THE EFFECT OF OXIDATIVE STRESS ON THE ATTACHMENT OF METASTATIC PROSTATE CANCER CELLS TO HUMAN BONE MARROW ENDOTHELIAL CELLS

by

Shannon Marshall

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Shannon Marshall

Approved: _____

Randall Duncan, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____

Carlton Cooper, Ph.D.
Co-Advisor and Committee member from the Department of Biological Sciences

Approved: _____

Gary Laverty, Ph.D.
Committee member from the Board of Senior Thesis Readers

Approved: _____

Hemant Kher, Ph.D.
Chair of the University Committee on Student and Faculty Honors

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ABSTRACT

Prostate cancer (PCa) is both the most common and the third deadliest cancer among American men. Most of the mortality associated with PCa stems from metastasis of the cancer to bone (1,2), leading to my previous hypothesis that prostate cancer cells preferentially adhere to human bone marrow endothelial (HBME) cells. Previous experiments in our lab have confirmed this preference and I investigated the physiological factors that contribute to PCa metastasis. The initial goal of this project was to determine if fluid shear stress would increase expression of membrane adherence proteins on HBME cells thereby increasing the ability of PCa cells to adhere to these cells. I further postulated that adhesion of prostate cancer cells to HBME cells is regulated by nitric oxide (NO), with reduced levels of NO leading to increased PCa adhesion. To evaluate the relationship of NO to cell adhesion we performed adhesion assays under fluid shear and in the presence of NG-nitro-L-arginine methylester (L-NAME), an inhibitor of NO synthase (3). Using calcein loaded PCa cells, I found that the number of labeled cells was higher in the non-sheared, or static, samples that when compared to sheared cells. Indicating that PCa cell attachment to the HBME cell monolayer was also higher in these samples. Inhibition of NO synthesis with L-NAME also increased PCa cell attachment in sheared samples but was not effective in the static samples. Because my data did not

support my original hypothesis, I proposed that Oxidative Stress (OS) could be another potential factor that could influence PCa adhesion to HBMECs. OS results from an imbalance in the production of reactive oxygen species (ROS) and the antioxidants required to metabolize ROS (4). I hypothesized that OS could play a role in increasing the expression of cell adhesion molecules (CAMs) on the membrane of HBMECs therefore increasing the ability of PCa cells to attach to them. To test my hypothesis, I used DCFH-DA to confirm that we could artificially induce OS in HBMECs and an adhesion assay similar to our previous studies to measure its effect on adhesion. I found that inducing OS in endothelial cells triggered increased PCa cell attachment to HBMEC cells however further studies will be needed to confirm that the increased attachment is due to increased CAMs. Future studies will also include experimental design modifications such as increasing the duration that the endothelial cells are sheared to determine if long term shear produces a different effect than short term shear. Completion of these studies may lead to insight into the racial differences in OS and NO production and the impact of this racial disparity on prostate cancer occurrence and metastasis.

Chapter 1

INTRODUCTION

1.1 Rationale

Prostate cancer (PCa) is both the most common and the third deadliest cancer among American men with 161,360 new cases and 26,730 deaths predicted for 2017. In fact, about 1 in 39 American men die as a result of this disease (9). The Centers for Disease Control and Prevention found that compared to Caucasian Americans, African Americans exhibit both a higher rate of prostate cancer incidence and prostate cancer mortality (10). In addition to the greater rate of incidence, African American men show a more aggressive form of the disease at diagnosis with ~7% of cases being metastatic. The cause of this reported disparity in prostate cancer metastasis remains enigmatic. Numerous factors could contribute to this disparity such as diet, environment, and previous healthcare discrimination decreasing the likelihood that African Americans visit doctors. In these studies, I propose to determine if physiological differences between African Americans and Caucasian Americans could be a direct cause. One physiological difference that I initially proposed could be responsible for the difference in PCa metastasis is hypertension. Hypertension or high blood pressure is defined as a systolic pressure of 140mmHg or above and a diastolic pressure of 90mmHg or above. A study by the Centers for Disease Control and Prevention showed that African American males exhibited the highest percentage of persons with hypertension when compared to Caucasian American men and Mexican American men. Along with the highest in percentage of hypertension African

American men also have the lowest percentage of individuals with their hypertension under control by medication or other methods (11). This means that African American men are more likely to experience adverse effects from uncontrolled chronic hypertension. My initial hypothesis was that increased fluid shear on endothelial cells due to hypertension would increase the expression of cell adhesion molecules (CAMs) on these cells to increase PCa cell attachment. I proposed that fluid shear stress initiates transcription factors responsible for the expression of CAMs such as ICAM-1 (12,13,14). Counter to my hypothesis, I found that subjecting endothelial cells decreased the attachment of PCa cells to human bone marrow endothelial cells (HBMECs). Upon further review of the literature I found that acute fluid shear increases endothelial nitric oxide synthase (eNOS) activity and therefore nitric oxide (NO) concentration in the endothelial cell. The NO produced may be vasoprotective since it has been shown to significantly decrease the expression of the VCAM-1, ICAM-1, and E-selectin (15). This knowledge shifted my reasoning and prompted me to look for other racial differences especially those that could also decrease the bioavailability of NO. Central to many racial disparate diseases was the level of oxidative stress (OS) of the population. Compared to those of Caucasian Americans, endothelial cells in African American men exhibit greater oxidative stress. Among the adverse effects of OS, increased reactive oxygen species (ROS) have been shown to increase the expression of ICAM-1, VCAM-1, and PECAM (16,17). With this knowledge, I formed a new hypothesis that increased OS will increase PCa attachment to HBMECs via the increased expression of CAMs. To assess whether or not oxidative stress plays a role in the adhesion of PCa cells to HBMECs I have devised a method of inducing oxidative stress in endothelial cells in a way that does not

negatively affect cell survival using the free radical generator, SIN-1. The decay of SIN-1 is induced by biological oxidizers and this decay results on the production of NO and O2- that combine to form ONOO-. After inducing oxidative stress, I measured a significant increase in PCa to HBMEC adhesion, suggesting that oxidative stress may contribute to prostate cancer metastasis. I postulate that the mechanism for this contribution is the upregulation of the CAMs ICAM-1, VCAM-1, and E-selectin, however further studies are required for confirmation.

1.2 Background

One of the most crucial factors leading to patient mortality in prostate cancer is the ability of the cancer to metastasize. The term metastasis describes the action of cancer cells leaving the site of the primary tumor then spreading through either the lymphatic system or blood stream and ultimately forming tumors in another part of the body. Metastasis begins when cancer cells phenotypically change and develop the ability to invade nearby tissues. Metastatic invasion is possible due to alterations in cell to cell adhesion properties which will allow the malignant cells to detach from the primary tumor (5). This dissociation causes changes in the tumor cells interaction with the extracellular matrix including the production of matrix metalloproteases and expressing genes responsible for cell motility. When the cell ultimately reaches the surrounding blood or lymphatic vessels it then secretes molecules necessary for degrading the basement membrane in order to enter the circulatory or lymphatic system. Cancer cells circulating the bloodstream or lymphatic system then attach to endothelial cells, mainly through integrin mediated adhesion (5). In a process known as extravasation the cancer cells then exit the circulatory or lymphatic system into a distant tissue similar to the process used in the leukocyte endothelial interaction. Once

in the new tissue the cancer cells must survive and proliferate to form a secondary tumor. Metastatic cancer cells maintain the features of the primary tumor. For example, if lung cancer metastasizes to the brain it is metastatic lung cancer and not brain cancer (5).

1.2.1 Cell Adhesion Molecules Involved in Prostate Cancer Metastasis

PCa attachment to endothelial cells is mediated by several attachment factors known as cellular adhesion molecules (CAMs). CAMs are transmembrane proteins and fall into four categories; integrins, selectins, IG superfamily members, and cadherins. Integrins are used for cell attachment to the ECM and in some cell-to-cell interactions. Each integrin is a heterodimer formed from an α and β subunit. There are 18 α subunits and 8 β subunits that can come together in 24 different combinations. Once bound as a dimer, these subunits undergo a Mg^{2+} and Ca^{2+} dependent conformational change to expose the binding site of the integrin resulting in cell attachment (6). Integrins are heterophilic, meaning they can bind to molecules in the ECM like fibronectin, collagen, and laminin, and other CAMs like IG superfamily members and cadherins but not to other integrins. Integrins also play a role in the transduction of signals both from the ECM to the cell and from the cell to the ECM. Cadherins are transmembrane proteins that form the main adhesive interactions responsible for holding cells together to form tissues. Cadherins have three domains; an extracellular domain which consists of repeating subunits bridged by Ca^{2+}, a transmembrane segment, and cytoplasmic domain that interacts with catenins inside of the cell (6). Cadherins only bind in a homophilic manner, meaning they are only able to bind to other cadherins. In order for cadherins to bind Ca^{2+} is required. Like integrins, cadherins are involved with signal transduction in cells for example, in

cancer cells lack of E-cadherin based attachment to other cells drives the change to a malignant phenotype (6). IG superfamily (IGSF) members are CAMs that play a role in inflammation and the immune response. The structure of IGSF members includes a large extracellular domain containing immunoglobulin, a helical transmembrane segment, and a cytoplasmic tail. IGSF members are responsible for the activation of B and T cells in response to the presence of antigens. IGSF members require both Ca^{2+} and Mg^{2+} to function and are capable of binding in an homophilic and heterophilic manner (6). Selectins are CAMs that form very weak attachments which usually serve as the initial step for stronger attachment. One common example of the role of selectins is their function in the interaction between leukocytes and endothelial cells in the inflammatory response (7). Selectins form transient bonds that cause leukocytes to "roll" along the endothelial cell layer until stronger bonds such as those from integrins form. Selectins are heterophilic and only bind to glycoproteins on the surface of the target cell (6,7). Several CAMs are expressed on the surface of endothelial cells such as E-selectin, VCAM-1, ICAM-1, PECAM, and vWF. All of which could play a role in the attachment of circulating PCa cells to vascular endothelial cells.

1.2.2 Relevance of Nitric Oxide Normal and Pathologic Function

Recent studies examining the adhesion of cancer cells to the vascular endothelium have focused on Nitric Oxide (NO). NO is a signaling molecule involved in many cellular processes such as cell adhesion, neurotransmission, relaxation of blood vessels and the immune response (8). NO synthesis and release is stimulated by a variety of factors, including fluid shear stress on endothelial cells. The production of NO occurs via the enzymatic activity of Nitric Oxide Synthase (NOS). There are three types of NOS, neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS

(iNOS) (8). Since prostate cancer spreads throughout the body via the circulatory and lymphatic systems NO produced by eNOS in endothelial cells could play a role in PCa attachment during the initial stages of the extravasation. eNOS is composed of two domains, a Reductase Domain where NADPH is reduced to NADP+ and an Oxygenase Domain where L-Arginine and O_2 is converted to L-Citrulline and NO. In order for proper function, eNOS must be associated with other factors such as calmodulin and BH4 (8). Any disruption of this association will lead to dysfunctional eNOS activity and could contribute to racial differences in prostate cancer metastasis. In African Americans, dysfunctional eNOS converts O_2 to O_2^- thereby increasing the amount of reactive oxygen species (ROS) in the cell leading oxidative stress (24). The resulting loss in endothelial NO has been shown to upregulate the expression of the CAMs P-Selectin, ICAM-1, and VCAM-1 that could increase the adhesion of PCa cells (8).

1.2.3 Oxidative Stress

Oxidative Stress (OS) is defined as an imbalance between the production of ROS in the cell and the cell's ability to neutralize the ROS with antioxidants. This imbalance could be caused by depletion of antioxidants or increased production of ROS. The term ROS describes oxygen containing free radicals which have one or more unpaired electrons making them highly reactive. The most common ROS in the body is O_2^- which is produced in several cellular reactions including electron transfer from electron transport chains in the mitochondria, protein folding in the endoplasmic reticulum, and NADPH oxidase function (4). One of the body's defense mechanisms against high O_2^- concentration is superoxide dismutase which converts O_2^- to H_2O_2 which can easily be detoxified to water by other enzymes. However, when

concentrations are very high, O_2^- can react with H_2O_2 to form the much more reactive hydroxyl ion (OH). Hydroxyl ions present danger because they will quickly react with nearby molecules and have no known scavengers (4). Excess O_2^- in cells can also react with NO to form peroxynitrite that exacerbates the oxidative stress in the cell and reducing the amount of free NO. OS has many adverse effects on cell function many of which result in necrosis or apoptosis. The presence of ROS activates transcription factors required for the production of pro-inflammatory enzymes. Although the inflammatory response is acutely protective, the presence of chronic inflammation has been linked to many diseases such as cancer. OS also disrupts intracellular Ca^{2+} levels by activating Ca^{2+} release from the ER. The released Ca^{2+} will flood into the cytoplasm activating Ca^{2+} dependent process and result in the loss of Ca^{2+} dependent chaperones in the ER, causing an accumulation of unfolded and misfolded proteins (4). The OH radical can initiate a particularly dangerous process known as lipid peroxidation. Lipid peroxidation occurs when OH reacts with a hydrogen from the fatty acid chains of phospholipids that make up cell and organelle membranes. This generates free radicals from fatty acid chains which is easily propagated to adjacent fatty acids ultimately resulting in the destruction of the membrane (4). Finally, OS can also contribute to DNA damage by ROS interacting directly with deoxyribose sugars causing strand breakage or by reacting with histones which creates problems with chromatin folding and can adversely affect DNA repair processes as well as transcription (4). OS may play a role in both the development and metastasis of PCa by reducing in available NO, activating pro-inflammatory enzymes, damaging DNA, and upregulating CAMs.

Chapter 2

MATERIALS AND METHODS

2.1 Chemicals and Solutions

2′,7′-dichlorofluorescin diacetate (DCFH-DA) was purchased from Cayman Chemical company. DCFH-DA shows OS levels in cells by crossing the cell membrane where esterases cleave the diacetate (DA) trapping the DCFH portion inside of the cell. DCFH then donates a Hydrogen to ROS present in the cell creating the fluorescent DCF molecule. A 20mM stock solution was made by dissolving DFCH-DA in dimethyl sulfoxide (DMSO). When loading the cells the DFCH-DA, the stock solution was diluted to a final concentration of 20µM in KRH Buffer. 3-morpholinosydnonimine hydrochloride (SIN-1) was purchased from Abcam. A 50mM stock solution of SIN-1 was made by diluting SIN-1 in sterile water. During treatment SIN-1 stock was diluted to smaller concentrations using KRH buffer. KRH buffer was made from specifications by Cold Spring Harbor using the following reagents; 160mM NaCl, 4mM KCl, 1mM $MgCl_2$, 1.8mM $CaCl_2$, 25mM Dextrose, 10mM HEPES acid. The fluorescent label calcein-AM was purchased from Molecular Probes and was diluted in DMSO prior to loading. Calcein-AM functions by crossing the cell membrane where the AM portion is cleaved by esterases trapping the calcein portion which fluoresces. Cells were cultured in either RPMI (C4-2) or M199 (HBMEC) medium supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin (P/S). During experiments, the FBS concentration was reduced to 0.2%.

2.2 AdhesionAssay: Fluid Shear

To begin this experiment, HBME cells were grown to a monolayer on the bottom of 8 T-25 flasks. Four of the flasks were placed on a rocker and subjected to oscillatory fluid shear stress (FSS) at 5 dynes/cm^2 for 2 hrs at 37˚C and 5% CO_2. The other four were left static for 2 hrs at 37˚C and 5% CO2 to serve as a control. Two flasks in both the shear and control groups received L-NAME to block NO synthesis mediated by eNOS. During the 2 hrs, C4-2 cells were labeled with calcein AM so they could be distinguished from the HBME cells. After the 2 hours, 100,000 C4-2 cells were seeded onto the HBME cell monolayer and given an hour to adhere at 37˚C and 5% CO_2. An experimental diagram is presented below. The flasks of HBME cells and C4-2 cells were then washed to remove non-adherent C4-2s then quantified using flow cytometry.

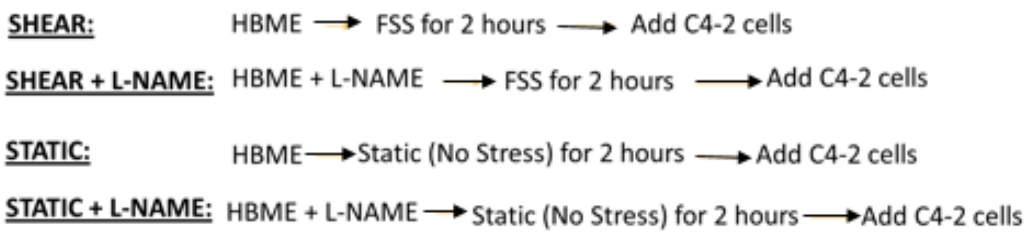


Figure 1. Schematic showing the experimental groups for our shear studies. Sheared groups consisted of HBMECs subjected to oscillatory shear stress for two hours either in the presence or absence of the NO inhibitor L-NAME then 1 hour for the labeled C4-2s to adhere. In the static samples HBMECs remained static with or without L-NAME treatment and also had C4-2s added for 1 hour to adhere. Immediately following the experiment all samples were analyzed using flow cytometry to determine how many C4-2s were in each sample based on the calcein label.

2.3 DCF assay for OS measurement

2.3.1 Cell Preparation

HBME cells were counted using a hemocytometer. $2.5x10^5$ were seeded into a 96-well plate and left to adhere overnight. The following day the cells were serum starved using serum reduced M199 media (0.2%FBS/5%PS) for 12 hrs. Following serum starvation, the reduced media was removed and the cells were washed with KRH buffer then incubated at 37˚C in 20µM DCFH-DA for 30 minutes. After the incubation with DCFH-DA the cells were washed with KRH Buffer then treated with either 100µM H2O2, 100µM SIN-1, 320µM SIN-1, or 500µM SIN-1 and the fluorescence was measured and recorded. Three different concentrations of SIN-1 were used to determine which dosages of SIN-1 would be best for future experiments. To create a time course showing how OS changed, measurements were taken at three different times.

2.3.2 Measurement

The SIN-1 treated cells were placed in a Beckman Coulter DTX-880 plate reader with a constant temperature of 37 ˚C. The excitation filter was set to 485nm and the emission filter was set to 530nm. The fluorescence was measured at 15 min, 45 min, and 60 min intervals and a plot was constructed to show how the fluorescence intensity changed with treatment. Negative controls were cultured cells in KRH Buffer without treatment. To observe the auto fluorescence in the wells, fluorescence was measured in well without cells or buffer.

2.4 Sustained OS Experiment

2.4.1 Cell preparation

$2.5x10^5$ HBMECs were plated in the wells of a 96-well plate, allowed to attach overnight then serum starved using serum reduced M199 medium. HBMECs were then washed with KRH Buffer and given one of the following treatments: 100µM H_2O_2 for 1 hour, 100µM SIN-1 for 1 hour, or no treatment for 1 hour. Cells were then viewed under a microscope to assure that none of the treatments resulted on cell death or detachment from the surface of the wells.

2.4.2 Measurement

Fluorescence intensity was measured using the Beckman Coulter DTX-880 plate reader. Measurements of the OS in each well were taken at 6, 12 and 24 hours. After all the measurements were taken the cells were viewed under a microscope to check for cell detachment or death.

2.5 Adhesion Assay: OS

2.5.1 Cell preparation

HBMECs were grown to a monolayer in six T-25 flasks with M199 media, then serum starved reduced M199 media 18 hours prior to the experiment. Following this half of the flasks were treated with SIN-1 for 18 hours to induce OS and the other half remained untreated, then all were incubated at 37˚C and 5% CO_2. During this incubation C4-2 cells were loaded with the fluorescent molecule calcein- AM. Prior to loading a 1mM stock solution by diluting 50mg of calcein-AM in 50µL of DMSO which was then diluted in RPMI to 1µM then added to C4-2 cells 30 mins. After loading, the C4-2 cells were washed twice with PBS and detached from their flask

using cell stripper. Labeled C4-2 cells (~100,000) were then added to the treated HBMECs and given 2 hours to adhere. Following this hour, the flasks were washed to remove non-adherent and loosely adherent cells. The remaining cells were trypsinized then transferred to a 15mL conical tube for analysis.

2.5.2 Analysis

Samples were analyzed using a Becton Dickinson FACSCalibur Flow Cytometer. Gating was set up using HBMECs, unlabeled C4-2s, and unlabeled C4-2s. The number of C4-2 cells that attached was determined by measuring fluorescence from each sample.

Chapter 3

RESULTS

3.1 Adhesion Assay for Fluid Shear

In this study, I found that subjecting HBMECs to oscillatory fluid shear decreased the ability of C4-2 cells to attach. In static samples, we measured an average of 1475 calcein labeled C4-2 cells in each flask and this number decreased to 1133 labeled cells when we subjected the endothelial layer to fluid shear stress (Fig. 2). When treating with the NO inhibitor L-NAME, we saw the average number of adherent cells in sheared samples increase to 1697. An increase in attachment due to L-NAME treatment was not observed in static samples which may be due to less NO production and therefore less NO to be inhibited. The decreased attachment in sheared samples contradicted our hypothesis and we believe that this may be due to only shearing the endothelial cells for 2 hours which could induce acute shear stress responses. This was problematic since changes in cell attachment stem from endothelial cells experiencing chronic shear stress. One of the responses in HBMECs to acute fluid shear stress is increased NO production, which can be vasoprotective and decrease the presence of adhesion molecules on the cell surface. Our results seem to support this theory of a NO based protective response because the decrease in attachment after shear was noticeably reversed when we inhibited NO.

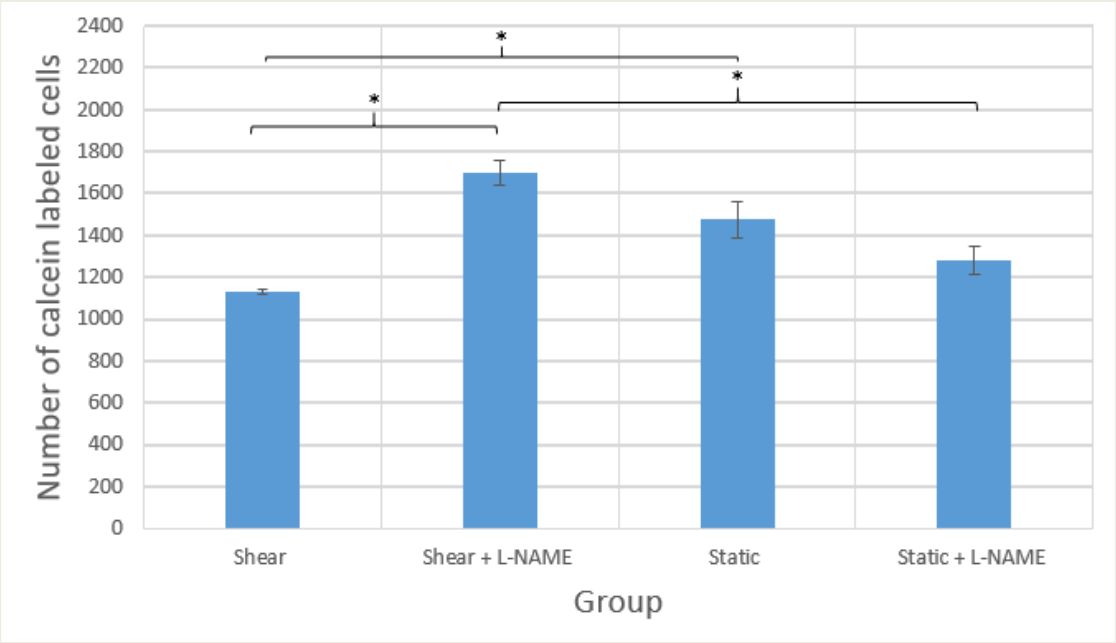
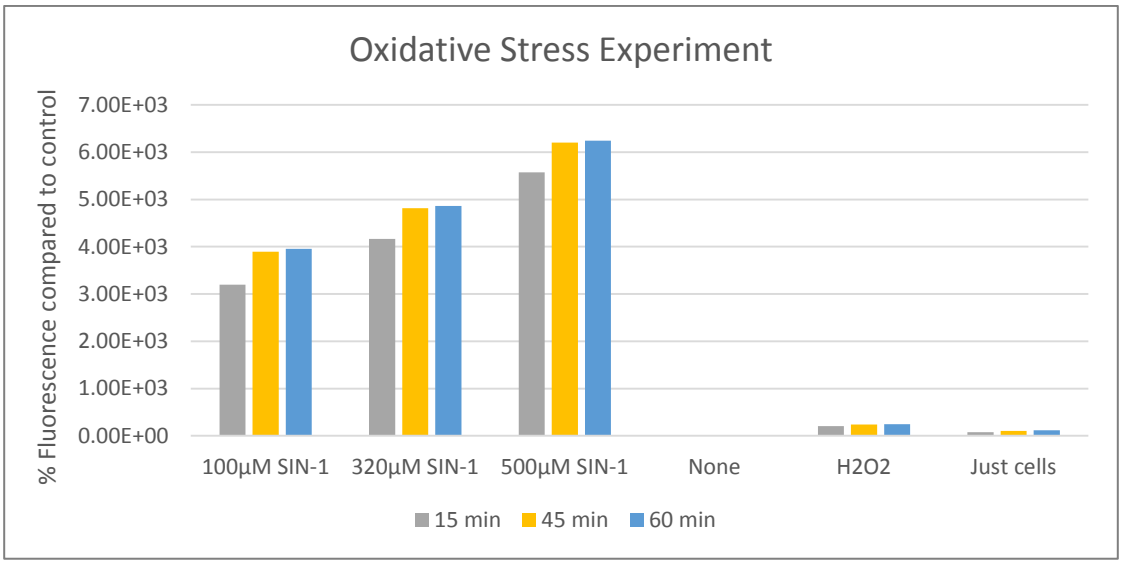


Figure 2. Results from our studies observing the effect of fluid shear on PCa attachment to HBMECs. We noticed a decrease in attachment when HBMECs were subjected to fluid shear compared to static samples. Also, we noted that when sheared samples were treated with the NO inhibitor L-NAME adhesion increased. We suspect that shearing endothelial cells for 2 hours may have acutely increased NO release which decreases adhesion molecule expression. Adhesion in static samples did not increase due to L-NAME treatment which may be due to less NO being produced in these samples and therefore less NO it be inhibited. *$p < 0.002$, Anova.

3.2 DCF assay for OS measurement

SIN-1 significantly induced OS in endothelial cells (Fig. 3). At the lowest concentration of 100µM, the fluorescence intensity of the cells treated with SIN-1 was about 3,000% greater than the cells that were not treated in the first 15 minutes and increased to about 4,000% higher after 60 minutes of treatment. Cells treated with 320µM SIN-1 displayed a fluorescence intensity that was 4,000% higher than untreated cells in 15 minutes which increased to 5,000% by 60 minutes. The highest level of OS came from the endothelial cells treated with 500µM SIN-1. The fluorescence intensity of these cells was more than 5,000% higher than untreated cells at 15 minutes and after 60 minutes were 6,000% more fluorescent (Fig. 3A). Results from this experiment showed that we were able to induce high OS on endothelial cells using the free radical generator SIN-1. These results also demonstrated that the level of OS induced by SIN-1 was greater than that of H_2O_2 which only fluoresced twice as much as untreated cells (Fig. 3B). The difference in fluorescence intensity confirmed our belief that SIN-1 is ideal for use in our studies. We observed that there was very little auto-fluorescence from the wells of the 96-well plate indicating background fluorescence is unlikely to have affected our results.

A



B

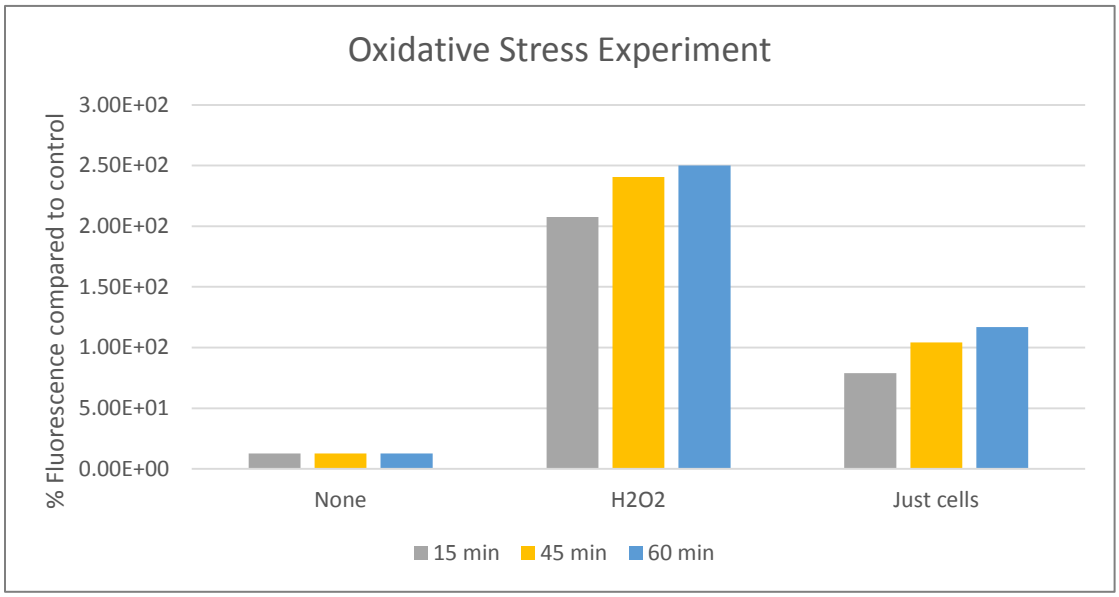


Figure 3. A. Measurement of the OS induced by SIN-1 and Hydrogen Peroxide. The OS in the samples treated with SIN-1 dwarfs that of the samples treated with Hydrogen Peroxide and the untreated samples. B. The results from the same experiment excluding SIN-1 treated samples to show the contrast between the H_2O_2 and untreated samples. This experiment confirmed that SIN-1 could induce OS in our HBMECs and would be ideal for future studies.

3.3 Sustained OS Experiment

SIN-1 mediated OS was sustained in endothelial cells (Fig. 4). At the 6, 12, and 24 hour time points, the samples treated with SIN-1 exhibited the greatest fluorescence intensity of all of the experimental groups. Even though the fluorescence in the SIN-1 samples was greater than the others, the intensity at 6, 12, and 24 hour time points was about ten times less than cells treated with SIN-1 then measured immediately. This suggests that within the 6 hours after treatment the cells may be responding to decrease their level of OS. Even with this supposed cellular response the SIN-1 treated cells still maintained a fluorescence intensity of close to 300% of the average intensity of samples containing untreated cells. At each of the time points the fluorescence intensity of the samples containing H_2O_2 treated cells and untreated cells was relatively the same. This is most likely due to the cells ability to easily metabolize H_2O_2 in order to protect themselves from damage resulting from increased OS. The rapid breakdown of H_2O_2 shows that it would not be an effective treatment for further experiments studying the response of endothelial cells to extended periods of oxidative stress. As time progressed after the 6 hour mark oxidative stress in the H_2O_2 and untreated samples began to increase. It is possible that this increase may be due to the endothelial cells being kept in KRH Buffer instead of supplemented M199 media that they are cultured in. Once again, there was very little auto-fluorescence from the wells of the plate. Since the samples containing SIN-1 remained in a state of high OS compared to the untreated cells through 24 hours, we concluded that it is an effective treatment to measure the cellular responses to extended periods of OS. Both immediately after treatment with SIN-1 and after 24 hours of incubation post SIN-1 treatment no HBMEC detachment from the 96-well plate was seen indicating that our treatment conditions did not induce apoptosis.

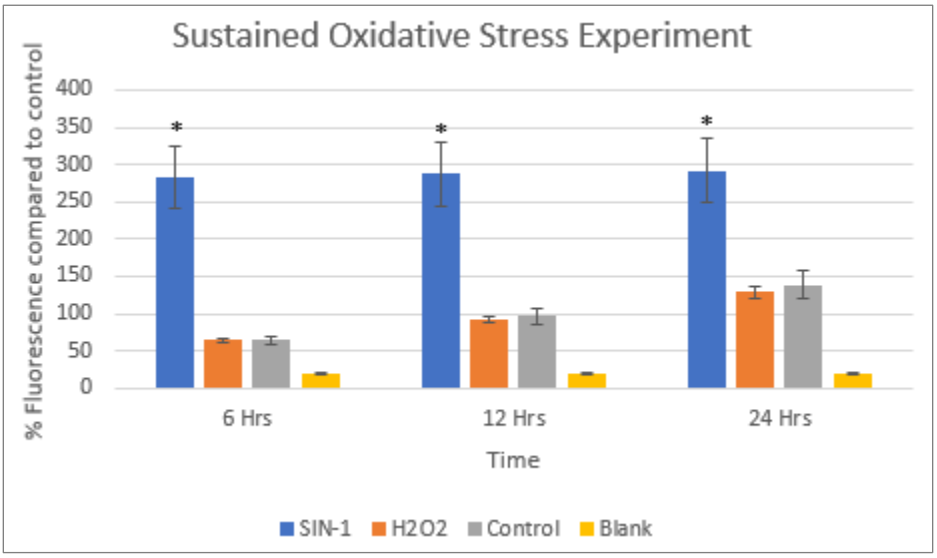


Figure 4. Time course of the duration of OS after being treated with SIN-1 or H_2O_2. Samples treated with SIN-1 displayed a significantly higher fluorescence intensity at all three timepoints indicating that the OS induced by treatment was sustained for 24 hours (*p > 0.001, Anova). In samples treated with H_2O_2 the fluorescence intensity was relatively equal to that of untreated cells indicating that the cells were no longer in a state of OS.

3.4 Adhesion Assay for OS

After inducing OS in HBMECs using the peroxynitirite generator SIN-1 we observed a significant increase in the adhesion of PCa cells (Fig. 5). In SIN-1 treated samples we observed that about 1.07% of the 100,000 cells analyzed were fluorescently labeled C4-2 cells. In untreated samples, we saw this percentage decrease to about 0.625%. Using these percentages, we were able to determine that there was a 1.7 fold increase in attachment in the SIN-1 treated samples. The greater number of adherent PCa cells in the samples with HBMECs under OS supports our hypothesis that OS increases HBMEC to PCa cell attachment.

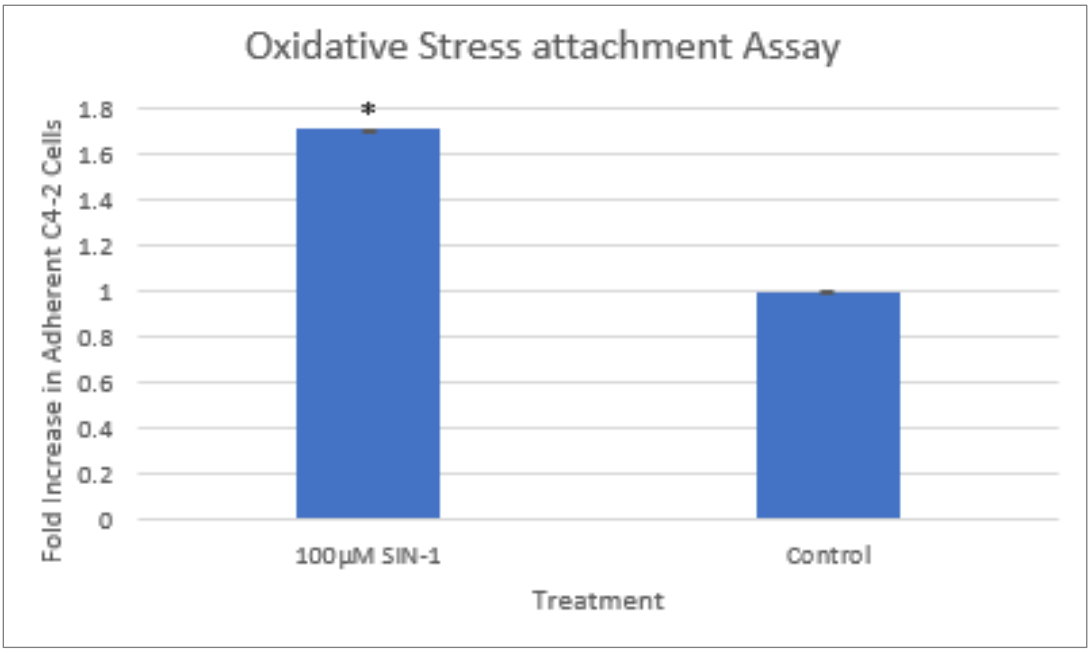


Figure 5. My adhesion assay shows that there is a significant increase in attachment with HBMECs that are experiencing OS compared to those in normal conditions (*$p < 0.005$, Anova). This increase confirms my hypothesis that OS plays a role in PCa cell to HBMEC attachment. However, to confirm that the observed increase is the result of altered CAM expression further studies will be required to observe how CAMs respond to OS.

Chapter 4

CONCLUSION AND DISCUSSION

4.1 Conclusion:

In this study, I sought to uncover physiological factors that could potentially affect the ability of prostate cancer cells to metastasize to the bone marrow endothelium. Since African Americans exhibit the greatest incidence of and mortality from prostate I chose factors that are more common in the African American population. Initially I proposed that fluid shear increased PCa cell adhesion since African Americans experience greater rates of hypertension and therefore greater shear on their vascular endothelial cells. After subjecting endothelial cells to oscillatory fluid shear for 2 hours we observed a slight decrease in the adherence of PCs cells. Although initially this decrease led us to believe that fluid shear did not play the role in cell adhesion that we expected, further analysis of literature suggested differential responses of endothelial cells to short term and long term fluid shear (20, 21). Short term or acute fluid shear stress such as that experienced when a person exercises has been shown to have beneficial and protective effects on the body like inducing vasodilation and increasing the permeability of the endothelial cell layer (22). Another effect is an increase in eNOS activity and NO production which could decrease the expression of CAMs (15). Long term or chronic shear such as that experienced by a person who suffers from hypertension leads to adverse effects including signaling cascades that result in the activation of transcription factors that increase ICAM-1 and PECAM -1 expression (20). Increasing the presence of these molecules not only increases PCa metastasis by providing more sites for attachment

and extravasation but will also contribute to chronic inflammatory processes which have been linked to many pathologic conditions. The second physiological factor we chose to study was OS which many African American endothelial cells are under due to elevated O_2^- levels. The excess O_2^- comes from a variety of sources including a dysfunctional eNOS enzyme and increased NADPH oxidase activity both of which are found more often in African Americans (23,24). Excess O2- can interact with NO produced by cells which decreases the bioavailability of NO and causes oxidative damage(24). The free radical generator SIN-1 was chosen to induce OS in our endothelial cells because its decay mechanism results in ONOO- which is the product of the O2- and NO reaction. Using the fluorescent molecule DCFH-DA we were able to show that SIN-1 increased the presence of ROS in endothelial cell culture, that ROS levels remained high over 24 hours, and that the endothelial cells did not die in response to treatment. Initially my protocol to measure the change in PCa to HMBEC attachment in response to OS consisted of 1 hour of SIN-1 treatment followed by a 6 hour incubation to allow for the transcription of new CAMs then 2 hours of PCa attachment. Following this protocol, I observed no increase in adhesion. However, after increasing the time of SIN-1 treatment from 1 hour to 18 hours I was able to observe an increase in PCa attachment to endothelial cells. My results do indicate that PCa cells exhibit a greater ability to attach to endothelial cells that are under oxidative stress which could provide some context to the greater rate of metastasis in African Americans. I also saw that there is a difference between how fluid shear and OS effect cellular responses in acute vs. chronic situations.

4.2 Clinical Significance

My overarching goal of these studies was to provide insight into the racial disparity in prostate cancer metastasis that exists between African Americans and Caucasian Americans. Knowledge of which physiological factors contribute to PCa metastasis is crucial to developing novel treatments to improve the prognosis for PCa patients. Besides medicinal treatments knowledge that OS contributes to metastasis indicates that lifestyle changes to reduce OS can be beneficial to early stage PCa patients. Some lifestyle changes decrease OS include reducing sugar intake, preventing infections, reducing stress, avoiding certain chemical compounds, and consuming foods and supplements that increase the production of antioxidants (25). All of these changes work to restore the balance between ROS and antioxidants in the body and a few such as stress reduction and avoiding certain chemical compounds like those in cigarettes also decrease rates of hypertension. The implementation of these practices in early stage prostate cancer patients could combat metastasis and increase survival rates which plummets by over 70% when PCa metastasizes (19).

4.3 Future Directions

In order to determine the mechanism by which OS increases prostate cancer metastasis further studies must be performed. First, by using a method such as qPCR we will measure how the expression of certain CAMs change after the induction of OS. After observing the difference in cellular attachment due to extended SIN-1 treatment we will repeat the shear experiment with longer shear times to determine if we can induce a chronic response that increases PCa attachment. Future shear studies will also aim to more accurately mimic physiologic conditions which includes developing methods for subjecting endothelial cells to laminar flow inducing levels of

shear that are representative of normal and hypertensive conditions. One of the more difficult aspects of these experiments that our lab has battled with is shearing the cells without causing them to detach from the substrate that they are seeded on. A potential solution to this issue is coating the surface with some molecule such as collagen to strengthen the adherence of endothelial cells. To more accurately study racial disparity, we will obtain endothelial cells from both African American and Caucasian American individuals with and without hypertension. A protocol developed by Yi Lin et al. (2000) explains using beads coated in P1H12, an antibody specific to endothelial cells, to isolate them from blood draws (18). Using these isolated endothelial cells, we would measure their base levels of ROS to confirm that African American cells are in fact under oxidative stress. Finally, we will use the isolated endothelial cells to measure the difference in PCa adherence to African American versus Caucasian American their respective ability to compensate with treatments that induce OS.

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