telomeric fluorescence intensity in the cells of the hLF-hTERT$^+$ clone as compared with the telomerase negative controls as measured by fluorescence in situ hybridization (Fig. 2B). The results verified that expression of telomerase did extend the endogenous telomeres.

3.4. Resistance to $^\bullet$OH-induced apoptosis in telomerase expression cells

Since expression of telomerase in normal fibroblasts delays senescence of these cells, further experiments were to show if the expressed telomerase and elongated telomeres would affect apoptosis. Normal hLF, fibroblasts transfected with the control vector and hLF-hTERT$^+$ cells were treated with $FeSO_4$/H_2O_2 (0.1 mM/0.6 mM) for 3 h. Hallmarks of apoptosis were detected. As illustrated in Fig. 3A, all three types of cells in the control group showed normal morphology. In the $^\bullet$OH-treated group, however, both the normal cells and the cells transfected with control vector rounded up, lost contact with surrounding cells and finally detached from the culture flask surface. On the contrary, the hLF-hTERT$^+$ cells maintained the normal morphology.

The DAPI staining also showed that the nuclei of all three kinds of cells in control group demonstrated normal shape and size while only the hLF-hTERT$^+$ cells in the $^\bullet$OH-treated group maintained a normal nuclear morphology. The nuclei of both the normal fibroblasts and the fibroblasts transfected with the vector shrank and condensed (Fig. 3B).

The TUNEL procedure which enables in situ detection of DNA breaks via visualization of DNA 3′-OH nicks showed the apoptosis-specific DNA fragmentation in $^\bullet$OH-treated normal cells and cells transfected with the vector but not in $^\bullet$OH-treated hLF-hTERT$^+$ cells. Cells in the control group showed no DNA fragmentation (Fig. 3C).

Annexin V assay, a quantitative analysis of apoptosis, also provided evidence that cells transfected with vector encoding hTERT are resistant to $^\bullet$OH-induced apoptosis. Only 6% of the cells of the hLF-hTERT$^+$ clone were apoptotic whereas about 30% of the cells of the normal fibroblasts and fibroblasts transfected with the control vector underwent apoptosis with a background of 4.0% cell death in untreated cells (Fig. 3D(a)). A similar result was obtained by the flow cytometric analysis using PI which provides quantitative measurements of apoptosis. As shown in Fig. 3D(b), $^\bullet$OH treatment induced 33.3% apoptosis in cells transfected with the control vector while only 6.4% of the hLF-hTERT$^+$ cells underwent apoptosis.

Fig. 4 illustrates the dose–response of hLF-hTERT$^+$ cells resistant to apoptosis induced by $^\bullet$OH. The number of apoptotic cells significantly decreased in hLF-hTERT$^+$ cells as compared with that of hLF-hTERT$^-$ and hLF cells.

The data provide convincing evidence for the apoptosis resistance of hLF-hTERT$^+$ cells.

3.5. Telomere shortening occurred during apoptosis in human fibroblasts expression of telomerase

It has been well established that the role of telomerase in senescence and apoptosis is based on its modulation of the

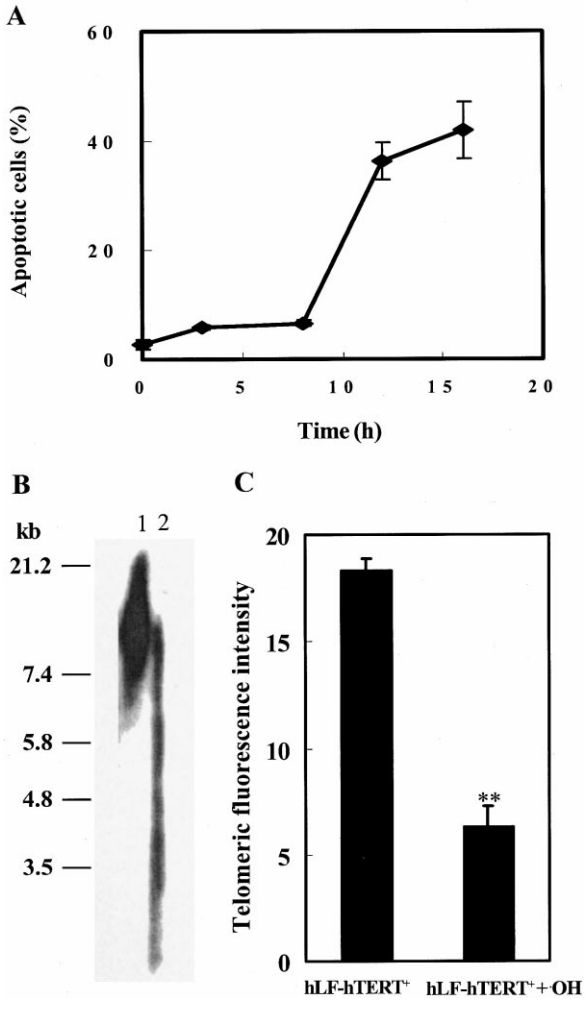

Fig. 5. Telomere shortening in apoptotic hLF-hTERT$^+$ cells. Cells were treated with 0.1 mM $FeSO_4$/0.6 mM H_2O_2 for 12 h. A: Time course of apoptosis induction in hLF-hTERT$^+$ cells. The apoptotic cells were detected using annexin V analysis. B: Southern assay of TRF in hLF-hTERT$^+$ cells cultured with or without $^\bullet$OH. Lanes: 1, untreated; and 2, treated with $^\bullet$OH. C: Telomere length determined by the method of fluorescence in situ hybridization in $^\bullet$OH-treated hLF-hTERT$^+$ cells. Values are means ± S.D. of three separate experiments done in triplicate. Error bars indicate standard deviations.

telomere length. Telomere shortening has been found to account for the limitation of the number of divisions in normal human fibroblasts where telomerase activity is not detectable leading to cellular senescence. At the same time, it has been documented that telomere shortening is related to apoptosis in human tumor cells in which telomerase was inhibited. However little is known about the interrelation between telomere shortening and apoptosis in human normal (not tumor) cells. Although telomerase-expressing fibroblasts are resistant to •OH-induced apoptosis when the treatment lasted for 3 h, an extended treatment may finally induce these cells to undergo apoptosis. To determine the linkage between telomere shortening and apoptosis in normal human fibroblasts, a much longer treatment of •OH was used to trigger apoptosis in hLF-hTERT$^+$ cells. When the hLF-hTERT$^+$ cells were treated with •OH for 12 h, 36% of the cells died through apoptosis (Fig. 5A). To investigate the effect of •OH on telomere length, the size of the TRF from hLF-hTERT$^+$ cells treated with the drugs was examined using Southern blot. As shown in Fig. 5B, the cells treated with •OH demonstrated pronounced telomere shortening. Quantitative analysis showed that the telomeric fluorescence intensity in cells treated with •OH decreased 66.3% as measured by fluorescence in situ hybridization (Fig. 5C). The results thus indicate that shortened telomeres are associated with an increased susceptibility to apoptosis as well as senescence in human fibroblasts.

3.6. •OH-induced caspase-3 independent apoptosis in human fibroblasts

We further investigated if •OH-induced apoptosis in normal cells is associated with the well characterized caspase-related apoptotic pathway. In this study, the caspase-3 activity was measured using a synthetic tetrapeptide Ac-DEVD-AMC as a specific substrate. No caspase-3 activation was found when cells were treated with hydroxyl radicals, and the activity remained unchanged throughout the whole incubation course (Fig. 6). The results indicate that •OH-induced fibroblast cell death is not related to caspase-3 signaling cascade.

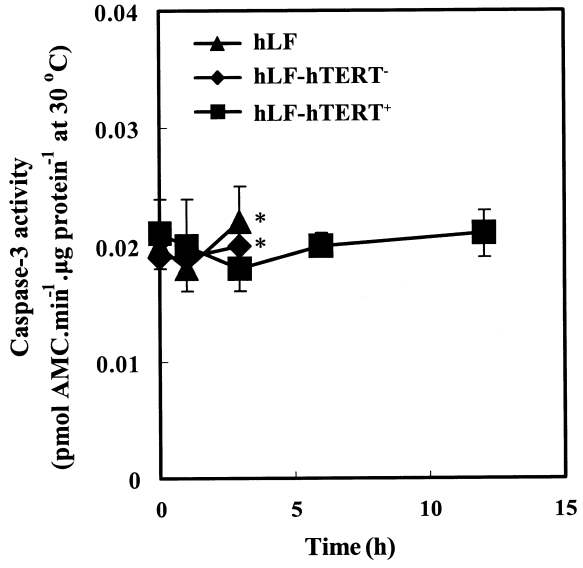

Fig. 6. Independence of caspase-3 in •OH-induced apoptotic cell death. Caspase-3 activities were determined at different times during •OH treatment. (▲) hLF cells; (♦) hLF-hTERT$^-$ cells; and (■) hLF-hTERT$^+$ cells. *Cells at this time died through apoptosis. Error bars indicate standard deviations.

4. Discussion

Discovery of the suppression of replicative senescence upon transfection of the hTERT gene in normal human fibroblasts that are telomerase negative has contributed greatly to our understanding of the mechanism of cellular senescence [2]. At the same time, telomerase was found to play a central role in cellular resistance to apoptosis of cancer cells [9]. To further study the mechanism by which telomerase acts to resist apoptosis in normal cells and also the possible linkage between cellular senescence and apoptosis, we introduced hTERT into normal hLF and found that telomerase expression led to the elongation of telomeres and resistance to •OH-induced apoptosis in normal cells. Our data indicated that the maintenance of telomere length is crucial to the resistance to both replicative senescence and apoptosis of human fibroblasts. We found that a significant telomere length shortening took place when a longer •OH treatment was used to trigger apoptosis in hLF-hTRT$^+$ cells. The results thus raise the possibility that cellular senescence and apoptosis induced by oxidative stress may share a common link, i.e. telomere shortening before the pathway branches. In human fibroblasts, rate of telomere shortening was increased by mild hyperoxia and hydrogen peroxide [18,19]. Henle et al. [20] found that •OH-mediated DNA oxidations had preferential cleavage sites which are at the nucleoside 5′ to each of the dG moieties in the sequence RGGG, a sequence commonly found in telomeres. Their results support the possibility that telomeres may be a direct target of •OH and the shortened telomeres can no longer protect DNA and oxidative damage will accumulate leading to cellular senescence as well as apoptosis.

Fu et al. [9] reported that overexpression of Bcl-2 and caspase inhibitor zVAD-fmk protected PC12 cells against apoptosis in the presence of telomerase inhibitors including DODCB and TTAGGG. Their results suggest that telomerase acts prior to caspase activation. However, we found that when 0.1 mM $FeSO_4$/0.6 mM H_2O_2 reaction was carried out to generate •OH, caspase-3 activity remained unchanged throughout the whole incubation course. It is apparent that the •OH-induced apoptosis in telomerase-expressed cells is following a caspase independent pathway.

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