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Low oxygen tension alleviates oxidative damage and delays cellular senescence in G6PD-deficient cells

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Abstract

Previous studies have shown that glucose-6-phosphate dehydrogenase (G6PD)-deficient cells are under increased oxidative stress and undergo premature cellular senescence. The present study demonstrates that G6PD-deficient cells cultured under 3% oxygen concentration had an extended replicative lifespan, as compared with those cultured under atmospheric oxygen level. This was accompanied by a reduction in the number of senescence-associated β-galactosidase (SA-β-Gal) positive and morphologically senile cells at comparable population doubling levels (PDL). Concomitant with the extension of lifespan was decreased production of reactive oxygen species. Additionally, lifespan extension was paralleled by the greatly abated formation of such oxidative damage markers as 8-hydroxy-deoxyguanosine (8-OHdG) as well as the oxidized and cross-linked proteins. Moreover, the mitochondrial mass increased, but the mitochondrial membrane potential $\Delta\Psi m$ decreased in cells upon serial propagation. These changes were inhibited by lowering the oxygen tension. Our findings provide additional support to the notion that oxidative damage contributes to replicative senescence of G6PD-deficient cells and reduction of oxidative damage by lowering oxygen tension can delay the onset of cellular senescence.

Keywords: *Glucose-6-phosphate dehydrogenase(G6PD), cell senescence, oxygen tension, 8-OHdG, lipofuscin, mitochondria*

Introduction

Glucose-6-phosphate dehydrogenase (G6PD), the key regulatory enzyme in the hexose monophosphate shunt (HMS), catalyzes the oxidation of glucose-6-phosphate (G6P) to 6-phosphogluconolactone and the production of reducing equivalents in the form of NADPH. This meets the cellular needs for reductive biosynthesis and maintenance of the cellular redox balance. The importance of G6PD is illustrated by clinical manifestations of its deficiency. G6PD deficiency, a common enzymopathy affecting over 200 million people worldwide, can cause neonatal jaundice, drug- or infection-induced hemolytic crisis, favism and, less commonly, non-spherocytic hemolytic anemia [1,2]. The hemolytic nature of all these syndromes is attributed to the inability of erythrocytes under oxidative stress to maintain NADPH in its reduced form. This subsequently causes oxidation of cellular components and eventual removal of the damaged cells from circulation [2].

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Our recent studies have shown that G6PD has a cell growth regulatory role in cells other than erythrocytes: G6PD deficiency causes growth retardation and premature senescence in human foreskin fibroblasts (HFF) during their continual propagation in culture [3]. Additionally, the G6PD-deficient cells show an increased propensity for oxidant-induced senescence [4]. Consistent with these findings, G6PD-nullizygous ES line are unable to cope with exogenous oxidants [5,6]. These lines are unable to generate viable mice completely devoid of G6PD activity [7]. These studies advocate the unequivocal role of G6PD in cell growth as well as development.

Normal human diploid cells have a finite lifespan in culture [8]. This phenomenon, termed "replicative senescence", is defined as an irreversible state of growth arrest in which cells assume an enlarged, flattened morphology, and exhibit a number of molecular and biochemical changes [9]. Among the senescence-associated changes in fibroblasts are reduction in telomere length, changes in gene expression, altered composition of extracellular matrix proteins, accumulation of oxidative damage, decline in mitochondrial function, and a positive stain for senescence-associated β-galactosidase (SA-β-Gal). It has been suggested that telomere uncapping and shortening acts as a trigger for cellular senescence [10,11]. In addition to such telomere-dependent senescence mechanism, different forms of stress can elicit senescence. Oxidative stress represents one of such stresses. The vast majority of reactive oxygen species are generated by mitochondria, and if unchecked, cause indiscriminate macromolecular damages. Accumulation of oxidative damage leads to senescence over time. Oxidative DNA damage has been shown to accumulate during cellular senescence [12,13]. Lipofuscin-like material, which represents the oxidatively-modified protein and lipid degradation residues, is deposited intracellularly in senile cells [14]. Exposure to sub-lethal oxidative stress, such as *tert*-butyl hydroperoxide [15], hydrogen peroxide [16], or hyperbaric atmosphere with high O_2 partial pressure, induces cellular senescence [17,18]. Our previous findings have shown that G6PD-deficient cells display increased fluorescence of dichlorofluorescin stain, implying the presence of chronic oxidative stress. The intracellular antioxidant reserve, as indicated by the ratio of reduced to oxidized form of nicotinamide adenine dinucleotide phosphate (i.e. NADPH/$NADP^+$ ratio) and the ratio of reduced to oxidized form of glutathione (i.e. GSH/GSSG), is lower in these cells than in their normal counterpart [3,4]. Moreover, the G6PD-deficient cells show increased propensity for oxidant-induced cellular senescence [4]. Our findings suggest that reactive oxygen species and oxidative damage may play important roles as mediators of cellular senescence in G6PD-deficient cells. This also raises the possibility that a reduction in oxidative stress could delay the onset of cellular senescence.

In this article, we report that the G6PD-deficient cells (HFF1) and their normal counterpart (HFF3) grown under 3% of oxygen had extended lifespan, as compared with those cultured under 20% oxygen tension. Lifespan extension was associated with decreases in number of SA-β-Gal^+ senescent cells as well as diminution of oxidative stress and damages, as indicated by the levels of 8-OHdG and lipofuscin. Additionally, the accompanying increase in mitochondrial mass and reduction in $\Delta\Psi$ were alleviated by lowering oxygen tension. These findings support the notion that oxidative damage is a driving force for cellular senescence, and reduction of intracellular oxidative stress can delay the onset of cellular senescence.

Materials and methods

Cell culture

Primary human foreskin fibroblasts (HFF3) and its G6PD-deficient counterparts HFF1 were isolated as previously described [3,4]. HFF1 was derived from an individual carrying Taiwan–Hakka ($G6PD^{1376T}$) variant of the G6PD gene. The 1376 G → T transversion, the most common G6PD mutation in Taiwan, changes the corresponding amino acid from arginine to leucine, leading to a marked reduction in G6PD activity. The cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% FCS, 100 units/ml of penicillin, 100 μg/ml of streptomycin and 0.25 μg/ml of amphotericin at 37°C. To test the effect of different oxygen tensions on the lifespan, about 5×10^5 cells were seeded in 10 cm tissue culture dish. Cells were cultured in incubators gassed with a mixture of either 3% O_2/5% CO_2/92% N_2 or 20% O_2/5% CO_2/75% N_2. The cultures were split once the cells reached confluence. The cell number was determined by the trypan blue dye exclusion method. The number of population doubling (PD) is defined as $\log_2 N/N_o$, where N is the cell number at the time of passage and N_o is the cell number at the time of seeding. Population doubling level (PDL) refers to the cumulative number of PD, which cells have undergone since their isolation from primary culture. Cells are considered senescent if they do not show any sign of proliferation for at least 1 month in culture.

Senescence-associated β-galactosidase (SA-β-Gal) staining

The staining was carried out as previously described [19]. Cells were washed in phosphate-buffered saline (PBS), fixed for 3 min at room temperature in 2% formaldehyde/0.2% glutaraldehyde, washed, and then

incubated at 37°C with fresh stain solution (0.1% (w/v) 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal)/40 mM citric acid/sodium phosphate (pH 6)/5 mM potassium ferrocyanide/5 mM potassium ferricyanide/150 mM NaCl/2 mM $MgCl_2$). Staining proceeded for around 12 h.

Detection of oxidative stress by dihydrorhodamine 123 staining

Dihydrorhodamine 123 (D-632; Molecular Probes, Eugene, OR, USA) was prepared as a 10 mM stock solution in DMSO and stored at 20°C. Cells were loaded in medium containing 3 μM dihydrorhodamine 123 at 37°C in a 5% CO_2 incubator for 1 h. After loading, the cells were briefly washed twice with PBS and trypsinized for flow cytometric analysis as previously described [20].

Determination of the ratio of 8-hydroxy-2-deoxyguanosine (8-OHdG) to deoxyguanosine

DNA was extracted from cultured human fibroblast cells [21]. The DNA was dissolved in 200 μl of 20 mM sodium acetate (pH 5.2), and digested to nucleotide level at 37°C with 20 units of nuclease P1 for 2 h. It was followed by addition of 6 units of alkaline phosphatase in 1 M Tris buffer (pH 8.5) for 1.5 h at 37°C. The hydrolysate was filtered through microcon YM-10 (Millipore, Bedford, MA, USA) prior to HPLC analysis to remove enzymes and other macromolecules. Nucleosides in the filtrate were separated by a reverse phase HPLC system (ESA, Inc., Chelmsford, MA, USA) equipped with a C8 column (3 μm, 4.6 × 150 mm, YMC-BD), and were eluted at the flow rate of 1.0 ml/min with a mobile phase of 5% methanol in 100 mM sodium acetate buffer (pH 5.2) [22]. The amount of 8-OHdG and dG were monitored with an ESA Coluchem II electrochemical detector; the high sensitivity analytical cell 5010 was set at 150 mV for electrode 1, 300 mV for electrode 2 (with a full range deflection of 100 nA), 700 mV for electrode 3 and 800 mV for electrode 4 (with a full range deflection of 100 μA). The 8-OHdG and dG were used as standards. The 8-OHdG levels are expressed as the number of 8-OHdG molecules per 10^6 dG.

Determination of oxidized and cross-linked proteins content

Oxidized/cross-linked proteins (lipofuscin-like material) in samples of 5×10^5 cells were determined by measuring the cellular autofluorescence within the spectral range from 563 to 607 nm by flow cytometry, as described previously [23].

Assessment of mitochondrial mass and membrane potential $\Delta\Psi m$

The mitochondrial mass was monitored using mitochondrion-selective fluorescent dye Mitotracker Green FM (Molecular Probes, Eugene, OR, USA). Briefly stated, about 5×10^5 cells were incubated in medium containing 75 nM of Mitotracker Green FM at 37°C under the indicated oxygen tension. Sixty minutes later, cells then trypsinised and resuspended in 2% BSA/PBS. The MitoTracker fluorescence was analyzed by a Becton Dickinson FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) and CellQuest software.

The mitochondrial membrane potential was determined using the cationic, lipophilic dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide) (Molecular Probes, Eugene, OR, USA), as described elsewhere [24]. In brief, about 5×10^5 cells were incubated with 0.5 μM of JC-1 in culture medium at 37°C under the indicated oxygen tension. After 60 min, the adherent cell layer was then washed three times with PBS and dislodged with trypsin–EDTA. Cells were collected in 2% BSA/PBS, washed twice by centrifugation, and resuspended in 0.3 ml of 2% BSA/PBS for analysis using a Becton Dickinson FACScan. JC-1 monomers emit at 527 nm (FL-1 channel), and J-aggregates emit at 590 nm (FL-2 channel). Cytometer settings were optimized for green (FL-1) and red (FL-2) fluorescence, and the data were analyzed with CELLQuest software.

Statistical analysis

Data were analyzed by *t*-test or analysis of variance (ANOVA). When necessary, Tukey test or Dunnett test was used to compare the mean values of groups. *p* values of less than 0.05 were considered significant.

Results

Low oxygen tension delays senescence in G6PD-deficient and normal cells

G6PD-deficient fibroblasts, cultured under standard culture condition, are prone to premature senescence upon serial passage and challenge with oxidants. Conceivably, these antioxidant-deficient cells, when exposed to atmospheric oxygen tension (~20%), suffer from increased oxidative damage and undergo senescence. To further test this idea, we cultured G6PD-deficient HFF1 and normal HFF3 cells in 3 and 20% O_2, and compared their growth kinetics. At PDL 20, HFF1 cells grew with essentially the same doubling time as HFF3 cells, irrespective of the oxygen tension (Table I). However, the oxygen tension had differential effect on growth of the later-passage cells. At PDL 40, the doubling time of HFF1 cells

Table I. Effects of oxygen tension on growth of HFF1 and HFF3 cells.

		Doubling time (hour) (mean ± SEM)	
Cell strain	PDL	20% O_2	3% O_2
HFF1	20	18.89 ± 0.42	18.42 ± 0.11
	40	50.96 ± 6.64	38.49 ± 1.51⋆
	60	197.23 ± 40.98	65.77 ± 10.92⋆
HFF3	20	18.21 ± 0.16	17.99 ± 0.95
	40	30.24 ± 2.64	27.45 ± 0.85
	60	42.58 ± 4.87	30.57 ± 2.53⋆

Results are the mean ± SEM for three determinations
⋆$p < 0.05$ vs. the same cell strain at the corresponding PDL and grown under 3% O_2.

grown under 3% O_2 (38.49 ± 1.51 h, $n = 3$) was significantly lower than those under 20% O_2 (50.96 ± 6.64 h, $n = 3$). On the other hand, the difference between the growth rates of HFF3 cells (PDL 40) cultured under 3 and 20% O_2 remained relatively small. Such difference widened as PDL increased. The doubling times of HFF1 cells at PDL 60 increased to 65.77 ± 10.92 h ($n = 3$) and 197.23 ± 40.98 h ($n = 3$), respectively, when these cells were cultured under 3 and 20% O_2. Likewise, cultivation at lower oxygen tension increased the growth rate of HFF3 cells by 1.39-fold. On close examination of their growth histories, we found that HFF1 cells grown under 20% O_2 became senescent at PDL 62.0 ± 2.5, whereas HFF3 cells did so after about 20 more PD. When grown under 3% O_2, HFF1 and HFF3 cells achieved 26.0 and 14.6% more PD, respectively (Figure 1). Thus, lower oxygen conditions offer growth advantage and allow the lifespan extension, especially in cells with G6PD deficiency.

Enhancement of growth rate and extension of cellular lifespan under 3% oxygen tension were accompanied by the morphological changes of cells.

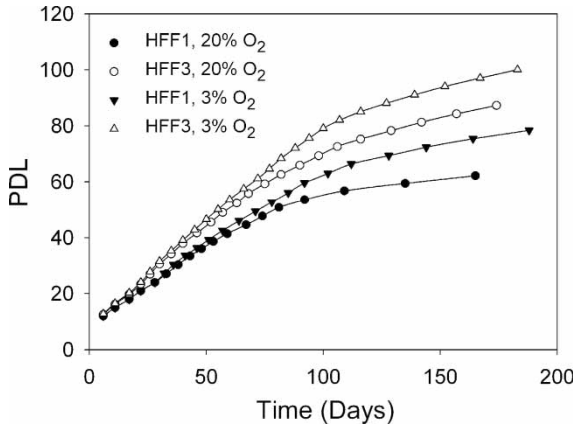

Figure 1. Cumulative growth curves of HFF1 cells and HFF3 cells cultured under 3 or 20% O_2. HFF1 and HFF3 cells at PDL 12 were serially passaged under 3% O_2/5% CO_2/92% N_2 or 20% O_2/5% CO_2/75% N_2 until they became senescent. Data show the average of triplicate samples and standard deviations are smaller than symbols.

As shown in Figure 2B,I, over 90% of HFF1 cells at PDL 60, which had been incubated under 20% O_2, were positive for SA-β-Gal staining. These cells were enlarged and flattened and acquired an increased cytoplasm-to-nucleus ratio; all these features are characteristic of senescent cells. Lowering the oxygen tension to 3% significantly reduced the number of SA-β-Gal positive and morphologically senescent cells (Figure 2F,I). A similar trend was also observed in the case of HFF3 cells, despite that only few cells were SA-β-Gal positive even at PDL 60 (Figure 2D,H,I).

Reduction in oxidative stress and damage by lowering oxygen tension

Previous experiments have revealed increased intracellular ROS production and lowered resistance to oxidant-induced senescence in G6PD-deficient cells [4]. It follows that the growth-enhancing effect of low oxygen tension may be a consequence of lower oxidative stress and damage. To test such possibility, we compared the extent of ROS production in HFF1 and HFF3 cells grown under 3 and 20% O_2. Consistent with our previous findings [3], the levels of oxidative stress, as shown by mean fluorescence intensities of rhodamine 123 (Rh123), were higher in HFF1 cells than in HFF3 at the corresponding PDL and oxygen tension (Figure 3). For HFF1 cells grown under 20% O_2, an overt oxidative stress was observed in the early-passage (PDL 20) cells (Figure 3). As PDL increased, the relative intensity of Rh123 in HFF1 cells increased, being 1.3-times as high for cells at PDL 40 and 6.3-times as high for cells at PDL 60. When oxygen tension was reduced to 3%, the intracellular ROS level dwindled in cells at PDL 40 and 60. For the early-passage (PDL 20) cells, an insignificant reduction was also noted. In comparison, the oxidative stress level in HFF3 cells grown under 20% O_2 increased modestly throughout the PDL range from 20 to 60. Less ROS were generated when cells were cultured under 3% O_2 (Figure 3). Experiments with dichlorofluorescin diacetate yielded similar results (data not shown).

Increased oxidative stress in cells was associated with accumulation of such radical damage end-products as 8-OHdG and lipofuscin (Figure 4). Upon serial passage under 20% O_2, HFF3 cells at PDL 60 accumulated 8.73 times more 8-OHdG than the early-passage (PDL 20) cells (Figure 4A). Likewise, the level of lipofuscin-like materials, as indicated by cellular autofluorescence, increased by nearly 7.5-fold during the same period (Figure 4B). Both damage markers decreased when oxygen tension was reduced (Figure 4). The effect was particularly pronounced for the later-passage cells. Under 3% O_2, the levels of 8-OHdG and lipofuscin-like substances in cells at PDL 60 decreased by 53 and 63%, respectively. On the contrary, these damage markers accumulated at a

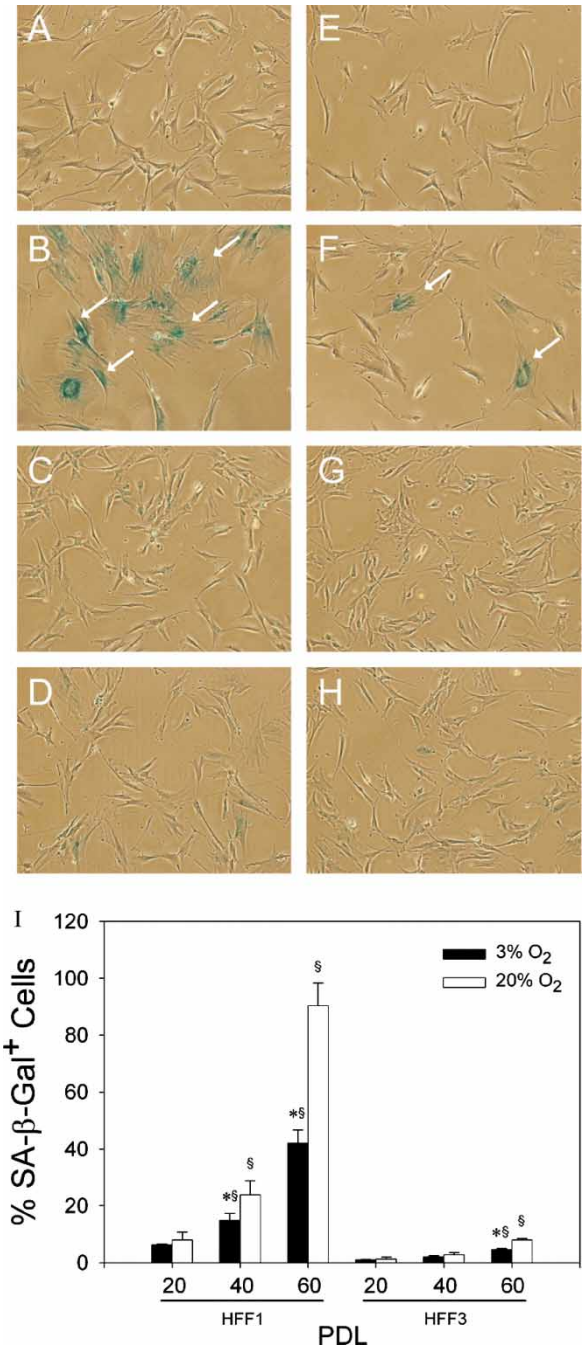

Figure 2. Senescence-associated (SA)-β-galactosidase staining of HFF1 and HFF3 cells grown under 3% or 20% O_2. The HFF1 (A, B, E, F) and HFF3 (C, D, G, H) cells at PDL 20 (A, E, C, G) and 60 (B, F, D, H) were cultured under 20% O_2/5% CO_2/92% N_2 (A–D) or 3% O_2/5% CO_2/75% N_2 (E–H), and stained for SA-β-Gal activity, which was manisfested as a blue coloration of cytoplasm. Typical SA-β-Gal$^+$ cells with flattened morphology are indicated by white arrows. The representative photographs shown here are taken from one of three experiments (Original magnification: 200 ×). (I) The percentage of SA-β-Gal$^+$ cells in cultures gassed with 20% O_2/5% CO_2/92% N_2 or 3% O_2/5% CO_2/75% N_2 was calculated. Over 10^4 cells within 20 microscopic views were counted. The results are mean ± SD, $n = 3$, $^\star p < 0.05$, cells grown under 3% O_2 vs. the same cells at the corresponding PDL and under 20% O_2; $^\S p < 0.05$, cells at the indicated PDL and cultured under the indicated oxygen tension vs. the same cells at PDL 20 and cultured under the corresponding oxygen tension.

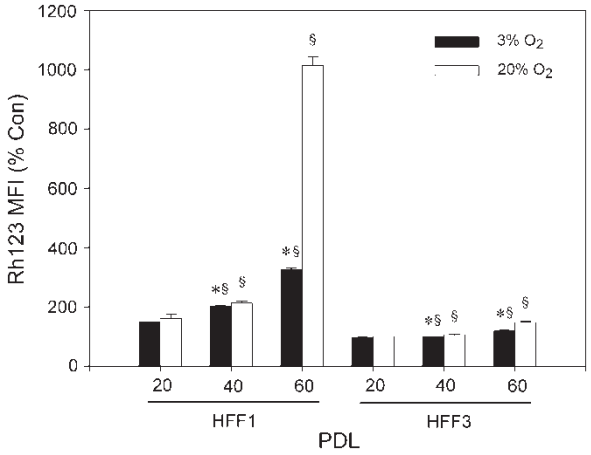

Figure 3. Low oxygen tension reduces ROS generation during cellular senescence. HFF1 and HFF3 cells at PDL 12 were serially passaged under 20% O_2/5% CO_2/92% N_2 or 3% O_2/5% CO_2/75% N_2. Cells at PDL 20, 40 and 60 were stained with the leuco dye dihydrorhodamine 123, and analyzed by flow cytometry as described in Materials and Methods. The ROS level is indicated by the mean fluorescence intensity (MFI) of rhodamine 123 (Rh123), and is expressed relative to the Rh123 MFI of the early-passage (PDL 20) HFF3 cells grown under 20% O_2. The results are mean ± SD, $n = 4$, $^\star p < 0.05$, cells grown under 3% O_2 vs. the same cells at the corresponding PDL and under 20% O_2; $^\S p < 0.05$, cells at the indicated PDL and cultured under the indicated oxygen tension vs. the same cells at PDL 20 and cultured under the corresponding oxygen tension.

much slower pace in HFF3 cells than in HFF1 cells (Figure 4). The cellular effect of 3% O_2, even though significant, was less pronounced as compared with that of HFF1 cells. These results indicate that the lower is the oxygen tension, the less are ROS and oxidative damage produced.

Low oxygen tension lessens senescence-associated changes in mitochondrial mass and $\Delta\Psi m$

Mitochondria are the major intracellular source and target of ROS, and their integrity and function decline as a function of age [25]. It is probable that low oxygen tension may suppress ROS generation through its effect on mitochondria. To test such hypothesis, we examined the mitochondrial mass and $\Delta\Psi$ of HFF1 and HFF3 cells cultured under 3 and 20% O_2. The mitochondrial mass, as indicated by fluorescence intensity of MitoTracker Green, in HFF1 cells increased during serial passage under 20% O_2 (Figure 5). As these cells reached PDL 60, their mitochondrial mass was 75% higher than that of early-passage (PDL 20) cells (Figure 5A). HFF3 cells, on the other hand, showed a relatively modest increase in mitochondrial mass as they were passaged from PDL 20 to 60 (Figure 5A). Reduction in oxygen level prevented the senescence-associated increment of mitochondrial mass. At 3% O_2, the relative intensities of MitoTracker Green of HFF1 and HFF3 cells at

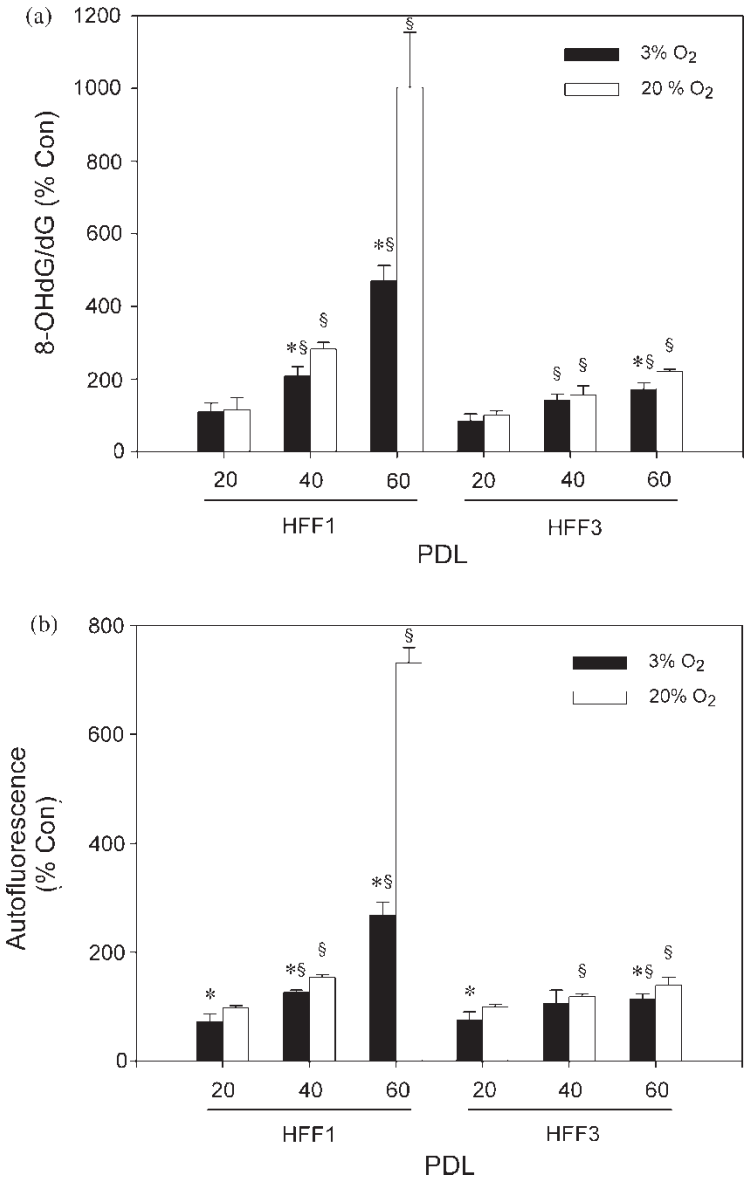

Figure 4. Reduction in oxidative damages in cells cultured under 3% O_2. HFF1 and HFF3 cells at PDL 12 were serially passaged under 20% O_2/5% CO_2/92% N_2 or 3% O_2/5% CO_2/75% N_2. Cells at PDL 20, 40 and 60 were analyzed for levels of (A) 8-OHdG and (B) lipofuscin as described in Materials and Methods. Levels of 8-OHdG and lipofuscin are expressed relative to the corresponding values of the early-passage (PDL 20) HFF3 cells grown under 20% O_2. The results are mean ± SD, $n = 4$, $^{*}p < 0.05$, cells grown under 3% O_2 vs. the same cells at the corresponding PDL and under 20% O_2; $^{\S}p < 0.05$, cells at the indicated PDL and cultured under the indicated oxygen tension vs. the same cells at PDL 20 and cultured under the corresponding oxygen tension.

PDL60 were 54 and 30% lower, respectively. These results indicate that low oxygen tension prevents the increase in mitochondrial mass during the course of senescence.

We proceeded to assess the effect of oxygen tension on $\Delta\Psi m$, an indicator of mitochondrial function. Cells were loaded with JC-1, analyzed by flow cytometry, and the FL1/FL2 ratio determined. As cells were serially passaged, the $\Delta\Psi m$ dropped gradually: the $\Delta\Psi m$ values of HFF1 and HFF3 cells at PDL 60 were 12.5 and 10% lower as compared with those of respective cell strains at PDL 20 (Figure 5B). Under 3% O_2, their $\Delta\Psi m$ values increased significantly, and remained stable throughout the PDL range from 20 to 60. Such findings show that mitochondria depolarize during senescence, and low oxygen tension can block such functional deterioration.

Discussion

In the present study, we show that reduction of the O_2 level led to decreased intracellular oxidative stress and damage (Figures 3 and 4). This was accompanied by enhanced cell growth, delayed acquisition of senescent morphology, and lifespan extension (Figures 1 and 2; Table I). Such effect was prominent

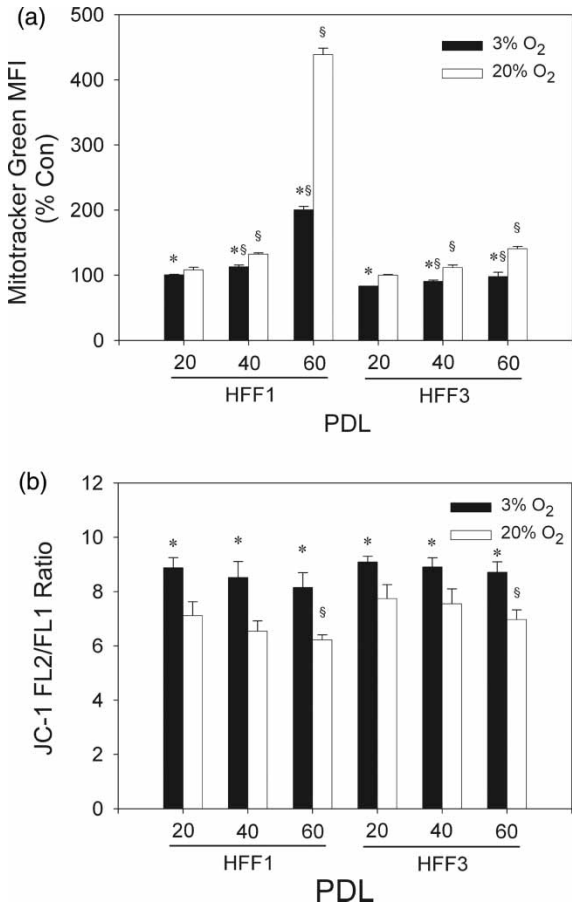

Figure 5. Low oxygen tension reverses senescence-associated increase in mitochondrial mass and mitochondrial depolarization. HFF1 and HFF3 cells at PDL 12 were serially passaged under 20% O_2/5% CO_2/92% N_2 or 3% O_2/5% CO_2/75% N_2. Cells at PDL 20, 40 and 60 were analyzed for: (A) mitochondrial mass, and (B) mitochondrial membrane potential $\Delta\Psi m$ as described in Materials and Methods. The mitochondrial mass is indicated by MFI of MitoTracker Green, and is expressed relative to the corresponding values of the early-passage (PDL 20) HFF3 cells grown under 20% O_2. The $\Delta\Psi m$ is indicated by the ratio of MFI of FL2 channel to MFI of FL1 channel (i.e. JC-1 FL2/FL1 ratio). The results are mean ± SD, $n = 4$, $^{\star}p < 0.05$, cells grown under 3% O_2 vs. the same cells at the corresponding PDL and under 20% O_2; $^{\S}p < 0.05$, cells at the indicated PDL and cultured under the indicated oxygen tension vs. the same cells at PDL 20 and cultured under the corresponding oxygen tension.

for G6PD-deficient fibroblasts. These findings support the notion that oxidative stress and damage is a contributory factor in cellular senescence. Additionally, increased ROS generation was concomitant with increased mitochondrial mass and decreased $\Delta\Psi m$ during the course of senescence (Figure 5). Exposure of G6PD-deficient and normal cells to low oxygen tension retarded such changes. These findings suggest that mitochondrial dysfunction may lead to ROS generation and possibly the onset of cellular senescence.

There appears to be an intimate relationship between oxygen tension and cellular lifespan. In the standard tissue culture condition, 20% atmospheric oxygen tension is generally considered normoxic. However, this is far above the physiological level in most tissues. Ambient air has a partial oxygen pressure of ~159 mmHg; in human subjects, the partial oxygen pressure in alveoli is ~100 mmHg, while it is ~95 mmHg in arterial blood and 40 mmHg in venous blood. The partial oxygen pressure in the basal layer of the epidermis is ~20 mmHg [26]. This corresponds to 3% O_2, the physiological oxygen level which the foreskin fibroblasts encounter *in vivo*. Reducing the oxygen tension from the standard 20 to 3% significantly enhanced cell growth, and effectively extended the replicative lifespan of G6PD-deficient and normal cells. Similar results on the beneficial effect of lower oxygen tension have also been reported [12,27,28]. The degree of such beneficial effect may depend on other factors. It has been recently found that low oxygen tension abrogates the replicative senescence in murine embryonic fibroblasts [29]. Human fibroblasts HFF1 and HFF3 cells, on the other hand, underwent delayed senescence under 3% O_2. Our findings are consistent with earlier reports [12,17,30,31]. Thus, strain differences may exist with respect to the oxygen sensitivity of cells. Additionally, the developmental stage at which cells are derived may affect the outcomes of cultivation under reduced oxygen tension. Fetal skin fibroblasts are more sensitive to oxygen, and can undergo proportionally more doublings in the atmosphere of 3% O_2 than the adult ones [27]. Consistent with this, reduced oxygen tension is necessary to successful immortalization of fetal lung fibroblasts, while it is helpful but not essential for immortalization of adult cells [32]. It is conceivable that the normoxic condition is toxic, and low oxygen tension is beneficial to the long-term proliferation of human fibroblast cultures. Still, a number of factors may affect the growth advantage by low oxygen level. As would be expected, lowering oxygen tension prevents the ROS production, and therefore reduces the level of oxidative damage. It follows that some of these modulatory factors are related to the cellular antioxidant status [27,33]. The antioxidant defenses of fetal skin fibroblasts are much lower than that of adult cells [32], probably explaining the developmental differences in their oxygen sensitivities.

The kinetics of ROS generation and damage accumulation is intriguing. The G6PD-deficient cells, which had lowered $NADPH/NADP^+$ ratio, were laden with increased oxidative stress [4]. At 20% O_2, the level of oxidative stress, as shown by rhodamine 123 fluorescence, increased gradually in the early proliferative stage (PDL 20–40), and at a greatly increased rate in the late proliferative stage (PDL 60) (Figure 3). This was paralleled by the greatly accelerated accumulation of 8-OHdG and lipofuscin as these cells approached senescence (Figure 4). This phenomenon could be expounded

in terms of a balance between ROS generation, clearance by antioxidants, macromolecular oxidative damage, and repair or replacement of the damaged cellular components. In normal cells, ROS generation is limited by antioxidants. On the contrary, in G6PD-deficient cells, such balance is tilted towards ROS generation. The clearance process is far from perfect, and the reactive species may set up a positive feedback loop for biomolecular damage and further free radical production. Damage to DNA may cause defects in such processes as transcription and translation of repair protein- or antioxidant enzyme-encoding genes [34]. Lipofuscin-induced inhibition of lysosomal proteases and proteasome [14,35], as well as decreased expression of proteasomal subunits [36], may lead to defective turnover of organelles and oxidized protein. It has been shown that inhibition of proteasome leads to alteration in mitochondrial turnover and increased ROS production [37]. The positive feedback loop (vicious cycle) is particularly important when mitochondria are taken into consideration. Mitochondria themselves are among the targets accessible to ROS. Initial ROS-induced mitochondrial impairment leads to ROS production, which, in turn, causes further mitochondrial damage [38]. Added to the complexity of such scenario, the worn out mitochondria are refractory to autophagocytosis by lipofuscin-loaded lysosomes, and continue to produce ROS [39]. Such self-amplifying loop probably accounts for robust increase in levels of oxidative stress and damages in cells under 20% O_2, and may provide an explanation for cellular effect of low oxygen level. At 3% O_2, less ROS would be available to initiate and fuel such a vicious cycle.

There is growing evidence that mitochondrion is a critical player in the vicious cycle of ROS-mediated oxidative damage and cellular senescence. Replicative senescence of G6PD-deficient and normal cells was associated with decreased $\Delta\Psi m$ and increased mitochondrial mass (Figure 5). Mitochondrial depolarization, together with increased ROS generation, indicates mitochondrial dysfunction during continual passage of these cells. This is in consistence with previous reports that $\Delta\Psi m$ is reduced in cells from aged animals [40,41]. The rate of decline in $\Delta\Psi m$ was modest as compared to the rate of accumulation of lipofuscin and 8-OHdG. This might reflects the cell's metabolic need to maintain a relatively constant $\Delta\Psi m$. The increase in mitochondrial mass may represent compensatory mechanism in response to the decline in mitochondrial respiratory function [42]. It can be inferred that low oxygen tension protects mitochondria from oxidative damage, helps to maintain mitochondrial function, and prevents the increase in mitochondrial mass.

Our findings have intriguing implications in the pathophysiology of G6PD deficiency and provision of a better health care plan for G6PD-deficient individuals. For instance, normal preterm infants had increased G6PD activities in blood cells as compared to full term ones [43]. Such increase is viewed as a compensatory mechanism to cope with the oxidatively stressful situation in preterm infants. It follows that the G6PD-deficient infant born preterm might have insufficient capability to combat an adverse environment. This is particularly important when the premature infants have respiratory distress syndrome and are put on supplemental oxygen. Bronchopulmonary dysplasia (BPD), which develops as a complication of such therapy, has been found to be associated with increased oxidative damage [44]. Being detrimental to antioxidant capacity, G6PD deficiency may further increase the severity of BPD in the affected infants. A number of studies have suggested that antioxidant therapies may have beneficial effects on infants with BPD [45]. Such therapies may be particularly helpful for those with G6PD deficiency.

In conclusion, we have demonstrated that low oxygen tension can delay replicative senescence and lessen ROS generation and oxidative damage. The effects are more pronounced for G6PD-deficient cells. Moreover, low oxygen tension prevents the senescence-associated mitochondrial dysfunction and increase in mitochondrial mass.

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