Growth kinetics rather than stress accelerate telomere shortening in cultures of human diploid fibroblasts in oxidative stress-induced premature senescence

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Received 29 June 2001; accepted 2 July 2001

First published online 18 July 2001

Edited by Barry Halliwell

Abstract WI-38 human diploid fibroblasts underwent accelerated telomere shortening (490 bp/stress) and growth arrest after exposure to four subcytotoxic 100 μM *tert*-butylhydroperoxide (t-BHP) stresses, with a stress at every two population doublings (PD). After subcytotoxic 160 μM H_2O_2 stress or five repeated 30 μM t-BHP stresses along the same PD, respectively a 322 ± 55 and 380 ± 129 bp telomere shortening was observed only during the first PD after stress. The percentage of cells resuming proliferation after stress suggests this telomere shortening is due to the number of cell divisions accomplished to reach confluence during the first PD after stress.

Key words: Telomere; Aging; Senescence; Oxidative stress; H_2O_2

1. Introduction

The most commonly accepted mechanism of induction of replicative senescence by telomere shortening is that, when critically short, telomeres activate a DNA damage response pathway, which in turn leads to irreversible growth arrest in the G1 phase of the cell cycle. The recombination of critically short telomeric ends increases the number of dicentric chromosomes with population doublings (PDs). Upon breakage during mitosis, dicentric chromosomes generate single- and double-strand breaks recognized as DNA damage [1,2].

Two types of protocols are used for inducing stress-induced premature senescence (SIPS) in human diploid fibroblasts (HDFs): continuous chronic stress such as hyperoxia for several weeks, or short repeated discontinuous subcytotoxic stress (H_2O_2, *tert*-butylhydroperoxide (t-BHP), etc.) followed by recovery periods of several days. Multiple biomarkers of replicative senescence are observed in SIPS among which a senescent morphology and senescence-associated β-galactosidase (SA β-gal) activity (for review see [3]). WI-38 HDFs exposed to 40% O_2 undergo SIPS and accelerated telomere shortening (500 bp/PD) within three PDs [4]. We tested whether accelerated telomere shortening takes place in WI-38 HDFs exposed discontinuously to SIPS-inducing concentrations of H_2O_2 and t-BHP. Then we tested whether SIPS-inducing discontinuous stress makes it possible to discriminate between stress-dependent or DNA duplication-dependent telomere shortening occurring after stress arguing respectively in favor of a telomere-dependent or a telomere-independent induction of SIPS.

2. Materials and methods

2.1. Stress

WI-38 HDFs (American Type Culture Collection, USA) were cultivated [5] in BME medium+10% fetal calf serum (FCS) (Flow Laboratories, UK). Confluent HDFs at 50% of proliferative life span were exposed to five stresses of 1 h under 30 μM t-BHP diluted in BME+10% FCS (Merck, Germany) with a stress/day for 5 days, or to a 2 h 160 μM H_2O_2 stress (Sigma, USA). The cultures were rinsed twice with BME and incubated in BME 10% FCS. Control cultures at the same early PDs followed the same schedule of medium changes. After 2 days of recovery the cells were seeded at a 1:2 ratio and subcultivated until exhaustion of proliferative potential (replicative senescence). In other experiments, confluent cells were exposed to a 1 h stress under 100 μM t-BHP at every two PDs. All stress conditions were non-cytotoxic [6,7].

2.2. SA β-gal activity

At 48 h after stress, the cells were seeded at 700 cells/cm^2. After 24 h, the proportions of SA β-gal-positive cells were determined [8] in four samples of 400 cells/dish from different cultures. To avoid non-specific staining due to confluence, SA β-gal histochemical staining was performed on non-confluent cells.

2.3. [^{3}H]Thymidine incorporation

Cells were seeded at 15 000 cells/well. 1 μCi [^{3}H]thymidine (2 Ci/mmol, NEN, USA) was added for 24 h. The incorporated radioactivity was quantified (Beckman, USA) on triplicates from four different cultures.

2.4. Determination of telomere length

Genomic DNA was digested (25 U *Rsa*I and *Hin*fI, Pharmingen, USA), electrophoresed, transferred to nylon membrane, pre-hybridized and hybridized with a 51-mer biotinylated telomere probe (Telo-

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

PII: S0014-5793(01)02679-5

mere Length Assay kit, Pharmingen, USA). After scanning of the autoradiographies with a densitometer, the mean terminal restriction fragment (TRF) length was calculated. The signal intensity above background was integrated over the entire TRF distribution as a function of TRF length, using the formula: $L = [\Sigma(OD_i\ L_i)]/\Sigma OD_i$ where OD_i and L_i are respectively the signal intensity and TRF length at position i on the gel image.

3. Results

3.1. Telomere shortening after exposure to t-BHP at every two PDs

HDFs were exposed for 1 h to 100 μM t-BHP at every two PDs, allowed to recover for 48 h and plated at a 1:4 ratio. When confluent, half of the cultures were exposed to a further stress while the other half were used for TRF length measurements. A 490 ± 71 bp TRF shortening/stress was found in the confluent cells previously exposed to t-BHP stress. Four stresses (eight PDs) were sufficient to reach growth arrest in the stressed cultures. The non-stressed cells proliferated for more than 20 PDs and underwent a 214 ± 48 bp TRF shortening per two PDs, thus about 107 bp/PD, in agreement with previous results [4]. Therefore a value of accelerated TRF shortening of 490−214 = 276 bp was obtained after each stress. Control and stressed cells stopped growing when the mean TRF length was between 4.8 and 5.0 kb.

Oxidative stress with t-BHP or H_2O_2 generate single-strand breaks responsible for TRF shortening [9,10]. However, exposure of HDFs to subcytotoxic oxidative stress also involves that a small proportion of the cells recover their proliferative capability many days after stress [3]. These cells must divide a greater number of times before the culture reaches confluence again after plating. Thereby an increased TRF shortening should occur when compared to non-stressed cultures. Using models of short oxidative stresses (single H_2O_2, repeated t-BHP exposures) we wished to discriminate these two possibilities.

3.2. Telomere shortening after single H_2O_2 or repeated t-BHP exposures within the same PD

After a single stress with 160 μM H_2O_2 or five repeated stresses with 30 μM t-BHP, which induced SIPS, the percentages of HDFs positive for SA β-gal activity reached similar values of respectively 50 ± 5% and 45 ± 4%, which corresponds to presenescent HDFs at 80–90% of their proliferative life span [6]. Given this similarity with presenescent HDFs, we expected that only a small fraction of the HDFs recover their proliferative capability after stress and thereby escape SIPS.

We followed the incorporation of [^{3}H]thymidine into DNA. Low [^{3}H]thymidine incorporation is known to take place until day 6 after five repeated stresses with 30 μM t-BHP in culture medium plus serum [6]. In this study, the level of incorporation of [^{3}H]thymidine was estimated by 24 h labellings starting at each day after the five stresses with t-BHP from day 3 to day 12. From day 3 to day 6 after stress, a low level of [^{3}H]thymidine incorporation was obtained in the t-BHP-treated cells (346 ± 70 cpm/15 000 cells), as compared to the control cells (1739 ± 180 cpm/15 000 cells), confirming previous findings. This low level is comparable to that of presenescent HDFs at 95% of their proliferative life span [6]. Interestingly, an increase in the level of incorporation of [^{3}H]thymidine into DNA was observed from day 8 to day 12 after the t-BHP stresses. At day 12 after the stresses, it reached the mean value obtained in control HDFs at early PDs. About 20% of the cells were proliferative between day 3 and day 6 after stress since the mean value of cpm (346 ± 70) obtained between day 3 and day 6 after stress represented a fifth of the value of cpm (1739 ± 180) obtained at day 12 after stress. This estimation must be even lower than 20% since Cristofalo and Sharf have shown that, at 50–60% of proliferative life span, only 80% of the HDFs still proliferate [11], giving a final estimation of 16% of cells resuming mitosis after stress. Individual cells at low density were followed for 12 days after stress to know whether data on individual cells confirm the values obtained on populations of cells. After t-BHP stress, thymidine incorporation was observed in 20 ± 4% of the cells at day 7 after stress. Very few cells (3 ± 2%) showed thymidine incorporation over the 12 days following the H_2O_2 stress.

A proportion of 16% proliferative cells should require about three rounds of division to reach confluence after cell plating at a 1:2 ratio at day 2 after stress. Theoretically, a similar mean value of 12.5% proliferative cells should give respectively 25, 50, and 100% of the cell population after one, two and three rounds of division. Therefore in confluent cells exposed to those stresses and plated at a 1:2 ratio after stress, one should find a TRF shortening equal to the TRF shortening observed in control HDFs after three PDs. Confluent cells were exposed to five 1 h stresses with 30 μM t-BHP with one stress/day, allowed to recover for 48 h after stress

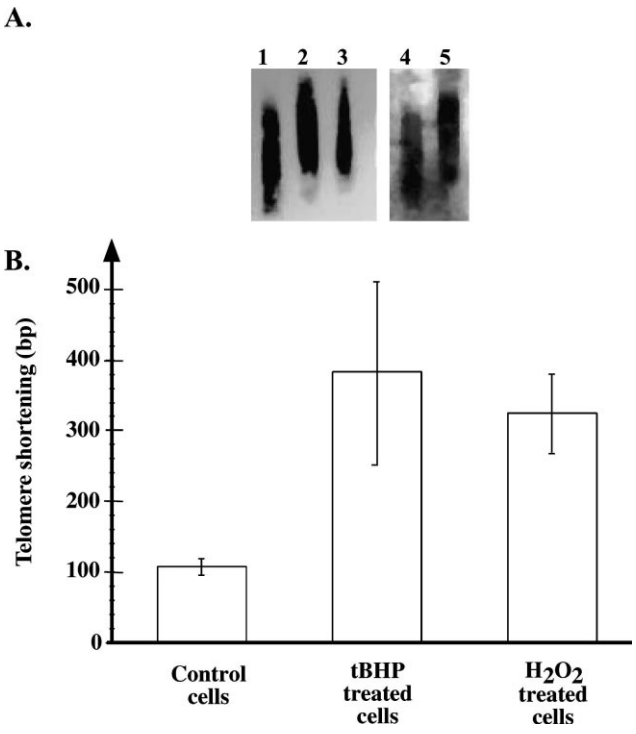

Fig. 1. TRF length in WI-38 HDFs after stress with H_2O_2 or five t-BHP stresses repeated within the same PD. WI-38 HDFs were exposed at PD 30 to five repeated stresses of 1 h under 30 μM t-BHP or to a single 2 h stress under 160 μM H_2O_2. After recovery for 48 h, the cells were plated at 1:2. TRF length was analyzed at confluence. A: Autoradiography. TRF length before stress (lane 2), after five repeated t-BHP stresses (lane 1) or control cells following the same medium changes without t-BHP (lane 3). TRF length after a single H_2O_2 stress (lane 4), control cells following the same medium change without H_2O_2 (lane 5). B. Quantification of TRF shortening. Results are means ± S.D. of four independent experiments.

(these total 7 days representing non-proliferative conditions [6]), and plated at 1:2. When confluence was reached, TRF length was determined. In the control cells, a TRF shortening of 105 ± 12 bp was observed. In the HDFs exposed to t-BHP and plated at a 1:2 ratio after stress, confluence was reached on day 12 after stress and a TRF shortening of 381 ± 139 bp was observed. After 160 μM H_2O_2 stress, a low level in [^{3}H]thymidine incorporation was observed within 12 days after 1:2 plating. The cells became confluent on day 18 after stress and the TRF shortening was 322 ± 55 bp (Fig. 1). These results can be corroborated with the hypothesis that the cell populations were multiplied about by three to reach confluence since the expected mean shortening after three PD is 105 × 3 = 315 bp. Statistical significance was found using Student's *t*-test.

The accelerated TRF shortening observed during the first subculture after stress was no longer observed when the stressed cells were subcultivated until exhaustion of proliferative life span. Similar telomere shortening rates of 108, 93 and 109 bp/PD were observed respectively in the t-BHP-treated, H_2O_2-treated and control cells (Fig. 2).

4. Discussion

After four stresses with 100 μM t-BHP, with one stress per two PDs, the cells stopped proliferating while the control cells always proliferated for more than 20 extra PDs. Both stressed and control cultures stopped growing when the mean telomere length was around 5 kb. This growth arrest of the stressed cells can be interpreted as premature senescence.

A very low [^{3}H]thymidine incorporation takes place for 6 days after five 30 μM t-BHP stresses. This level is similar to that observed in WI-38 HDFs at 95% of proliferative life span [6]. After 150 μM H_2O_2 stress, a very low level of incorporation was observed for 12 days. After plating at a 1:2 ratio after the (last) stress, the cultures needed respectively 18 and 12 days to become subconfluent after H_2O_2 and t-BHP stress. TRF shortening is expected to take place when some cells recover their proliferative capability, e.g. between day 6 and 12 after t-BHP stress and between day 12 and 18 after H_2O_2 stress. These durations might be sufficient for repair of telomere single-strand breaks to occur. This would explain why no telomere shortening occurs when the stressed cells are further subcultivated after one PD after stress. Petersen et al. [10] showed that 50% of the H_2O_2-induced single-strand breaks are not repaired at day 19 after stress. According to the data presented here, it is possible that these 50% single-stranded breaks are mostly present in the cells which did not resume mitosis.

The levels of [^{3}H]thymidine incorporation made it possible to estimate that about 16% of cells recover their proliferative capability after the successive t-BHP stresses. Several other reasons reinforce this estimation. First, the proportion of HDFs positive for SA β-gal, with staining performed or non-confluent cells obtained after the stresses, is very similar to that observed in presenescent HDFs at 80–90% of their proliferative life span [6]. Second, the proportions of the various morphotypes of HDFs showed that the stressed cultures behaved like presenescent cultures [11]. Third, cells treated with subcytotoxic concentrations of t-BHP and H_2O_2 present a long-term overexpression of the cyclin-dependent kinase inhibitor $p21^{waf-1}$ and retinoblastoma protein hypophosphorylation at levels observed in presenescent cultures [6,12]. Four, previous studies followed single cells up to 28 days after such stress and showed that about one cell out of 10 resumes mitosis [13]. As a direct consequence of the low percentage of 16% cells recovering proliferative capability, these cells underwent a telomere shortening close to that observed after about three PDs to reach culture confluence. If stress-induced DNA damage was responsible for telomere shortening, a much more important telomere shortening would be observed. Indeed, H_2O_2 and t-BHP generate DNA damage stochastically, e.g. not only in these portions of the telomere which will be erased when DNA duplication takes place.

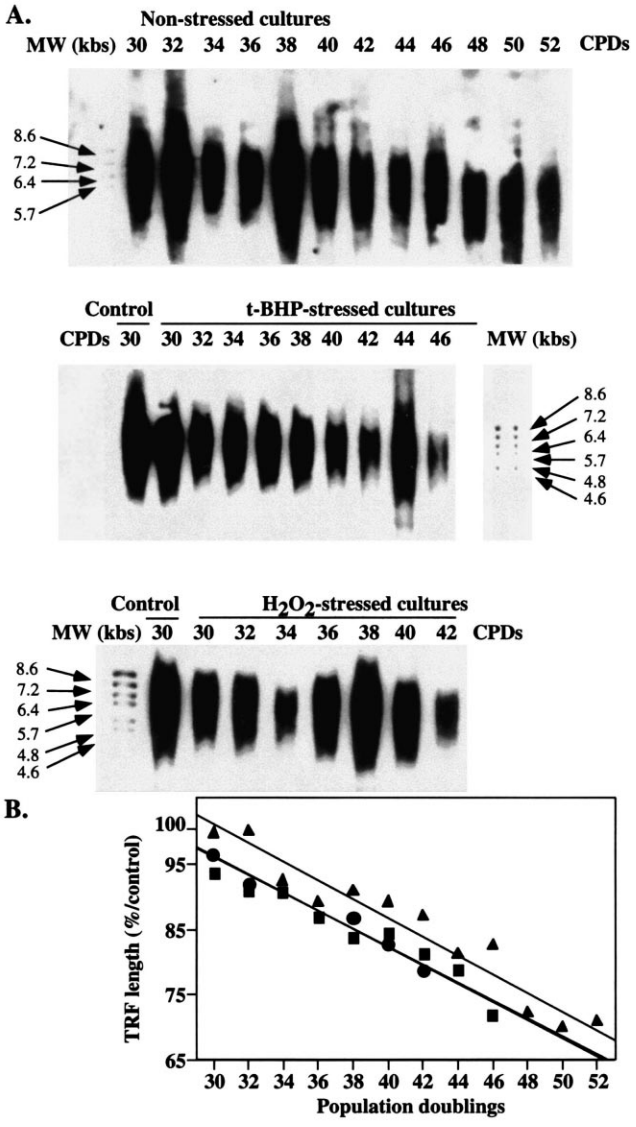

Fig. 2. TRF length in WI-38 HDFs exposed to one H_2O_2 stress or five t-BHP stresses, and subcultivated until exhaustion of proliferative life span. A: Autoradiographies. TRF length in the control, t-BHP- and H_2O_2-treated cells. HDFs were exposed at PD 30 to five repeated stresses of 1 h with 30 μM t-BHP or to a single 160 μM H_2O_2 stress. Cells were subcultivated at a 1:4 ratio until exhaustion of proliferative potential. TRF length was determined every two PDs. B: Quantifications of the TRF shortening. HDFs exposed to five t-BHP stresses (■) or to H_2O_2 stress (●), control HDFs (▲). The results are expressed as percentages of TRF length at PD 30. Results are means ± S.D. of four independent experiments.

This study implies other mechanisms at work, which would explain that a majority of cells irreversibly lose their proliferative capability in SIPS-inducing conditions, before telomere shortening takes place. It has recently been shown that transforming growth factor-β1 (TGF-β1) secreted protein levels are increased within 36 h after subcytotoxic H_2O_2 stress. Incubation of H_2O_2-treated HDFs with antibodies against TGF-β1, or against TGF-β receptor II abrogates the stress-induced increase in the proportion of SA β-gal-positive HDFs. These antibodies also abrogate the stress-induced increase in the mRNA level of these four senescence-associated genes [7], long before telomere shortening takes place. The next step is to identify the mechanisms whereby DNA damage leads to TGF-β1 overexpression in conditions of subcytotoxic stress.

Acknowledgements: J.-F.D. and C.F. have a fellowship from the FRIA, F.C. is a Research Assistant and O.T. a Research Associate of the FNRS, Belgium. V.R. has a post-doctoral fellowship of the European Union, 5th Framework Programme, Quality of Life, R&D (QLK6-CT-1999-02193). J.P. de M. work is funded by the FCT, Portugal.

References

[1] Vaziri, H. and Benchimol, S. (1996) Exp. Gerontol. 31, 295–301.
[2] Sherwood, S.W., Rush, D., Ellsworth, J.L. and Schimke, R.T. (1989) Proc. Natl. Acad. Sci. USA 85, 9086–9090.
[3] Toussaint, O., Medrano, E.E. and Von Zglinicki, T. (2000) Exp. Gerontol. 37, 925–945.
[4] Von Zglinicki, T., Saretzki, G., Döcke, W. and Lotze, C. (1995) Exp. Cell Res. 220, 186–193.
[5] Hayflick, L. and Moorhead, P.S. (1961) Exp. Cell Res. 25, 585.
[6] Dumont, P., Burton, M., Chen, Q.M., Gonos, E.S., Frippiat, C., Mazarati, J.-B., Eliaers, F., Remacle, J. and Toussaint, O. (2000) Free Radical Biol. Med. 28, 361–373.
[7] Frippiat, C., Chen, Q.M., Zdanov, S., Magalhaes, J.-P., Remacle, J. and Toussaint, O. (2001) J. Biol. Chem. 276, 2531–2537.
[8] Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scotti, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., Pereira-smith, O. and Peacocke, M. (1995) Proc. Natl. Acad. Sci. USA 92, 9363–9367.
[9] Altman, S.A., Zastawny, T.H., Randers, L., Lin, Z., Lumpkin, J.A., Remacle, J., Dizdaroglu, M. and Rao, G. (1994) Mutat. Res. 306, 35–44.
[10] Petersen, S., Saretzki, G. and von Zglinicki, T. (1998) Exp. Cell Res. 239, 152–160.
[11] Cristofalo, V.J. and Sharf, B.B. (1973) Exp. Cell Res. 76, 419–427.
[12] Chen, Q.M., Bartholomew, J.C., Campisi, J., Acosta, M., Reagan, J.D. and Ames, B.N. (1998) Biochem. J. 332, 43–50.
[13] Toussaint, O., Houbion, A. and Remacle, J. (1992) Mech. Ageing Dev. 6, 65–83.