Stress-induced premature senescence in BJ and hTERT-BJ1 human foreskin fibroblasts

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Abstract To test the involvement of the telomeres in the senescent phenotype, we used telomerase-immortalized human foreskin fibroblasts (hTERT-BJ1). We exposed hTERT-BJ1 and parental BJ cells to either UVB or H_2O_2 subcytotoxic stress(-es). Both cell lines developed biomarkers of replicative senescence: loss of replicative potential, increase in senescence-associated β-galactosidase activity, typical senescence-like morphology, overexpression of $p21^{WAF-1}$ and $p16^{INK-4a}$, and decreased level of the hyperphosphorylated form of pRb. Telomere shortening was slightly higher under stress for both BJ and hTERT-BJ1 but still much lower than that reported for other cell lines. We conclude that pathways alternative to telomere shortening must cause the appearance of the senescence phenotype.

Key words: Cellular senescence; Fibroblast; Telomere; Telomerase; H_2O_2; UVB

1. Introduction

Telomeres are repeated sequences at the end of chromosomes that shorten, normally by 20–200 bp, with each cell division. Previous research suggested that telomere shortening represents a mechanism for counting cell division triggering cellular senescence [1]. Short telomeres might activate a p53 DNA damage response pathway that in turn leads to growth arrest [2]; cellular senescence can also be induced by the pRb pathway [3]. Telomerase is a reverse-transcriptase enzyme that elongates the telomeres [4]. The catalytic subunit of human telomerase was transfected into normal human fibroblasts. Cell lines were thereby established that failed to reach cellular senescence without transformation [5].

Stress-induced premature senescence (SIPS) is characterized by a cell cycle arrest similar to cellular senescence. SIPS can be triggered by way of single or repeated subcytotoxic stress such as UVB [6] or H_2O_2. After at least 48 h of recovery, the cells begin to display biomarkers of cellular senescence: senescence-associated β-galactosidase (SA β-gal) activity, changes in the expression of several genes, similarities in the regulation of the G1 growth arrest, cellular morphology, etc. (for a review see [7]).

WI-38 fetal lung human diploid fibroblasts (HDFs) kept under 40% O_2 for three population doublings (PDs) undergo SIPS. An accelerated telomere restriction fragment (TRF) shortening of 500 bp per PD is observed [8]. When subcytotoxic H_2O_2 stress or five repeated subcytotoxic *tert*-butylhydroperoxide (t-BHP) stresses are performed on cells during a given PD, respectively, a 322 ± 55 bp and a 381 ± 139 bp TRF shortening are observed during the first PD after stress [9]. HDFs at early PD exposed to 150 μM H_2O_2 once or 75 μM H_2O_2 twice in 2 weeks display biomarkers of senescence. Two treatments with 75 μM H_2O_2 fail to induce significant TRF shortening, suggesting that SIPS can emerge without telomere shortening [10].

In this work, we compared SIPS induced by H_2O_2 or UVB in human diploid BJ fibroblasts expressing telomerase (hTERT-BJ1) and in parental BJ cells to know if major differences would be found in the proportion of cells entering SIPS when telomerase activity is present.

2. Materials and methods

2.1. Cell culture and exposure to UVB and H_2O_2

hTERT-BJ1 HDFs were purchased from Clontech (USA) at 111 PD and stressed around 130–140 PD. BJ HDFs were a kind gift of Dr. E.E. Medrano, Baylor College (USA). HDFs were routinely subcultivated as previously described [11].

Confluent cultures of BJ HDFs at early PD and hTERT-BJ1 HDFs were submitted to five repeated subcytotoxic exposures to UVB stress with one stress per day for 5 days as described previously [6]. Confluent cultures were submitted to a single 2 h exposure to H_2O_2 diluted in medium+10% fetal calf serum (FCS), as was described previously [12]. Control cultures at the same PD followed the same schedule of medium changes without UVB or H_2O_2 treatment.

2.2. Cytotoxicity assays

At 48 h after the (last) stress, cells were washed twice with phosphate buffer saline (PBS) and lysed with NaOH 0.5 N.

To measure cytotoxicity, five exposures to UVB or a single exposure to H_2O_2 stress were performed at increasing doses. Cytotoxicity was measured at 48 h after the (last) stress. The cellular protein content was assayed by the Folin method [13], which has proven to give results similar to those found with the MTT assay and cell counts [6,14,15]. Triplicates were always performed. Results are expressed as mean values ± S.D.

2.3. SA β-gal activity and [^{3}H]thymidine incorporation

At 48 h after the (last) stress, the cells were seeded at a density of 700 cells/cm^2. After 24 h, SA β-gal activity was assessed as described in [16]. The proportion of cells positive for SA β-gal was determined in three samples of 400 cells per dish, each sample in a different dish. Results are expressed as mean values ± S.D.

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Abbreviations: SIPS, stress-induced premature senescence; SA β-gal, senescence-associated β-galactosidase; HDF, human diploid fibroblast; PD, population doubling; TRF, terminal restriction fragment; t-BHP, *tert*-butylhydroperoxide

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At 24 h after the (last) stress, cells were seeded at 10 000 cells/2 cm^2 well. 1 μCi [^{3}H]thymidine (specific activity: 2 Ci/mmol, Du Pont NEN, USA) was added to the culture medium for 48 h. The incorporated radioactivity was quantified by a scintillation counter (Beckman Coulter Inc., USA). The procedure was repeated each day for 4 days after stress. Triplicates were always performed. Results are expressed as mean values ± S.D.

2.4. Telomere length and telomerase activity

Two days after the (last) stress, cells were trypsinized and seeded at a 1:2 ratio. When cells became confluent, genomic DNA was extracted, digested with 25 U *Rsa*I and *Hin*fI (Pharmingen, USA), electrophoresed, transferred to a nylon membrane, pre-hybridized and hybridized with a 51-mer biotinylated telomere probe using the reagents and buffers of the Telomere Length Assay kit (#559838, Pharmingen, San Diego, CA, USA), as used previously [9]. The mean terminal restriction fragment (TRF) length was calculated for each sample by integrating the signal density above background over the entire TRF distribution as a function of TRF length, using the formula $L = \Sigma(OD_i \times L_i)/\Sigma(OD_i)$, where OD_i and L_i are respectively the signal intensity and TRF length at position i on the gel image, as described in the manufacturer's recommendations. We also determined telomere length before the (first) stress. Results are expressed as mean values ± S.D. on three experiments. Telomerase activity was studied by TRAP assay (Intergen Inc., USA) following the manufacturer's recommendations.

2.5. Western blot detection of p53, $p21^{WAF-1}$, $p16^{INK-4a}$ and pRb

The cells were washed twice with ice-cold PBS and protein extraction was conducted as previously described [12]. Samples of 20 μg protein were electrophoresed and transferred overnight on Immobilon-P membrane (Millipore, Bedford, MA, USA). Each protein was detected with its specific antibody: anti-p16, anti-p21, anti-Rb (Santa Cruz, Germany) and anti-p53 (Pharmingen). After incubation with horseradish peroxidase-linked secondary antibody, the bands were visualized after incubation with chemoluminescent substrates using the ECL detection kit (Pharmacia, Belgium). Results are expressed as mean values ± S.D. on three experiments.

3. Results

3.1. Cytotoxicity after UVB irradiation or H_2O_2 stress

The results were expressed as percentages of the controls, which were subjected to the same conditions for the same period of time without being subjected to H_2O_2 or UVB. As expected, the cytotoxicity increased with the UVB or H_2O_2 doses (Fig. 1A,B). BJ and hTERT-BJ1 cells behaved similarly. From day 0 to day 3 (= 48 h after H_2O_2 stress) and day 7 (= 48 h after the last UVB stress), we found that the cellular protein content of the controls increased roughly by, respectively, 20 and 40%. Thus an apparent decrease of 20 or 40% of cellular protein shown at 48 h after stress with respectively H_2O_2 or UVB corresponds to absence of growth rather than cell death. These results suggested to use the doses of 1.2 mM H_2O_2 and 600 mJ/cm^2 in our experimental conditions and to consider them as subcytotoxic doses.

3.2. SA β-gal activity, cell morphology and cellular proliferation

SA β-gal activity was described to appear in replicative senescence and SIPS [16]. After a single H_2O_2 stress at 1.2 mM H_2O_2, the percentage of SA β-gal positive cells increased by 15–20% in both BJ and hTERT-BJ1 cells (Fig. 2). When hTERT-BJ1 cells were exposed to H_2O_2 at a cytotoxic concentration (1.5 mM) the percentage of positive cells increased only by 7% (not shown). After five UVB stresses, the percentage of cells positive for SA β-gal activity increased by 30–35% at 600 and 800 mJ/cm^2 UVB for BJ cells and reached similar values at 500 and 800 mJ/cm^2 UVB for hTERT-BJ1 cells (Fig. 2). An appreciation of cellular morphology shows that after a H_2O_2 stress, both BJ and hTERT-BJ1 cells develop a higher incidence of abnormal morphology, resembling a senescent morphology. The differences between controls and stressed cells are not so intense following five UVB stresses.

Subcytotoxic stress with agents such as H_2O_2, t-BHP or UVB triggers irreversible growth arrest of a large proportion of a population [6,9,17]. In this work we tested whether cells transfected with hTERT would show a different behavior after being stressed with UVB or H_2O_2 as far as [^{3}H]thymidine incorporation is concerned. After a single H_2O_2 stress, the level of incorporation fell by 50% in BJ and hTERT-BJ1 HDFs, indicating that only about 50% of the cells were still able to divide when compared to non-stressed controls, from day 3 to day 7 after stress (on the graph, day 1 indicates the first day of measurement of incorporation corresponding to 72 h after stress) (Fig. 3). After five consecutive UVB stresses, the difference was smaller, being respectively 20–25% from day 3 to day 7 after the last stress for BJ and for hTERT-BJ1 cells (Fig. 3). The time-dependent decrease observed in hTERT-BJ1 cells, stressed or unstressed, was highly reproducible and will be considered in Section 4.

3.3. Telomere shortening

A very low and similar TRF shortening occurred in both BJ and hTERT-BJ1 cells without stress: 37 ± 18 bp/PD and 43 ± 13 bp/PD, respectively. These results are within those described earlier [18,19]. After H_2O_2 stress, TRF shortening was multiplied by two in BJ cells and by four in hTERT-BJ1

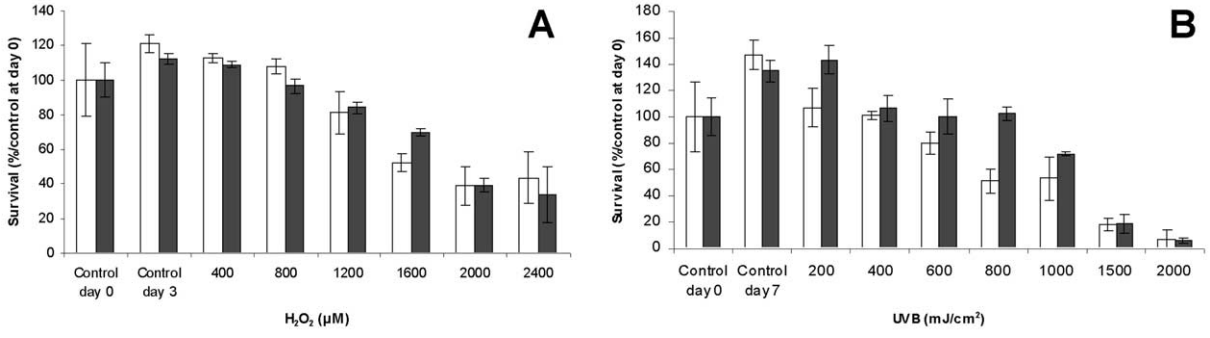

Fig. 1. Cytotoxicity of a single exposure to H_2O_2 (A) or five repeated exposures to UVB (B) with one stress per day in BJ (white columns) and hTERT-BJ1 (gray columns) HDFs. The results are expressed as percentages of the values found in control cells at 48 h after the (last) stress. Results are given as mean values ± S.D. from three independent experiments.

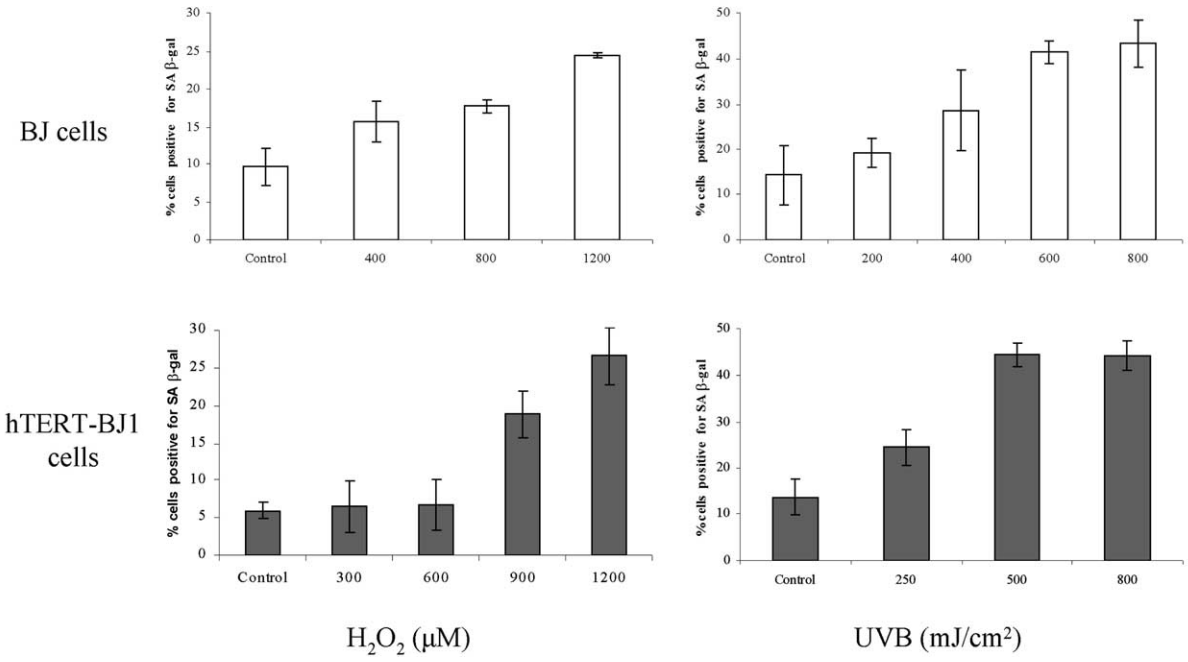

Fig. 2. Effects of a single H_2O_2 stress or five repeated UVB exposures on the proportion of BJ and hTERT-BJ1 HDFs positive for SA β-gal activity. Results represent the proportion of cells positive for SA β-gal. The results are presented as mean values ± S.D. from three independent experiments.

cells, indicating that H_2O_2 stress affected telomere length (Fig. 4A). We did not explain that difference between the two types of cells. After repeated UVB stresses, BJ cells underwent a TRF shortening of 70 ± 27 bp, while TRF shortening in hTERT-BJ1 cells was 38 ± 30 bp. It might be considered that about 25–30% of the cells do not resume mitosis after the stress, from the data obtained with [^{3}H]thymidine incorporation and SA β-gal histochemistry. Calculations of expected TRF shortening due to compensatory cycling of the fraction of cells recovering mitotic capability suggest that the limited shortening observed cannot be accounted for by the compensatory cycling and is also supposed to be caused by DNA damage.

3.4. Telomerase activity

Although we cannot discard minor telomerase activity fluctuations, our results suggest that telomerase activity is not as affected by the stress as when SIPS is established (Fig. 4B). Indeed, we detected similar telomerase activity before and after each type of stress in hTERT-BJ1 cells. No telomerase activity was detected in BJ cells. These findings confirm previous results obtained in different experimental conditions [19].

3.5. Expression level of p53, $p21^{WAF-1}$, $p16^{INK-4a}$ and phosphorylation level of Rb

It was shown that p53 is overexpressed by IMR-90 HDFs after subcytotoxic H_2O_2 stress and comes down to basal level within 44 h after the stress [17]. Only minimal overexpressions of p53 were found in BJ cells at 72 h after H_2O_2 stress (about 20%) and after UVB stress (about 40% overexpression). No overexpression of p53 was found in hTERT-BJ1 cells at 72 h

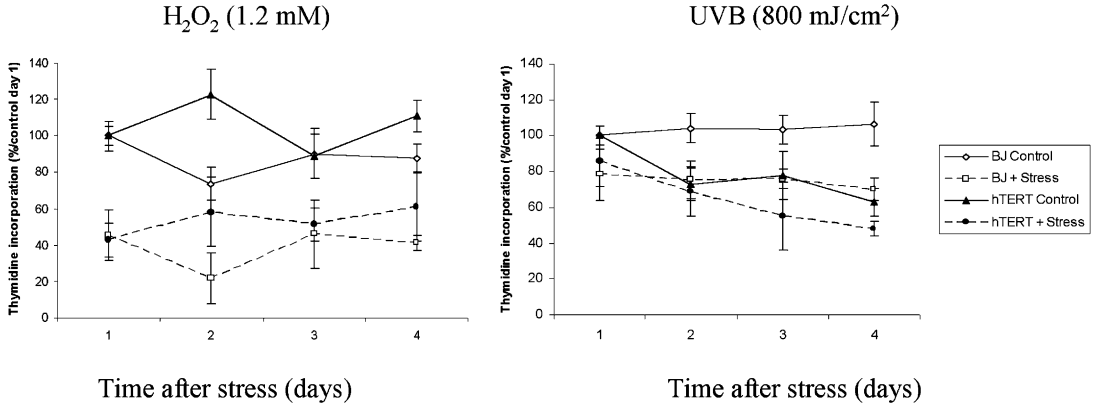

Fig. 3. Estimation of the proliferative potential of BJ and hTERT-BJ1 HDFs exposed to a single H_2O_2 stress or five consecutive exposures to UVB by measurement of the incorporation of [^{3}H]thymidine into DNA between day 1 and day 4 after stress. The results obtained are expressed as percentages of the cpm incorporated by the control cells. The results represent the mean values ± S.D. from three independent experiments.

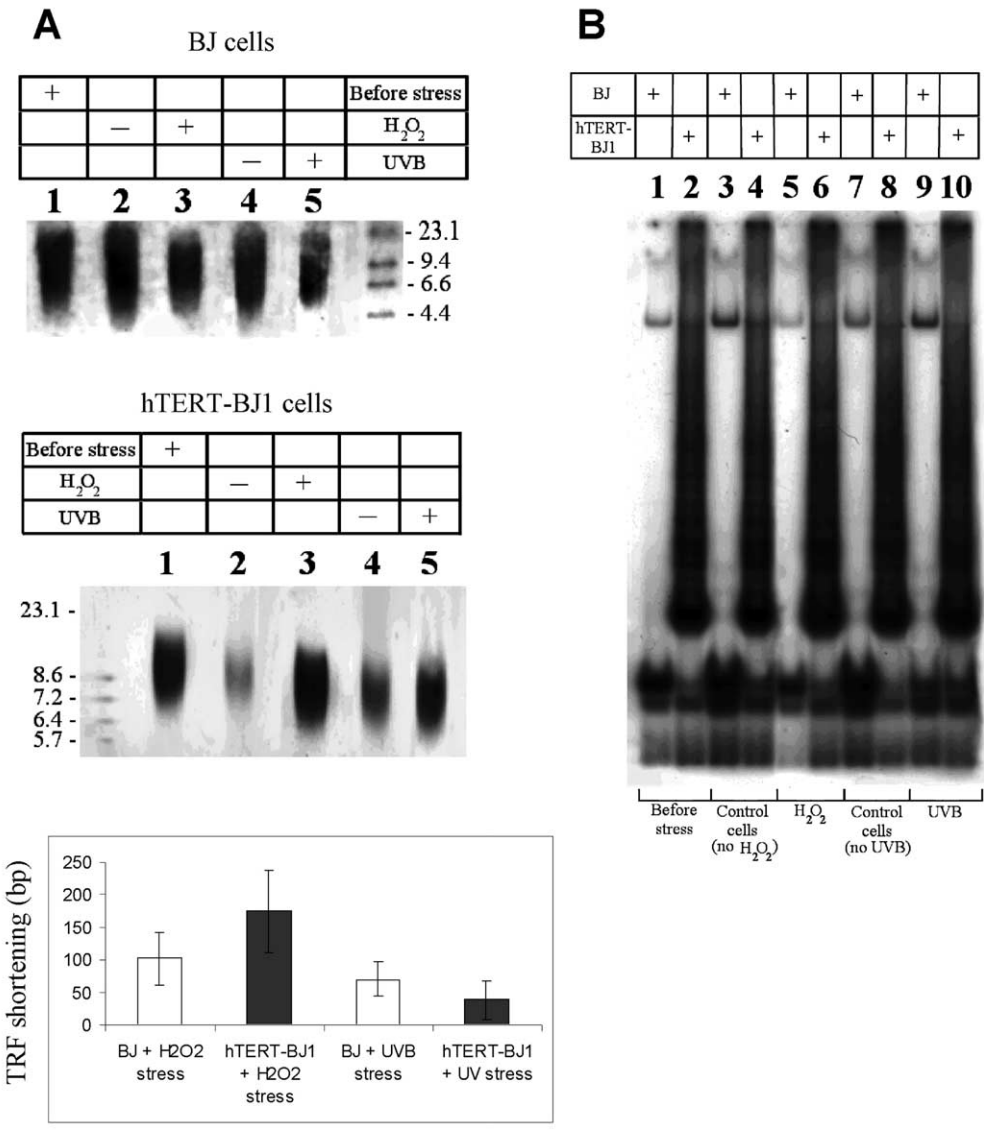

Fig. 4. A: Effect of a single H_2O_2 or five UVB exposures on telomere shortening in BJ and hTERT-BJ1 HDFs. Lane 1: cells at confluence before the stress. Lane 2: control cells for H_2O_2 experiment. Lane 3: cells exposed to H_2O_2. Lane 4: control cells for UVB experiment. Lane 5: cells exposed to UVB. Telomere shortening of stressed cells was measured by comparing telomere length of stressed cells to controls. Controls correspond to HDFs submitted to the same culture conditions as the stressed cells but without any H_2O_2 or UVB. The results represent the mean values ± S.D. from at least three independent experiments. B: No effect of a single H_2O_2 or five UVB exposures on telomerase activity. Lane 1: BJ cells before the experiment. Lane 2: hTERT-BJ1 cells before the stress. Lane 3: control BJ cells for H_2O_2. Lane 4: control hTERT-BJ1 cells for H_2O_2. Lane 5: BJ cells exposed to H_2O_2. Lane 6: hTERT-BJ1 cells exposed to H_2O_2. Lane 7: control BJ cells for UVB. Lane 8: control hTERT-BJ1 cells for UVB. Lane 9: BJ cells exposed to UVB. Lane 10: hTERT-BJ1 exposed to UVB.

after both types of stress (Fig. 5A.1). This suggests that p53 remains overexpressed a little longer in BJ cells compared to IMR-90 cells and the presence of telomerase activity blocks this overexpression.

$p21^{WAF-1}$ is overexpressed at 72 h after five repeated t-BHP stresses in WI-38 HDFs [14] and after a single H_2O_2 stress in IMR-90 HDFs [15,17]. A 2.1- and 1.8-fold overexpression of $p21^{WAF-1}$ was found at 72 h after subcytotoxic H_2O_2 stress and five repeated UVB stresses on BJ cells, respectively (Fig. 5A.2). In hTERT-BJ1 cells, a 1.8- and 1.5-fold overexpression of $p21^{WAF-1}$ protein was found at 72 h after, respectively, H_2O_2 or UVB stress. Measurements of the DNA binding capability of p53 would give information on the dependence of this overexpression of $p21^{WAF-1}$ toward p53. No difference of $p16^{INK-4a}$ protein level was observed in BJ cells exposed to H_2O_2 and in hTERT-BJ1 cells exposed to H_2O_2 or UVB (Fig. 5A.3). A limited 35% overexpression was observed in BJ cells exposed to UVB. A similar change in the phosphorylation status of pRb was observed after stress in all four situations (UVB and H_2O_2 in BJ and hTERT-BJ1 cells) (Fig. 5B).

4. Discussion

SIPS has previously been demonstrated to occur after subcytotoxic stress with, for example, H_2O_2 in IMR-90 HDFs, t-BHP and hyperoxia in WI-38 HDFs (for a review see [7]) and in FS skin HDFs exposed to UVB [6]. BJ HDFs are extremely resistant to hyperoxia and H_2O_2 [19]. This work also shows the remarkable resistance of these cells to H_2O_2 and UVB, with respective subcytotoxic doses of 1.2 mM and

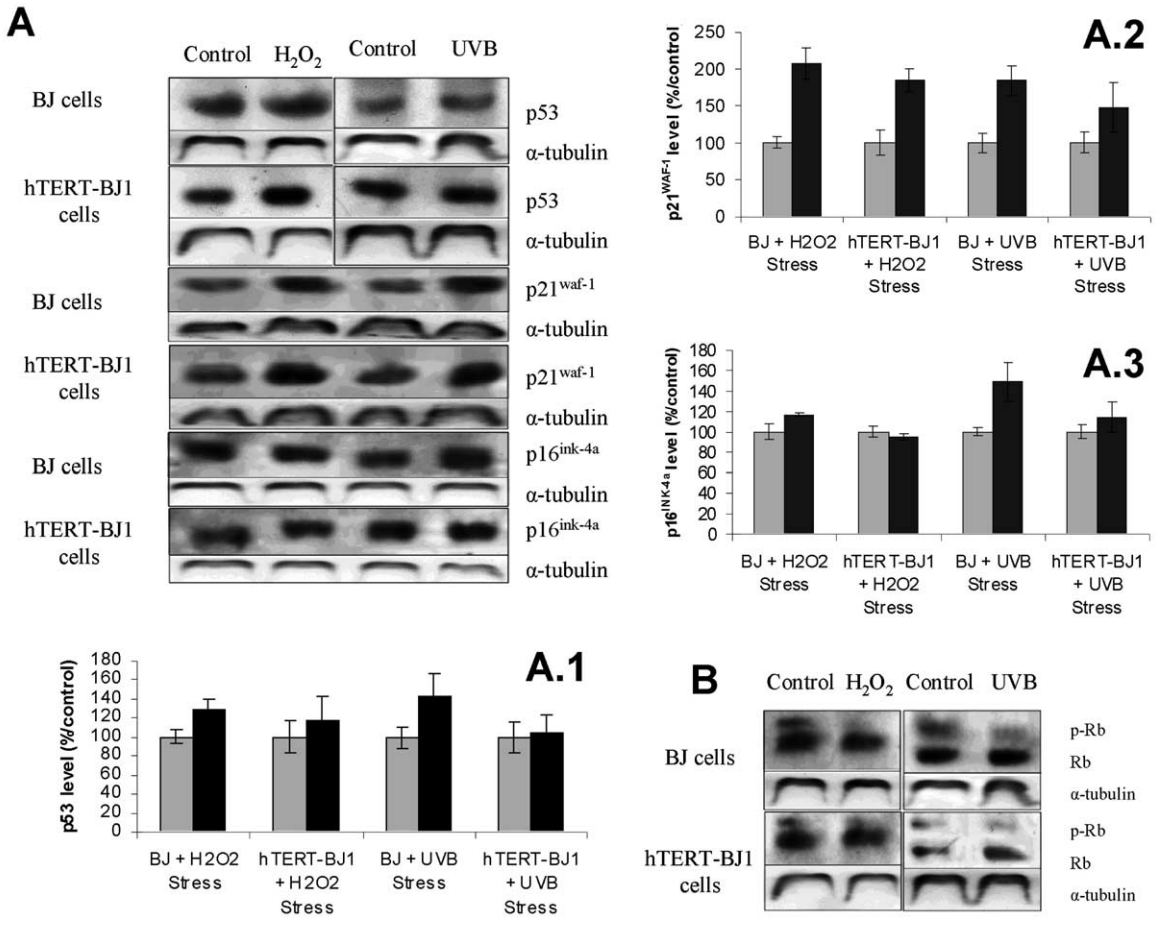

Fig. 5. A: Western blots and quantifications of the protein level of p53 (A.1), $p21^{WAF-1}$ (A.2), and $p16^{INK-4a}$ (A.3) when BJ and hTERT-BJ1 cells are exposed once to H_2O_2 or five times to UVB. B: Phosphorylation status of the Rb protein at 72 h after stress. P-Rb stands for phosphorylated Rb; Rb for hypophosphorylated Rb. The results represent the mean values ± S.D. from three independent experiments.

0.15 mM H_2O_2 in BJ and IMR-90 HDFs [12], and respective subcytotoxic doses of 600 and 500 mJ/cm^2 UVB in BJ and FS HDFs [6], these two strains being derived from the skin.

The growth of BJ HDFs and their telomerase positive derivates was only slightly reduced under hyperoxia. A high resistance of BJ cells to oxidative damage is known [19]. We found a lower proportion of BJ cells to enter SIPS after subcytotoxic H_2O_2 stress. Indeed, the percentage of SA β-gal positive cells after exposure of two strains of fetal lung HDFs, IMR-90 and WI-38, to, respectively, 150 and 160 μM H_2O_2 was around 55% [9,12]. However, similar results were obtained after UVB stress at the respective subcytotoxic doses of 600 and 500 mJ/cm^2 in BJ and FS HDFs [6].

Both BJ and hTERT-BJ1 cells showed a decrease in proliferative capability on the long term after H_2O_2. The level of [^{3}H]thymidine incorporation was not much different in the BJ and hTERT-BJ1 cells after UVB. There might be a lag time before unstressed BJ and hTERT-BJ1 cells resume mitosis after the many days of confluency occurring between and after the UVB stresses (7–11 days before [^{3}H]thymidine incorporation finishes). This could explain the small difference of thymidine incorporation between the UVB-stressed and non-stressed BJ and hTERT-BJ1 HDFs.

After a period of extension of telomere length following ectopic expression of telomerase, telomere length starts to fall slowly over time, reaching a length similar to the parental cells [19], as confirmed in this work. The remaining low telomerase activity appears to stabilize predominantly the shortest telomeres, allowing the growth of these cells despite the average telomere length becoming eventually shorter than in the parental cells at senescence [20]. Therefore, these cells were ideal to test whether SIPS could still appear despite the presence of an hTERT activity that would maintain the telomeres at a subcritical length, thereby allowing the maintenance of the cell cycle.

The limited telomere shortening after stress was found to be about two and four times higher after H_2O_2 stress than in control cells, in BJ and hTERT-BJ1 cells, respectively. This is intriguing since telomerase remained active in the stressed cells and control hTERT-BJ1 cells. It could be that this TRF shortening affected those telomeres which were at a subcritical length, thereby triggering growth arrest. After the UVB stress, the presence of telomerase activity seems to have offered some protection against any further telomere shortening. Anyway, these different shortenings did not lead to a different percentage of cells in UVB-induced SIPS in BJ or hTERT-BJ1 cells. It might, however, have affected the kinetics of recovery of the proportion of the cell population not in SIPS. This result can be correlated with the slight increase in $p16^{INK-4a}$ level after UVB only in BJ cells.

It is possible to induce the senescence of HeLa cells, which have short telomeres, by repressing the human papillomavirus

type 18 E6 and E7 genes, resulting in reactivation of the dormant p53 and pRb tumor repressor pathway. Stable clones of HeLa cells that express hTERT have elevated telomerase activity and extended telomeres. These clones gave an identical response when the E6 and E7 proteins were repressed: growth arrest, SA β-gal activity, altered morphology and increased autofluorescence. Therefore, HeLa senescence induced by these means was not triggered by short telomeres [21]. pRb has been shown not only to be involved in the control of the cell cycle but also in the appearance of different biomarkers of senescence after H_2O_2 stress in IMR-90 HDFs (SA β-gal activity, altered morphology, overexpression of fibronectin, osteonectin, apolipoprotein J) [12,22]. In this cell strain, overexpression of transforming growth factor-β1 (TGF-β1) starting at 24 h after subcytotoxic H_2O_2 stress triggered the appearance of these biomarkers. TGF-β1 overexpression disappeared when E7 was stably expressed in these cells [12]. However, we failed to find any increase in TGF-β1 after UVB or H_2O_2 stress in BJ or hTERT-BJ1 cells (results not shown).

H_2O_2 might have modified the level of the telomeric DNA binding proteins TRF-1 or TRF-2. It was recently shown that overexpression of TRF-2 protects critically short telomeres from fusion and represses chromosome-end fusions in presenescent HDFs, even if accelerated telomere shortening is observed [23]. UVB might not affect the TRF-2 expression level. In addition, it was already known that the introduction of TTAGGG oligonucleotides into HDFs induces a p53- and $p21^{WAF-1}$-dependent long-term growth inhibition [24]. Binding of TRF-2 protein by these oligonucleotides might also explain these results.

In conclusion, this study shows that, besides telomere shortening and TGF-β1 overexpression, other mechanisms might exist which can trigger SIPS.

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