DIRECT EXERCISE METABOLITE INHIBITION OF BREAST CANCER CELL GROWTH

An Undergraduate Research Scholars Thesis

by

ALEXANDRA CHRISTINE SCHNEIDER

Submitted to Honors and Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as an

UNDERGRADUATE RESEARCH SCHOLAR

Approved by
Research Advisor: Dr. Steven Riechman

May 2014

Major: Kinesiology

TABLE OF CONTENTS

ABSTRACT

Direct Exercise Metabolite Inhibition of Breast Cancer Cell Growth. (May 2014)

Alexandra Christine Schneider
Department of Health and Kinesiology
Texas A&M University

Research Advisor: Dr. Steven Riechman
Department of Health and Kinesiology

The purpose of this study is to determine the effect of lactate released from contracting muscle on breast cancer cell growth. Epidemiological studies clearly show that physically active women have a significantly reduced risk of developing breast cancer but the mechanism is unknown. It is well known that during exercise, contracting muscle releases many different substances, including hydrogen ions, lactate, adenosine diphosphate, ammonia, as well as many proteins. Therefore, it is hypothesized that an exercise-induced, muscle specific factor directly suppresses breast tumor growth. Likely candidates include a protein or exercise related metabolite. This research will specifically address lactate's role in breast cancer growth.

DEDICATION

I dedicate this research first, to my parents. Without their love and continued support for my education, this would not be possible. They have been by my side through it all, and their words of love and encouragement have helped me through the best and the worst of times. I would like to next dedicate this research to all those affected by breast cancer, specifically my Aunt Tara Schneider. A battle with cancer is not an easy one, and I hope to one-day make that battle a little bit easier for those subjected to such an unfortunate diagnosis.

ACKNOWLEDGEMENTS

I would first like to thank Dr. Steven Riechman for allowing me to collaborate with him in his endeavor to further his research in preventative chemotherapy for breast cancer. His guidance and knowledge in his field of work has been crucial in the development of this thesis. His support and teaching have been invaluable in my growth as a student and as a person. I would also like to thank Dr. James Fluckey for his knowledge, guidance, and patience throughout my research experience.

CHAPTER I

INTRODUCTION

Influence of exercise on breast cancer

An increased prevalence in breast cancer has lead to an increase in the amount of research done regarding breast cancer prevention and treatment. Although many believe that breast cancer is much more curable, it is likely related to the fact that breast cancer is diagnosed in earlier stages of its development, therefore leading to higher survival rates due to early treatment implementation. It is also known that there are a variety of risk factors that play a role in an individual's likelihood of developing breast cancer. Unfortunately, for example, age, family history, and race are aspects of one's life that cannot be changed. Physical inactivity, however, is a modifiable risk factor that, when altered, can significantly influence the likelihood of not only developing breast cancer, but also preventing the growth and spreading of it. This fact alone is the basis for conducting this experiment, along with the hope of developing a cost effective treatment that is available to all individuals. During exercise, contracting skeletal muscle releases a number of metabolic factors into circulation, one of which has shown to directly suppress the growth of breast tumor cells in preliminary data. In particular, this study focuses on the role of lactate in tumor growth suppression.

Exercise metabolites and energy pathways

During exercise, skeletal muscle excretes a number of metabolic by-products, including proteins, hydrogen ions, ammonia, lactate, and many more. When subjected to different exercise bouts, skeletal muscle activates different metabolic pathways depending on the energy requirements for

that particular exercise session. The metabolic pathways of concern in this study are those that involve the release of lactate. Anaerobic Glycolysis and Oxidative Phosphorylation both involve the production of lactate. Anaerobic glycolysis relies on the breakdown of glycogen to provide energy. Glycogen is converted to glucose, which is then broken down in order to synthesize the energy molecule, ATP. During this synthesis, the breakdown of glucose results in the creation of the metabolic by-product pyruvate as well as excess hydrogen ions. These substances will combine in order to prevent metabolic acidosis, thus creating lactate. When lactate begins to accumulate in the blood, the body will eventually reach its lactate threshold. Concentrations past this threshold indicate that the body has crossed over into anaerobic metabolism and has maximized oxygen utilization. This is in part due to the fact that the brain is only able to use glucose for energy. The rest of the body must accommodate the brains' demands, and therefore spare its glycogen stores in order for the brain to maintain its minimal energy requirements. Then, the process of oxidative phosphorylation will take place. In this process, pyruvate is converted to Acetyl CoA, which will yield a large amount of ATP to sustain the energy demands of the body, specifically contracting skeletal muscle.

The Warburg Hypothesis

Although the systems of the body can function on many different energy pathways, the Warburg Hypothesis suggests that tumor cells can only survive on glycolytic energy metabolism (Chen, 2008). Warburg suggested that by blocking the tumor's ability to use glycolytic metabolism, an antiproliferative and apoptotic effect is observed. As previously mentioned, in response to higher energy demands, the body must shift in to different metabolic pathways to sustain energy requirements. During exercise, the increases in metabolism in skeletal muscle typically require a

rapid shift in energy partitioning and mobilization. Thus, the overarching hypothesis is that skeletal muscle, in response to accelerated metabolism due to muscle stimulation, releases a metabolite that acts directly on all uninvolved tissues in suppressing glycolytic metabolism in order to spare glucose for essential and immediate functions (primarily brain and skeletal muscle). Suppression of glycolytic metabolism compels oxidative metabolism and mitochondrial adaptation. In cancer cells, compulsory activation of dysfunctional mitochondria results in apoptosis. It is recognized that skeletal muscle releases multiple metabolites such as lactate, hydrogen ion, potassium, adenosine and nitric oxide that have many local and systemic effects to adapt to acute exercise challenge mainly by redistribution of blood flow. I am hypothesizing that the tumor suppressing effects will only be seen if lactic acid is present, which would be consistent with Warburg's hypothesis, as lactate can specifically cause these cellular shifts in metabolism.

Preliminary data

Preliminary data gathered from initial studies by Dr. Riechman provide evidence that a muscle specific factor is released into circulation and directly suppresses tumor growth**.** In a rat hindlimb that is perfused with a red blood cell free artificial blood and stimulated to contract, the resulting perfusate coming out of muscle inhibits breast cancer cells in vitro and tumors in vivo. In cell culture, a 40-50% reduction in total cell number is observed in the MCF7 bioassay in stimulated perfusate vs. unstimulated muscle perfusate. In two separate experiments where muscle perfusate was injected into MCF7 tumor bearing mice, tumor size was significantly suppressed only in those injected with stimulated muscle perfusate. This was consistent with the significantly elevated markers of apoptosis.

CHAPTER II

METHODS

Hindlimb perfusion

Female wistar rat hindlimbs, following a hemicorpus preparation, will be perfused using an oxygenated, red blood cell-free Krebs-Henseleit (K-HB) medium (pH 7.4), containing a Muscle Biology (Fluckey) and Human Countermeasures Laboratory (Riechman) developed protein free osmoregulator additives as well as standard albumin osmoregulator and 5 mM glucose. After an initial control period of 10 minutes of perfusion, rat hindlimbs will be stimulated with high frequency pulses to elicit high intensity muscle actions. During muscle actions, flow rate will be elevated to 21 ml per minute to accommodate activity. Perfusate will be collected separately during the initial control perfusion period and after the onset of muscle contractions. This 10 minute control/resting condition followed by 10 minute stimulation/exercise is our basic design but we will repeat the experiment two additional ways. The time under perfusion control will be 10 minutes control/resting followed by another 10 minutes of control/resting. Finally, we will perform the stimulation/exercise in the first 10 minutes followed by 10 minutes of control/resting. For the MCF7 assays we will also add a perfusate control group that will be unused red blood cell-free Krebs-Henseleit (K-HB) medium otherwise treated the same as experimental samples. These additional controls will ensure that our observed effects are due to muscle contraction and not just due to muscle contractions coming at the end of the experiment when it is possible that the experimental conditions are causing a detrimental effect of the muscle.

Lactate analysis

As previously mentioned, the experiment is designed to monitor the levels of various exercise metabolites, the primary interest being in lactic acid. Aliquots of perfusate will be taken every 30 seconds over the course of the 20 minutes to be analyzed for changes in lactate levels. This will be important in determining whether or not lactate plays a key role in the inhibition of MCF7 breast cancer cells. The concentration of lactate will also be compared to the changes in pH exhibited in the muscle perfusate. This will help in attributing the chemotherapeutic effects to pH change, lactate concentration increase, general metabolic changes, or to some other metabolite, primarily the interest being in a protein.

MCF7 Bioassay

These experiments will use cell culture as a bioassay for the determination of the antiproliferative/apoptotic effects of our muscle perfusion isolates. The breast cancer cell lines selected will be one estrogen receptor positive (ER+) (MCF7+E_2 pellet) and one negative (ER-) (MDA MB 231). Muscle perfusate from the various conditions described under hindlimb perfusion will be lyophilized and resuspended in sterile water for the bioassay. Additionally, some control samples will have lactate added to equivalent levels as exercise perfusate to determine lactate specific effects. The extracts will first be tested for mitotic and apoptotic effects *in vitro* in the two cell lines using 5-bromo-2-deoxyuridine incorporation (BrdU) and TUNEL assays (Dupont-Versteegden et al., 2006b). Harvested cells will be rapidly frozen to the temperature of liquid nitrogen, pulverized, lysed and homogenized in a specially designed lysis buffer to maintain phosphorylation states of specific proteins at the time of harvest for cell signaling studies. We will then load equal amounts of protein from lysates to a traditional

discontinuous Tris-Glycine buffer system developed by Ornstein and Davis (Ornstein L, 1964,Davis J, 1964)) and further perfected by Laemmli (Laemmli U, 1970) to separate proteins using SDS-PAGE. Thereafter proteins will be transferred to nitrocellulose membranes and probed for proteins regulating growth/survival in the muscle cell (Akt/mTOR pathway, and the mitogen activated protein kinase family), as well as specific markers of apoptosis and proteolysis. Specifically, cell nuclei will be partitioned in order to measure levels of BrdU incorporation (a marker of cellular/nuclear proliferation), and specific proteases, including ubiquitin and ubiquitin ligases, will be assessed as proteolytic markers using methodologies we have previously employed in skeletal muscle (Dupont-Versteegden et al., 2006a). Apoptosis will be measured using TUNEL assay, and the expression of signaling intermediates, including Bcl and Bax and I-kappa-B will be measured.

CHAPTER III

RESULTS

Setback

As our timeline indicated, our experiments were planned for early Spring 2014. However, our IACUC approval required some revisions before we were able to place the order for our animals. We promptly made the necessary changes, however, Dr. Riechman continued to receive notifications indicating that additional revisions were needed, although we were certain these corrections had been handled. After much disdain, Dr. Riechman contacted the head of the IACUC board to find out why revisions were still necessary and what still needed to be done in order to receive final approval. We were informed that there had been a glitch in the system and our IACUC proposal was continually being submitted as a new proposal, rather than as a revised proposal. Our proposal was eventually approved and we were able to order animals at the beginning of March. As you can imagine, this set our project back quite some time and we have been struggling to meet our deadlines.

Before any experiments can begin, the Wistar rats had to be put in isolation for at least one week in order to adjust to their new environment. After this one-week isolation period, we hoped to finally begin our experiment. Unfortunately, scheduling conflicts continued to impede the beginning of our experimentation. Although our team could not all be present for the "ground-breaking" surgery of sorts, Dr. Riechman was able to successfully perform a trial surgery on Friday, March 28, 2014. This was important technical, procedural practice to firmly establish the coordination of these complex experiments. We were able to establish very precise lactate and

glucose measurements that fell in normal physiological ranges. April 4th experiments are planned to complete the muscle perfusate and lactate determination for the objectives of this study.

Our team has continued working diligently to prepare the bioassays and ensure that our lactate analyzer is ready for the first successful perfusate collection. As with any research project, things never go according to plan and our team has done the best we can with the hand we have been dealt. April 4th experiments are planned to complete the muscle perfusate and lactate determination for the objectives of this study.

CHAPTER IV

CONCLUSION

Looking to the future

Without any usable data to run through the lactate analyzer or use in our bioassays, it is difficult to make a definitive conclusion regarding the chemotherapeutic effects of lactate at this point in time. However, data will continue to be collected over the next few weeks and a conclusion will be reached on the role of lactate in MCF7 breast cancer cell growth inhibition.

Current research supports that blood lactate concentration will increase as a result of exercise. We can, without any reasonable doubt, hypothesize that the lactate concentrations will consequently vary according to muscle stimulation levels. Our experiment model currently states that we will be collecting aliquots every 30 seconds during the rest period as well as during the stimulation period. This is to ensure that we are able to directly attribute the change in lactate concentration to the stimulation, and not to any other source, such as the length of time the perfusion has been running.

During the trial consisting of 10 minutes of rest followed by 10 minutes of rest, we can assume that there will be no changes in the level of lactate present in our aliquots. In the trial combining 10 minutes of rest followed by 10 minutes of exercise, we will expect to see a sharp increase in the lactate concentration in each subsequent aliquot beginning at the 10 minute, 30 second collection. In the final trail, combining 10 minutes of stimulation, followed by 10 minutes of rest, we expect to see a gradual decline in the lactate concentration during the resting period, which can be attributed, with much certainty, to the lack of muscle stimulation.

Following the collection of all data, we plan to run the aliquots through the lactate analyzer in order to track the changes in lactate concentrations. This analysis will be compared to the results we obtain from the bioassays. Should we find that the tumor cell growth is inhibited by the perfusate collected during the stimulation periods, we can assume that lactate likely has some role in the suppression of cell growth. However, is cell growth is inhibited by perfusate collected from rest periods, as well as from stimulation periods, then it is likely that the source of the chemotherapeutic effects does not come from contracting muscle. Either of these results would help further our research by either confirming out negating my hypothesis, along with Dr. Riechman's hypothesis.

Expectations

When I originally began working with Dr. Riechman on this project, I had very high expectations of coming out on the other side of this with a hugely expanded knowledge of the chemotherapeutic effects of exercise on breast cancer tumor cell growth. I was also somewhat naïve to think that we would not run in to any hold-ups or to think that everything would go according to plan. This is science – how could I expect that? Although my expectations in that regard have not yet been met, I have no doubt that over the course of the next month we will gain a great deal of knowledge and will have taken one step closer to pinpointing the source of these chemotherapeutic effects. I have indeed come out with a more mature and realistic mindset regarding research; as a scientist we must be prepared for the worst while hoping for best, and anything in between is just another stepping stone to reach our final goal. It has been hard for me to understand how there can be so much research conducted to find a cure for cancer, but yet we still don't have a cure. I now see the light, so to speak.

REFERENCES

Chen JLY, JE Lucas, T Schroeder, S Mori, J Wu, J Nevins, M Dewhirst, M West, JT Chi. The Genomic Analysis of Lactic Acidosis and Acidosis Response in Human Cancers. PLOS Genetics 4: 2008.

Dupont-Versteegden E E, Fluckey J D, Knox M, Gaddy D, Peterson C A. Effect of flywheel-based resistance exercise on processes contributing to muscle atrophy during unloading in adult rats. J Appl Physiol 2006a; (101): 202-212.

Schulz TJ, Thierbach R, Voigt A, Drewes G, Mietzner B, Steinberg P, Pfeiffer AF, Ristow M. Induction of oxidative metabolism by mitochondrial frataxin inhibits cancer growth: Otto Warburg revisited. J Biol Chem 281: 977–981, 2006.

Weinhouse, S. "The Warburg Hypothesis Fifty Years Later." *Zeitschrift Fur Krebsforschung Und Klinische Onkologie* 87.2 (1976): Print.