Impairment of Lon-Induced Protection Against the Accumulation of Oxidized Proteins in Senescent Wi-38 Fibroblasts

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Oxidative damage to mitochondrial proteins is thought to contribute to the aging process, but the Lon protease normally degrades such proteins. In early-passage WI-38 human lung fibroblasts, Lon expression is rapidly induced during H2O2 stress, which prevents the accumulation of oxidized proteins and protects cell viability. In contrast, middle passage cells exhibit only sluggish induction of Lon expression in oxidative stress, and oxidized proteins initially accumulate. Late-passage, or senescent, cells have low basal levels of Lon and high levels of accumulated oxidized proteins; in response to oxidative stress, they fail to induce Lon expression and exhibit continually increasing accumulation of oxidized proteins. Senescent cells separated into two populations, one exhibiting normal mitochondrial mass and a second displaying significant loss of mitochondria; both populations had diminished mitochondrial transmembrane potential. These senescent changes are similar to the effects of Lon silencing in young cells. We suggest that loss of Lon stress inducibility is part of a pattern of diminishing stress adaptability that predisposes cells to senescence.

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Mitochondria are major sources of intracellular free radical generation, a problem that increases with age (1). Mitochondrial proteins are highly susceptible to oxidative damage, and the targeted removal of damaged proteins is important in maintaining mitochondrial function (2). Early work in our laboratory revealed that the Lon protease selectively degrades oxidatively modified mitochondrial aconitase at a much higher rate than the unoxidized control protein (3). So far, Lon is the only known enzyme that selectively breaks down oxidatively modified proteins within mitochondria.

The physiological relevance of the Lon protease in mammalian mitochondria is not well understood. In yeast, loss of Lon function results in irregularly shaped mitochondria in which the matrix is filled with electron dense inclusions that are thought to be damaged protein aggregates (4,5). We have reported the generation of similar mitochondrial structures when Lon synthesis was blocked (with antisense) in human WI-38 VA-13 fibroblast cells (2). In addition, the silencing of Lon resulted in impaired mitochondrial function and cell death (2). These data strongly suggest that Lon is crucial in maintaining mitochondrial function and may be doing so by regulating the levels of protein aggregates within the mitochondria.

Mitochondrial Lon plays an important role at the interface between aging and oxidative stress. Mice that are heterozygous for mitochondrial manganese-superoxide dismutase (SOD2−/+ mice) suffer considerable oxygen radical damage, resulting in altered mitochondrial function (6). We compared Lon protein levels in skeletal muscles of young and old, wild-type (SOD2−/−), and heterozygous (SOD2−/+), and showed that Lon protein levels were lower in old and oxidatively challenged animals (SOD2−/−) compared with SOD2−/+ mice). Lon deficiency was associated with increased levels of carbonylated proteins, one of which was identified as aconitase (7). In light of these murine observations, we next decided to study Lon in human cells. When we challenged human cells with hydrogen peroxide, there was little accumulation of oxidized proteins; however, when Lon synthesis was silenced with siRNA, there was a dramatic increase in the accumulation of oxidatively damaged proteins (8). Taken together, these data strongly suggest that human Lon is relevant for mitochondrial function and for protection against oxidative protein damage during oxidative stress.
One of the prominent theories of aging suggests that oxidative stress is a major contributor to cellular senescence. Cellular defense systems in older cells are thought to be weakened, resulting in a vicious cycle of oxidative protein damage, which, eventually, results in a cell’s demise. It is estimated that some 30%–40% of proteins exhibit oxidative damage as a part of normal aging (9,10). We wondered if there would be a deficiency of Lon-induced protection in aging human cells, which might contribute to the increased levels of oxidative protein damage commonly observed. Replicative senescence of human fibroblasts has been widely studied and displays, at least some of, the important aspects of late-stage aging phenomena (11). In our previous report, we attempted to model an aging phenotype in the immortalized WI-38 VA-13 lung fibroblasts by downregulating the Lon protease. In this report, we now follow up by analyzing the original nonimmortalized WI-38 fibroblasts at various population doubling stages (early, middle, and late or senescent) for differences in both basal Lon and inducible protein levels, as well as the accumulation of oxidatively damaged cell proteins. Lung cells are an especially good model in which to study the effects of oxidative stress on the aging process because the lung is constantly exposed to high oxygen concentrations and a host of environmental (oxidative) toxins.

**Materials and Methods**

**Materials**

All reagents were obtained from VWR unless otherwise stated.

**WI-38 and WI-38 VA-13 Human Lung Fibroblast Cell Culture**

The WI-38 parental cell line was purchased from ATCC and maintained at 37°C/19.9% O2/5% CO2 in modified Eagle’s Medium supplemented with 10% fetal bovine serum.

**Quantification of Population Doublings**

WI-38 cells were cultured until confluent. Cells were then trypsinized and counted using a particle cell counter. Population doublings were calculated using the equation

\[ A = B \times 2^X \]

with A as the final cell count, B as the initial number of cells seeded, and X as the population doubling number. Cells were then propagated at a seeding density of 750,000 cells per 75 cm² flask until confluency was reached again.

**Staining for Cellular Senescence**

Once WI-38 cells exhibited growth arrest for at least 1 month, they were stained using the Sigma senescence cell staining kit (CS0030). Briefly, 5 × 10⁶ cells were seeded on a 6-well plate and cultured for 24 hours. Cells were then stained with the senescence staining kit, according to the manufacturer’s instructions, for an additional 24 hours. Stained cells were quantified under a bright field microscope.

**Ki-67 Staining**

WI-38 cells of early, middle, and late passages were pelleted at a concentration of 2 × 10⁷ cells. Cells were fixed and then stained with Fluorescein isothiocyanate (FITC) Ki-67 antibody (BD Biosciences) according to manufacturer’s instructions (BD Biosciences). The samples were then subjected to fluorescence-activated cell sorting analysis and measured under the FITC fluorescence channel. Data were collected using a SORP LSR II flow cytometer and subsequently analyzed using FACSDiva V 6.1.x.

**Mitochondrial Mass and Membrane Potential Measurements**

WI-38 cells were cultured until they reached confluence. Cells from each age group were then trypsinized and then stained with either MitoTracker Red CMXRos (Molecular Probes) or MitoTracker Green FM (Molecular Probes), in suspension, according to the manufacturer’s instructions. The samples were then subjected to fluorescence-activated cell sorting analysis and measured under the FITC fluorescence channel. Data were collected using a SORP LSR II flow cytometer and subsequently analyzed using FACSDiva V 6.1.x.

**Treatment With Hydrogen Peroxide**

WI-38 cells were seeded at a density of 3 × 10⁶ cells per 75 cm² flask 24 hours prior to H₂O₂ treatment. A stock concentration of 8.8 M hydrogen peroxide was diluted (just in advance of each experiment) in modified Eagle’s Medium to the concentrations needed. Cells were incubated in modified Eagle’s Medium with H₂O₂ for 1 hour and then washed twice with phosphate-buffered saline. Fresh modified Eagle’s Medium, supplemented with 10% fetal bovine serum, was then added to the cells during incubation for the designated recovery hours.

**Western Blot Analysis**

WI-38 cells were collected by trypsinization and then Western blot analysis was performed on polyvinylidene difluoride membranes. After the Western blot transfer, gels were stained with coomassie brilliant blue dye in 10% acetic acid and 90% acetic acid. The gels were then destained in 10% acetic acid and 10% methanol and used as a reference for total protein loading.

**Results**

WI-38 VA-13 subline cells are derived from the parental human pulmonary cell line, which has the ability to reach replicative senescence. Because our initial experiments on
the WI-38 VA-13 cells indicated that Lon downregulation induced a similar phenotype to that expected of senescent cells, we decided to further investigate whether senescent cells from the parent WI-38 line would also display reduced Lon levels. WI-38 cells were cultured until they reached senescence at about 40–45 cumulative population doublings, a point where they can no longer divide. WI-38 cells were classified into early-passage fibroblasts corresponding to cumulative population doublings <25, intermediate or middle-passage cells with CPD between 25 and 39, and senescent fibroblasts corresponding to late-passage cells with CPD >40, as classified previously by Ahmed and colleagues (12). To confirm the senescence phenotype, β-galactosidase activity was measured by fixing and staining each group of cells and examining them under bright field microscopy at 40× magnification. Representative photographs of stained cells are shown. When quantified, β-galactosidase staining revealed that at least 85% of the cells exhibited positive staining in senescent cultures, whereas less than 10% were positive in the early-passage cultures.

We also tested if this decline in growth fraction was accompanied by a detectable endogenous β-galactosidase activity, which has been shown to be associated with senescent human fibroblasts (14). In staining the cells for senescence-associated β-galactosidase activity, we observed that at least 85% of the cells exhibited positive staining in senescent cultures, whereas less than 10% were positive in the early-passage cultures (Figure 1B).

After confirmation of cellular growth fraction, the cells were harvested and lysed for Western blot analysis of Lon protein and total carbonyl (oxidized proteins) content. To assess oxidative protein damage, carbonylated proteins were first derivatized with dinitrophenylhydrazine, and carbonyls were detected after gel electrophoretic separation and Western blot analysis using the “OxyBlot” method. Although levels of carbonyls were unchanged in middle-passage cells (compared with early-passage cells), we observed an approximate 2.4-fold increase in the accumulated levels of carbonylated proteins in senescent cells (Figure 2A). Western analysis of senescent cells revealed approximately a 66% decrease in Lon protein levels when comparing early- and late-passage cells (Figure 2B and C). We have recently shown that Lon induction and de novo synthesis during oxidative stress adaptation is protective against the accumulation of oxidative protein damage and that such (Lon) adapted cells also survive better (than do nonadapted cells) under conditions of high oxidative stress.
Lon inducibility and stress protection are lost in senescence

(or heat or starvation) stress (8). We wondered if the inducibility of Lon might be attenuated in senescent cells because they exhibit higher levels of even basal oxidative damage than do early-passage cells (Figure 2). To test this possibility, we treated WI-38 cells with 40 μM H₂O₂ for 1 hour, then allowed them to recover and adapt in complete medium without peroxide for an additional 24 hours; this protocol has previously been shown to induce adaptation to stress and greatly increased Lon expression (8). We tested early-passage, middle-passage, and senescent cells, as confirmed by both population doubling, Ki-67 staining and β-galactosidase staining, as indicated in Figure 1.

Western blot analysis of early-passage cells clearly revealed significant (twofold to threefold) Lon induction within 5 hours of H₂O₂ treatment, and Lon levels remained elevated for at least 25 hours after treatment (Figure 3A). Cells expressing higher levels of Lon also exhibited lower levels of protein carbonylation. Interestingly, after 7 and 25 hours of H₂O₂ adaptation, early-passage cells exhibited carbonyl levels that were even slightly lower than those of control cells, which were not adapted to H₂O₂ (Figure 3A). Middle-passage cells exhibited a gradual induction of Lon protein levels (up to twofold) starting at 1 hour after treatment that remained elevated for at least 25 hours after treatment (Figure 3B). It will be noted, however, that the pattern of Lon induction was rather sluggish in the middle-passage cells, for example, it took 25 hours for middle-passage cells to reach the level of Lon induction seen after only 5 hours in early-passage cells (Figure 3A and B). The carbonyl content of middle-passage cells increased significantly following H₂O₂ treatment and remained much higher than that of early-passage cells, returning to basal levels only between 7 and 25 hours after H₂O₂ treatment (Figure 3B).

Senescent cells exhibited low baseline levels of Lon, with only a very small Lon induction between 7 and 25 hours after H₂O₂ treatment; the maximal Lon induction seen in senescent cells (at 25 hours) only reached the basal (preinduced) Lon level observed in early-passage cells (Figure 3A and C). The basal carbonyl content of untreated senescent cells was much higher than that of either early- or middle-passage cells (between 2.5-fold and fourfold) and evinced an almost steady rise for at least 25 hours following H₂O₂ treatment (Figure 3C). It should, thus, be noted that senescent cells actually exhibited no adaptive Lon induction in response to oxidative stress and no adaptive resistance to the accumulation of oxidatively damaged cell proteins.
Lon silencing (Lon siRNA) results, from our previous work in divisionally competent cells, revealed that the induction of Lon protects cells against the accumulation of oxidatively damaged proteins after treatment with a challenge dose of H$_2$O$_2$ (8). When cells were subjected to siRNA Lon knockdown, they exhibited virtually no Lon induction following H$_2$O$_2$ treatment and no induced protection against protein carbonyl production; in contrast, cells with no siRNA treatment and cells treated with scrambled (control) siRNA sequences underwent significant Lon expression after H$_2$O$_2$ treatment and evidenced strongly induced protection against the accumulation of oxidized proteins (8).

The reduction in Lon levels and the loss of oxidative stress protection observed in senescent cells made us wonder if mitochondrial function and levels would be altered. Previously, we showed that a WI-38 immortalized cell line, silenced with Lon siRNA, exhibited an early loss of transmembrane potential, eventually leading to an emergence of cells with lower mitochondria mass (2).

In this study, the reverse experiment was performed, whereby the parental WI-38 cell line was cultured until replicative senescence, and the mitochondrial mass and membrane potential were analyzed. Cells from early, middle, and late passage were stained with MitoTracker Green FM, a dye that binds to mitochondrial membranes, regardless of mitochondrial membrane potential and that, thus, reflects mitochondrial mass (15). These cells were analyzed by flow cytometry, and total fluorescence is reported. There was no significant difference in total mitochondrial mass between early- and middle-passage cells but almost a 40% reduction in total mitochondrial mass in late-passage cells as seen in Figure 4A. However, a population of cells exhibiting approximately a 10-fold reduction in mitochondrial mass can be seen in the fluorescence-activated cell sorting histogram (denoted by the arrow) in the late-passage cells. Late-passage cells formed two distinct populations as judged by mitochondrial density: Approximately 60% of the cells exhibited relatively normal mitochondrial mass, but some 40% of the late-passage cells had a mitochondrial density only 10% of that seen in early-passage cells. Thus, on average, there was actually almost a 40% decline in mitochondrial mass in the whole population (Figure 4A). Although total mitochondrial mass was approximately the same in early- and middle-passage cells, it can be seen, even in the histogram for middle-passage cells, and much more clearly in late-passage cells, that the mitochondrial mass peak is much broader and less well defined than that of early-passage cells, indicating that, as the cells replicate, mitochondrial mass apparently becomes progressively more heterogeneous, finally resulting in two distinct populations of cells at replicative senescence.

Mitochondrial transmembrane potential ($\Delta\psi_m$) was analyzed using MitoTracker Red FM, a dye whose accumulation is dependent upon transmembrane electrical potential (15). The cells were stained and then analyzed by flow cytometry. There was no significant difference between early and middle passage cells; however, in late-passage cells, a reduction of 36% was observed, when compared with early passage cells (Figure 4B). This major drop in $\Delta\psi_m$ would be expected to have significant metabolic effects.

**Discussion**

An age-dependent decline of mitochondrial Lon messenger RNA (mRNA) and protein levels has been reported in vivo for mice and rat tissues and in cells from such animals (7,16,17). Lon is a multifunctional protein that is required for the proteolytic removal of oxidatively damaged mitochondrial matrix proteins (2,3,7,18) and for both mitochondrial biogenesis and the maintenance of the mitochondrial genome (5,19,20). We have recently shown that Lon responds to stress largely via Lon translational activation, with only small increases in lon transcription. Increased mitochondrial Lon protein levels and proteolytic activity in oxidative stress-adapted cells are associated with decreased...
accumulation of oxidatively damaged proteins and with improved mitochondrial bioenergetic functions and cell survival during stress; all such improvements are lost, however, when Lon synthesis is blocked with siRNA (8).

We are interested in determining the cause and effects of diminishing Lon levels during aging. In rats and mice, Lon seems to decline precipitously toward the end of life span, suggesting, perhaps, that this may be a senescence-associated deficit. We have previously reported that down-regulation of Lon in WI-38 VA 13 immortalized cells led to alterations in mitochondrial morphology that strongly resemble the mitochondria of aged cells, namely loss of cristae, and appearance of both giant mitochondria and electron dense inclusion bodies (Bota, 2005 #58). In addition, despite significant apoptosis, cell growth studies revealed that a subpopulation of “survivor cells” were able to escape the detrimental fate of Lon downregulation and continue to survive (2). In the present study, we wondered if these “survivor cells” could be similar to the senescent cells that are often studied as a model for the final stage of cell life span. The immortalized WI-38 cells, however, have limited potential for the study of aging because of their (essentially) unlimited replication potential. In order to proceed with this model, we next examined the parental WI-38 cell line, which naturally undergoes replicative senescence. Indeed, we found that senescent cells exist in two cell populations, where a subpopulation displays an approximate 10-fold reduction in mitochondrial mass when compared with the major population, characteristic of the survivor cells in the immortalized WI-38.

We next examined Lon levels and Lon stress inducibility in the parental WI-38 cell line. Replicative senescence of human fibroblasts has been widely studied and clearly recapitulates, at least some of, the important aspects of late-stage aging phenomena (11). An accumulation of oxidized proteins has been documented in senescent fibroblasts, whereas damage removal systems, such as the proteasome, and repair systems like methionine sulfoxide reductases are impaired during replicative senescence of human WI-38 fibroblasts (21,22). Oxidized proteins have also been found to accumulate during serial passaging of WI-38 fibroblasts, and the level was reduced when stimulated with 20S proteasome activity through a mild heat stress (23). Other studies show that the types of oxidized protein damage found in these serially passaged, or senescent, cells that accumulate with cell “age” are also observed in animal tissues during aging in vivo (24–26).

The age-related accumulation of oxidized proteins depends upon the balance between generation of oxidatively modified proteins and their removal by protein degradation and/or repair systems. Oxidized proteins are degraded by the proteasome in the cytosol, nucleus, and the endoplasmic reticulum (27,28), whereas the Lon protease performs this function in the mitochondrial matrix (2,3). Mitochondrial integrity declines with age (29), and this appears to result in increased O$_2^-$ and H$_2$O$_2$ production. Even in young healthy cells, mitochondria constitute the greatest source of intracellular metabolic oxidant generation (30). The accumulation of oxidized mitochondrial proteins has been observed in aged tissues. Cultured human fibroblasts from individuals between 17 and 60 years of age show little or no change in protein oxidation, whereas cells from older individuals (60–80 years) contained significantly higher levels of oxidized proteins (30). In addition, these authors indicate that the rate of protein carbonyl accumulation is significantly higher in the mitochondrial fraction than in the whole-cell lysate (30). An age-related accumulation of altered (i.e., oxidized and glycoxidized) liver mitochondrial matrix proteins has also been associated (temporally) with diminishing Lon-like ATP-stimulated proteolytic activity in old (27-month) rats compared with young animals (16).

In this report, we show that the carbonyl content of WI-38 cells increases with replicative senescence, which agrees well with published data (12). In addition, we show that the level of Lon protein in early-passage and middle-passage cells is higher than senescent cells. This supports previous work in mice in which Lon mRNA, protein, and activity was significantly reduced in aged tissues (3,17,31). We also show that the Lon protease is inducible in young cells that have adapted to treatment with a low dose of H$_2$O$_2$, but this inducibility is only sluggish in middle-passage cells and completely lost in senescent cells. In the same experiments, H$_2$O$_2$-adapted early-passage cells exhibited no accumulation of oxidized proteins (carbonyls), middle-passage cells accumulated significant amounts of oxidized proteins, and senescent cells accumulated more than twice the accumulated oxidative protein damage of early- or middle-passage cells. The inducibility of Lon was previously shown by our laboratory to be protective against the accumulation production of oxidized proteins in Rhabdomascoma cells (7). In the present study, we conclude that, in early-passage WI-38 senescent cells, both high basal Lon levels and Lon inducibility contribute to the low levels of oxidative protein damage. In contrast, sluggish Lon induction (with stress) in middle-passage cells seems to permit increased accumulation of oxidized proteins. Finally, the low basal Lon level of senescent cells and the apparent complete loss of Lon stress inducibility predisposes these cells to extremely large accumulations of oxidized proteins that can compromise cell function and, even, survival.

Interestingly, in a similar study by Ahmed and colleagues (32), there was no apparent change in total Lon levels measured in mitochondrial isolates. The activity of Lon, however, actually increased with replicative senescence in the isolates (32), possibly through stress induction. The apparent contradictions between our results and that of Ahmed and colleagues (32) deserve serious consideration. In this context, it should be noted that Ahmed and colleagues (32) first isolated intact mitochondria, then measured Lon in the mitochondrial isolates. In the present study, we have measured Lon levels, Lon induction, and protein carbonyls in lysates of whole healthy WI-38 cells.
cells. Our results demonstrate significant alterations in mitochondrial mass and mitochondrial bioenergetics ($\Delta\psi_{m}$) with senescence. Our results are consistent with previous work showing that mitochondrial integrity declines with age (29). Thus, we think it likely that, in studying only intact mitochondria, Ahmed and colleagues (32) actually missed the senescent fraction of cells in which loss of mitochondrial integrity may have prevented successful mitochondrial isolation. In other words, Ahmed and colleagues (32) may well have only studied the remaining healthy mitochondria in senescent cells, thus missing the decline in Lon that we now report.

Our new studies further strengthen the important role of Lon inducibility in protecting cells against the fluctuating levels of stress experienced in vivo. Lon expression is essential for healthy mitochondria, and Lon downregulation causes alterations in mitochondrial morphology that strongly resemble the mitochondria of aged cells, including loss of cristae and appearance of both giant mitochondria and electron dense inclusion bodies (2). Lon silencing in "young" cells also causes significant apoptosis. It is, therefore, tempting to suggest that declining Lon synthesis and inducibility may actually contribute to the process of senescence. Although organismal aging is complex and a single round of cellular replicative senescence in vitro is not a direct analogy to aging, it does replicate important aspects of late-stage aging phenomena. These results may help explain why protein oxidation is seen to significantly increase in vivo during the last quarter of life span. In the broader sense, Lon may be only one of a number of stress-protective enzymes whose inducibility, or stress-responsiveness, is lost at the end of a life span, contributing to the rapid senescence that often precedes death.

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**References**


