An Accumulation of Senescent Cells May Contribute to Chronic Inflammation of the Mammalian Liver

by
Aaron Sugiyama

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Biochemistry and Biophysics
(Honors Associate)

Presented August 23, 2016
Commencement June 2016

AN ABSTRACT OF THE THESIS OF

Aaron Sugiyama for the degree of Honors Baccalaureate of Science in Biochemistry and Biophysics presented on August 23, 2016. Title: An Accumulation of Senescent Cells May Contribute to Chronic Inflammation of the Mammalian Liver.

Abstract approved:_____

Tory Hagen

ABSTRACT: The aging process is a biological phenomenon in mammals that heavily contributes to the onset of chronic diseases. While there are many theories that attempt to explain the biological onset of aging, one emerging theory involves chronic inflammation. Chronic inflammation has been previously linked to several age-related diseases and is a known driver of chronic inflammation. To examine chronic inflammation and cellular senescence in the context of aging, we examined hepatocytes harvested from rat livers as a model. We found that rat hepatocytes not only undergo cellular senescence, but that senescent cells accumulate with age. This accumulation of senescent cells with age was also accompanied by a large increase in miR-146a and miR-155 (a seven fold and a forty-five fold change respectively) in the old rat exosomes, which indicate chronic inflammatory insult. These findings corroborate with our findings regarding an accumulation of senescent cells in the liver, and suggests that the liver undergoes an inflammatory stress. We then examined a possible treatment for cellular senescence: the drug known as dasatinib. This drug showed promising results in both reducing senescent cell populations and preventing the onset of cellular senescence in HepG2 cells.

Key Words: inflammation, aging, senescence, dasatinib, hepatocytes.

Corresponding e-mail address: aaron.m.sugiyama@gmail.com

An Accumulation of Senescent Cells May Contribute to Chronic Inflammation of the Mammalian Liver

by
Aaron Sugiyama

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Biochemistry and Biophysics
(Honors Associate)

Presented August 23, 2016
Commencement June 2016

Honors Baccalaureate of Science in Biochemistry and Biophysics project of Aaron Sugiyama presented on August 23, 2016.

APPROVED:

_____

Tory Hagen, Mentor, representing Biochemistry and Biophysics

_____

Viviana Perez, representing Biochemistry and Biophysics

_____

Kate Shay, representing Linus Pauling Institute

_____

Toni Doolen, Dean, Oregon State University Honors College

I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

_____

Aaron Sugiyama, Author

Acknowledgments

I would not be here today without the support of my entire family: my parents Calvin and Karen Sugiyama, my sister Amy, my brother Allen, my brother-in-law Dan, my sister-in-law Kim, my nephew Declan, and my niece Jocelyn.

I would also not be here today without the help and support of the entire Hagen Lab, including Dr. Tory Hagen, Dr. Kate Shay, Nicholas Thomas, Amanda Kelley, Judy Butler, and Tong Wu. I would also like past Hagen lab members, Brian Ceballos and Chloe Donegan. I would also like to thank two teachers, Dr. Kari Van Zee and Dr. Viviana Perez for their mentorship and guidance.

Table of Contents

List of Figures

Chapter 1

Aging, Inflammation, and Cellular Senescence

Introduction

The aging population and chronic disease

Advancements in science and technology have extended human lifespan significantly over the last century alone. The average lifespan in the United States is approaching 74 years in men and 79 years in women, an increase from 48 and 50 years respectively at the turn of the century (National Center for Health Statistics, 2004). In a report published by the U.S. Census Bureau, it is projected that by the year 2030, the U.S. population over the age of 65 will constitute 19.6% of the total population. This shift is unprecedented when one considers that this age group only constituted 12.4% of the total population in 2010 (U.S. Census Bureau, 2010). This change in demographics ushers in new challenges and complications, many of which revolve around the increased prevalence of chronic disease.

While biological aging is not considered a disease, advancing age significantly increases the risk for chronic diseases. The Centers for Disease Control reports that in 2010, seven of the top ten leading causes of death in the U.S. were chronic illnesses, such as cardiovascular disease, cancer, respiratory disease, stroke, Alzheimer's, and diabetes. Both the transition in demographics and increase in chronic disease occurrence together pose an immenint threat on the United States' health care system, especially since it is estimated that 25% of individuals over the age of 65 have two or more chronic diseases (Death and Mortality, 2016). Considering that treatments for said chronic diseases accounts for nearly two-thirds of

the nation's health care spending, the significant increase in the aging population will place an ever-increasing burden on the national public healthcare costs and infrastructure (Public Health and Aging, 2010). As chronic illness is both debilitating to the nation's healthcare budget and reduces the quality of life of millions of Americans, it is pertinent that steps must be taken to better understand the mechanisms responsible for the pathogenesis of chronic diseases.

Chronic Inflammation

A major hallmark of aging is an overall decline of bodily functions. For mammals, this degeneration may lead to heart failure, neurodegeneration, muscular atrophy, and several other disorders (Campisi, 2013). Moreover, aging is one of the greatest risk-factors for chronic diseases (López-Otín, 2013). While there is no definitive cause for aging and chronic disease acquisition, several theories exist that attempt to explain both of these phenomena. Chronic inflammation is one such process that may play a significant role in tissue damage and disease. Chronic inflammation should not be confused with acute inflammation, which is the immune response that is characterized by a rapid infiltration of macrophages at a wound or infection (Diegelmann, 2004). This acute response contributes to wound healing, pathogen clearance, and is essential for survival (Feghali, 1997). Chronic inflammation, which is known as "sterile inflammation" occurs in the absence of pathogens (Kubes, 2012). Furthermore, chronic inflammation is a low-grade form of inflammation that accompanies advanced age and may play a critical role in tissue

fibrosis, dysfunction, and death (Baylis, 2013). The mechanism responsible for tissue death is attributed to inadequate clearance of both damaged cells and immune cell from the tissue, leading to an increased deposition of dysfunctional cells, destructive factors, and reactive oxygen species (Deigelmann, 2004). Additionally, the factors released by immune cells as part of the chronic inflammation response can manipulate the microenvironment of the tissue, resulting in further cellular dysfunction by disrupting nearby healthy cells (Rodier, 2011). Previous investigations have elucidated a correlation between chronic inflammation and several chronic diseases such as heart disease, cancer, neurodegeneration, and diabetes (Salminen, 2012). Although it has been established that chronic inflammation accompanies the aging process and may play a part in its onset, it is still unknown as to which factors contribute the most to the promotion of age-related inflammation.

Cellular senescence

One process that may exacerbate chronic inflammation is cellular senescence. Cellular senescence was first described by Hayflick, who found that there is a finite number of cellular divisions before cells would cease dividing *in vitro* (Hayflick, 1961). While cellular senescence was first thought to only be a cell culture artifact, it has been since shown to occur *in vivo*. Cellular senescence is one cell fate that can occur in response to a stressor such as oxidative stress, DNA damage, genotoxic stress, and mitochondrial dysfunction among other things (Coppé, 2010). A major

hallmark of cellular senescence is the essentially permanent arrest in cellular proliferation (Nair, 2015). Once a cell is in this non-replicative state, it remains metabolically active, and can release pro-inflammatory agents as part of its Senescent Associated Secretory Phenotype, or SASP (Coppé, 2010). Chemicals released as part of the SASP (which includes peptide hormones, exosomes, cytokines etc.) helps recruit immune cells to the site of the damaged tissue (Re, 2009). This response has been shown to not only speed up wound repair, but also promote tissue formation in young, healthy organisms (Demaria, 2014). It is hypothesized that cellular senescence is a mechanism that suppresses cancer proliferation by inhibiting cellar division and by promoting inflammation at the site of tissue damage, which, supposedly, should result in clearance of the potentially cancerous cells (Coppé, 2008). While this mechanism is important for the survival of an organism, particularly in young organisms, it seems apparent that senescent cells are not cleared as efficiently as an organism ages, thus they have been observed to accumulate in certain tissues with increasing age (Nair, 2015). For instance, it has been previously determined that human fibroblasts are capable of undergoing cellular senescence and have been found to accumulate with advanced age (Dimri, 1995).

Markers for cellular senescence

One hallmark of cellular senescence is increased lysosomal activity at sub-optimal pH levels (Rayess et al, 2015). Lysosomes of most cells contain β-galactosidase, an enzyme that normally participates in the catabolism of complex

carbohydrates. In normal, non-senescent cells, this enzyme has an optimal pH between 4.0-4.5. However, in senescent cells, this enzyme is able to fully function at pH levels around 6.0 (Kurz, 2000). A staining solution at a pH between 5.9 and 6.1 can be applied to a sample of cells; if senescent cells are present, the β-galactosidase in these lysosomes will cleave a substrate in the staining solution within this pH range, producing a blue precipitate (Kurz, 2000). While β-galactosidase is commonly used as a marker for senescence, it has also received criticism for not being highly specific for senescent cells (Severino, 2000). For the purposes of this investigation, β-galactosidase will serve as an acceptable marker of senescence. This investigation also examined components of the SASP response as a marker of senescence and inflammation.

Additional markers for cell senescence that were not employed in our studies includes elevated levels of p53 and p16 (proteins involved in cell cycle regulation), telomere shortening, pro-inflammatory agents (such as cytokines and interleukins) and senescence-associated heterochromatic foci (Jesus, 2012).

Exosomes and miRs 146a and 155

Exosomes are small vesicles (40-100 nm) that are released via endocytosis by various cell types including but not limited to: immune cells, tumor cells, epithelial cells, and dendritic cells (Xu, 2013). It has also been found that senescent cells release a variety of contents as part of the SASP response, and one of these released products includes exosomes (Lehmann, 2008). Exosomes are formed intracellularly

and are released by the inward budding of the endosomal membrane, which can then fuse to the plasma membrane of nearby cells (Esser, 2010). Furthermore, exosomes are believed to play a role in cell-signaling (Xu, 2013) and have also been found to be released during the early stages of apoptosis (Mathivanan, 2010). Prior studies have found that exosomes released by macrophages and dendritic cells can contain enzymes that contribute to the synthesis of leukotrienes, which are a class of proinflammatory lipid mediators (Esser, 2010). Additionally, exosomes are known to contain a variety of different types of cargo, including but not limited to: cytosolic proteins (such as tubulin and actin), intracellular membrane fusion and transport proteins (such as annexins and Rab proteins), metabolic enzymes (such as peroxidases, pyruvate kinases, and lipid kinases), heat shock proteins (HSPs, such as HSP70 and HSP90), tetraspanins, integrins, mRNA, and miRNA (Théry, 2002). The last component listed, miRNA, is of particular interest to this thesis project.

miRNAs are short (approximately 19 to 22 nucleotides long), non-coding segments of RNA that serve a variety of regulatory functions (Doxaki, 2014). Exosomes have been shown to contain large quantities of miRNAs and are believed to play a role in preventing the degradation of miRNAs and to help facilitate their transport to neighboring cells (Han, 2015). Two specific miRNAs were of particular interest to our endeavors: 146a and 155. miR-146a is known to be involved in the negative feedback loop that prevents overexpressing and secretion of proinflammatory agents (Olivieri, 2015). miR-146a also plays an important part in macrophage activation (Vergadi, 2014). miR-155 is known to regulate T-cell fitness and acts as an anti-inflammatory factor (Huffaker, 2012).

Using QPCR, it is possible to detect relative levels of the miRs 146a and 155 from exosomes found in young and old rat hepatocytes. These miRs will not only serve as additional markers of cellular senescence in the liver, but will also give insights as to what pro-inflammatory products are being released by aged liver cells. We hypothesize that there is an upregulation of both miR-146a and miR-155 in the aging rat liver.

Objectives

It is unknown at this time as to what factors contribute the most to chronic inflammation. We suspect that cellular senescence plays a pivotal role in chronic inflammation and the aging process. While it is known that senescent cells accumulate in human skin fibroblasts, there is still relatively little evidence on senescent cells accumulating in metabolic organs. The primary objective of this investigation was to determine if cellular senescence occurs in the liver, a highly metabolic organ. The liver is an appropriate organ system to study in the context of aging, since it is known to have a progressive decline in functionality with age. Our studies suggest that there is indeed not only senescent cells present in the rat liver, but that senescent cells accumulate in the liver with age. The next objective was to determine if two important pro-inflammatory agents, miR-146a and miR-155, were elevated in the aging rat liver. After exosome and miRNA isolation, it was determined that there was an elevation in both of those miRNAs in the exosomes from old rat hepatocytes compared to levels found in exosomes isolated from young

rats. The final objective was to evaluate the effectiveness of a potential therapy for cellular senescence.

References

1. Baylis, D., Bartlett, D. B., Patel, H. P. & Roberts, H. C. Understanding how we age: insights into inflammaging. *Longev Healthspan* **2,** 8 (2013).
2. Campisi, J. Aging, Cellular Senescence, and Cancer. *Annu Rev Physiol* **75,** 685–705 (2013).
3. Centers for Disease Control and Prevention. Death and Mortality. NCHS FastStats Web site. http://www.cdc.gov/nchs/fastats/deaths.htm. Accessed July 28, 2016.
4. Coppé, J.-P. *et al.* Senescence-Associated Secretory Phenotypes Reveal Cell-Nonautonomous Functions of Oncogenic RAS and the p53 Tumor Suppressor. *PLOS Biol* **6,** e301 (2008).
5. Coppé, J.-P., Desprez, P.-Y., Krtolica, A. & Campisi, J. The Senescence-Associated Secretory Phenotype: The Dark Side of Tumor Suppression. *Annu Rev Pathol* **5,** 99–118 (2010).
6. Demaria, M. *et al.* An Essential Role for Senescent Cells in Optimal Wound Healing through Secretion of PDGF-AA. *Dev Cell* **31,** 722–733 (2014).
7. Diegelmann, R. F. & Evans, M. C. Wound healing: an overview of acute, fibrotic and delayed healing. *Front. Biosci.* **9,** 283–289 (2004).
8. Dimri, G. P. *et al.* A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci U S A* **92,** 9363–9367 (1995).
9. Doxaki, C., Kampranis, S. C., Eliopoulos, A. G., Spilianakis, C. & Tsatsanis, C. Coordinated Regulation of miR-155 and miR-146a Genes during Induction of Endotoxin Tolerance in Macrophages. *J Immunol* **195,** 5750–5761 (2015).
10. Esser, J. *et al.* Exosomes from human macrophages and dendritic cells contain enzymes for leukotriene biosynthesis and promote granulocyte migration. *Journal of Allergy and Clinical Immunology* **126,** 1032–1040.e4 (2010).

11. Feghali, C. A. & Wright, T. M. Cytokines in acute and chronic inflammation. *Front. Biosci.* **2,** d12–26 (1997).
12. Han, C. *et al.* Exosomes and Their Therapeutic Potentials of Stem Cells. *Stem Cells International***2016,** e7653489 (2015).
13. Hayflick, L., Moorhead, P.S. The Serial Cultivation of Human Diploid Cell Strains. Experimental Cell Research, 1961, Dec;25:585-621.
14. Huffaker, T. B. *et al.* Epistasis between microRNAs 155 and 146a during T cell-mediated antitumor immunity. *Cell Rep* **2,** 1697–1709 (2012).
15. Jesus, B. B. de & Blasco, M. A. Assessing Cell and Organ Senescence Biomarkers. *Circ Res* **111,**97–109 (2012).
16. Kubes, P. & Mehal, W. Z. Sterile Inflammation in the Liver. *Gastroenterology* **143,** 1158–1172 (2012).
17. Kurz, D. J., Decary, S., Hong, Y. & Erusalimsky, J. D. Senescence-associated (beta)-galactosidase reflects an increase in lysosomal mass during replicative ageing of human endothelial cells. *J. Cell. Sci.* 113 (Pt 20), 3613–3622 (2000).
18. López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M. & Kroemer, G. The Hallmarks of Aging.*Cell* **153,** 1194–1217 (2013).
19. Mathivanan, S., Ji, H. & Simpson, R. J. Exosomes: Extracellular organelles important in intercellular communication. *Journal of Proteomics* **73,** 1907–1920 (2010).
20. Nair, R. R., Bagheri, M. & Saini, D. K. Temporally distinct roles of ATM and ROS in genotoxic-stress-dependent induction and maintenance of cellular senescence. *J Cell Sci* 128, 342–353 (2015).
21. National Center for Health Statistics (US) . Health, United States, 2004: With Chartbook on Trends in the Health of Americans. Hyattsville (MD): National Center for Health Statistics (US); 2004 Sep. Chartbook on Trends in the Health of Americans.
22. Olivieri, F. *et al.* DNA damage response (DDR) and senescence: shuttled inflamma-miRNAs on the stage of inflamm-aging. *Oncotarget* **6,** 35509–35521 (2015).

23. Prescription Drug Use Among Midlife and Older Americans. Available at: http://assets.aarp.org/rgcenter/health/rx_midlife_plus.pdf. (Accessed: 1st August 2016).
24. Public Health and Aging: Trends in Aging --- United States and Worldwide. Available at: http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5206a2.htm. (Accessed: 17th March 2016)
25. Rayess, H., Wang, M. B. & Srivatsan, E. S. Cellular senescence and tumor suppressor gene p16. *Int. J. Cancer* 130, 1715–1725 (2012).
26. Re, R. N. & Cook, J. L. Senescence, apoptosis, and stem cell biology: the rationale for an expanded view of intracrine action. *Am J Physiol Heart Circ Physiol* **297,** H893–H901 (2009).
27. Rippo, M. R. *et al.* MitomiRs in human inflamm-aging: A hypothesis involving miR-181a, miR-34a and miR-146a. *Experimental Gerontology* **56,** 154–163 (2014).
28. Rodier, F. & Campisi, J. Four faces of cellular senescence. *J Cell Biol* **192,** 547–556 (2011).
29. Salminen, A., Kauppinen, A. & Kaarniranta, K. Emerging role of NF-κB signaling in the induction of senescence-associated secretory phenotype (SASP). *Cellular Signaling* **24,** 835–845 (2012).
30. Severino, J., Allen, R. G., Balin, S., Balin, A. & Cristofalo, V. J. Is β-Galactosidase Staining a Marker of Senescence in Vitro and in Vivo? *Experimental Cell Research* **257,** 162–171 (2000).
31. Smith, E. J. *et al.* Age-related loss of hepatic Nrf2 protein homeostasis: Potential role for heightened expression of miR-146a. *Free Radic. Biol. Med.* **89,** 1184–1191 (2015).
32. Théry, C., Zitvogel, L. & Amigorena, S. Exosomes: composition, biogenesis and function. *Nat Rev Immunol* **2,** 569–579 (2002).
33. U.S. Census Bureau. International database. Table 094. Midyear population, by age and sex. Available at http://www.census.gov/population/www/projections/natdet-D1A.html.
34. Vergadi E, Vaporidi K, Theodorakis EE, Doxaki C, Lagoudaki E, Ieronymaki E, Alexaki VI, Helms M, Kondili E, Soennichsen B, Stathopoulos EN, Margioris AN, Georgopoulos D,

Tsatsanis C. Akt2 Deficiency Protects from Acute Lung Injury via Alternative Macrophage Activation and miR-146a Induction in Mice. J Immunol. 2014; 192:394-406.

35. Xu, D. & Tahara, H. The role of exosomes and microRNAs in senescence and aging. *Advanced Drug Delivery Reviews* **65,** 368–375 (2013).

Chapter 2

Cellular Senescence and the Aging Liver

Introduction

The liver as a model for aging

Prior investigations have suggested that the liver experiences several drastic changes with age, many of which lead to damage and loss of function (Sersté, 2006). We chose to study aging in the context of the liver because the liver is such an essential organ that serves may vital functions. These functions, which include metabolism, glucose storage, detoxification, medication clearance, bile production, and protein synthesis, are known to decline with age. Given this known patter of decline and the significance of the liver for maintaining overall health and wellbeing, it becomes necessary to investigate the liver in the context of aging in order to better understand the mechanisms that leads to its inevitable decline in functionality.

The liver and its microanatomy

The liver is organized in functional units known as lobules (Fig. 1). At the center of each lobule is the central vein. Branches leading to the central vein are called sinusoids, and are designed to channel blood from the portal veins and hepatic artery to the central vein (Bowen, 2003). At the periphery of the lobules are portal triads, which contain a hepatic artery, portal vein, bile duct, lymphatic vessels, and a branch of the vagus nerve (Bowen, 2003). The structures listed above are illustrated in Figure 1.

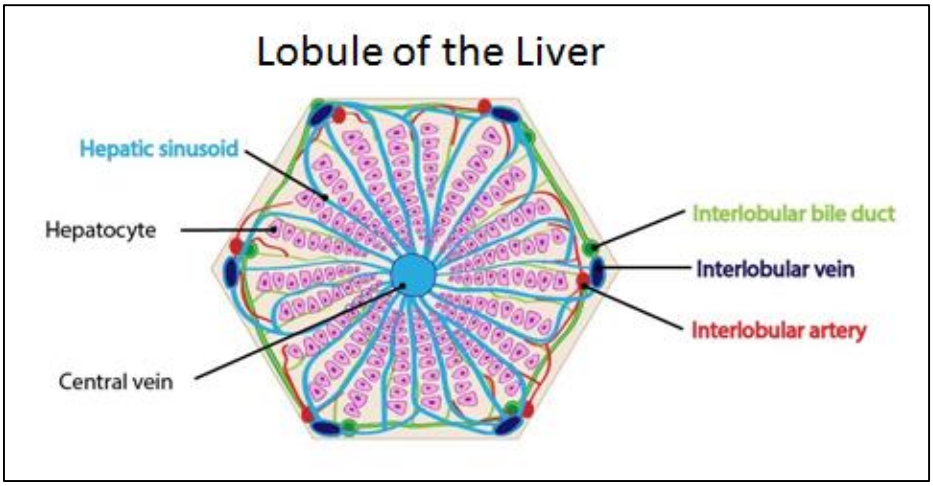

Figure 1: View of a single lobule, the functional unit of the liver. Hepatocytes (parenchymal cells) reside peripherally to the sinusoids, which are lined with endothelial cells. Prior studies have found increased infiltration of immune cells around the areas near the interlobular and central veins associated with age. Image Courtesy of Fontana et al., 2015.

Lobules are composed of two major cell types: parenchymal and non-parenchymal cells (Krishna, 2013). Parenchymal cells (hepatocytes) constitute nearly 80% of the cell volume of the liver and have several functions including bile production, protein synthesis, and detoxification (Kmieć, 2001). In addition to the lobule, there is also the microvascular unit of the liver is known as the acinus (not shown in Fig. 1). This unit is a collection of parenchymal cells that are grouped around the terminal branches of arterioles and the portal venule. It is through this region that oxygen and nutrients are exchanged between the blood vessels and the liver (Lautt, 2009).

Non-parenchymal cells includes Kupffer cells, sinusoidal endothelial cells, stellate cells, and pit cells (Knolle, 1995). Among their many functions, non-parenchymal cells play an important role in the inflammatory response in the liver by releasing a large amount of cytokines (Hasmall, 2000). Cytokines are inflammatory

mediators that have been found to influence hepatocyte functions (Ramadori, 2001) and are associated with the development of chronic liver diseases such as alcoholic liver disease (Seo and Jeong, 2016). Additionally, prior studies have also shown that there are significant immune cell clusters located primarily near the central and portal veins and appear to accumulate with age (Singh, 2008). This infiltration supports the idea that the mammalian liver is under an inflamed state that can potentially lead to cell dysfunction and apoptosis.

We chose to focus primarily on hepatocytes in the context of aging in the liver. It is known that the liver has decreased functionality with age and this loss of functionality may be attributed to the dysfunction and death of hepatocytes (Sersté, 2006). This investigation aims at determining if hepatocytes are capable of undergoing cellular senescence. While non-parenchymal cells are important for liver function and immunity, our current model of isolation only allows for obtaining hepatocytes. Future studies could be pursued if we developed a model for non-parenchymal cell isolation, however, that was beyond the scope of this thesis project. For the purposes of this investigation, cellular senescence was only investigated in the context of parenchymal cells.

Inflammation in the mammalian liver

The Hagen Lab has previously shown that there is a significant change in hepatic gene expression within the aging rat liver. Specifically, they have found that there is at least a twofold difference in expression of almost 700 distinct genes with

advanced age (Finlay, 2012). The Hagen Lab has also shown that of all the gene changes that occur in the liver with age, nearly 44% are necroinflammatory in nature. The lab has also shown that Nrf2, a protein that regulates the expression of many anti-oxidant and anti-inflammatory genes, is decreased in the aging liver, suggesting that the organ is more prone to damage (Smith, 2015).

These changes in gene expression and levels of Nrf2 can drive increased immune cell infiltration and inflammation. Chronic inflammation is associated and implicated in the overall decrease in liver function with age due to an increased release of proinflammatory agents, which can then lead to hepatic cell death (Baylis, 2013), as well as cirrhosis of the liver and the subsequent deterioration of normal liver functions (Brenner, 2013). It is still relatively unknown as to what major forces are driving the decline of functionality in mammalian livers with age. It does appear, however, that the liver experiences age-related inflammation, and this inflammation may contribute to the overall decline of liver function with age (Braylis, 2013). The exact cause of this inflammation response is still unknown.

The purpose of this study is to answer two questions: do liver cells undergo cellular senescence, and if they do, is there an accumulation of senescent cells with age? We hypothesized that not only do mammalian liver cells undergo cellular senescence, we postulate that there is an accumulation of senescent cells in liver tissue, leading to increased inflammation and tissue death. To test this, we used rat hepatocytes as a model and used β-galactosidase activity (which is active at a sub-optimal pH in senescent cells), as well as miRNA isolated from exosomes for markers of senescence and tissue stress.

Objectives

The first part of this investigation has two primary questions: do mammalian liver cells undergo cellular senescence, and if so, is there a discern accumulation that is associated with aging? The second part of this investigation focuses on the result of such accumulation on the liver. Specifically, is there a relationship between an increased presence of senescent cells in the liver and an increased pro-inflammatory state. In order to determine this, we will look at two miRs that will serve as markers for inflammation: miR-146a and miR-155.

Methods

Hepatocyte isolation and culture

Six well plates were treated with a collagen solution consisting of 975 µl PBS and 25 µl of 2 µg/ml collagen dissolved in 0.1 M hydrochloric acid one day prior to isolation. Hepatocytes were isolated by collagenase perfusion from young (3 months) and old (24-26 months) Fischer 344 rats were isolated as described by Shenvi et al. After the isolation, 200,000 cells per well were plated in 2 ml of William's E full media: 500 ml William's E, 25 ml FBS, 5 mL antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin), 5 ml L-glutamine, 5 µl insulin, and 2 µl 0.25 M dexamethasone, and incubated at 37 °C in 5% carbon dioxide. Media was changed four to six hours after the initial plating.

Determination of Cell Senescence with β-galactosidase

Isolated hepatocytes were cultured as described above and were stained for β-galactosidase the day following isolation. The protocol provided by Cell Signaling was followed with only one adjustment: cells were incubated for twenty three hours. After incubation, cells were counted and a percent senescence was determined.

Exosome isolation

Hepatocytes were isolated as described above. Cells were plated on three collagen-coated, T-175 flasks. 15 million cells were added to 35 mL of William's E Full media. Media was changed four to six hours after the initial isolation and the cells were incubated overnight. The day following isolation, media from all three flasks was pooled. The media was briefly spun at room temperature at 500 x g for five minutes and pellets were discarded. The media was then sterile filtered. After the filtration, the media were spun at 9,000 x g at 4 °C for 10 minutes. Any pellets were then discarded. The media was carefully massed in Beckman ultracentrifuge tubes and were then spun at 100,000 x g for ninety minutes at 4 °C. Pellets were collected and pooled into a single tube, and were then spun again at 100,000 x g for ninety minutes at 4 °C. Pellets were collected and resuspended in exosome buffer provided by Life Technologies and stored at -20 °C.

miRNA isolation and QPCR

After exosomes were isolated, miRNA was extracted using the Total Exosome RNA and Protein Isolation kit provided by Life Technologies (catalog number: 4478545). The protocol provided by the kit was followed. miRNA yield was determined via nanodrop.

Real time quantitative polymerase chain reaction (QPCR) was conducted on the exosomes obtained from the young and old rat hepatocytes in order to determine the relative amounts of miR-146a and miR-155 present in the exosome samples. TaqMan primer probes for mir-146a (UGAGAACUGAAUUCCAUGGGUU) and mir-155 (UUAAUGCUAAUUGUGAUAGGGGU), which were provided by Thermo Fisher, were used for QPCR. The protocol for the TaqMan MicroRNA Assay was followed.

Results and Discussion

For this investigation, we chose to utilize the cellular senescence marker β-galactosidase. While many other markers exist, we chose this one specifically because of its convenience due to time constraints. The β-galactosidase staining revealed that hepatocytes do indeed undergo cellular senescence. Furthermore, the staining elucidated that cellular senescence was seen in both the young and the old rat livers. Furthermore, the results of the β-galactosidase staining showed that there is a difference between the senescent cell populations of young and old rats (Fig. 2). It

was determined that senescent cells composed 6.135 percent of the total young rat hepatocytes (±0.7989, n=6). In contrast, the old rat hepatocytes had more than double the senescent cells present at 13.53 percent (±1.097, n=3). A Student's t-test yielded a p-value of 0.001.

Closer examination of Figure 2B and 2C shows that many cells are mildly stained for β-galactosidase. However, these cells were not counted as being senescent. Since β-galactosidase is present in all cells, there is a chance the enzyme may still be functioning at a non-ideal pH. Arrows in Figure 2B and 2C show deep blue staining, which was interpreted as a positive senescent cell. Given the subjectivity of this method, there is potential room for error in calculating percent senescence. It is because of this subjectivity that additional markers should be utilized to confirm the presence of senescent cells.

These findings answer two big questions: do mammalian liver cells undergo cellular senescence, and if so, is there a noticeable accumulation with age? The β-galactosidase exhibits a correlation between advanced age and senescent cells presence in the mammalian liver. Given that senescent cells promote inflammation, ultimately leading to tissue damage, it is intuitive that the increase in senescent cells with age coincides with the chronic inflammation and subsequent decrease in liver functionality. There is still uncertainty as to what extent senescent cells play a role in the decreased liver functionality with age, and future studies should be designed to answer this question.

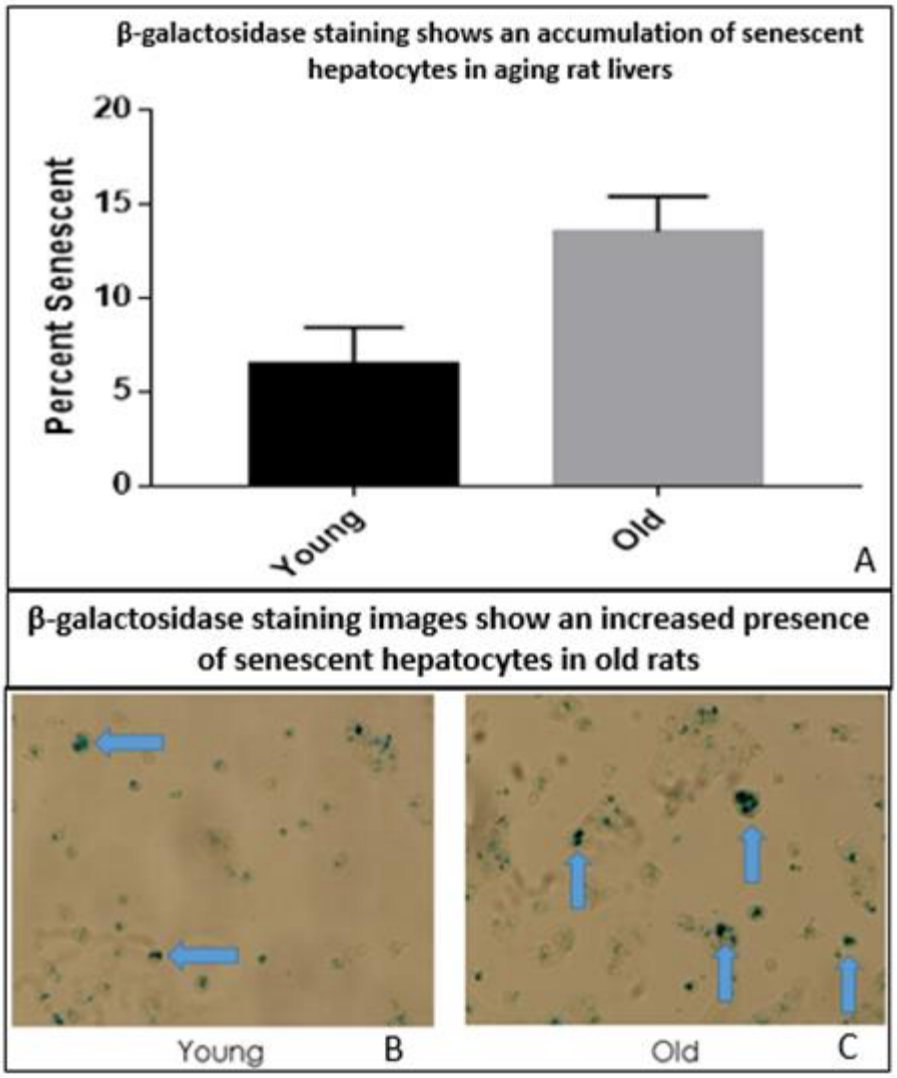

Figure 2: β-galactosidase staining was used as a marker for cellular senescence. (A) β-galactosidase staining reveals that 6.135% of young rat hepatocytes are senescent (±0.7989, n=6). This is nearly half the population of senescent cells found in old rat hepatocytes, which was found to be 13.53% senescent (±1.097, n=3). A Student's t-test yielded a p-value of 0.001. (B) Images taken after staining of young rat hepatocytes and (C) old rat hepatocytes. Arrows pinpoint the positive β-galactosidase stains.

QPCR analysis revealed that both miR-146a and miR-155 were significantly elevated in exosomes isolated from old rat hepatocytes (Fig. 3). These findings reinforce our previous findings regarding an increase in senescent cells in the liver. Analysis showed a significant difference in levels of both miR-146a and miR-155

between young and old rats (a seven fold and a forty-five fold change respectively). The reason why these miRs are elevated may be because there is a significant immune response that is ongoing within the liver, thus increased levels of these miRs are released in an attempt to reduce the inflammation. These findings are still preliminary as there is only a single n-value, thus replications of this experiment are warranted. A microarray on exosomes collected may also be beneficial in order to ascertain a broader sense of the contents of senescent hepatocyte exosomes.

To reiterate, the findings thus far have found three key findings: senescent cells are present in rat livers, there is an accumulation of senescent cells with age in the rat liver, and there is a significant elevation of miR-146a and miR-155 released via exosomes in old rat hepatocytes compared to young rat hepatocytes. Together, these findings suggest that the rat liver is under a state of stress and inflammation and hepatocytes are releasing miRs in an attempt to reduce the inflammation. Now that it is firmly established that the rat liver is experiencing stressors that can be attributed to senescent cells, the next question that must be addressed is: what can be done to combat the effects of cellular senescence?

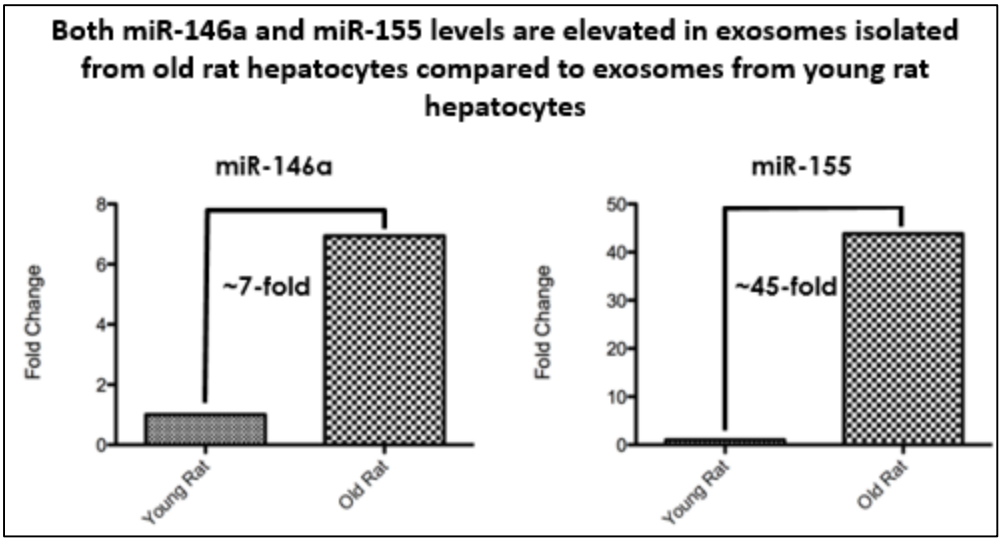

Figure 3: QPCR analysis reveals that old rat hepatocytes release exosomes that contain larger quantities of both miR-146 and miR-155 compared to levels found in young rat hepatocytes (seven fold and forty-five fold difference). There are no additional statistical data on these findings, as there is only a single n-value.

References

1. Baylis, D., Bartlett, D. B., Patel, H. P. & Roberts, H. C. Understanding how we age: insights into inflammaging. *Longev Healthspan* **2,** 8 (2013).
2. Bowen, R. Hepatic histology: sinusoids. Pathophysiology of the Digestive System, 2003.
3. Brenner, C., Galluzzi, L., Kepp, O. & Kroemer, G. Decoding cell death signals in liver inflammation. *Journal of Hepatology* **59,** 583–594 (2013).
4. Fontana, J. et al. Liver and biotransformation of xenobiotics. Functions of Cells and Human Body, 2015
5. Hasmall, S. C., West, D. A., Olsen, K. & Roberts, R. A. Role of hepatic non-parenchymal cells in the response of rat hepatocytes to the peroxisome proliferator nafenopin in vitro. *Carcinogenesis* **21,** 2159–2165 (2000).

6. Kmieć, Z. Cooperation of liver cells in health and disease. *Adv Anat Embryol Cell Biol* **161,** III–XIII, 1–151 (2001).
7. Knolle, P. *et al.* Parenchymal and nonparenchymal liver cells and their interaction in the local immune response. *Z Gastroenterol* **33,** 613–620 (1995).
8. Krishna, M. Microscopic anatomy of the liver. *Clinical Liver Disease* **2,** S4–S7 (2013).
9. Lautt, W. W. Hepatic circulation: physiology and athophysiology . (Morgan & Claypool Life Sciences, 2009).
10. Ramadori, G. & Armbrust, T. Cytokines in the liver. *Eur J Gastroenterol Hepatol* **13,** 777–784 (2001).
11. Senescence β-galactosidase Staining Kit. Available at: https://media.cellsignal.com/pdf/9860.pdf. (Accessed: 5th August 2016).
12. Seo, W. & Jeong, W.-I. Hepatic non-parenchymal cells: Master regulators of alcoholic liver disease? *World J Gastroenterol* **22,** 1348–1356 (2016).
13. Sersté, T. & Bourgeois, N. Ageing and the liver. *Acta Gastroenterol. Belg.* **69,** 296–298 (2006).
14. Shenvi, S. V., Dixon, B. M., Petersen Shay, K. & Hagen, T. M. A rat primary hepatocyte culture model for aging studies. *Curr Protoc Toxicol* Chapter 14, Unit 14.7 (2008).
15. Singh, P. *et al.* Lymphoid Neogenesis and Immune Infiltration in Aged Liver. *Hepatology* **47,**1680–1690 (2008).
16. TaqMan Fast Advanced Master Mix Protocol. Available at: https://tools.thermofisher.com/content/sfs/manuals/cms_084554.pdf. (Accessed: 5th August 2016)

Chapter 3

Dasatinib as a Potential Therapy for Cellular Senescence

Introduction

Reducing cellular senescence

Thus far, we have established three important conclusions: the rat liver undergoes cellular senescence, there is an accumulation of senescent cells with age in the rat liver, and old rat hepatocytes release exosomes containing larger quantities of both miR-146a and miR-155. These findings support the idea that cellular senescence may play a critical role in chronic inflammation and therefore chronic disease. Our next question therefore becomes: what, if any, treatment options are there to combat cellular senescence? This question ultimately lead us to investigate a drug that may potentially reduce senescent cell populations.

Dasatinib as a possible treatment for cellular senescence

The chemotherapy drug known as dasatinib (Fig. 4) is a multikinase inhibitor that is currently being employed to treat resistant chronic myeloid leukemia and resistant Philadelphia Chromosome positive acute lymphoblastic leukemia (Lindauer, 2010). Dasatinib has been shown to inhibit cell proliferation as well as promote cell death in various tumor cells by suppressing the Src family nonreceptor kinases (Johnson, 2005). Previous studies have found that dasatinib is a relatively safe drug for human use, eliciting some reversible and non-life-threatening side effects, such as headaches, diarrhea, peripheral edema, effusions (which were managed with

diuretics), and temporary abnormalities in liver function tests (Talpaz, 2006). Dasatinib is of particular interest to studies in cellular senescence since it has been found to preferentially induce apoptosis in senescent human preadipocytes and endothelial cells (Zhu, 2015). Dasatinib is believed to induce senescent cell death in a similar fashion to how it induces cancer apoptosis: by interfering with anti-apoptotic and pro-survival mechanisms, mechanisms that are overexpressed in both senescent and cancer cells (Zhu, 2015). No studies to date have attempted to examine if dasatinib can reduce cellular senescence in rat hepatocytes or in HepG2 cells, therefore it is uncertain if dasatinib can be utilized as a means to slow down or inhibit chronic inflammation in the liver.

Objectives

This investigation aims at answering whether the chemotherapy drug dasatinib can be used to effectively reduce senescent cell populations in the metabolically active liver.

Methods

Young rat hepatocyte dasatinib treatment

Hepatocytes from a young rat were harvested via collagenase perfusion as outlined in Shenvi et al (see Chapter 2). Cells were plated on a collagen-coated, six

well plate (200,000 cells per well) in William's E full media (see Chapter 2). Media was changed four to six hours after the initial isolation. The day after isolation, media was changed and a dasatinib gradient of 0 nM, 200 nM, 400 nM, 600 nM, and 800 nM was added to the wells. Cells were incubated for twenty-four hours. After the incubation period, the media was removed and a β-galactosidase stain was conducted (see Chapter 2).

Results and Discussion

The dasatinib treatments for the young rat hepatocytes (Fig. 4) shows a decrease in senescent cells after a one day treatment. The control hepatocytes were 4.79% senescent. All hepatocytes from the same rat treated with dasatinib exhibited decreased levels of cell senescence. The 200 nM treatment hepatocytes were 2.69% senescent, the 400 nM treatment hepatocytes were 2.91% senescent, the 600 nM treatment hepatocytes were 2.78% senescent, and the 800 nM treatment hepatocytes were 3.54% senescent. At this time, there is no way of knowing if these results are significant, as no statistical analysis could be conducted since there is only one n-value.

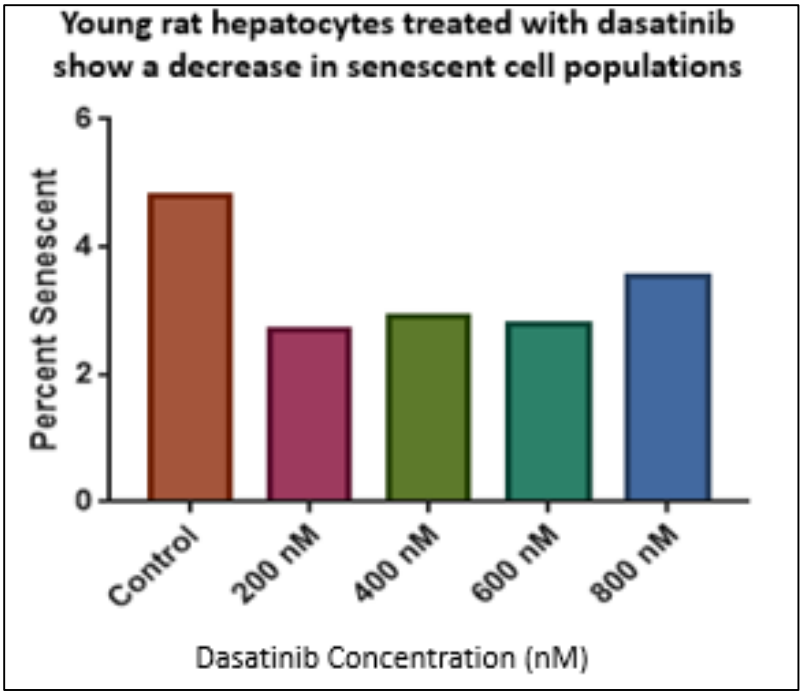

Figure 4: Young rat hepatocytes treated with dasatinib. Percent senescence was determined using β-galactosidase staining. The percent of hepatocytes found to be senescent were as follows: 4.79% in the control, 2.69% in the 200 nM treatment, 2.91% in the 400 nM treatment, 2.78% in the 600 nM treatment, and 3.54% in the 800 nM treatment. Since there is only a single n-value, statistical analysis could not be conducted.

References

1. Johnson, F. M., Saigal, B., Talpaz, M. & Donato, N. J. Dasatinib (BMS-354825) Tyrosine Kinase Inhibitor Suppresses Invasion and Induces Cell Cycle Arrest and Apoptosis of Head and Neck Squamous Cell Carcinoma and Non–Small Cell Lung Cancer Cells. *Clin Cancer Res* **11,** 6924–6932 (2005).
2. Lindauer, M. & Hochhaus, A. Dasatinib. *Recent Results Cancer Res.* **184,** 83–102 (2010).
3. Shenvi, S. V., Dixon, B. M., Petersen Shay, K. & Hagen, T. M. A rat primary hepatocyte culture model for aging studies. *Curr Protoc Toxicol* Chapter 14, Unit 14.7 (2008).
4. Talpaz, M. *et al.* Dasatinib in Imatinib-Resistant Philadelphia Chromosome–Positive Leukemias. *New England Journal of Medicine* **354,** 2531–2541 (2006).

5. Zhu, Y. *et al.* The Achilles' heel of senescent cells: from transcriptome to senolytic drugs. *Aging Cell* **14,** 644–658 (2015).

Conclusion

The liver is an essential organ that has a known loss of functionality associated with aging. This loss of functionality may be linked to chronic inflammation. One hypothesis regarding the cause of this inflamed state is the accumulation of senescent cells. This study provided insight on senescent cell accumulation in rat livers. Specifically, it has shown that there is an accumulation of senescent hepatocytes with age. Our studies found that 6.135% of young rat hepatocytes are senescent, whereas 13.53% of old rat hepatocytes are senescent. This accumulation leads to an increased release of pro-inflammatory agents via exosomes, specifically miR-146a and miR-155 (seven fold and forty-five fold changes respectively), both of which are known to exacerbate cell senescence and subsequent liver tissue damage. An important unanswered question regarding this accumulation is: to what extent do senescent cells contribute to the chronic inflammation phenomena? One way to elucidate this answer is by selectively removing senescent liver cells and to observe what effects, if any, this has on the overall health of the organism.

Next, we investigated abilities of the drug known as dasatinib in killing senescent cell populations. Dasatinib, which is normally implemented as a chemotherapy agent, was found to reduce senescent liver cell populations in a young rat. The hepatocytes treated with 200 nM, 400 nM, 600 nM, and 800 nM dasatinib exhibited 2.69%, 2.91%, 2.78%, and 3.54% senescence respectively. This was a reduction from 4.79% in the control hepatocytes. This shows a mild decline in

senescent cell populations. However, since there is only one n-value, no statistical analysis could be performed, thus there is currently no way of determining if this decline in senescent cell populations is significant.

Since it has been shown that senescent cells accumulate in the liver with age, it seems that the most important future study regarding these findings are *in vivo* studies with dasatinib. Using a rat model, supplements containing dasatinib can be administered both to young and old rats. This will help further demonstrate the potential benefit of dasatinib reducing, and maybe even preventing, chronic inflammation. Similarly, this drug may one day be used in humans as a way to potentially reduce the likelihood of obtaining age-related liver diseases, any maybe even other chronic illnesses. While many studies are still needed at this time, this project helped establish future research endeavors that may lead to treatments to slow down the aging process in humans.