Senescence or apoptosis? The choice bovine fibroblasts make in the presence of increasing concentrations of extracellular H₂O₂

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Reactive oxygen species (ROS) are potent inducers of oxidative damage and have been linked to the regulation of specific cellular functions, including apoptosis. In response to specific signals, mitochondrial H₂O₂ can trigger mitochondrial swelling and apoptosis; however, as a protective measure, cells can also enter a state of senescence. The purpose of this study was to determine the effects of extracellular H₂O₂ on cellular senescence induction via the mitochondrial ROS-signaling pathway. Bovine fibroblasts were synchronized at the G1/S phase using a thymidine-nocodazole mitotic block and treated for two hours with H₂O₂ concentrations: 25µM, 50µM, 100µM, 150µM and 200µM. Post H₂O₂ treatment, the number of floating dead cells was quantified using a hemocytometer. Cells sticking to the bottom of the culture dish were then washed with fresh media and cultured for 24 hours at 39°C at 5% CO₂ under humidified conditions and assayed for senescent cell quantification using SA-β-galactosidase staining assay. Preliminary data indicates that the number of dead cells increased with increasing H₂O₂ dosage, with the maximum number of dead cells at 200µM in both cell lines. Along with the increase in number of dead cells, the number of senescent cells decreased up to 50µM, and no senescent cells were detected at ≥ 50µM concentrations. This indicates that cells are sensitive to rising levels of H₂O₂ and make a choice to enter the senescent or apoptotic pathway based on the oxidative insult they face. Future studies would focus on the molecular mechanism of the mitochondrial-ROS signaling pathway.

Oxidative stress evolves from the production of reactive oxygen species (ROS), which are naturally occurring substances (oxygen ions, free radicals, peroxides) derived both exogenously through environmental factors and endogenously via mitochondrial production.¹ Within the mitochondria, a complex series of events leads to the generation of ATP via the electron transport chain, along with the production of ROS as a byproduct.¹ The presence of ROS leads to oxidative stress -- a common determinant in cellular aging. Currently, the free-radical hypothesis is one of the most accepted theories of aging, as research indicates that aerobic organisms are constantly producing ROS, causing oxidative damage to accumulate over time.¹ This accumulation can result in such damaging effects as cell death, chromosomal abnormalities and cancer-inducing mutations. While apoptosis has long been recognized as a cellular mechanism used to prevent cellular transformation and cancer, cellular senescence has also been examined for its potential protective effects; however, this protection is believed to come at the cost of cellular aging.²

Cellular senescence is a state of permanent cell growth arrest resulting from the inability to divide after a fixed number of cell divisions, as defined by the Hayflick limit. This limit applies a numerical value to somatic cell lines that corresponds to the number of replications cells can undergo before they enter a state of cellular senescence.³ Almost all

cellular systems exhibit a senescent state (excluding immortalized cells) after a given number of replications, although the onset and progression varies between organisms and tissues.⁴ Cellular senescence is believed to be involved in aging by directly contributing to the functional decline of aging tissues or by indirectly affecting the surrounding non-senescent cells.¹

Telomeres, located at the ends of mammalian chromosomes, appear to play a critical role in activating the senescent state. During somatic cell replication, the repetitive telomeric DNA sequence (TTAGGG)ⁿ shortens due to the lack of DNA repair mechanisms.⁵ Research has indicated that once telomere length shortens beyond a specific threshold, a p53 dependent pathway is activated that permanently halts cell division.⁶ Recently, it has been shown that telomere 'uncapping' itself can signal the senescent state⁷, which could explain how cells can prematurely enter a state of cell growth arrest despite having long telomeres *in vivo* or after exposure to oxidative stress.⁸ Research that examined the effects of oxidative stress on telomere length has shown evidence that oxygen free radicals can cause single-stranded breaks in telomeric DNA that accumulate and shorten the replicative lifespan of fibroblasts.⁹ This evidence is strengthened by a study demonstrating that increased antioxidant activity (associated with decreasing the

Figure 1. A (left) shows the number of dead bovine fibroblasts (M205 cell line) resulting from increasing H₂O₂ exposure. B (right) shows the number of bovine fibroblasts (M205 cell line) in a state of senescence following increasing H₂O₂ exposure.

rate of telomere shortening) led to a prolonged lifespan in human fibroblasts.¹⁰

Based on this evidence, it has been suggested that increased oxidative stress and ROS production can induce and/or accelerate the onset of cellular senescence, as cellular senescence is linked to the redox state of the environment.¹ In particular, redox enzymes can generate mitochondrial H₂O₂ (a type of ROS) in response to specific signals, resulting in mitochondrial swelling and apoptosis. Depending on the amount of mitochondrial ROS produced and its subsequent release from the mitochondria, it has been suggested that cells will either enter a senescent or apoptotic pathway. Therefore the purpose of this study is to determine the effects of extracellular H₂O₂ on cellular senescence induction via the mitochondrial ROS-signaling pathway. It is hypothesized that at low concentrations of H₂O₂, more cells will choose to enter a state of senescence, whereas at high concentrations of H₂O₂, more cells will enter a state of apoptosis.

MATERIALS AND METHODS

Materials

Hydrogen peroxide, thymidine, nocodazole and DMEM were obtained from Sigma-Aldrich (Oakville, ON). Senescence β-galactosidase Staining Kit was obtained from Cell Signaling Technology, Inc. (Pickering, ON).

Cell Culture

Cryogenically frozen bovine fibroblast cells from two different cell lines (1007 and M205) in liquid nitrogen at -150°C were thawed in a 37°C waterbath, washed and

centrifuged to remove remaining frozen media. Cells were grown on six 35 mm well culture plates in 2ml of Dulbecco's modified Eagle medium (DMEM) with 10% FBS, 1% Pen-Strep and 2% L-glutamine.

Cell Synchronization

When cells reached 40% confluency, old media was removed and 2ml of DMEM (10% FBS, 1% Pen-Strep, 2% L-glutamine) containing 2mM thymidine was added for 24 h (S-phase block). After thymidine block, thymidine was removed by washing with 2ml PBS and fresh DMEM (10% FBS, 1% Pen-Strep, 2% L-glutamine) was added for 3h to release cells. After 3h, 100ng/ml nocodazole was added to the media for 12h (mitotic block). Nocodazole was removed by washing with 2ml PBS and 2ml of fresh DMEM (10% FBS, 1% Pen-Strep, 2% L-glutamine) was added. Cells then progressed synchronously through G1- and S-phase.

H₂O₂ Treatment

When cells reached 70% confluency, concentrations of 0, 50, 100, 150 and 200 μl of H₂O₂ in 2ml DMEM (10% FBS, 1% Pen-Strep, 2% L-glutamine) were added individually to each of the six wells for 2h. After 2h, the media containing H₂O₂ was removed and stored separately in 15ml tubes. Each well was replenished with fresh media and returned to the incubator at 39°C at 5% CO₂ under humidified conditions for 24 hours.

Dead cell quantification

Using a hemocytometer and 11μl of media containing H₂O₂, the number of dead cells were counted for each H₂O₂ concentration (already stored separately in 15ml tubes) in

Figure 2: A (left) demonstrates the number of dead bovine fibroblasts (1007 cell line) resulting from increasing H₂O₂ exposure. B (right) demonstrates the number of bovine fibroblasts (1007 cell line) in a state of senescence following increasing H₂O₂ exposure.

triplicates. An average was calculated in order to determine the number of dead cells resulting from a specific concentration of H₂O₂. This process was repeated for each of the concentrations of H₂O₂ used.

SA-β-galactosidase staining

24h post-H₂O₂ treatment, cells were treated with senescence associated (SA)- β-galactosidase staining. A 10x staining solution and 10x fixative solution were both diluted with distilled water to make 1x solutions. The media was removed from the cells and the wells were washed once with 2ml PBS. The cells were fixed with 1ml of 1x Fixative Solution for 12.5 min at room temperature (21°C). The wells were washed twice with 2ml PBS and 1ml of β-galactosidase stain (930 μl staining solution, 10 μl 500 mM potassium ferrocyanide, 10 μl 500 mM potassium ferricyanide, 50 μl 20 mg/ml X-gal in DMF). Cells were incubated overnight at 39°C at 5% CO₂ under humidified conditions.

Imaging

Images were obtained using phase contrast light microscopy. Four pictures were taken of each well. The number of β-galactosidase stained cells versus unstained cells were counted and used to calculate a final average ratio of the number of stained to unstained cells in each well.

RESULTS AND DISCUSSION

This study demonstrates that as the levels of H₂O₂ increase, bovine fibroblasts undergo less senescence yet exhibit higher apoptotic levels. The number of dead cells increased with increasing H₂O₂ dosage, with the maximum number of dead

cells at 200μM in both cell lines (**Figure 1A and 2A**). At the same time, as concentrations of H₂O₂ increase, the number of senescent cells in both cell lines decreased up to 150μM and no senescent cells were detected at concentrations of 200μM (**Figure 1B and 2B**). This suggests that at low levels of H₂O₂, cells can enter a senescent state as a protective mechanism, however as the level of H₂O₂ increases, it overwhelms the senescent state induced-protection causing an increase in cell damage. This results in the cell entering an apoptotic state instead. These results were found for both cell lines, suggesting that a variety of bovine fibroblast cells all respond in the same manner following exposure to H₂O₂.

One limitation of this study is the levels of senescence were found to be higher than expected in untreated cells. This could be due to the number of cell passages completed, as the cells were treated with H₂O₂ at passage twelve. Research has shown that the level of senescence of cultured bovine fibroblasts does not vary greatly until they have been passaged over twenty times, indicating that high passage rates most likely are not the cause of the high senescence levels. As well, unpublished data from this lab of younger cells (passage 4/5) that were unsynchronized demonstrated similar results in the control group. Another potential cause of the high levels of senescence is oxygen tension levels, as high oxygen tension has been associated with an increase in senescence.¹ Attempts were made to control for high oxygen tension as cells were stored in low oxygen-incubators in order to limit the potential interaction between cells and high oxygen tension.

β-galactosidase staining also demonstrated some of the morphological changes that are characteristic of senescent cells such as gross enlargement and binucleation (**Figure 3**).

Figure 3: Demonstrates bovine fibroblasts (M205 cell line) treated with SA-β galactosidase post-H₂O₂ treatment. Top row from left to right: 0μM, 25μM, 50μM of H₂O₂. Bottom row, left to right: 100μM, 150μM, 200μM of H₂O₂.

As the concentration of H₂O₂ increased, the cells also began to appear more fragmented and of variable densities. This variation is indicative of a damaged cell undergoing apoptosis (as characterized by unpublished data obtained from this lab) and further strengthens the results from **Figure 1 and 2**.

In conclusion, these results demonstrate that an inverse proportional relationship exists between H₂O₂ exposure and cells in senescence; however, increasing levels of H₂O₂ elicit a directly proportional change in apoptotic levels. This indicates that cells are sensitive to rising levels of H₂O₂ and make a choice to enter the senescent or apoptotic pathway based on the oxidative insult they face. While this research has direct implications for all age-related diseases, such as Parkinson's and Alzheimers, and may have potential to prevent, reverse or slow down cellular aging, mechanisms by which cellular senescence occurs need to be further elucidated.

FUTURE EXPERIMENTATION

Future studies will look at MitoSox staining to directly examine changes in superoxide levels in the mitochondria in order to determine the specific level of mitochondrial ROS production. In order to further characterize the mitochondrial ROS signaling pathways, changes in specific gene expression of proteins involved in the pathway should be examined. Flow cytometry can also be used to further

characterize the dead cells in order to unequivocally demonstrate that they are in a state of apoptosis.

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