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Free Radical Control of Senescence

by

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Abbreviations

BCNU	1,3-bis[2-chloroethyl]-2-nitrosourea
BSO	L-buthionine-[S,R]-sulphoximine
CPD	Cell Population Doublings
G6PD	Glucose-6-Phosphate Dehydrogenase
GCLS	Gamma-Glutamate Cysteine Ligase Synthase
GPx-1	Glutatihone Peroxidase-1
HUVEC	Human Umbilical Vein Endothelial Cells
HEDS	Hydroxyethyldisulfide
MnSOD	Manganese Superoxide Dismutase
PDL	Population Doubling Level
t-BHP	*tert*-Butyl Hydroperoxide

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Abstract

Eukaryotic cells have a finite lifespan which ends with senescence. Oxidative stress accelerates this process by increasing the rate of telomere decay. However, the mechanism responsible for this remains unknown. One probable explanation for this is free radical induced damage, or regulation of telomere associated proteins. The ribonucleoprotein Telomerase, and the DNA repair protein Ku70/80 are both pivot players in telomere maintenance. Recent studies have shown that shown that the activities of these two proteins can be changed by their redox state. Oxidative stress in cells has also been shown to decrease the activity of both *in-vivo*. The goal of this mini-proposal is to put forth the hypothesis that free radicals regulate telomere stability by altering the reduced thiols status of cells and influencing telomere maintenance proteins. Using published data from other studies I will describe the effect increased production of reactive oxygen species has on telomere biology. This will then be related to the function of telomere associated proteins that are also negatively regulated under the same circumstances. Finally, I propose three specific aims that will investigate our long term goal of understanding how free radical biology can affect aging.

Background and Significance

Cell immortalization and telomeres

Normal eukaryotic cells have a limited replicative lifespan. This was first described by Hayflick in 1965 where, in his landmark, paper he demonstrated that cells grown both in-vivo have a finite lifespan (1). Also early on it was noted that normal cells eventually capitulate to senescence, while cancer cells do not. Not until 1990 did it become apparent that telomeres might play a role in senescence (2). Telomeres are located at the ends of eukaryotic chromosomes with the repeat sequence of TTAGG (3). Originally their function was believed to be the protection of chromosome ends and maintain genomic stability (4). Work from Levy and colleagues showed that telomeres resolve the end replication problem associated with linear chromosomes, and have an association with aging (5). What leads to senescence in normal cells? The relationship between telomeres and aging seemed to be the breakthrough to explain how cells become older, serving as a "mitotic clock" for cell aging because they shorten by approximately 65 bp with each replication of the genome (2). With the discovery of Telomerase by Greider and coworkers in 1987, and the realization that immortalized cells have increased telomerase activity, made it seem like the mythical fountain of youth was becoming a reality (6, 7). However, we now know this is not the case because the mechanism that regulates the maintenance and function of telomeres in normal cells remains unknown.

Proteins maintain telomeres during cell division

Proteins involved in regulating telomere length have mushroomed beyond that of Telomerase. TRF1, and TRF2 are located at telomeres are have opposite effects on maintaining their stability. TRF1 seems to block telomere elongation by blocking the binding of other proteins. TRF2 binds the TTA repeat and interacts with many proteins during telomere

elongation (3). A novel class of poly(ADP-ribose) polymerases, Tankyrase-1 and Tankyrase-2, also have an important role in telomere maintenance (8-10). The last major contributor of telomere maintenance is the DNA repair protein Ku70/Ku80. It was originally believed to be involved in non-homologous end-joining but, in 1999 Hsu and coworkers found it localized to telomeres and postulated it might function in their maintenance (11).

Telomere associated proteins are controlled by redox

Cellular redox status influences the function of many proteins in cells. As a general rule proteins are more active when they are reduced. As described earlier in this review the apparatus controlling telomere growth contains many diverse proteins. Some of which have been shown to be governed by their redox state. Most important among these are Telomerase (12) and Ku70/80 (13). Being controlled by redox state makes them excellent candidates to link free radicals with telomere associated senescence. Telomerase is ribonucleoprotein that has reverse transcriptase activity and adds new telomeric repeats to the end of chromosomes to compensate for their loss during replication (4). It is composed of two subunits, hTERT, the enzymatic subunit, and hTR, the subunit that binds the RNA complementary to the telomeric repeat. Expression of Telomerase subunits is also increased in cell lines immortalized by SV40 transduction (7). Oxidative stress has been shown to inhibit Telomerase activity. Kurz *et al.* demonstrated this by treating HUVEC cells with BSO and measuring Telomerase activity (12). This blocked the production of glutathione and resulted in a three fold loss in telomerase activity which coincided with accelerated telomere shortening.

Ku70/80 is a heterodimer of consisting of a 70 kDa and a80 kDa subunits . It is most well known for its role in repairing double strand breaks. Since its identification as a telomere associated protein (11) it has been shown to physically interact with Telomerase (14), TRF2 (3),

and DNA associated protein kinase C (15). Yeast model systems have revealed that Ku70/80 is essential for telomere maintenance and the transcription of genes located near chromosomal ends (16). Moreover, Ku knockout mice have increased levels of cancer and exhibit a severe aging phenotype and radiation sensitivity. These phenotypes are further exacerbated in TERT/Ku double knockout mice (15, 17). The presence of 12 oxidizable cysteines in Ku make it an excellent candidate for redox regulation (13). A connection between Ku function and thiol status was first established by Ayene and coworkers using a Chinese hamster ovary cells null for G6PD. Oxidative stress significantly reduced Ku DNA binding in G6PD null cells, but had no effect in wild type or revertant lines (18). This offers a direct link between free radicals and the function of a protein that governs telomere maintenance.

Free radicals influence telomeres

Reactive oxygen species can be produced in mitochondria. The primary site for these leaks is in the electron transport chain. The common mtDNA mutation G11778A is located in the coding sequence of NADH dehydrogenase subunit 4 and manifests itself as Leber's hereditary optic neuropathy. Another mtDNA mutation, A3243G, encodes a defective tRNA. When the mutant tRNA is used by cells during translation defective electron transport chain proteins area produced. Hence, both of these mutations lead to increased ROS production. Oexle and Zwirner exploited this to show that white blood cells from these patients have higher rates of telomere decay than their respective age matched controls (19). This coincide with the overall length the telomeric repeats in these mutants being shorter than cells from patients of equal age. They conclude that this observation is probably the result of an early event associated with the ROS produced in the disorder. **This would suggest that ROS production has direct influence on telomere maintenance.**

Studies using normal human fibroblasts have demonstrated that hyperoxia increases the rate of telomere shortening from ~60 bp/division to ~500 bp/division **(20)**. Under hyperoxia the primary method of telomere degradation is by the formation of ssDNA breaks. Such a dramatic change in telomere stability quickly forces cells in senescence. Mitochondrial ROS was observed to have an influence by Saretzki and coworkers in 2003. By blocking ROS production with the mitochondrially targeted antioxidant compound MitoQ, hyperoxia induced telomere shortening was stopped and senescence somewhat delayed (21). **These findings are significant because they show how that scavenging ROS can "slow the hands of the mitotic clock".**

Peroxides have been observed to exert a negative influence on telomere maintenance. Growing human umbilical vein endothelial cells (HUVEC) for multiple doublings in *tert*-butyl hydroperoxide (*t*-BHP) increases telomere decay by 2-fold and pushes cells into premature senescence (12). Hydrogen peroxide on the other hand does not appear to influence telomere length in human foreskin fibroblasts (HFF). Yet it still pushes cells into senescence (22). However the HFF cells were treated only transiently with hydrogen peroxide and allowed undergo only two population doublings prior to telomere analysis. Therefore treatment schedules account for the disparity between these studies. The slow generation of ROS in cells is most likely similar to the first study. **Thus, reactive oxygen species negatively influence telomere growth. It also demonstrates a mechanism by which superoxide produced during electron transport can influence this process.**

Increased free radical production could possibly deplete the reduced glutathione pool and account for telomere decay. Cells harboring a Glucose-6-phosphate dehydrogenase (G6PD), and enzyme in the oxidative pentose phosphate cycle, lack the ability to maintain high steady state levels of NADPH, and quickly change their redox state after oxidative stress. HFF's derived

from individuals with mutations in this enzyme increase their population doubling level (PDL). This measures how well cells can divide, with a large value indicating poor cell proliferation (23). Entrance into senescence also occurred earlier for the G6PD deficient cells and is accompanied by a well-defined increase in doubling time. Lower levels of NADPH would hinder the cells from effectively turning over oxidized glutathione. Inhibition of glutathione synthesis by L-buthionine-[S,R]-sulphoximine (BSO) pushes HUVEC cells into early senescence, and activates telomere shortening (12). What we cannot infer from these findings is that maintaining a reducing environment would increase cell lifespan, and postpone senescence indefinitely. **However, this does allow us to ponder the possibility that a reducing environment in cells counters the influence of free radicals and allow them to reach their maximum life expectancy.**

Summary

Reactive oxygen species and reduced glutathione affect telomere maintenance. It is clear from the examples above that cells under the influence of oxidative stress senescence faster, and can be attributed to an increased rate of telomere degradation. Conversely reducing potential in the form of NADPH, or reduced glutathione, is probably needed to allow cells to actively grow until their telomeres are lost do to normal DNA replication processes. Chronic oxidative stress hastens telomere decay and entry into senescence (12, 19-21). At this time the exact mechanism behind this phenomenon remains unclear. What can push cells towards senescence? According to the unified theory of cell differentiation, aging, and cancer put forth by Oberley and collaborators, the production of superoxide has deleterious effects on cells (24). At the time of its formulation telomeres were not yet known to have roles in aging. Studies have shown that overexpression of MnSOD allows organisms to reach their maximal lifespan. Investigations into

what effect the expression of antioxidant enzymes have on telomere maintenance have yet to be undertaken. Production of ROS from the electron transport seem to initiate telomere shortening (19). The goal of this proposal will be to investigate the influence free radicals exert on telomere biology by altering the mechanism that maintains them. This will be done by citing specific examples of how free radicals control the machinery regulating telomere growth. My overlying hypothesis is that free radicals regulate telomere stability by altering the reduced thiols status of cells. **This hypothesis is grounded in the aforementioned studies demonstrating that both telomeres, and the proteins that regulate them are regulated by free radicals.**

Specific Aims

Senescence of normal cells can be linked to their rate of telomere degradation. This process is influenced by oxidative stress and the redox status of cells. However, the exact mechanism of how this occurs has yet to be established. The goal of this proposal is to investigate how oxidative stress can influence the function of the telomere maintenance proteins and lead to telomere shortening. ***My primary hypothesis for this application is that production of free radicals changes the activity of telomere maintenance proteins.*** In order to address this question we will alter the levels of superoxide and reduced thiols in primary cell lines. Assays will then be carried out to test the effects on telomere stability and the activity of telomere maintenance proteins. Previous studies have shown that reactive oxygen species and thiol status influence senescence by regulating telomeres and that the primary telomere associated proteins Telomerase and Ku are regulated by their redox state. My long term goal is to understand how free radical biology influences cell growth and aging. Understanding the regulatory mechanism of senescence will have direct benefits to the fundamental principles governing cellular aging. I therefore propose the following specific aims.

1) To investigate how superoxide production can influence telomere maintenance. The production of reactive oxygen species in mitochondria hastens the decay of telomeric repeats. Previous studies have shown that overexpression of manganese superoxide dismutase (MnSOD) can scavenge excess superoxide. One recent study has shown that oxidative stress can compromise telomere integrity and accelerate cellular senescence. Therefore *I hypothesize that increased superoxide scavenging by MnSOD will delay senescence after oxidative stress.* This hypothesis will be tested by measuring telomere maintenance in primary endothelial cells after the overexpression of MnSOD and induced oxidative stress by blocking the mitochondrial electron transport. This approach will demonstrate how superoxide produced in the mitochondria can influence telomere biology.

2) To measure the activity of telomere associated proteins in response to oxidative stress. Oxidative stress accelerates telomere shortening and premature entry into senescence. Current publications have demonstrated that oxidative stress initiated by peroxides can affect the activities of Telomerase. *My hypothesis for this aim states that oxidative stress leads to increased production of reactive oxygen species that inhibit the activity of telomere maintenance proteins.* This hypothesis will be addressed by subjecting cells to oxidative stress and measuring the activity of Telomerase and Ku70/80. This approach will demonstrate that metabolically produced reactive oxygen species disrupt telomeres by inhibiting the activity of the proteins that maintain them.

3) To evaluate the effects of thiol status on telomere maintenance. The redox status of Telomerase and Ku70/80 regulates their activities. Recent publications have demonstrated that a reduction in reduced thiols hastens cellular senescence and reduces the activity of Telomerase and Ku70/80. *I hypothesize that maintaining cells in a reducing environment will delay*

senescence by increasing the activities of Telomerase and Ku70/80. This hypothesis will be tested by growing primary cells in the presence of a reducing agent and measuring their changes their doubling times. Assays will also be carried out to determine the activities of Telomerase and Ku70/80 in these cells. This approach will demonstrate the mechanism by which thiols control senescence.

Preliminary Data (Adopted from other published works)

The production of reactive oxygen species in mitochondria hastens the decay of telomeric repeats. Initial studies have demonstrated that either the increased production of reactive oxygen species accelerates telomere degradation. The results from Oexle *et al.,* shown in **Figure 1,** demonstrate that white blood cells harvested from patients with respiratory chain mutations have shorter telomeres (19). Such mutations would increase the level of reactive oxygen species produced by cells. Notice the median telomere length of white blood cells from individuals harboring the A3243G mutation (odd numbered lanes **Figure 1A**) is less than their respective age matched control from normal subjects (even numbered lanes **Figure 1B**). Similarly WBCs

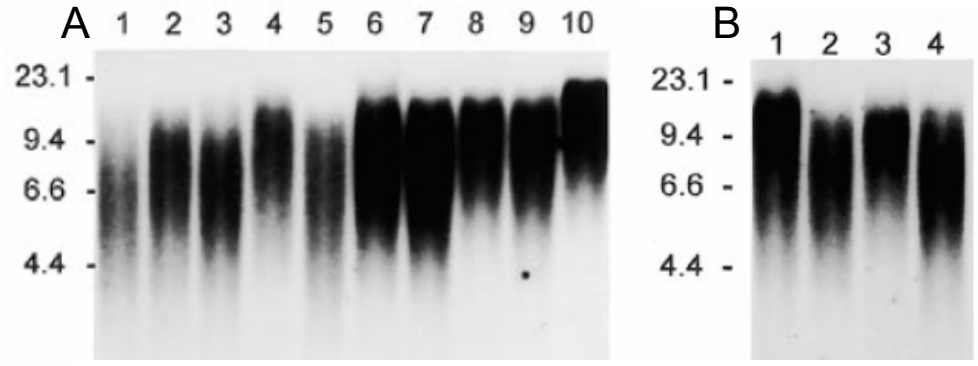

Figure 1. Telomere lengths in WBC DNA of five patients with the A3243G (A) or G11778A (B) mutation. A. Telomeric repeat analysis from patients aged 60 years (lane 1), 38 years (lane 3), 36 years (lane 5), 12 years (lane 7) and 4 years (lane 9) as compared to age-matched controls (lanes i + 1). B. Telomeric repeat analysis from patients aged 25 years (lane 2) and 30 years of age (lane 4) as compared to age matched controls (lanes i – 1) (19).

from patients with the mtDNA mutation G11778A, which is located in the coding sequence of NADH dehydrogenase subunit 4, have decreased telomere length (even lanes **Figure 1B**).

Conversely, the age matched controls seem to have longer telomeric repeats (even lanes **Figure 1B)**. Work by Saretzki *et al.* has shown that hyperoxia (40% oxygen) can increase the rate of telomere decay (21). **Figure 2** illustrates that treatment of MRC-5 fibroblasts with the non-reactive compound DPPT could not slow the accelerated rate of telomere decay under hyperoxia. Notice how the median telomere length in these cells (represented by the white line on the telomere southern blot) slowly declines with each population doubling under hyperoxic conditions. However, treatment of hyperoxic cells with MitoQ, a blocker of mitochondrial oxidative damage, protected their telomeric repeat. *These preliminary results by Oexle et al. and Saretzki et al. are important to this proposal by demonstrating that reactive oxygen species can play a role in accelerating telomere breakdown, which is essential for the proposal of Aim #1.*

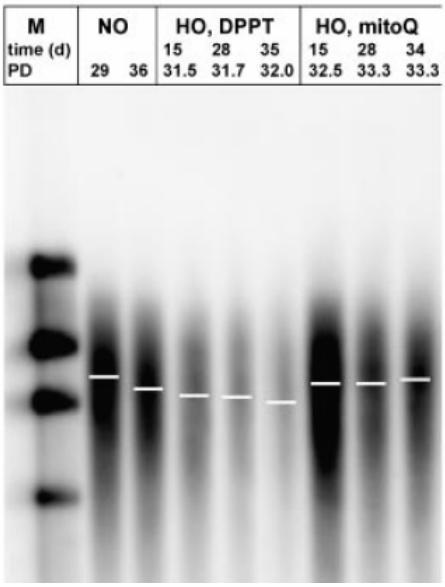

Figure 2 MitoQ counteracts telomere shortening under hyperoxia. Cells were grown under normoxia (NO) and, after one week pretreatment with either DPPT or MitoQ (50mM), under 40% hyperoxia (HO) (21).

Oxidative stress accelerates telomere shortening and premature entry into senescence. Work by Kurz and colleagues has shown that treatment of cells with peroxides pushes them into premature senescence (12). HUVEC cells were treated with 0.1μM *tert*-butyl

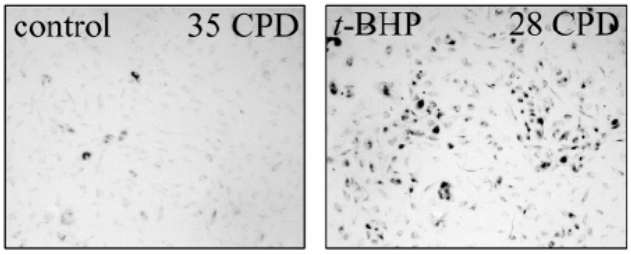

Figure 3: Premature onset of senescence under chronic oxidative stress. Brightfield photo-micrographs showing senescence-associated b-galactosidase staining of control and stressed HUVECs at similar CPD levels (12).

hydroperoxide (*t*-BHP) continually to investigate the affects of peroxides on telomere maintenance. Cells were stained to determine the number of β-galactosidase positive cells (**Figure 3**). Notice that cells treated with *t*-BHP contain high levels of β-galactosidase activity at 28 cell population doublings (CPD), while at 35 CPD the untreated cells have yet to begin senescence. This means that peroxide treated cells are entering senescence faster. A role for peroxides in regulation telomere maintenance proteins can be inferred from their results in **Figure 4** (12). From their results it can be seen that treatment with *t*-BHP increased the rate of telomere decay. Their subsequent analysis showed it had increased the rate of telomere decay by two fold (Not shown). *These described results from Kurz et al. are important to the proposed Aim #2 by demonstrating that oxidative stress by peroxides can accelerate a cells entry into senescence and possibly be attributed by inactivation of telomere maintenance proteins.*

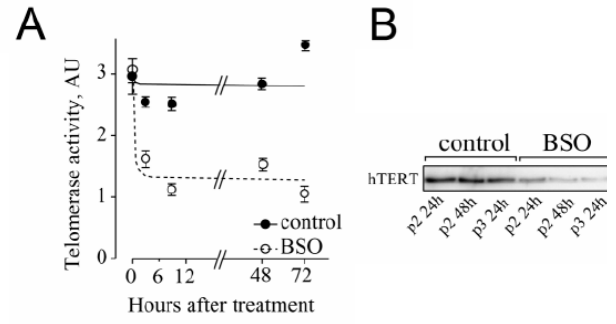

Figure 4: Rapid decrease in telomerase activity following exposure to oxidative stress. (A) Time course of telomerase activity in young HUVEC following a single exposure to 10 mM BSO. Values represent the mean (± s.d.) of duplicate cultures from representative experiments. (B) Immunoblots showing levels of hTERT in nuclear extracts at the indicated time points after the initial oxidant treatment at passage 2 and 3 (12).

The redox status of Telomerase and Ku70/80 regulates their activities. Altering the thiol status in cells leads to decreased telomerase activity. The results from Kurz *et al.,* shown in **Figure 4A** demonstrate that blocking the production of glutathione by BSO decreases Telomerase activity. Immunoblot analysis of hTERT in nuclear extracts show that BSO actually decreases the level of hTERT in these cells (**Figure 4B**). This indicates that premature senescence was not the cause of decreased telomerase activity, but is caused by a direct effect of

limited glutathione production on telomerase. Ayene *et al.* have shown that cells with an altered thiol status have decreased binding of Ku70/80. Using Chinese hamster ovary cell lines null for G6PD allowed them to decrease the level of reduced glutathione in cells with mild oxidative treatment with hydroxyethyldisulfide (HEDS). This works by limiting the production of NADPH which in turn inactivates the function of Glutathione Disulfide Reductase and lower the reduced glutathione pool in cells. Their results are shown in **Figure 5**. The binding activity of Ku70/80 is easily measured in gel mobility shift assays using total nuclear proteins. This has the great advantage of allowing **direct** measurement of Ku binding after changes have been made in cellular redox how cellular redox status. It can be clearly seen that the wild type K1 or A1A reverent cells do not respond to HEDS treatment, and hence maintain their DNA binding activity. They also determined that there was not change in the ratio of GSH/GSSG. Conversely the G6PD mutant E89 cells show a 75% reduction in DNA binding activity following HEDS treatment, and is accompanied by a decrease in the radio of GSH to GSSG ratio. *These findings allow us to propose Aim #3 by providing a direct link between the regulation of a telomeric maintenance proteins and cellular redox.*

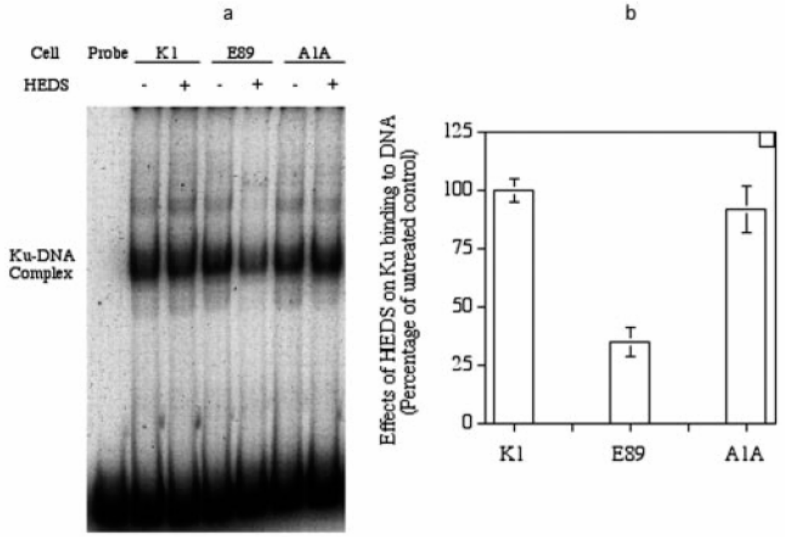

Figure 5: Ku binding is decreased in G6PD defieient E89 cells after HEDS treatment. Cells were pretreated with HEDS after which total cell extracts were obtained. EMSA were performed to selectively measure Ku binding activity (18).

Methods of Experimentation

Specific Aim 1: To investigate how superoxide production can influence telomere maintenance.

Rationale: The production of superoxide is a byproduct of normal respiration. During oxidative stress the level of superoxide produced by normal cells can increase. Mitochondrial superoxide dismutase scavenges superoxide and by dismutation converts it into hydrogen peroxide and molecular oxygen before further damage can occur. Previous studies have shown that chronic oxidative stress accelerates telomere decay (12, 19). I therefore believe that increased expression of MnSOD in primary cell lines will have a protective effect during oxidative stress and slow the rate of telomere decay. Recently it has become apparent that hydrogen peroxide can have deleterious effects on telomere biology (23). Therefore, the role of hydrogen peroxide in MnSOD experiments must also be examined by of overexpression of Glutathione peroxidase-1 (GPx-1) and mitochondrially targeted Catalase.

Experimental Design: Primary human umbilical vein endothelial cells (HUVEC) will be grown and subjected to oxidative stress by light inhibition of electron transport in the mitochondria. To test the role of superoxide cells in regulating telomere biology we will infect these cells with an Adenoviral vector that expressing MnSOD. Since the production of H_2O_2 must also be addressed, MnSOD will be coinfected with either GPx-1 or mitochondrially targeted Catalase. After approximately 4 population doublings cells will be reinfected with the Adenoviral vectors to keep the levels of these antioxidant proteins high. After approximately 30 population doublings the length of telomeres in these cells will be determined along with the activity of MnSOD.

Anticipated results and alternative approaches: I anticipate that cells overexpressing MnSOD

will have decreased decay of telomeric repeats and increased levels of MnSOD. **I acknowledge that successive infections with an Adenovirus could induce artifacts in my system.** However, this will be controlled by infection cells with a virus that contains no payload. **If cells cannot be efficiently transduced, stably transfected cell lines overexpressing MnSOD alone or in combination with Catalase or GPx-1 will be created.** This approach will demonstrate the regulatory role superoxide has on telomere stability.

Specific Aim 2: To measure the activity of telomere associated proteins in response to oxidative stress.

Rationale: Accelerated telomere decay has been observed in cell lines exposed to oxidative stress (12). It has also been observed that the activity of telomerase and DNA binding by Ku70/80 can be inhibited under oxidizing conditions (18). Therefore, on the basis of published results, I believe that oxidative stress must be inhibiting the function of Telomerase and Ku70/80 and lead to the accelerated decay of telomeres. Therefore the activity of these proteins will be measured after cells have been subjected to long term oxidative stress.

Experimental Design: HUVEC cells will be grown in the presence of 0.1μM *t*-BHP for 30 population doublings. The length of telomeric repeats will then be analyzed using Southern blotting. To determine how oxidative stress influences the enzymatic activity of Telomerase assays similar to those employed by Kruz *et al.* will be used (12). The DNA binding activity of Ku70/80 will be assayed using selective conditions in an electron mobility shift assay (EMSA) similar to that used in the preliminary results from Ayene *et al.* (18).

Anticipated results and alternative approaches: I expect that the activity of Telomerase and DNA binding of Ku70/80 will be decreased after exposure to chronic oxidative stress by *t*-BHP.

This result would be in agreement with other published showing their decreased activity *in-vitro*. **We do not foresee any difficulties in employing these assays under these growth conditions. Both have been successfully used in multiple times in published studies in peer reviewed journals.** These approaches will effectively determine the effect of oxidative stress on telomere associated proteins.

Specific Aim 3: To evaluate the effects of thiol status on telomere maintenance.

Rationale: Many proteins have increased activities in a reduced state. Recent published accounts from other laboratories have shown that altering the cellular thiol pool by blocking gamma-glutamate cysteine ligase synthase (GCLS) using BSO, or disrupting the function of G6PD can significantly inhibit the function of both Telomerase and Ku70/80 (12, 18). It would therefore seem acceptable that maintaining cells under reducing conditions by exogenous means would keep these proteins operating at their maximal levels and delay senescence.

Experimental Design: HUVEC cells will be maintained in the presence or absence of reducing agents and the number of population doublings to reach senescence will be determined. Cells will also be treated with 1,3-bis[2-chloroethyl]-2-nitrosourea (BCNU) to lower their level of reduced glutathione. Total and reduced glutathione will also be quantified to determine what effect these culture conditions have on the redox status in these cells. The role of reduced thiols on telomere stability will be measured using Southern blot analysis on cells that have undergone several population doublings.

Anticipated results and alternative approaches: I expect that cells grown in the presence of a mild reducing agent will have delayed senescence and stabilized telomeres. **Growing cells under reducing conditions could have cytotoxic effects. We would then attempt to increase**

the levels of reduced glutathione by growing cells in high Glucose media to increase the production of NADPH by the oxidative pentose phosphate pathway. Together experimental approaches will demonstrate the role of redox in regulating senescence.

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