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Role of Periprostatic Adipose Tissue in Prostate Cancer Cells

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ROLE OF PERIPROSTATIC ADIPOSE TISSUE IN

PROSTATE CANCER CELLS

by

Massa Mafi

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ABSTRACT

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CANCER CELLS

by

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Under the Supervision of Professor Jennifer A. Doll

Background: Prostate cancer (PCa) is a leading men's health concern. It is the most common cancer diagnosed in American men. Obesity, especially excess visceral adipose tissue, increases the risk of PCa progression; however, the mechanism is yet unknown. In addition, studies have demonstrated that the locale of the visceral adipose depots affects its function and change the levels of secreted products. In PCa patients, increased thickness of the periprostatic adipose tissue (PPA), a type of visceral adipose tissue which covers the prostate organ, is positively correlated with progression, suggesting that this depot may specifically stimulate PCa progression in obese patients. In this study, I tested the hypothesis that visceral adipose tissue (VAT) secretions, and specifically PPA tissue secretions, from a mouse model will induce pro-proliferative activity of microvascular endothelial and PCa cells. This hypothesis was tested by two aims. The first was quantifying the pro-proliferative activity of subcutaneous (SQA) and VAT secretions on normal human endothelial cells. The second aim was to measure the pro-proliferative activity of adipose tissue secretions on PC-3 PCa cells.

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Method: SQA and two types of VAT, including visceral fat pad (VFP), and PPA tissues from wildtype mice C57BI/6 and obese mice were collected. Tissues were minced and placed in explant cultures in serum-free media. Conditioned media (CM) was collected after 48 hours of incubation. Human microvascular endothelial cells (HMVEC) were treated with concentrated CM of each adipose tissue depot. HMVEC and PC-3 cells were also treated with unconcentrated CM of each adipose depot. The proliferative activity and total protein concentration of each adipose tissue sample was analyzed via MTT proliferation and protein quantification assay, respectively. Data from the MTT assay were analyzed by normalizing them to total protein concentration per gram weight of each adipose tissue sample. Lipolytic activity of each adipose tissue sample was also measured by performing a free glycerol assay.

Result: With HMVEC cells, the unconcentrated 100% VAT CM treatment group had significantly higher cell numbers as compared to the SQA 100% CM or negative control groups (P-value < 0.001). However, when the CM was concentrated no HMVEC proliferation was observed with any adipose tissue types that were tested. The 30% diluted SQA tissue CM also significantly induced proliferation of PC-3 PCa cells (P-value ≤ 0.05) as compared to the negative control. However, no proliferation was seen when a larger sample size was used. Finally, a significant increase in PC-3 PCa cell proliferation was observed with PPA tissue CM treatment when data was normalized to total protein concentration per gram weight of each sample (P-value < 0.001 vs. the negative control). There was also no detectable difference in lipolytic activity between the SQA, VAT, or PPA tissues.

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Conclusion: My results demonstrated that treatment with the PPA tissue secretions increased PC-3 cell number over that of either the VFP or the SQA tissue secretions. These data suggest that unique characteristics of the PPA tissue may contribute to PCa progression.

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Finally, but most importantly, I would like to thank my dear husband and my beloved daughter, Arash and Delara, for their un-ending support and unconditional love.

INTRODUCTION

The Prostate and Prostate cancer

The prostate is a walnut-sized gland that constitutes a part of the male reproductive system. The prostate is located under the bladder and in front of the rectum. The main function of the prostate is to secrete some of the fluid content of semen (CDC, 2013). Family history, older age, and genetic background can increase the likelihood of developing prostate cancer (PCa) (Hsing et al., 2007). PCa is a leading men's health concern. It is the second-most common cancer diagnosed in American men (CDC, 2013a). According to the Centers for Disease Control and Prevention (CDC), PCa is more common among men older than 65, but the chance of developing PCa increases after age 50 (CDC, 2014). It has been estimated that 238,590 men are diagnosed with PCa and 29,270 men among these patients die from the disease in America annually (Siegel et al., 2013). Genetics, race, and advanced age are the most common risk factors that are involved with developing PCa, as well as lifestyle factors such as a high fat diet (CDC, 2002). A study by Xue et al. showed that feeding a Western-style diet (low calcium and vitamin D, but high in fat content) to C57BL/6J mice for 5 and 16 weeks increased the proliferation of prostate epithelial cells in the anterior and dorsolateral lobes of the mouse prostate, but not in ventral lobe of the prostate (Xue et al., 1997). Low grade PCa is a slow-growing cancer (CDC, 2013); hence, men are usually diagnosed when they are old and/or they might die from other diseases without even being diagnosed with PCa (CDC, 2013b). However, high-grade PCa which also affects younger men (Roach et al., 1999) tends to grow quickly (Gleason 1992). The rate of dying from the disease is also 3% higher in young patients than in older patients (Lin et al., 2009).

Digital rectal exam (DRE) and the prostate specific antigen (PSA) test are the two common screening tests that are available for PCa detection (CDC, 2013c). In a DRE, a doctor can physically check the prostate by inserting his finger into the patient's rectum and examine any abnormalities (CDC, 2013c) such as presence of nodules (Simmons et al., 2011). In PSA testing, elevated levels of PSA in the blood can indicate the presence of PCa (CDC, 2013c). A PSA level of above 2.5-4 ng/mL is considered to be abnormal and it is linked to an increased chance of having PCa. Some factors, including enlarged prostate or inflammation (Nadler et al., 1995), age, race, infection, and certain medications (CDC, 2013c), can increase the level of PSA in non-prostate cancer men; thus, the PSA test alone cannot be used to diagnose PCa. Therefore, further follow-up screening and biopsy is needed to confirm the presence of the tumor (Simmons et al., 2011). A high PSA level is correlated with a higher grade (Gleason score) PCa, meaning that men with higher PSA levels have more aggressive PCa (Shih et al., 1992; Thompson et al., 2004).

The Gleason score is a system used for PCa pathological grading (Andren et al., 2006; Gleason, 1992). This technique indicates the survival rate based on the histology of the cancer tissue (Andren et al., 2006; Gleason, 1992). In this technique, the primary and the secondary patterns, which are the most and second most common patterns of cancer tissue in the prostate, are assigned a grade ranging from one to five. The sum of these two grades makes a range of two to ten, which is called the Gleason score. A score of six is considered to be cancer, and a score of seven or above represents a more aggressive

tumor (Witte et al., 2000). D'Amico introduced a classification method to aid in the identification of more aggressive PCa. In the D'Amico classification method, the combination of the Gleason score, the PSA level, and the size of the tumor were used to predict the risk of PCa recurrence (D'Amico et al., 1998; Kane et al., 2005).

Obesity in Prostate Cancer

Obesity, the prevalence of which is rapidly growing in the United States, is known to be associated with aggressive PCa, which tipically leads to death (Freedland and Aronson, 2005; Freedland et al., 2009; Hsing et al., 2007). Obesity is defined as a body mass index (BMI) of greater than or equal to 30 (Buschemeyer and Freedland, 2007). The majority of PCa studies examining the relationship with obesity have used BMI and/or waist to hip ratio for evaluating the amount of adipose tissue in the body (Buschemeyer and Freedland, 2007). Although these methods cannot differentiate between types of adipose tissues or their locations in body, they are reasonable methods to measure overall adiposity (Hsing et al., 2007; Pouliot et al., 1994). Supporting this, increased BMI correlates with more aggressive PCa (Baillargeon and Rose, 2006; Kane et al., 2005). In a study by Kane et al., 2,952 normal weight, overweight, and obese PCa patients and PCa survivors participated. They were classified based on low to high risk PCa using D'Amico classification and the PSA level that was measured. The study then compared the groups based on BMI and demographics such as age, ethnicity, education, income, relationship status, smoking, alcohol, and comorbidities using univariate and multivariate analysis. The result indicated that normal and even overweight patients are at low risk,

while obese patients are at high risk, for high-grade PCa (Kane et al., 2005). A review of large prospective cohort studies by Freedland and Aronson, which monitored healthy men for 13 to 16 years for medical problems such as obesity and cancer, demonstrated that the rate of dying from PCa increases as the BMI increases (Freedland and Aronson, 2005). For example one of these studies showed that 20% of obese men with a BMI of 30.0-34.9 kg/m^2 died from PCa, while the rate of death from PCa was 14% more among even more obese men with a BMI of 35.0-39.9 kg/m^2 (Freedland and Aronson, 2005). Some research also suggests that obesity is also a risk factor for PCa, but because of the complex association, the exact mechanism that is involved is not known yet (Baillargeon and Rose, 2006).

A high fat diet often leads to obesity, and obesity plays an important role in increasing the risk of developing many cancers, such as colon, gallbladder, kidney, and pancreatic cancer (Hsing et al., 2007). While in PCa, where risk is still being studied, it is well established that a high fat diet and obesity are correlated with more aggressive disease. Excess body fat is associated with altered metabolism, which may contribute to cancer progression (Hsing et al., 2007). More than one-third of adults in the United States are obese (CDC, 2013d); therefore, understanding the interaction between obesity and PCa is important.

Adipose tissue depots

There are two types of adipose tissue in mammals' bodies, white and brown (Gustafson, 2010). White adipose tissue is made up of adipocytes, which are responsible for storing excess energy as lipids (Fonseca-Alaniz et al., 2007). White adipose tissue can be generalized by its location as subcutaneous adipose (SQA) tissue, located beneath the skin, or visceral adipose tissue (VAT), which covers the organs (Fain et al., 2004). These adipose tissues consist of adipocytes, fibroblasts, macrophages and some other stromal-vascular fraction cells. Macrophages and fibroblasts constitute about one-half of the total cell number (Hausman, 1985).

Adipose tissue depots differ in their production of cytokines and adipokines that are involved in autocrine, paracrine, and endocrine systems in the body (Greenberg and Obin, 2006; Tritos and Mantzoros, 1997). Some fat depots are more associated with the incidence or progression of some diseases than other fat depots (Bjorndal et al., 2011). For example, excess visceral adipose tissue in the body is linked to many diseases, such as cardiovascular and type 2-diabetes (Hajer et al., 2008). Hence, the differences between SQA tissue and VAT are not only limited to their location, but also vary in function and metabolism (Fonseca-Alaniz et al., 2007). Excess visceral adipose tissue in men is strongly related with some type of cancers, including colon, esophagus, gallbladder, kidney, pancreas, rectum, and thyroid (Murphy 2013), and it increases the risk of high-grade PCa (Gong et al., 2006).

It is now recognized that adipose tissue is an endocrine organ because it produces some hormones and cytokines such as leptin, interleukin-6 (IL-6), and tumor necrosis factor - α (TNF-α) (Ahima and Flier, 2000; Fain et al., 2004; Fruhbeck et al., 2001). IL-6 is a key regulator in immune system. It also plays a role in lipolytic activity and maintains body weight (Hoene and Weigert, 2008). TNF-α is one of the main mediators of inflammation (Kern et al., 2001; Suganami et al., 2005), and leptin is a hormone that controls appetite and regulates energy expenditure to maintain body weight homeostasis (Benoit et al., 2004; Saglam et al., 2003). Adipose tissue's endocrine function can be altered by excess body adipose tissue. The adipose tissue in obese individuals has different levels of adipokine production, lipid metabolism, and adipocyte differentiation (Kershaw and Flier, 2004). This is mostly related to individuals' age, sex, nutrition, and also the ability to maintaining energy balance by the body's adipose tissues (Bjorndal et al., 2011; Wajchenberg, 2000).

The periprostatic adipose (PPA) tissue, which covers the prostate organ, is one type of visceral adipose tissue. Some studies link excess PPA tissue to aggressive PCa. PPA tissue thickness increases in obesity, and a clinical study by Bhindi et al. has shown that patients with thicker PPA tissue have more aggressive PCa (Bhindi et al., 2012). They indicated that measuring the PPA tissue thickness via transrectal ultrasonography can be a stronger predictor for aggressive PCa than overall obesity (Bhindi et al., 2012). Another study has shown that high levels of IL-6 are secreted from the PPA tissue of patients with a high grade of PCa (Finley et al., 2009). However, the molecular mechanism that is involved and the unique characteristics of the PPA adipose tissue that promote this

activity are still unclear. A pilot study by Venkatasubramanian et al., the authors collected SQA and PPA tissue from lean, overweight, and obese PCa patients during prostate removal surgery. They found that PPA tissue secretions from obese PCa patients were more inductive in both endothelial and PC-3 PCa cells in an MTT proliferation assays than PPA secretions from lean or overweight men and SQA tissues, but lean PPA tissues were also inductive over the negative control (Venkatasubramanian et al., 2014). Therefore, PPA tissue seems to contribute to PCa progression.

Obese versus lean adipose tissue

White adipose tissue works as a thermal insulator to maintain body temperature (Fonseca-Alaniz et al., 2007). In a lean body, white adipose tissue, including SQA and visceral adipose tissue, can store 200,000- 300,000 kcal for energy. Adipose tissue also has an endocrine function and it secrete high levels of adipokines such as TNF-α, IL-6, and leptin. The functional role of these adipokines is diverse and induces regulating inflammation, cell growth, apoptosis, and motility (Toren et al., 2013; Wang et al., 2012). However, the secretion level of the adipokines is altered when adipose tissue deposition increases, as occurs with obesity. Adipose tissue can be expanded by both adipocyte cell number and size. Each fat cell has a capacity to store 0.7 to 0.8 µg of lipid. If more lipids are produced in the body, then more fat cells will be generated (Gustafson, 2010). Therefore, by increasing the number and size of the adipocytes in obesity, the expression level of some adipokines such as IL-6, TNF-α, and leptin from the adipocytes can also be elevated.

Although only 10% of the total body fat is composed of visceral adipose tissue, it has been shown to be metabolically more active than subcutaneous adipose tissue (Hsing et al., 2007). Visceral obesity has an influence on cellular pathways and alters hormone circulations (Mistry et al., 2007). The effect of VAT on cellular pathways could be either local through autocrine and paracrine mechanisms or via endocrine pathways mechanism. It could also be related to the metabolic and lipolytic activity of the VAT (Deveaud et al., 2004; Wajchenberg, 2000; Yang et al., 2008). Alteration in androgen metabolism and insulin resistance (metabolic syndrome) are examples of these effects (Hsing et al., 2007; Mistry et al., 2007). Cytokine and hormones are the key regulators in these mechanisms; therefore, changes in the regulation and secretion level of each adipokine are associated with some health issues (Hsing et al., 2007; Mistry et al., 2007). Some adipokines expression is increased with obesity, and a subset of these is known to enhance the aggressiveness of PCa such as IL-6, leptin and TNF-α (Mistry et al., 2007).

Angiogenesis and Obesity

When adipose tissue expands in the body, the tissue must induce angiogenesis, the formation of new blood vessels from existing blood vessels. The new blood vessels are required to transfer oxygen and nutrients to the growing adipose depot (Fried et al. 1998). Angiogenesis is also a required step for tumor growth and development (Folkman, 1992). In a normal tissue, there is a balance between pro-angiogenic factors and inhibitory factors; however, in tumor tissues, alterations in this equilibrium might lead to higher

production of pro-angiogenic factors and result in tumor growth (Kerbel, 2000). Furthermore, a high level of cytokines in the tumor microenvironment could modulate the expression of some growth factor molecules which enhance tumor growth (Wu, 2012). For example, high levels of leptin cause proliferation of vascular endothelial cells in vitro and also initiates angiogenesis in vivo (Mistry et al., 2007). Other secreted factors from obese adipose tissue could also contribute to a net pro-angiogenic function.

Leptin

Circulating leptin in a lean body not only functions to maintain energy balance and immune system function, but it also plays a role in growth and maintenance of reproductive tissues, such as prostate tissue (Considine et al., 1996; Mistry et al., 2007). Expression of leptin has been shown to be elevated with increasing total body adiposity. Average leptin serum levels in a normal weight body is about 4 ng/ml, while in an obese body, this level is 10 times higher (40 ng/ml) (Considine and Caro, 1996; Frankenberry et al., 2004). This elevation might be related to higher insulin levels, which do impact leptin production (Fantuzzi, 2005). High levels of leptin have been shown to induce vascular endothelial cell proliferation in vitro. For example, a vitro study by Frankenberry et al. demonstrated increased DU145 and PC-3 cell proliferation with increasing leptin levels (Frankenberry et al., 2004). They also observed higher migration activity in these PCa cells with increasing leptin levels (Frankenberry et al., 2004). Another study, by Onuma et al., also examined leptin-stimulated growth of different cell lines, including DU145, PC-3, and LNCaP-FGC. The results, indicated that leptin induces proliferation of

androgen-independent cells, DU145 and PC-3, but not androgen dependent cell line, LNCaP-FGC (Onuma et al., 2003).

Interleukin-6 (IL-6)

Plasma IL-6 levels have been shown to be positively correlated with obesity (Kern et al., 2001). Moreover, a study by Fried et al. on obese human adipose tissues has shown that for the same number of adipocytes, IL-6 expression is three fold higher in omental VAT as compared to SQA tissue (Fain et al., 2004; Fried et al., 1998). Circulating IL-6 is positively associated with adipocyte size and a high BMI. The expression of IL-6 is also higher in VAT than SQA tissue (Gustafson, 2010). In an in vitro study by Chung et al., two androgen- independent PCa cell lines (DU-145 and PC-3) and two androgen-dependent PCa cell line (LNCaP-ATCC and LNCaP-GW) were tested for cytokine production levels. The result from this study showed that the androgen-independent PCa cell lines express higher levels of IL-6 (Chung et al., 1999). In addition, an in vitro study by Okamoto et al. demonstrated that IL-6 has the ability to induce proliferation in PC-3 and DU-145, via autocrine activity, while IL-6 inhibits LNCaP, via the paracrine system (Okamoto et al., 1997).

Tumor Necrosis Alpha (TNF-α)

TNF-α is another multifunctional adipokine that is produced by the macrophages in adipose tissues (Fonseca-Alaniz et al., 2007; Wisse, 2004). Similarly to leptin and IL-6, the expression of TNF-α is also increased by obesity (Fonseca-Alaniz et al., 2007).

Unlike the expression of IL-6 and leptin, which is mostly related to visceral obesity, increased TNF-α expression is related to overall obesity (Cartier et al., 2008). TNF-α has been shown to increase the production of leptin (Trayhurn and Beattie, 2001) and IL-6 (Fonseca-Alaniz et al., 2007). An in vitro study by Gasparian et al., the authors showed that TNF-α can induce proliferation of androgen-independent PCa cells via activation of NF-□B which prevents cell apoptosis. In this case NF-□B promotes survival signaling, and therefore promotes PCa proliferation (Gasparian et al., 2002). The precise mechanism through which obese adipose tissue promotes PCa progression is still unclear, but theories include (1) alteration in adipokines and hormones such as estrogen, testosterone and androgen, (2) high levels of pro-inflammatory cytokines such as TNF-α, (4) through signaling mechanisms associated with insulin resistance, and/or (5) through increased dietary fat intake which alters free fatty acid levels and function (Mistry et al., 2007).

Several in vitro studies have shown that, for the same weight of lean adipose tissue, SQA tissue produces more leptin, IL-6 (Wisse, 2004) and TNF- α (Fonseca-Alaniz et al., 2007; Fruhbeck, 2006; Steppan et al., 2001) as compared to VAT. A recent study by Fjeldborg et al. comparing lean and obese human SQA tissue demonstrated that obese SQA tissue produces more IL-6 and TNF-α than lean SQA tissue (Fjeldborg, 2014). In a study by Fried et al., IL-6 expression was compared between omental VAT and SQA tissue of 10 obese humans. The result from the explant cultures of the adipose tissue demonstrated that omental adipose tissue produces three times more IL-6 than SQA tissue (Fried et al., 1998). The release of IL-6 was also higher from omental isolated adipocytes than SQA

isolated adipocytes; however, this secretion was only about 10% of the total IL-6 release from the omental tissue (Fried et al., 1998). Nevertheless, in the case of obesity, abdominal visceral adipose tissue, which typically makes up only 10% of the total body fat, is metabolically more active than subcutaneous fat (Hsing et al., 2007). In addition to the metabolic activity, the rate of lipolysis is also higher in VAT than SQA (Jensen, 2006). Moreover, visceral adiposity is linked to many diseases, such as cardiovascular (Wajchenberg, 2000), insulin resistance, and type 2 diabetes (Virtanen et al., 2005).

A study by Kern et al. also examined the level of TNF-α expression from both lean and obese human adipose tissue (Kern et al., 1995). The result revealed that TNF-α levels are elevated by obesity and the levels, can be decreased by weight loss (Kern et al., 1995). TNF-α also elevates the expression of other cytokines and hormones, such as IL-6 and leptin, by adipocytes (Greenberg and Obin, 2006; Kershaw and Flier, 2004; Margetic et al., 2002). Both IL-6 and TNF-α are secreted by infiltrating macrophages in adipose tissue and act as paracrine and / or autocrine factors (Gustafson, 2010; Hotamisligil et al., 1995; Xu et al., 2002). They are also involved in accelerating lipolysis and the secretion of free fatty acids (FFA) (Gustafson, 2010; Sacca et al., 2012b). Therefore, dysregulated secretion of cytokines and hormones by visceral adipose tissue, due to obesity, may be involved in PCa progression (Hsing et al., 2007). More research is needed to identify the most relevant molecular factors and cellular mechanism that are involved in PCa progression process during VAT dysfunction.

A subtype of visceral adipose tissue, the periprostatic adipose (PPA) tissue, which surrounds the prostate organ, has been investigated for its role in PCa progression. In a study by Bhindi et al., PPA tissue thickness was measured via transrectal ultrasonography in 931 patients who underwent a prostate biopsy. This study showed that the thickness of PPA tissue was a better predictor for developing PCa or for delivering an aggressive form of the disease in patients than measures of overall obesity (Bhindi et al., 2012). Furthermore, a study by Finley et al., on PPA tissue taken from PCa patients, demonstrated that IL-6 is highly expressed by PPA tissue and that the levels positively correlate with increasing Gleason score of the cancer. Therefore, they suggest that the high concentration of IL-6 might contribute to PCa aggressiveness (Finley et al., 2009). Another study, by Sacca et al., compared the activity of secretions collected from PPA tissues from patients with PCa to patients with benign prostatic hyperplasia on proliferation, migration, adhesion, and metalloproteinase expression in two PCa cell lines, LNCap and PC-3 in vitro (Sacca et al., 2012a). Their results indicated that secretion of pro-metalloproteinases (pro-MMP) from PPA is higher from patients with PCa than in patients with benign prostate hyperplasia. MMP-9 is an endopeptidase that is involved in tumor growth and aggressiveness by cleaving molecules such as cytokines and growth factor (Chambers and Matrisian, 1997; Mira and Gómez-Moutón, 2004; Nelson et al., 2000). MMP-9 is secreted as pro-MMP-9 which is then cleaved into an active enzyme (Wilcock et al., 2011). MMP-9 can induce the release of growth factors in addition to degrading the extracellular matrix; therefore, it creates an environment that maintains tumor growth and allows for invasion and metastasis (Bergers et al., 2000; Chakrabarti and Patel, 2005). In addition, MMP-9 expression and activity levels are directly related to

body adiposity (Bouloumie et al., 2001; Unal et al., 2010). Hence, understanding the role of factors secreted by the PPA tissue on PCa progression is crucial. Previous data have shown that the PPA from obese PCa patients is more pro-proliferative on PCa cells compared to lean PPA and lean or obese SQA tissue depots (Venkatasubramanian et al., 2014). Studies to date have not compared the effect of all these three adipose tissue secretions on human normal endothelial or PCa cells. The overarching hypothesis for this study is that *VAT secretions, and specifically PPA tissue secretions, from a mouse model will induce pro-proliferative activity of microvascular endothelial and PCa cells*.

Specific Aim 1

To quantify the pro-proliferative activity of subcutaneous (SQA) and visceral adipose tissue (VAT) secretions on normal human endothelial cells.

Working hypothesis: VAT will induce more proliferation as compared to SQA, and tissues from obese mice will have increased proliferative activity as compared to lean mice.

Specific Aim 2

To measure the pro-proliferative activity of VAT and SQA and PPA tissue secretions on PC-3 PCa cells.

Working hypothesis: PPA tissue induces more proliferation as compared to that of other adipose depots tested.

In the present study, I first analyzed the effect of obesity on pro-proliferative activity of VAT compared to the SQA tissue of lean and obese mice on human dermal microvascular endothelia cells (HMVEC). In the next part of the experiment, I compared the effect of SQA, VAT, and PPA tissue secretions on PCa cell line PC-3. The results from this research in vitro will show us if the PPA secretions cause more proliferation in PC-3 cells compared to other adipose depots.

MATERIALS AND METHODS

Adipose tissue harvests

Two strains of mice, wildtype (WT) C57Bl/6J and obese (ob/ob) mice, B6.Cg-Lepob/J, were used for this study. For the first aim, two experiments were performed, the first using three mice per group at 6 months of age and the second using five mice per group at 6 months of age. The adipose tissue secretion samples from the second group of mice were used in the second aim. The second aim used an additional 7 mice in   at 4 months of age. Mice were housed in the Animal Research Center (ARC). Mice were either obtained from current colonies (WT) or were purchased from Jackson Laboratories. Mice were fed a regular chow diet ad libitum (Teklad 7912) until they reached 4 or 6 months of age. Tissue harvests were conducted in the necropsy room in the ARC. Mice were fasted for 4-6 hours before the tissue harvest. Mice were weighed and data was recorded. The mice were then euthanized by 1-3% isoflurane inhalation in a desiccator jar followed by cervical dislocation. For cervical dislocation, the back end of a tweezers was pushed down and forward on the back of the neck of the animal at the base of the head. The tail was pulled by the other hand backward at 30ºC to the surface of the table for dislocation. No heartbeat was felt before a surgical cut was made. A midline incision through the abdominal skin was then performed. The SQA adipose tissues were harvested from both sides of animal's body (near the fore- and hind-limbs) under the skin. A midline incision was made through the peritoneal lining. The visceral fat pads in the abdomen were collected, and then the intestinal organs were moved aside to reveal

the prostate. The PPA tissue, which covers the prostate organ, was then dissected and collected. VAT, SQA, and PPA tissues were placed on ice in a 50 ml conical tube on ice and transferred to the lab for processing.

All procedures were approved by the University of Wisconsin Milwaukee Institutional Animal Care and Use Committee.

Adipose tissues explant cultures and conditioned media collection

Harvested tissues were immediately processed in the lab for explant cultures. Adipose tissues were first weighed, and then washed twice in sterile Dulbecco's phosphate buffered saline (PBS) in a tissue culture hood. Tissues were processed using sterile instruments. Tissues were minced into 0.5 mm pieces using scissors, and tweezers were used to place the minced adipose tissue pieces into a 10 cm or smaller cell culture dish. Five ml of serum-free Dulbecco's Modified Eagle Medium (DMEM) containing 1% penicillin/streptomycin (P/S) was added to each dish. Serum free conditioned media (CM) were then collected after 48 hours of incubation and stored at 4ºC until use in MTT assays.

HMVEC and PC-3 PCa cell culture

Human dermal microvascular endothelial cells are a normal human endothelial cell strain, (HMVEC-d; Lonza). The cells were grown in endothelial growth medium (EBM-2) containing 10% fetal bovine serum (FBS) and the bullet kit in pre 0.01% gelatinized T75 flasks. The flasks containing cells then were incubated at 37°C with 5% CO_2.

PC-3 is a PCa cell line derived from a human bone metastasis (Kaighn et al., 1979). Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% P/S (Sigma Aldrich) was used as a growth media to maintain PC-3 cells, which were grown at 37°C with 5% CO_2.

Proliferation assays

For the proliferation assay, cells were plated in 96 well plates (Corning Inc.). To enhance cell adherence to the surface of the 96-well plates, they were gelatinized with 0.01% gelatin (Difco TM Gelatin) overnight. The HMVECs were harvested and counted using a hemocytometer or a cellometer (Nexcelom). The HMVECs were then re-suspended at a concentration of 20,000 cells/cm². The growth surface of a 96 well plate was 0.32 cm²/well with a 0.2 ml volume; therefore, 32,000 cells/well were used. The HMVECs were then plated into a 0.01% gelatinized 96 well plate, that was prewashed with 200 µL PBS using a multichannel pipette. The plate was then incubated at 37°C with 5% CO_2 for 24 hours. The growth media were changed after the overnight incubation. The media were aspirated from each well and cells were gently washed with 200 µl per well of PBS. After aspirating the PBS, the cells were then treated with 100 µl per well basal EMB-2 media (serum free media) and incubated for four hours. This starvation step was performed to synchronize cells in the cell cycle. Meanwhile, the treatment samples (CM from each adipose tissue type with or without PBS) were prepared on ice at 30, 50, and 100% concentration of CM or at 1-50µg/ml for concentrated media. After 4 hours, the basal media was aspirated and the treatment samples were added to the appropriate wells. Samples were tested in duplicate for most samples. Growth media and basal media were

added each to three wells, as positive and negative controls, respectively. The plate was then incubated for four days. The plate was checked every day for any contamination. For the methylthiazol tetrazolium (MTT) assay, the treatment media was aspirated, and the HMVECS were labeled by adding 100 µL of fresh serum-free culture medium plus 10 µL of the 12 mM MTT stock solution was added to each well and the plate incubated at 37°C for 4 hours. After this labeling process, the medium, except for 25 µL, were removed from the wells and 50 µL of dimethyl sulfoxide (DMSO) was added to each well and mixed thoroughly with a pipette. After incubating at 37°C for 10 minutes, the plate was read at 540 nm absorbance on the BIO-Tek Synergy HT plate reader. The absorbance data for each sample was entered into an excel spreadsheet. Each test sample was normalized by dividing the absorbance by the gram weight of the corresponding tissue sample. Normalized data was entered into the Sigma plot program and the mean values were graphed.

The same proliferation assay was also performed for the PC-3 cells. However, in this assay, DMEM growth media or DMEM basal media were used to treat these cells. The concentration of 16,000 cells/ well (20,000 cells/ cm2) was used to plate PC-3 cells into a 0.01% gelatinized 96 well plate.

Lipolysis Assay

Lipolytic activity of conditioned media of each of the three adipose tissue type was performed via the free glycerol assay (Sigma Aldrich, Saint Louis MO, F6428). The rate of breakdown of triglycerides to a glycerol and three free fatty acids directly indicated the

level of lipolysis. Briefly, 800 µL of free glycerol reagent (Sigma F6428) was added to all the standards (Sigma G7793) and the sample cuvettes. Ten microliter of each standard was then added to the standards cuvette. 100 µL of each sample conditioned media was also added to the sample cuvettes. The samples and standards were mixed thoroughly with a pipette. Ten microliter of mQH$_2$O was also added to two empty cuvettes to be used as the reagent blanks. The absorbance was read at 540 nm using a spectrophotometer. The standard curve was performed using the glycerol standard (Sigma Cat# 03008), and the each sample lipolytic activity was then measured and compared with the standard curve. The free glycerol data were then normalized to the total cell number.

Concentration of CM

After collecting the conditioned media from each adipose tissue type (VAT, SQA, and PPA tissue) into a 15 or 50 ml conical tube, the samples were centrifuged at 2500 rpm at 4 °C for 10 minutes to remove any residual cells. The cell free supernatants were concentrated 5 fold using Millipore Ultra-free membrane (Amicon Ultra, Billerica, MA). Briefly, the membrane was wet with 5-10 ml of sterile PBS and centrifuged at 2000 x g at 4°C for 10 minutes. Approximately 10 ml of the conditioned media was then added into the filter unit, and it was centrifuged again at 2000 x g at 4°C for 30-60 minutes. The filtrate was discarded every 5-10 minutes. All the conditioned media were added to the membrane and washed with 1-2 volumes of PBS. When the phenol red color of the media had cleared, the media were centrifuged to concentrate the sample 10 fold compared with the original media volume. The conditioned media were then collected in a siliconized microfuge tube to eliminate any protein attachment to the tube wall.

Protein quantification of CM

The Coomassie dye binding assay was used to detect and quantify the total protein in samples. The Thermo-Pierce protocol was used to perform this assay. Briefly, about 10 ml of the Coomassie protein assay reagent, which is a dye, was aliquoted into a 15 ml conical tube. 490 µl of the dye was then added to pre-labeled microfuge tube for both samples and the standards. 10 µl of PBS then was added to the first standard tube and 10 µl of each standard was also added to the rest of the standard tubes based on the concentration respectively and mixed with the dye. Specific amount of total protein was calculated for each sample. 10 µl of each sample was added to the labeled microfuge tube. The color of the solution in the tubes was compared to the standards to assure the sample was within the range of the standard curve. If the color of any sample was darker than the standards, they were diluted with the autoclaved water. 225 µl of each sample and standard were then added to each well of a 96 well plate in duplicate. Finally, the plate reader (BioTek, Synergy HT) was used to read the plate at 595 nm. The KC-4 program produced the standard curve based on the standards and the mean concentrations of the samples.

STATISTICAL ANALYSIS

Student t-tests was used to compare the HMVEC and PCa cell proliferation induced by VAT, SQA, and PPA tissue secretions at each dose. In addition, each of these groups was compared between lean and obese tissues. Within in a tissue, a dose response of the

samples was assessed by analysis of variance (ANOVA). P-value ≤ 0.05 was considered statistically significant.

RESULTS

Concentrated adipose conditioned media (CM) did not induce proliferation of a human normal endothelial cell lines.

Direct direct effect of secretions from adipose tissues on PCa cells or endothelial cells has not been well studied, therefore, to evaluate the pro-angiogenic effects of obese adipose tissue on human normal endothelial cells, and to investigate if VAT has more of a pro-proliferative effect on these cells as compared to SQA tissue, I first treated HMVEC cells with either lean or obese concentrated VAT and SQA tissue CM at 1 – 50 µg/ml for five days and measured proliferation using an MTT assay.

In my initial experiment, I tested 10 and 50 µg/ml total protein. As seen in Figure 1a and 1c, not only did the samples not induce proliferation, at the 50 µg/ml dose, the CM slightly inhibited proliferation in both the wildtype and obese mice samples and in both the SQA and visceral fat pad (VFP) tissue samples CM (P-value ≤ 0.01). Therefore, I reduced the concentration of the CM to 1 and 5 µg/ml, and tested HMVEC proliferation again as above. With these doses, again, no pro-proliferative activity was observed (Figure 1b,d), and the obese SQA CM, at the 5 µg/ml dose, slightly inhibited proliferation (Figure 1b) (P-value ≤ 0.003).
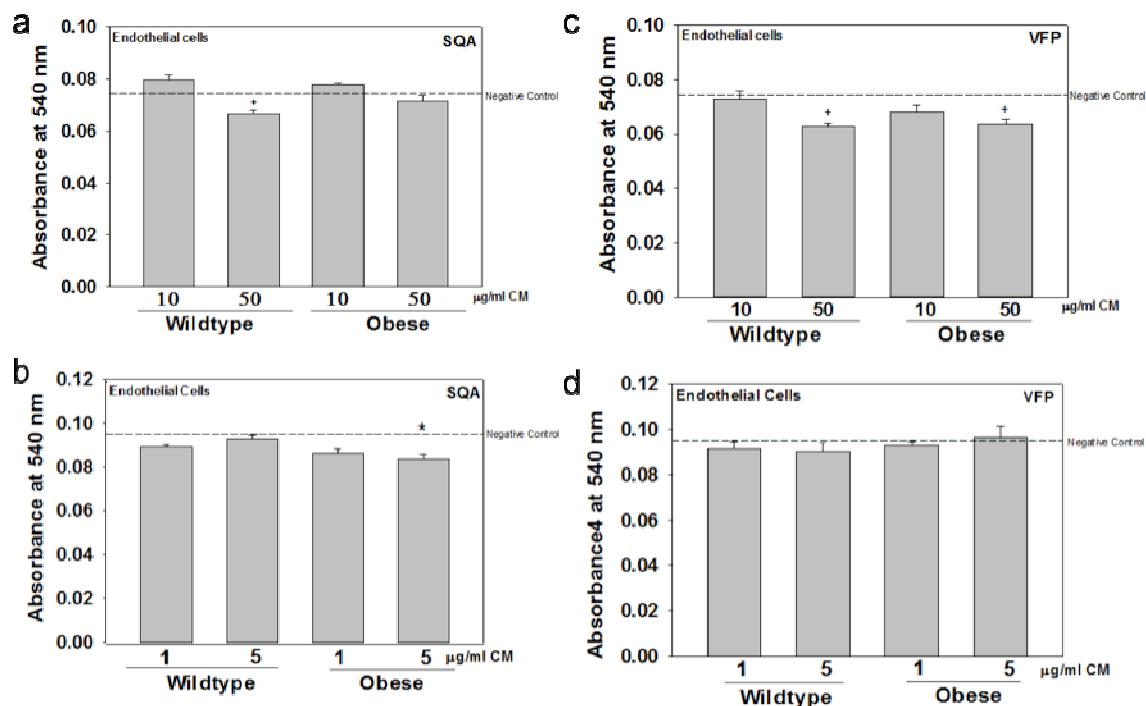
Figure 1. Concentrated adipose tissue serum-free conditioned media (CM) did not induce proliferation of human microvascular endothelial cells (HMVEC). Adipose tissue explant culture CM were collected from (a,b) subcutaneous (SQA) or (c,d) abdominal visceral fat pad (VFP) tissues from wildtype or obese mice (n=3 per group) and concentrated 10 fold. The concentrated CM were tested on HMVEC at different dosages [50, 10 (a,c), 5 and 1 (b,d) µg/ml] for 5 days, then an MTT assay was performed. In (a) WT SQA 50 µg/ml; +P-value ≤ 0.01 vs. the negative control. In (b) obese SQA 5 µg/ml; *P-value ≤ 0.003 vs. WT SQA 5 µg/ml. In (c) WT VFP 50 µg/ml; +P-value ≤ 0.001 vs. the negative control. In (c) obese VFP 50 µg/ml; +P-value ≤ 0.004 vs. the negative control. In (d) there was no significant result.

Unconcentrated visceral fat pad (VFP) conditioned media (CM) from lean mice induced proliferation of human microvascular endothelial cells.

In the initial experiments, with concentrated CM, I did not observe any proliferation in the HMVEC cells. While analyzing these results, one difference that I noted between my

 study and the Venkatasubramanian study (Venkatasubramanian et al., 2014), was that the authors used unconcentrated CM from the human adipose tissue samples in that study. Therefore, I tested the proliferation of HMVECs using un-concentrated CM of each adipose tissue type of lean (wildtype) mice at three different dosages, 30%, 50%, and 100% CM. As shown in figure 2, the VFP from WT mice at 100% significantly induced proliferation as compared to the negative control and to the SQA CM at 100% (P-value < 0.001, n=5). At the lower doses of VFP (30, 50%) and the 50% dose of SQA, there was statistically significant inhibition of HMVEC proliferation; SQA (Figure 2; P-value< 0.001), VFP at the 30% dose (P-value ≤ 0.02), and VFP 50% (P-value ≤ 0.001) (Figure 2). We showed that wildtype VAT secretions have the ability to induce HMVECs at high dose when it is not concentrated, but at lower doses, appeared to inhibit the proliferation. These data confirmed the effect of visceral adipose tissue on cell proliferation.
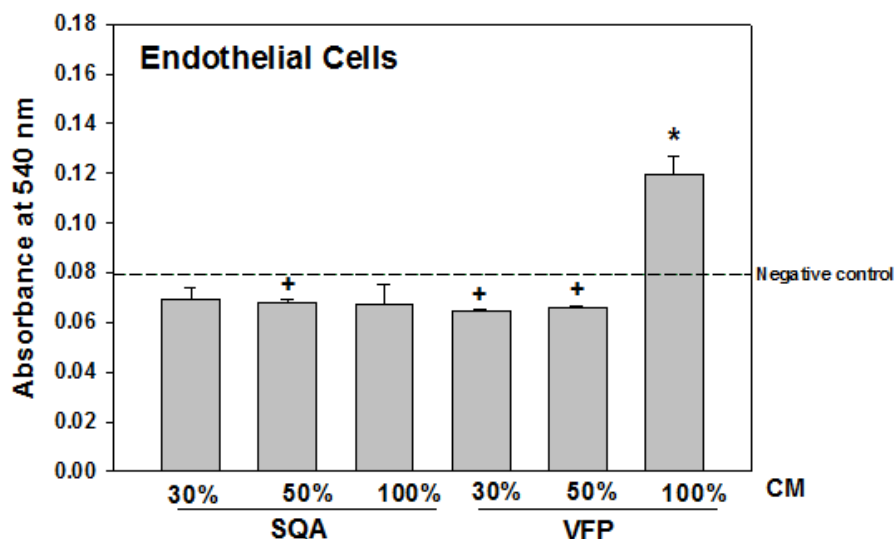
Figure 2. Unconcentrated visceral fat pad (VFP) conditioned media (CM) from lean mice induced proliferation of human microvascular endothelial cells. HMVEC cells were treated with unconcentrated VFP or subcutaneous adipose (SQA) tissue CM from wildtype or obese mice at different dosages (30%, 50%, and 100%; n=5 per group). An MTT assay was performed on day 5. There was a significant proliferation from VFP 100% *P-value < 0.001 vs. SQA 100% and the negative control. There was significant inhibition from SQA 50%; +P-value ≤ 0.001, VFP 30%; +P-value ≤ 0.02, and VFP 50%; +P-value ≤ 0.001 vs. the negative control.

Unconcentrated subcutaneous adipose (SQA) tissue conditioned media (CM) induced human PCa cell line, PC-3, proliferation cells at 30% dilution but not at full concentration.

As angiogenesis is just one mechanism of pro-tumorigenic activity of the adipose tissue secretions, I also tested their effects on PC-3 PCa cells. I plated the PC-3 cells at concentration of 16,000 cells/ well and treated them with the same VAT and SQA tissue secretions as used previously on the HMVECs (30%, 50% and 100%) for five days. I assessed proliferation using an MTT assay as above. While most samples were analyzed in duplication, for some samples, there was only enough CM to run in in a single well. However, a total of 5 mice were analyzed per group. As seen in figure 3, only SQA at the 30% dose induced PC-3 cell proliferation significantly as compared to the negative

control (Figure 3; P-value ≤ 0.05). No other concentration in the SQA CM or any of the

VFP samples had a significant effect (Figure 3).
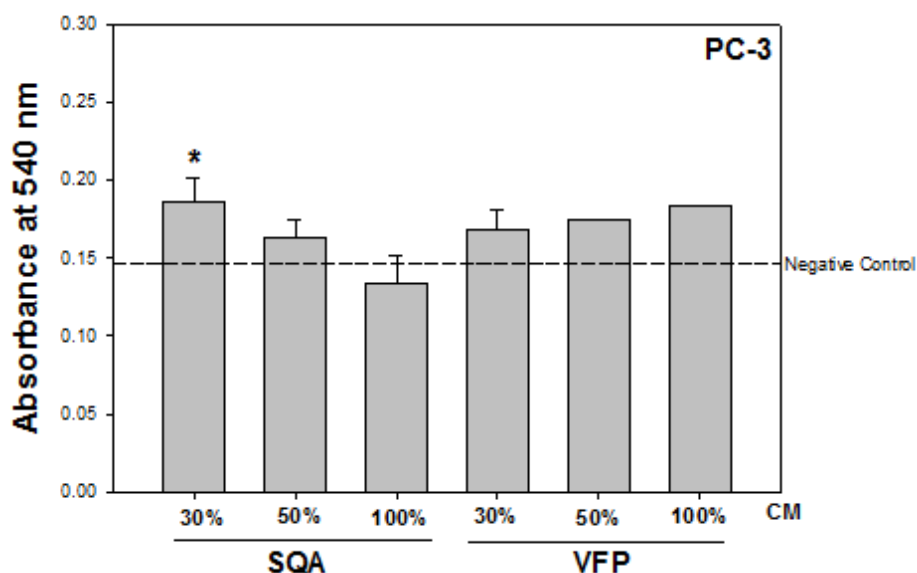
Figure 3. Unconcentrated subcutaneous adipose (SQA) tissue conditioned media (CM) induced human PC-3 prostate cancer cell proliferation only at 30% dilution. The PC-3 cells were treated with unconcentrated subcutaneous adipose (SQA) tissue or VFP CM from wildtype (lean) mice at different dosages (30%, 50%, and 100%; n=5 per group). An MTT assay was performed on day 5. Most samples were run in duplicate; however, the CM from some mice had only enough CM to run in a single well. However, multiple mice were included in each group. The significant proliferation was seen from SQA 30% *P-value <0.05 vs. the negative control.

Unconcentrated periprostatic adipose (PPA) tissue CM from lean mice induced human prostate cancer cell line, PC-3 cells.

The previous study by Venkatasubramanian et al. have demonstrated that both

endothelial and PCa cell proliferation were significantly induced when they were treated

with obese human PPA tissue secretions (Venkatasubramanian et al., 2014). In mice, very

little PPA tissue can be obtained. However, based on the discrepancy between my

analysis of VFP and the human study, I collected PPA and compared it to the effects of

both SQA and the VFP tissue secretions. PC-3 cells were plated as above, and then

treated with doses of 30%, 50%, and 100% CM issue type secretions for five days. An MTT proliferation assay was then performed. In this experiment, when raw data were analyzed only the SQA tissue CM at 30% induced PC-3 cell proliferation significantly (Figure 4; P-value < 0.04, n=2).
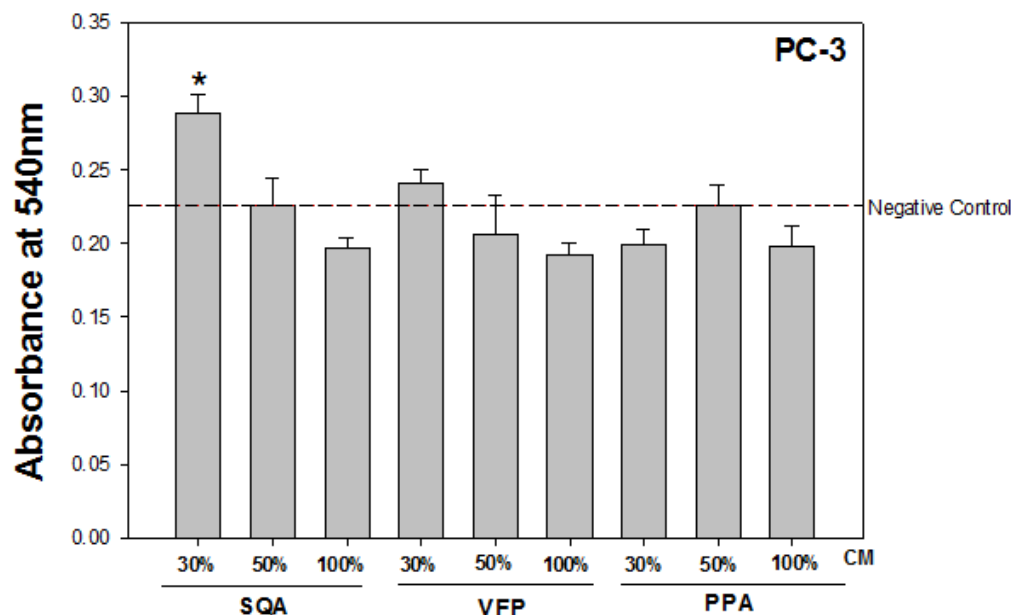
Figure 4. Unconcentrated SQA tissue conditioned media from lean mice induced human prostate cancer cell line, PC-3 cells at 30% concentration (4 months old mice, n=2 mice). The previous experiment was repeated, the only significant cause of PC-3 cells proliferation was SQA 30%; *P-value < 0.04 vs. the negative control. Significant differences in proliferation of PC-3 cells were also observed from SQA 30%; P-value ≤ 0.001 vs. VFP 30%. Also, SQA 30%; P-value ≤ 0.01 vs. PPA 30%. The VFP 30% was also significant as compared to the PPA 30% (P-value ≤ 0.01).

PC-3 cells were treated with conditioned media from five more wildtype mice, but no significant proliferation was seen.

The same experiment was repeated testing a larger sample size to achieve a more accurate result. The PC-3 cells were plated and treated the same way as mentioned above. No significant induction was observed in PC-3 cell proliferation after treating them with

the three doses of each adipose tissue types. Nevertheless, inhibition was observed in PC-3 cells treated with VFP 50% (Figure 5; P-value ≤ 0.04), PPA 50% and 100% (P-value ≤ 0.02) as compared to the negative control (Figure 5).

Based on figure 4 and 5, despite the study by Venkatasubramanian et al. my study did not show any proliferation of PC-3 cells by any adipose tissue type CM except SQA 30%. As the amount of tissue collected between depots varies, with PPA being the least amount of tissue, I normalized the proliferation data per total protein content in the CM samples.
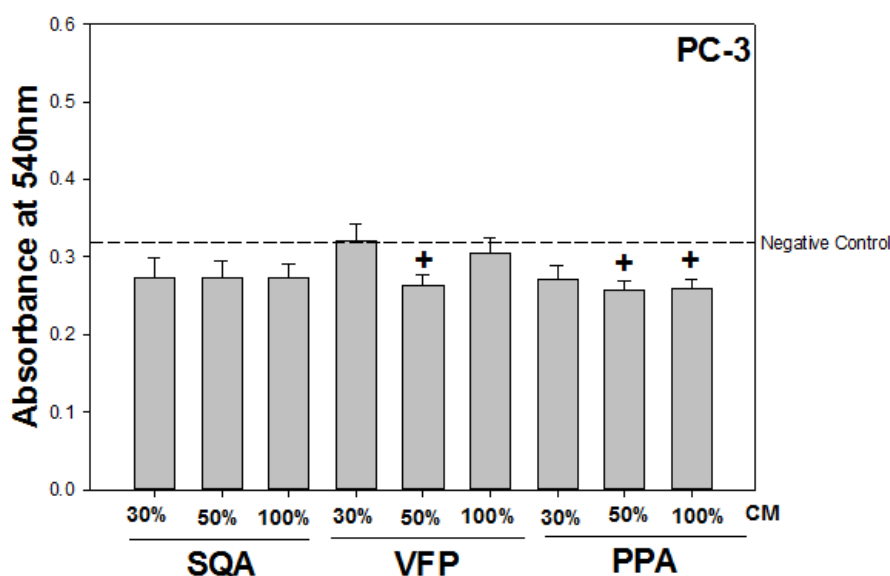
Figure 5. The same experiment was performed from different adipose tissues CM of 5 mice on PC-3 cells. The result indicated a significant Inhibition of PC-3 cells from PPA 50%; +P-value < 0.02 vs. the negative control. PPA 100%; +P-value <0.02 vs. the negative control. VFP 50%; +P-value ≤ 0.04 vs. the negative control. Significant inhibition was also seen from VFP 100%; P-value ≤ 0.05 vs. PPA 100%.

The wildtype PPA unconcentrated conditioned media induced proliferation of human PC-3 prostate cancer cells.

In order to determine the amount of total protein content in the CM samples, a Coomassie dye binding assay was performed for all seven mice that we had tested their adipose tissue secretions on PC-3 cells previously. The MTT proliferation assay data was then normalized to total protein concentration of each sample and graphed. Significant induction in PCa proliferation was observed via PPA CM at all three doses of 30, 50, and 100%. This induction was more than two fold than either SQA or VFP samples (Figure 6; P-value < 0.001, n=7).
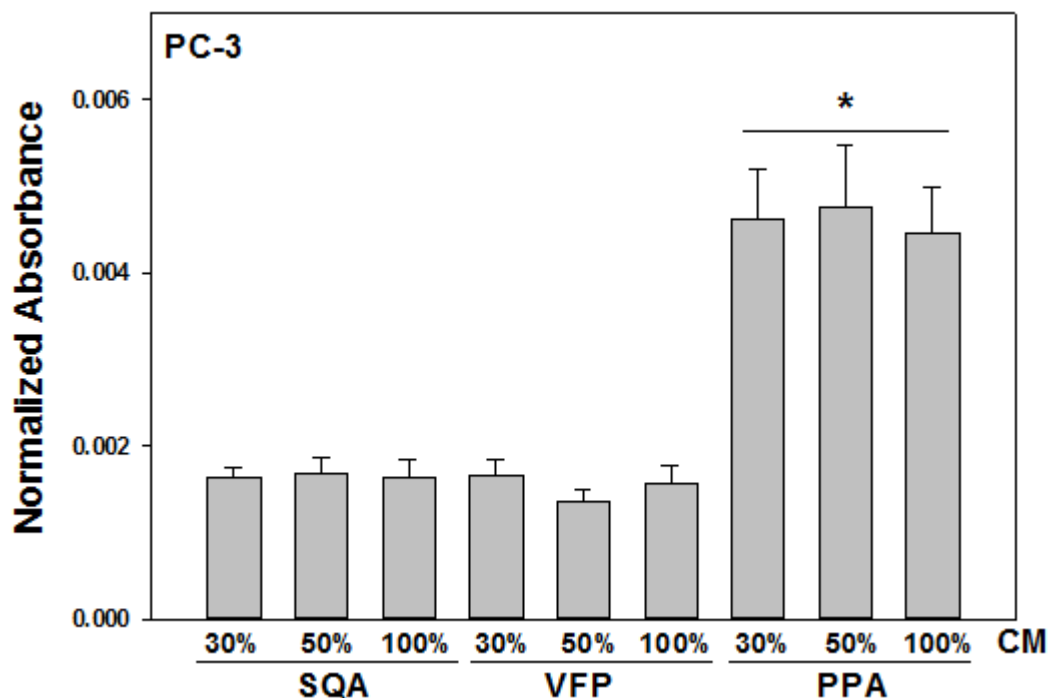
Figure 6. Normalized results of unconcentrated conditioned media (CM) demonstrate that periprostate adipose (PPA) tissues induced proliferation of human PC-3 prostate cancer cells. The PC-3 cells were treated with unconcentrated adipose tissue CM from wildtype mice or obese mice (n=7 per group) at different dosages (30%, 50%, and 100%) for 5 days, then the MTT assay was performed. Total protein concentration of each sample was obtained via a Coomassie dye binding assay. The MTT data was normalized to total protein concentration for each sample and graphed. *P-value <0.001.

There were no significant differences between lipid catabolic rates of each adipose tissue.

Free glycerol levels of SQA, VFP, and PPA tissue CM of five wildtype mice were measured via a lipolysis assay. The glycerol values were then normalized to gram weight of each adipose tissue sample. No differences in lipolytic rates were observed between the adipose tissue depots (Figure 7).
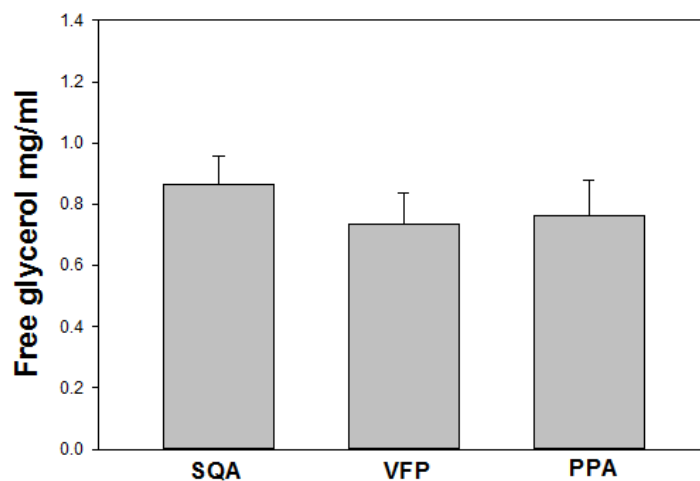
Figure 7. There were no significant differences between lipid catabolic rate of each adipose tissue.
Lipolysis, the rate of triglyceride breakdown, was measured for each adipose tissue depot using a free glycerol assay. The glycerol values were then normalized to gram weight of each adipose tissue sample. No significant differences in lipolytic rates were observed between the tissue depots.

DISCUSSION

Several studies demonstrate that visceral obesity is directly associated with aggressive PCa (Kane et al., 2005; Mistry et al., 2007). Some proteins including; leptin, IL-6, and TNF-α are produced by adipose tissues, and higher amounts of these substances are produced with obesity (Fonseca-Alaniz et al., 2007), when the size and number of the adipocytes increases. Several studies have linked PCa aggressiveness to the higher production of these cytokines and hormones via obese adipose tissues (Mistry et al., 2007). Recent research has shown that increases thickness of PPA tissue positively correlates with PCa aggressiveness (Bhindi et al., 2012) and that the PPA secretes high levels of IL-6 (Finley et al., 2009). In addition, one study has shown that human PPA secretions induce proliferation of both PCa cells and of endothelial cells (Venkatasubramanian et al., 2014). The goal of this study was to determine if PPA tissue secretions from a mouse model induce pro-proliferative activity in PCa cells and microvascular endothelial cells as compared to the abdominal visceral adipose tissue or subcutaneous adipose tissue.

Specific Aim 1

To quantify the pro-proliferative activity of subcutaneous (SQA) and visceral adipose tissue (VAT) secretions on normal human endothelial cells.

Working hypothesis: VAT will induce more proliferation as compared to SQA, and tissues from obese mice will have increased proliferative activity as compared to lean mice.

In my initial experiment, I treated normal human microvascular endothelial cells with either VAT or SQA tissue secretions. This experiment was performed for multiple reasons. First, I needed to determine if mouse adipose tissue CM could induce proliferation in human cells. Secondly, I specifically wanted to determine if the secretions from either tissue were pro-angiogenic. Thirdly, I wanted to establish if this angiogenic activity differed between adipose depots, SQA versus VAT secretions. Lastly, I aimed to establish a dose range for this activity and subsequent experiments. In the first experiment, concentrated CM (10-fold) was used. I tested a range of doses from 1 to 50 µg/ml of the CM. The result indicated that the doses of 10 and 50 µg/ml of the CM actually inhibited HMVECr proliferation, rather than induce it. The same experiment was then performed using 1 and 5 µg/ml, and again, there was no significant increase in proliferation at these lower doses. These data are not consistent with the study by Venkatasubramanian et al study, which showed that, CM from lean patients did induce proliferation of HMVECs above that of basal media. One difference between these studies is that I used visceral fat pad secretions while Venkatasubramanian et al. (2014) specifically used PPA tissue, which is a subtype of VAT. Another difference is that they used unconcentrated serum free CM in their study. Thus, it was possible that concentrating the CM might affect the CM activity as there maybe bioactive factors in unconcentrated CM that are destroyed or weakened during concentration or it may concentrate an inhibitory factor.

Therefore, in the next part of the study, I collected additional samples and assessed HMVEC proliferation using unconcentrated CM. Each CM was used undiluted (100% dose) or diluted with basal media to achieve the doses of 30% or 50% dilutions to obtain a dose response. In contrast to the first part of the study, a significant induction of proliferation was seen in the undiluted (100%) VAT secretions. This observation is consistent with that of Venkatasubramanian et al. (2014), and it supports the hypothesis that visceral adipose tissue secretions are more pro-angiogenic than SQA tissue secretions. This increased pro-angiogenic activity may be due to differences in the expression level of adipokines in these different adipose depots (Greenberg and obin, 2006). For example the expression level of IL-6 is higher from VAT than SQA tissue (Gustafson, 2010), and IL-6 is an established pro-angiogenic factor (Lazar-Molnar et al., 2000 and Wani et al., 2011). Thus, based on my observations and the results of reported studies, it is possible that the increased angiogenic activity I observed in the VAT samples is due to the high levels of IL-6 produced by this tissue. Future studies will be needed to specifically test this hypothesis. In addition, future studies should also compare tissues from lean versus obese mouse models to determine if VAT tissues from obese mice are more pro-angiogenic as compared to lean mouse tissues, as was shown for human tissues (Venkatasubramanian et al. 2014).

Specific Aim 2

To measure the pro-proliferative activity of VAT and SQA and PPA tissue secretions on PC-3 PCa cells.

Working hypothesis: PPA tissue induces more proliferation as compared to that of other adipose depots tested.

In the next part of the study, I treated human PC-3 PCa cells with the unconcentrated SQA tissue and VAT CM collected above. In this initial assay, CM of SQA tissue at the 30% dilution enhanced the proliferation of PC-3 cells significantly as compared to untreated cells. This was also significant as compared to the CM of VAT at the 30% dilution. However, the VAT secretions did not induce PC-3 cell proliferation as expected. I expected this result based on the Venkatasubramian et al. study where human VAT induced PC-3 cell proliferation (Venkatasubramian et al., 2014). However, in the Venkatasubramian et al. study, the authors specifically tested PPA tissue as the visceral adipose tissue source (Venkatasubramian et al., 2014). In my initial study, I used the visceral fat pad as the visceral adipose tissue source. Thus, I hypothesized that the locale that the visceral adipose tissue was taken was a key factor in activity level.

Although it is difficult to collect PPA from mice, and very little tissue can be obtained, to determine if PPA tissue is distinct from the abdominal VAT, I collected PPA, VAT, and SQA tissue from additional mice. I treated PC-3 PCa cells with unconcentrated SQA, VAT, and PPA tissue CM collected from five mice at three doses, 30%, 50%, and 100%. The raw data showed no increase in proliferation over that of the basal media, and in fact showed a decrease in cell number with the PPA secretions as compared to the negative control. This was not consistent with the published study on human tissues (Venkatasubramian et al, 2014). However, I also noted that in this study, they

normalized the pro-proliferative activity. Therefore, it seemed appropriate to normalize the data in my study to provide a more precise means of comparison between the tissues. Therefore, I normalized the proliferation data per total protein content in the CM samples per gram weight of tissue, both standard measures by which data are normalized. The graphed normalized data revealed that, per µg total protein, the PPA tissues secretions induced higher cell numbers as compared to the VAT or SQA tissue secretions. This proliferation from PPA tissue was more than two fold higher as compared to other adipose depots. These data are consistent with the Venkatasubramanian et al. study which showed that CM from PPA from lean PCa patients induced proliferation of PC-3 cells (Venkatasubramanian et al., 2014).

My study is the first to confirm the Venkatasubramian findings in a mouse model. While these data show that PPA tissue secretions support a pro-proliferative microenvironment, they do not delineate a mechanism of this activity. In a study by Finely et al., the authors showed that the PPA tissue produced high levels of IL-6 and this level correlated with high tumor grade (Finely et al., 2009). Moreover, Riberio et al. indicated that PCa provides a favorable microenvironment for PPA tissue to become metabolically more active. This cellular crosstalk between tumor cells and the PPA tissue cells stimulates and increases the secretion level of some adipokines, such as IL-6 and TNF-α which lead to PCa aggressiveness (Ribeiro et al., 2012). In another study, the level of pro-MMP-9 was reported to be higher in PPA tissue sample from PCa patients as compared to that of benign prostate hyperplasia (Sacca et al., 2012a), and increased MMP-9 has been

implicated in PCa metastasis (Johnson et al., 2010). Therefore, these characteristics of the PPA tissue may explain the results of my experiments.

Another mechanism that could be involved in tumor progression is increased fatty acid availability. Obese adipose tissue is known to secrete higher levels of fatty acids (de Ferranti and Mozaffarian, 2008). For example, elevated level of IL-6 was shown to induce lipolysis in VAT, which increases the release of FFA (Pradhan et al., 2001). Higher production of FFA is directly associated with insulin resistance (Boden G, 2011). Insulin is a key regulator of growth (Hsu et al., 2007); therefore, a higher amount of insulin due to insulin resistance could increase PCa cell proliferation. Moreover, high level of insulin can lead to activation of some growth hormones, including insulin-like growth factor (IGF) (Friedrich et al., 2012). IGF is known to have a mitogenic effect (Frasca et al., 1999; Hsu et al., 2007). Hence, these cellular mechanisms could enhance proliferation of some cancer cells. For example, an epidemiologic study by Manousos et al. demonstrated that there is direct link between serum IGF-1 and IGF-II and colorectal cancer (Manousos et al., 1999). The IGF-I can also increase the level of vascular endothelial growth factor (VEGF) which leads to angiogenesis (Fukuda et al., 2002; Stearns et al., 2005). Thus, increased levels of FFA from the PPA could contribute to PCa progression.

Therefore, in the last part of my research, I measured the lipolytic activity of each adipose tissue depot. The lipolysis assay was performed as a measure of the level of FFA produced from triglycerides by each adipose tissue type. Since in a human subjects study

the rate of lipolysis was reported to be higher in VAT than SQA tissue (Jensen, 2006), I expected to observe a higher lipolytic activity from the VAT and PPA tissues as compared to the SQA tissues. However, no significant differences were observed between the mean value of the SQA, VAT, and PPA tissue's lipolytic activity. One likely explanation for this difference might be related to the nature of the models. Smaller amount of adipose tissue can be collected from mice than human. Jensen used human subjects, while I used mouse tissues.

CONCLUSION

The role of secretions from three adipose tissue types, including SQA, VAT, and PPA were compared for pro-proliferative activity on human PCa cell line PC-3. It was hypothesized that secretions from VAT, and PPA tissue, a subtype of VAT, have a promoting effect on PCa progression as compared to that of abdominal VAT or SQA tissue secretions. For the first specific aim, the effect of abdominal VAT and SQA tissue secretions from lean mice on HMVEC was tested. The proliferation result from these samples at different concentrations did not indicate any significant proliferation of HMVECs. However, unconcentrated CM from the VAT at the 100% dose significantly induced HMVEC proliferation.

Specific aim 2 was focused on pro-proliferative activity of SQA, VAT, and PPA tissue secretions on PC-3 cells. The normalized proliferation data showed that the PPA tissue secretions at all three doses produced higher cell numbers, two-fold higher, as compared to the SQA and VAT secretions. Finally the lipolytic activity of SQA, VAT, and PPA tissue was measured, but no significant difference was seen between the FFA levels of these three adipose depots. In summary, my data supported my hypothesis that PPA tissue from a mouse model had similar characteristics to that taken from human tissues in that it induced pro-proliferative activity of PCa cells.

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