No Increase in Senescence-Associated β-Galactosidase Activity in Werner Syndrome Fibroblasts after Exposure to H_2O_2

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ABSTRACT: Normal human diploid fibroblasts (HDFs) exposed to a single H_2O_2 subcytotoxic stress display features of premature senescence, termed stress-induced premature senescence (SIPS). In this work, our aim was to study SIPS in Werner syndrome (WS) fibroblasts, derived from a patient with WS, a disease resembling accelerated aging. The subcytotoxic dose for WS fibroblasts was found to be inferior to that of normal HDFs, indicating WS fibroblasts are more sensitive to hydrogen peroxide than normal HDFs. SA β-gal activity has been shown to occur both *in vitro* and *in vivo*, and we studied the proportion of WS cells positive for SA β-gal. Intriguingly, the percentage of positive cells did not increase with the dose of H_2O_2 used. Contrary to other HDFs, the DNA-binding activity of p53 in WS fibroblasts did not increase in SIPS. We found, based on our results, that WS fibroblasts feature an altered stress response and do not reach SIPS from H_2O_2. We suggest that the proportion of cells that in normal HDFs would enter SIPS instead die in WS fibroblasts. Last, we propose that aging derives from a loss of integrity of the chromatin structure, which occurs faster in WS patients.

KEYWORDS: aging; cellular senescence; stress; Werner syndrome

Werner syndrome (WS) is an autosomal recessive disease resembling accelerated aging. The pathology of WS involves multiple organs, including skin changes, gray hair, alopecia, cataracts, and cancer susceptibility. The mutated gene, *WRN*, encodes a member of the RecQ helicase family unique in also featuring exonuclease activity. WRN interacts with several proteins involved in DNA metabolism. Alternate DNA structures serve as substrate to WRN, which is able to resolve aberrant DNA structures. Cells derived from patients with WS have a shorter proliferative capacity, an extended S phase, increased telomere loss, and feature genomic instabilities. WS fibroblasts are also hypersensitive to topoisomerase inhibitors and 4-nitroquinoline 1-oxide, but not to other DNA-damaging agents such as ultraviolet light. The accel-

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erated aging seen in tissues of patients with WS suggests that the premature *in vitro* senescence of cells derived from WS patients may be related events.[1]

Normal human diploid fibroblasts (HDFs) exposed to a single H_2O_2 subcytotoxic stress display features of premature senescence. After at least 48 hours of recovery, cells exhibit senescence-associated β-galactosidase (SA β-gal) activity, a senescent morphology, and the overexpression of a number of genes. This long-term response is termed stress-induced premature senescence (SIPS) and has been demonstrated in a wide variety of human cell lines. Understanding the mechanisms of SIPS may have implications for aging and cancer research.[2] In this work, our aim was to study SIPS in WS fibroblasts.

We obtained AG00780 WS dermal fibroblasts from ATCC, and cells were cultured in Eagle basal medium + 10% fetal calf serum at 37°C with 5% CO_2. Confluent cultures at population doubling (PD) 16 were exposed for 2 hours to H_2O_2 doses ranging from 10 to 500 μM diluted in medium plus serum. We determined the cytotoxic mortality using the Cytotoxicity Detection Kit from Roche (Basel, Switzerland). The subcytotoxic dose for AG00780 fibroblasts was calculated to be 75 μM, which is inferior to the subcytotoxic dose used in normal HDFs such as AG04431, BJ, IMR-90, and WI-38: respectively, 300, 1,200, 150, and 300 μM. These results indicate that WS fibroblasts are more sensitive to hydrogen peroxide than normal HDFs.

SA β-gal activity has been shown to occur both *in vitro* and *in vivo*, and we studied the proportion of WS cells positive for SA β-gal as described.[3] Previous reports indicated that SA β-gal activity increases drastically in AG00780 fibroblasts from PD 10.9 to PD 32.5.[4] The percentage of SA β-gal–positive cells also increases in HDFs at early PD under SIPS.[2] Our results show that although the percentage of WS fibroblasts positive for SA β-gal in the controls was relatively low (10.4 ± 3.8%), when compared with, for instance, senescent HDFs, the percentage of cells positive for SA β-gal staining did not increase with the dose of H_2O_2 used. For example, in foreskin BJ HDFs the SA β-gal activity in controls and at the subcytotoxic dose was respectively 9.7 ± 2.6% and 24.4 ± 0.3%. In addition, the WS fibroblasts grew slowly and showed the morphology of old cells both before and after a single H_2O_2 stress, which suggests an uncoupling between SA β-gal and the senescent morphogenesis.

One important pathway in SIPS and cellular senescence involves the activation of p53 in response to DNA damage. Indeed, recent results indicate that, as happens in normal HDFs, p53 plays a key role in the telomere-mediated signaling of senescence in WS fibroblasts.[5] Therefore, we used the TransAM kit from ActiveMotif (San Diego, CA, USA) to determine the influence of a single H_2O_2 stress on the DNA-binding activity of p53. Nuclear extracts were added to a 96-well plate, and the DNA-binding activity of p53 was determined according to the manufacturer's specifications. Our results suggest that the DNA-binding activity of p53 does not increase significantly in WS fibroblasts after a single H_2O_2 stress at either 24 or 72 hours after the stress: respectively, 39 ± 46% and 26 ± 41%. This is in contrast with other cell lines; for example, in BJ and hTERT-BJ1 cells p53's DNA-binding activity increases approximately twofold at 24 and 72 hours after a single subcytotoxic H_2O_2 stress (J.P. de Magalhaes *et al.*, in preparation).

We also used DNA microarrays to compare gene expression patterns between WS fibroblasts and BJ HDFs at early (young) and late (old) PD. We used the DualChip Human General from Eppendorf (Hamburg, Germany) containing 202 genes

involved in basic cellular processes. Although AG00780 and BJ cell lines were derived from the skin, alterations in gene expression may be caused by differences between the cell lines. Even so, we found a higher percentage of genes differentially expressed between WS fibroblast and young BJ HDFs than between senescent BJ HDFs and WS fibroblasts: 20% versus 12%. Genes affected by WRN included SHC1, SM22, CAV1, PKM2, PAI2, and PCNA, which were overexpressed in WS fibroblasts, and IL11RA, MSRA, NCOR1, and GSTP1, which were underexpressed. These results suggest that the WS fibroblasts' gene expression patterns are more closely related to senescent BJ cells than to BJ cells at early PD. The results are being further analyzed.

Based on our results, WS fibroblasts feature an altered stress response. Interestingly, p53-dependent apoptosis is attenuated in WS fibroblasts.[6] Our data confirm previous reports that WS fibroblasts are more sensitive to oxidative stress than normal cells. We propose that the proportion of cells that in normal HDFs would enter SIPS instead die in WS fibroblasts, perhaps through a p53-independent event such as necrosis.

The response to DNA damage is altered in WS fibroblasts, which helps explain the increased genomic instability. WRN functions as a key factor in resolving aberrant DNA structures and can induce p53 in response to DNA damage.[1,7] Recent results suggest that WRN plays a structural role in DNA repair independent of its enzymatic activities.[8] Herein, we propose that aging derives from a loss of integrity of the chromatin structure, which occurs faster in WS patients.

WRN may detect damage or changes in unusual DNA structures, facilitate the access of the DNA repair machinery to unusual DNA structures, or help maintain the integrity of the chromatin structure after repair. Indeed, WS fibroblasts are hypersensitive to topoisomerase inhibitors.[1] Changes in chromatin structure affect the expression of certain genes; DNA damage and repair are altered in regions where the chromatin structure changes, in turn disrupting the cell's response to stress/insults. Results from yeast already have implicated chromatin changes in aging and similar mechanism may operate in WS.[9] For example, it has been shown that changes in chromatin remodeling genes make yeast more susceptible to stress.[10] We propose that changes in chromatin structure, for example, condensation, accumulate with age, making our cell's ability to cope with insults decrease with age and thus resulting in an increase in age-related vulnerability. In patients with WS, the accumulation is presumably faster.

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