MODULATION OF PROSTATE CANCER CELL PROLIFERATION AND GENE EXPRESSION BY DIETARY FATTY ACIDS AND EFFECTS OF ADIPOCYTE CONDITIONED MEDIA

A Thesis in
Molecular Medicine

by
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ABSTRACT

Fish oil contains the marine omega-3 polyunsaturated fatty acids (n-3 PUFA) docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Consuming diets rich in these fatty acids has been associated with decreased incidence of prostate cancer, however much less is known about the non-marine n-3 PUFA alpha linolenic acid (ALA). To study which n-3 PUFA are more effective in prostate cancer prevention, and whether mechanisms of action are conserved between them, we tested DHA, EPA and ALA on the human prostate cancer cell line PC3. PC3 cells were treated with DHA, EPA, or ALA and changes in cell proliferation and gene expression examined. Different trends of inhibition of PC3 proliferation were observed for the three n-3 PUFA, with DHA exhibiting the most pronounced effects on PC3 cell proliferation and altered gene expression; ALA was the least efficacious of the three n-3 PUFA. All n-3 PUFA decreased fatty acid synthase (FASN) mRNA, and its regulator sterol response element binding protein 1c (SREBP-1c) mRNA to 50% of control levels. DHA, EPA and ALA decreased expression of macrophage chemotactic factor 1 (MCP1), an autocrine prostate cancer growth factor. Looking at genes involving inflammation, cell cycle and apoptosis, DHA regulated the greatest number of genes in all categories, followed by EPA and then ALA. In addition DHA and EPA increased gene expression of the pro-apoptotic protein activating transcription factor 3 (ATF3) mRNA by 11-fold and 3-fold, respectively while ALA had no effect. Moreover, DHA and EPA, but not ALA, significantly induced apoptosis. We conclude while some mechanisms of cancer cell inhibition are conserved among n-3 PUFA, the extent, magnitude, and duration of transcriptional changes vary for each individual fatty acid.
Obesity in men, particularly an abundance of abdominal fat deposits, has been correlated with increased incidence and poor prognosis of prostate cancer. Fat may also affect the activity of prostate cancer cells that metastasize to bone, as the bones of older individuals are composed predominantly of fatty tissue. N-3 PUFA are effective agents in cancer prevention and therapy, although their precise mechanisms of action and the differences between them have not been fully characterized. In addition to omega-3 PUFA, other fatty acids, including certain omega-6 (n-6) PUFAs, and both 10-trans,12-cis (10e12z) and 9-cis,11-trans (9z11e) isomers of conjugated linoleic acid (CLA), also inhibit proliferation of PC3 prostate cancer cells. To mimic fat cells in the body, and determine whether the presence of these fat cells would affect the inhibitory actions of fatty acids, we used a two-step model of media preconditioning and treatment. Culture media containing inhibitory concentrations of fatty acids was first conditioned on 3T3-L1 adipocytes, and then administered to PC3 cells. Here, we show that 3T3-L1 adipocytes modulate the effects of fatty acid treatment, generally rendering the fatty acids less effective in proliferative inhibition of PC3 prostate cancer cells. The mechanism of inhibition of the PC3 cells’ response to fatty acids in the presence of fat cells may be significant to understanding the correlation between obesity and resistance to prostate cancer therapy.
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<table>
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<th>Abbreviation</th>
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<tr>
<td>9z11e CLA</td>
<td>9-cis,11-trans conjugated linoleic acid</td>
</tr>
<tr>
<td>10e12z CLA</td>
<td>10-trans,12-cis conjugated linoleic acid</td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
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<tr>
<td>ALA</td>
<td>Alpha linolenic acid</td>
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<tr>
<td>ATF3</td>
<td>Activating transcription factor 3</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CLA</td>
<td>Conjugated linoleic acid</td>
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<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
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<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>FASN</td>
<td>Fatty acid synthase</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>GLA</td>
<td>Gamma linolenic acid</td>
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<tr>
<td>LA</td>
<td>Linoleic acid</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Macrophage chemotactic protein 1</td>
</tr>
<tr>
<td>n-3</td>
<td>omega-3</td>
</tr>
<tr>
<td>NPT</td>
<td>Normal prostate tissue</td>
</tr>
<tr>
<td>OA</td>
<td>Oleic acid</td>
</tr>
<tr>
<td>PCT</td>
<td>Prostate cancer tissue</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator activated receptor</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acids</td>
</tr>
<tr>
<td>SREBP-1c</td>
<td>Sterol regulatory element binding protein 1c</td>
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To my parents.
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Chapter 1

LITERATURE REVIEW
1.1 DIETARY FATTY ACIDS AND PROSTATE CANCER

1.1.1 Overview of Dietary Fatty Acids and Cancer

Omega-3 polyunsaturated fatty acids (n-3 PUFA) and their metabolites have been observed to inhibit the development and progression of a variety of cancers, including prostate cancer. N-3 PUFA elicit their effects through a variety of mechanisms, including growth inhibition and apoptosis induction (1). Consumption of n-3 fatty acids has protective effects against prostate tumorigenesis, thought to be largely due to suppression of the expression of pro-proliferative and anti-apoptotic genes (2). N-3 fatty acids are generally associated with production of anti-inflammatory eicosanoids and prostate cancer inhibition (3). As a result of their protective and anti-inflammatory properties, a diet high in omega-3 fatty acids is often recommended as a preventive measure against cancer development. They are also prescribed concurrently with several traditional chemotherapeutic agents, as they have been found to increase the effectiveness of the therapy. N-3 fatty acids have been recommended for use during hormone ablation therapy of prostate cancer, due to their inhibitory effects on proliferation of androgen dependent prostate cancer cells (4). The omega-3 fatty acid DHA has been found to enhance effects of chemotherapeutic drugs Docetaxel and Celecoxib in prostate cancer (5, 6). The anti-cancer effects of n-3 PUFA derive from their anti-inflammatory and anti-oxidant properties and their ability to modulate gene expression, allowing n-3 PUFA to specifically target neoplastic cells in a wide range of cancers (6).

Omega-6 (n-6) fatty acids are not as well characterized with regard to their role in cancer. Generally, n-6 fatty acids are associated with increased prostate tumorigenesis through their conversion to inflammatory eicosanoids (3). A diet containing a high ratio of
n-6 to n-3 PUFA is thought to predispose for cancer development (2, 3). Few studies have been conducted to determine differences between the mechanisms of action of different n-3 or n-6 fatty acids in prostate cancer.

Dietary fatty acids can be interconverted in the body, permitting synthesis of several non-essential fatty acids. N-6 fatty acid LA and n-3 fatty acid ALA are known as essential fatty acids because they cannot be synthesized in humans, and constitute essential building blocks for the synthesis of other fatty acids required by the body (Figure 1-1). Although the human body is capable of synthesizing AA, EPA, and DHA from the essential PUFA LA and ALA n-3 and n-6 conversion processes are highly inefficient and compete with each other (7). Therefore, dietary supplementation is necessary to provide consistent and biologically relevant doses of both essential and non-essential fatty acids.

1.1.2 Alpha Linolenic Acid (ALA)

ALA is derived from a variety of plants-based oils, and the major dietary sources of ALA are flaxseed, canola, and soybeans as well as tree nuts. Past studies show conflicting results regarding the role of ALA in prostate cancer risk. Serum levels of ALA have been correlated with increased risk of prostate cancer development (8). Another recent clinical study has shown there is no association between level of dietary ALA intake from different dietary sources and risk of prostate cancer development (9). Meta-analyses of clinical studies of the relationship between dietary ALA intake and risk of prostate cancer show conflicting results. Some studies report a weak positive association with ALA intake and risk of prostate cancer development (10) while others report that ALA has a slight protective effect against prostate cancer (11). Taken together, these findings show that there is likely no significant correlation—positive or negative—between ALA intake and prostate cancer risk (12).
Figure 1-1: Structure, metabolism, and interconversion of omega fatty acids (13-16)
Adapted from Teitelbaum et al (17).
1.1.3 Docosahexaenoic Acid (DHA)

The main dietary source of DHA is via fish consumption. *In vitro* and clinical studies have consistently shown a negative association between DHA administration and prostate cancer development and progression, due to induction of a wide range of cancer cell specific anti-proliferative and pro-apoptotic mechanisms (1, 2). Clinical studies have shown that elevated levels of DHA in the blood rather than in serum is particularly protective against prostate cancer development (7).

1.1.4 Eicosapentaenoic Acid (EPA)

Besides DHA, EPA is the other predominant fish oil. As with DHA, *in vitro* and clinical studies have consistently shown a negative association between EPA administration and prostate cancer development and progression (1, 2). Elevated EPA levels in the blood but not in serum have also been found to have a negative association with prostate cancer development (7). DHA and EPA are often grouped together as n-3 fish oils in mechanistic studies of cancer prevention.

1.1.5 Arachidonic Acid (AA)

The primary sources of AA in the western diet are animal products. AA is considered a pro-carcinogenic fatty acid due to production of inflammatory prostaglandins, resulting primarily from activity of COX-2, a rate-limiting enzyme in the arachidonic acid pathway (18). COX-2 overactivity has been linked with promotion of prostate tumors (19). N-3 are thought to elicit some anti-cancerous effects by inhibiting AA conversion to inflammatory prostaglandins through activation of competing processes (6). AA has also been shown to work through bone marrow adipocytes to promote prostate cancer metastasis (20).
1.1.6 Linoleic Acid (LA)

Dietary LA is primarily derived from plant oils. Although n-6 PUFA have been associated with cancer development (3), there is no conclusive epidemiological evidence specifically linking increased levels of LA ingestion to prostate cancer development (21).

1.1.7 Oleic Acid

Olive oil is the primary dietary source of OA, an omega-9 monounsaturated fatty acid (MUFA). Consumption of a diet rich in OA and increased concentrations of MUFA in the blood have been proposed to reduce risk of prostate cancer development (22, 23).

1.1.8 Conjugated Linoleic Acid

9-cis, 11-trans conjugated linoleic acid (9z11e CLA) and 10-trans, 12-cis conjugated linoleic acid (10e12z CLA) are geometric and positional isomers of LA (enzymatic conversion shown in Figure 1-2). The primary source of CLA in the western diet is consumption of meat and dairy products (7). Human gut has bacteria that are capable of producing CLA from LA through a similar isomerization reaction as encountered in ruminants, as well as through distinct enzymatic processes catalyzed in liver and mammary tissue (7, 24). CLA occurs naturally as a mix of several isomers, predominantly 9z11e and 10e12z (7). Reports are conflicting regarding the role of CLA in prostate cancer. Recent studies have shown 9z11e CLA to have anti-inflammatory effects while the 10e12z isomer elicits inflammation in some cell types (25-27). The general consensus with regard to cancer is that both 9z11e and 10e12z isomers are inhibitory, although they work through distinct mechanisms (7, 28).
Both human and ruminant gut bacteria have isomerase enzymes capable of converting linoleic acid to 9z10e and 10e12z isomers of conjugated linoleic acid. 9z11e CLA may also be produced from vaccenic acid (a CLA metabolite) in lactating mammary or liver tissue by human enzymes (24).

Figure 1-2: CLA biosynthesis in humans and ruminants (29)
1.2 OBESITY AND PROSTATE CANCER

Prostate cancer has been related to obesity in incidence, prognosis, and responsiveness to therapy (30). There is an increased incidence of prostate cancer among obese individuals, although this may in part be due to a bias in who comes to medical attention (31). Obesity has also been associated with decreased responsiveness to therapy, both surgical and drug therapy. The difficulties associated with treatment of obese men with prostate cancer are not entirely technical. Obesity correlates with development of more aggressive tumors that are more resistant to therapy and likely to relapse (31). The increase in prevalence and aggression of tumors with obesity is likely correlated with increased sex hormones (androgens promote proliferation and hyperplasia) and chronic inflammatory status (32). Obesity causes a chronic inflammatory state, and secretion of inflammatory factors affects surrounding tissues and may promote tumor progression (33).

In addition to promoting tumor progression, obesity results in resistance to therapy (30). Development of resistance is generally attributed to the development of more aggressive tumors, but it may also result from interference with normal mechanisms of drug action, or from propagation of pro-inflammatory signals that counteract the therapeutic function of drugs. The role of adipose in prostate cancer is of particular interest because prostate cancer is most prevalent among older males, who exhibit not only increased adiposity overall, but also increased bone marrow adiposity (34). This has important implications for metastatic prostate cancer, as increased adiposity at the metastatic site may promote the formation of more aggressive secondary tumors. Chapter 3 will address the implications of the presence of adipocytes for the prostate cancer with the goal of assessing the effects of adipocytes on the inhibitory action of several PUFA.
1.3 ADIPOCYTE DIFFERENTIATION

Mouse 3T3-L1 fibroblasts can be chemically induced to differentiate into adipocytes through treatment with 10 µg/mL insulin, 0.5 mM isobutylmethylxanthine (IBMX), and 1 µM dexamethasone (35, 36). Each of these chemicals targets specific receptors or transcription factors to initiate a series of changes in 3T3-L1 cells that eventually results in development of the adipocyte phenotype. Figure 1-3 details the best-characterized role of each chemical in inducing adipocyte differentiation. Several pathways have been noted as activated by insulin, dexamethasone, and IBMX, and only the fundamental pathway characterized in adipocyte differentiation is shown.

Although many pathways are involved in adipocyte differentiation, in vitro and in vivo, peroxisome proliferator-activated receptor γ (PPARγ) activation has been identified as crucial to differentiation (37). Adding the PPARγ agonist rosiglitazone to differentiating 3T3-L1 cells has been proposed as a measure to achieve more complete adipocyte differentiation. The effects of using rosiglitazone to achieve total differentiation of populations of adipocytes independently of passage have recently been tested and reported (38). Adipocytes differentiated with rosiglitazone appear phenotypically identical to adipocytes differentiated without rosiglitazone. In general, the effectiveness of rosiglitazone as a differentiation-inducing agent has only been characterized through observations of adipocyte phenotype and qualitative Oil Red O staining. The identical appearance of the two types of adipocytes is often construed as functional equivalence, although quantitative gene expression-based tests have not been completed to assess whether adipocytes generated with or without rosiglitazone are truly identical.
Figure 1-3: Role of chemical activators in adipocyte differentiation (35, 39-43)

Insulin primarily elicits its differentiation-promoting effects through activation of the insulin receptor in adipocytes, a tyrosine kinase receptor that is autophosphorylated to its active following ligand interaction. Active insulin receptor activates enzymes involved in uptake and synthesis of triglycerides for storage, and inhibits lipolysis (35). These effects promote adipocyte differentiation by leading to triglyceride accumulation and storage. Dexamethasone and IBMX activate transcription factors C/EBPδ and C/EBPβ, respectively. These two factors transcribe PPARγ and C/EBPα (39). Expression of C/EBPα is dependent on PPARγ expression (40). Conversely, C/EBPα promotes the expression of PPARγ, and both C/EBPα and PPARγ modulate insulin sensitivity of adipocytes (41). Insulin modestly activates PPARγ transcription (43). PPARγ is essential for differentiation, and mediates functions including the assembly of free fatty acids into triglycerides for storage (42). Rosiglitazone is a PPARγ agonist, and promotes adipocyte differentiation by activating PPARγ.
1.4 HYPOTHESIS OF THESIS

N-3 fatty acids have classically been grouped with regard to their role in cancer, with several studies prescribing a high n-3: n-6 ratio for health benefits (19, 44). This effect is thought to be at least in part due to competitive suppression of n-6 fatty acid conversion to inflammatory AA-derived eicosanoids by high n-3 intake (19). The omega-3 fish oils DHA and EPA have classically been associated with cancer prevention, while the plant-derived ALA has minimal association (positive or negative) with prostate cancer incidence. The hypothesis for the first part of these studies is that each omega-3 fatty acid affects prostate cancer proliferation and cell death differently by transcriptionally regulating different sets of genes and differentially activating specific pathways within the cells.

Increased adiposity has been strongly associated with prostate cancer development and diminished responsiveness to therapy (31). The hypothesis for the second part of this study is that adipocytes can directly alter the biological activities of n-3 and n-6 PUFA and CLA on prostate cancer cells. Specifically, the presence of fat cells will affect the activities of different dietary fatty acids on prostate cancer cells, and that the consequences of adipocyte conditioning for prostate cancer cell proliferation will vary among the fatty acids. If fatty acids exert specific effects on prostate cancer cells, it follows that the same fatty acids would have different effects on fat cells. Acting through these fat cells, the fatty acids will then elicit specific effects on prostate cancer cells. The adipocyte-conditioning model will serve to simulate a simplified microenvironment, through which the specific fat-mediated effects of different PUFA treatments can be determined and evaluated.
Chapter 2

DIETARY OMEGA-3 FATTY ACIDS MODULATE PROSTATE CANCER GENE EXPRESSION AND CELL PROLIFERATION IN VITRO
2.1 ABSTRACT

Fish oil contains the marine omega-3 polyunsaturated fatty acids (n-3 PUFA) docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Consuming diets rich in these fatty acids has been associated with decreased incidence of prostate cancer, however much less is known about the non-marine n-3 PUFA alpha linolenic acid (ALA). To study which n-3 PUFA are more effective in prostate cancer prevention, and whether mechanisms of action are conserved between them, we tested DHA, EPA and ALA on the human prostate cancer cell lines PC3 and LNCaP. Prostate cancer cell lines were treated with DHA, EPA, or ALA and changes in cell proliferation and gene expression examined. Different trends of inhibition of PC3 proliferation were observed for the three n-3 PUFA, with DHA exhibiting the most pronounced effects on PC3 cell proliferation and altered gene expression; ALA was the least efficacious of the three n-3 PUFA. All n-3 PUFA decreased fatty acid synthase (FASN) mRNA, and its regulator sterol response element binding protein 1c (SREBP-1c) mRNA to 50% of control levels. DHA, EPA and ALA decreased expression of macrophage chemotactic factor 1, an autocrine prostate cancer growth factor. Looking at genes involving inflammation, cell cycle and apoptosis, DHA regulated the most genes in all categories, followed by EPA and then ALA. In addition DHA and EPA increased gene expression of the pro-apoptotic protein activating transcription factor 3 mRNA by 11-fold and 3-fold, respectively while ALA had no effect. Moreover, DHA and EPA, but not ALA, significantly induced apoptosis. We conclude while some mechanisms of cancer cell inhibition are conserved among n-3 PUFA, the extent, magnitude, and duration of transcriptional changes vary for each individual fatty acid. In addition, while all n-3 PUFA inhibit FASN through SREBP1-c, fish oils affect multiple pathways regulating cell growth.
2.2 INTRODUCTION

Prostate cancer is one of the most commonly diagnosed cancers as well as the second leading cause of cancer death in men in the United States. Prostate cancer is a clinically heterogeneous disease that varies in its biological aggressiveness. Metastatic prostate cancer is incurable, and the primary treatment consists of androgen deprivation, which leads to apoptosis of cancer cells and regression of tumors (45, 46). However, response to treatment is temporary due to surviving tumor cells that emerge as androgen independent. Prostate cancer has long been linked to obesity and nutrition both in incidence and mortality (47, 48), although the role of dietary fatty acids in etiology or prevention of this disease is unclear.

Fatty acids are the primary energy source for prostate cancer cells and androgens up-regulate fatty acid synthase (FASN), the enzyme responsible for de novo synthesis of fatty acids (49). FASN is increased in prostate adenocarcinoma as compared to normal prostatic tissue and is a marker of prostate cancer recurrence, poor prognosis and higher Gleason’s grade (50). Sterol response element binding protein 1c (SREBP-1c) is a positive regulator of FASN expression through binding elements in the FASN promoter. Diets rich in n-3 polyunsaturated fatty acids suppress both SREBP-1 mRNA and the active nuclear form of the SREBP-1 protein (51-54). Consequent down-regulation of FASN has been linked to cell cycle arrest and induction of apoptosis due to nutrient deprivation in several types of tumors, including breast and prostate cancers, which rely on the activity of this enzyme as an energy source (49, 55-57).

Androgen ablation and androgen receptor (AR) antagonism therapy in patients with prostate cancer initially induces cell cycle arrest and apoptosis (58, 59). However, cancer cells eventually lose dependence on androgens, which leads to progression of the androgen-
independent tumors (60). Numerous mechanisms have been postulated to account for the conversion from the androgen-dependent to androgen-independent state, including the aberrant activation of androgen receptor by a variety of growth factors. Cytokines and chemokines, produced by activated resident immune cells, are the most important components regulating the tumor growth microenvironment (61-63). Many of these signaling molecules can also function in an autocrine manner. Both androgen dependent and independent prostate cancer cells produce high levels of macrophage chemotactic factor 1 (MCP-1) compared to normal prostate epithelial cells (64). MCP-1 acts as an autocrine growth and pro-metastatic factor in prostate cancer (65, 66). Also of interest, pro-inflammatory cytokines such as interleukin-1 (IL-1), IL-6 and tumor necrosis factor (TNF) can themselves affect prostate cancer risk (67-70).

Androgen-independent prostate cancer cells do not enter apoptosis upon androgen depletion, yet they do maintain the ability to enter the apoptotic pathway (71). An alternative way to modulate apoptosis is by regulating the expression levels of essential apoptotic genes. Anti-apoptotic factor B-cell leukemia/lymphoma 2 (Bcl-2) and tumor suppressor p53 are two such candidate genes (72). Additionally, activating transcription factor 3 (ATF-3) is a stress-response gene that is involved in several cellular processes, particularly growth regulation and apoptosis. Wild type ATF-3 is a transcription factor that regulates the activation of genes involved in cell growth regulation (73). Two isoforms of ATF-3 can induce apoptosis through different mechanisms. While the transcription factor ATF-3 derived from splice variant 1 of the gene directly elicits transcriptional changes, variant 4 lacks the leucine zipper region needed to associate with DNA (74). Instead, it induces apoptosis by suppressing
transcription factor NF-κB, which leads to subsequent inhibition of the downstream anti-apoptotic factors such as Bcl-2 (72, 75).

An increase in dietary n-3 fatty acids has been linked to good prostate health (47, 76) and prevention of prostate cancer progression to androgen independence (77). In general, there is good concurrence that fish oil is beneficial to reducing the risk of prostate cancer. However there are several studies which indicate that men with high dietary intake of ALA have a greater than 3 fold increase in prostate cancer (78, 79). Therefore it is important to understand how structurally similar fatty acids can have discordant effects on cancer risk and prognosis. The data on the efficacy of individual fatty acids and their effects on cellular mechanisms are less defined. To better understand the effects of individual fatty acids and to gain insight into potential mechanisms which may benefit prostate cancer patients, prostate cancer cell lines PC3 and LNCaP, which represent androgen independent and androgen dependent disease, respectively, were used in this study to determine if PUFA had a suppressive effect on proliferation in these cells. We found that although all the fatty acids decreased cell viability of both androgen dependent and independent cell lines, they exhibited different rates of activity. This finding has significance for dietary recommendations and potential design and use of supplements in the prevention of prostate cancer.
2.3 MATERIALS AND METHODS

2.3.1 Cell Culture

Prostate cell lines PC3 and LNCaP were obtained from Dr. John Araujo at M.D. Anderson Cancer Center. PC3 cells were grown in Dulbecco’s Modified Eagle’s Medium /Ham’s F-12 medium (DMEM/F-12; Sigma, Saint Louis, MO) supplemented with 10 % FBS (Hyclone, Logan, UT) and 100 units of penicillin and 100 µg/mL of streptomycin (Invitrogen, Carlsbad, CA). LNCaP cells were grown in Roswell Park Memorial Institute medium (RPMI; Sigma, Saint Louis, MO) supplemented with 10 % FBS and 100 units of penicillin and 100 µg/mL of streptomycin.

2.3.2 Fatty Acid-Albumin Conjugates

All fatty acids were conjugated to fatty acid-free bovine serum albumin (BSA). This was accomplished following established protocols maintaining a molar ratio of 4:1 (fatty acid: BSA). Fatty acid conjugates were stored under argon at -20°C.

2.3.3 Prostate Tissue Samples

Dr. John Araujo at M.D. Anderson Cancer Center obtained prostatectomy samples from untreated patients. Samples were flash frozen and stored at -80°C. Nine sections were obtained, each 10µm thick, and placed them on slides. One section was H and E stained to confirm pathology (either normal prostate or Gleason Grade 4 and 5). The remaining eight sections were examined under light microscopy and compared to the H & E stain. Tumor, which was present in >80% of the tissue, was macrodissected away from the remaining fibrous tissue and pooled for RNA isolation. Paraffin embedded samples from the same
prostatectomy samples were sectioned in 5 µm sections placed on charged slides for immunohistochemistry for FASN and SREBP-1. Dr. Jerry Thompson performed the RNA isolation from clinical samples, and Dr. Patricia Troncoso at M.D. Anderson assessed the immunohistochemistry slides.

2.3.4 In vitro Cell Viability Assay: Cell Titer-Glo

PC3 cells (1000 cells/well) and LNCaP cells (2500 cells/well) were seeded on white, 96-well cell culture plates (Greiner Bio-One, Frickenhausen, Germany) in the appropriate medium. The next day, the cell media was changed to the appropriate medium supplemented with 0.1 % FBS. The following day the media removed and fresh media containing 1% FBS and 100 µM conjugated fatty acid/BSA conjugate was added (n equal to or greater than 4). Cell viability or growth was measured using the Cell Titer-Glo Luminescent Cell Viability Assay (Promega, Madison, WI).

2.3.5 In vitro Cell Proliferation Assay: BrdU

PC3 cells (1000 cells/well) were seeded on white, 96-well cell culture plates (Greiner Bio-One, Frickenhausen, Germany) in Dulbecco’s Modified Eagle’s Medium/Ham’s F-12 medium (DMEM/F12; Sigma, Saint Louis, MO) supplemented with 10 % FBS (Hyclone, Logan, UT). The next day, the cell media was changed to DMEM/F12 supplemented with 0.1 % FBS. The following day the media removed and fresh media containing 1% FBS and 100 µM conjugated fatty acid/BSA conjugate was added (n equal to or greater than 4). Cell proliferation was measured using the BrdU Cell Proliferation Assay Kit (Cell Signaling Technology, Beverly, MA).
2.3.6 RNA Analysis

Cells were lysed in Tri-reagent (Sigma) and total RNA was extracted following the manufacturer’s protocol. For normal prostate tissue (NPT) and prostate cancer tissue (PCT), samples were snap frozen in liquid nitrogen and frozen tissue stored at -80°C. NPT samples were homogenized in Tri-reagent and total RNA extracted. Frozen PCT samples were cut into 20-micron sections and mounted on glass slides. A random slide from the dissected tissue was stained with hematoxylin for histological examination by Dr. Patricia Troncoso. Slides containing 80% or greater tumor were considered for analysis. Tissue from the adjacent 10 slides was scraped into Tri-reagent, and total RNA was isolated by Dr. Jerry Thompson. One µg of total RNA was reverse transcribed using the High Capacity cDNA Archive Kit (ABI, Foster City, CA.). cDNA was diluted and real time PCR was conducted with an ABI 7300 thermal cycler using Sybr Green PCR Master Mix (ABI). All mRNA expression data were corrected using RPL13a expression for normalization prior to graphing and analysis. Primer sequences used in this study are listed in Appendix A.

2.3.7 Immunohistochemistry of Prostatectomy Samples

Dr. Patricia Troncoso at M.D. Anderson Cancer Center carried out immunohistochemistry of prostatectomy samples. Paraffin-embedded tumor tissues were sectioned 5µm thick and mounted on positively charged gold plus microscope slides. Tissue slides were pre-heated at 60°C for 16 hours and dewaxed by immersion in xylene then successively diluted solutions of ethanol. Antigen retrieval was accomplished by heating the slides at 70°C for four hours, immersed in Borg decloaker solution (Biocare Medical Inc., Concord, CA). Endogenous peroxidase activity was blocked by incubating in 3% H2O2 in PBS for 12 minutes. After rinsing with PBS three times for three minutes each, non-specific tissue binding was blocked
for one hour in protein block solution (Cyto Q immuno-diluent buffer; Innovex, Richmond, CA). Primary antibody was diluted in protein block solution and incubated overnight at 4ºC. Dilutions of primary antibodies are as follows: Fatty Acid Synthase antibody (1:800, Novus Biologicals, Littleton CO, CAT # NB400-114), and SREBP-1 antibody (1:800, Novus Biologicals, Littleton CO, CAT# NB100-2215). Slides were washed with PBS three times for three minutes each followed by Mach 4 Universal HRP polymer (Biocare Medical Inc., Concord, CA) application for 20 minutes as a secondary antibody. The stain was visualized by incubation in 3, 3’-diaminobenzidine (DAB) and counterstained with Gill’s No. 3 Hematoxylin. Internal negative control samples were exposed to protein block solution instead of the primary antibodies and demonstrated no specific signaling. Slides were dried and mounted with Universal Mount solution (Research Genetics, Invitrogen Co., Carlsbad, CA). Slides were viewed on a Leica DM100 microscope with Leica objectives HCX PL Fluotar 10x/0.30 and HCX PL Fluotar 20x/0.50 with an Applied Scientific Imaging MS-2000 motorized, xy encoded stage. Photos were captured with a Qimaging camera and QCapture 2.90.1 imaging software.

2.3.8 Annexin Staining

PC3 cells were plated at 300,000 cells per plate in 60 mm dishes and serum starved overnight prior to treatment with fatty acids. Following 24, 48, and 72 hours of fatty acid treatment, growth medium was removed from each culture dish and transferred to conical tubes. Cells were removed from plates by scraping, pooled with media from the corresponding plate, and pelleted through centrifugation. The resulting pellet was stained with annexin CF 488 using components from a MitoDamage kit from Millipore (EMD Millipore, Billerica, MA). The manufacturer’s instructions were followed with the exception of omitting steps for the
staining with Mitosense Red. 7-AAD DNA intercalating dye was added to identify dead cells, which stained positive for 7-AAD only, and cells in late apoptosis, which stained positive for both 7-AAD and Annexin V. Stained samples were analyzed on the Beckman Coulter Cytomics FC. Results represent the mean of three independent experiments.

2.3.9 Statistical Analysis

Figures are displayed as the mean of replicates with error bars representing population SEM. Statistical significance was determined with all groups using one-way ANOVA followed by the Tukey post-test.
2.4 RESULTS

2.4.1 Omega-3 Fatty Acids Inhibit Prostate Cancer Cell Growth

To determine if all omega-3 fatty acids suppressed proliferation to a similar extent, we tested the effect of individual fatty acids, (ALA, DHA and EPA) on the growth of prostate cancer cell lines PC3 and LNCaP. Since the Cell Titer-Glo system used for viability assays quantitates total ATP rather than cell number, we first tested it alongside a BrdU DNA-based proliferation assay to ensure that changes observed following fatty acid treatments truly represented differences in cell number rather than ATP production (Appendix B). Androgen independent PC3 cells treated with 100 µM omega-3 fatty acids exhibited a marked decrease in proliferation and survival over a four-day time course experiment (Figure 2-1 A). While all fatty acids caused a decrease in cell number, the fatty acids worked at different rates, resulting in different overall inhibition patterns. DHA was the most rapidly acting and effective fatty acid in inhibiting cell viability, followed closely by EPA. ALA was the least active of the omega-3 fatty acids tested at inhibiting cell growth.

The androgen dependent LNCaP cells exhibited a similar, though less pronounced, trend of inhibition of proliferation over the same time course (Figure 2-1 B). The differences in inhibition are attributed to the slower rate of cell cycle/division of the LNCaP cell line. Since the inhibitory trends were more pronounced in the more rapidly dividing PC3 cells, cell cycle arrest, gene analysis and apoptosis experiments were conducted on this cell line.
Figure 2-1: Omega-3 fatty acids differentially inhibit androgen independent and androgen dependent prostate cancer cell line viability

PC3 and LNCaP cells were treated with 100 µM ALA, DHA, or EPA conjugated to BSA following overnight serum starvation. Cell viability was measured using the Cell Titer-Glo Luminescent Cell Viability Assay. Different dietary fatty acids exhibited different efficacies and potencies when administered at the same dose, however the effectiveness of each fatty acid was conserved across cell lines. Three trends of inhibition emerged in PC3 and LNCaP cells treated with the omega-3 PUFA. DHA inhibited both cell lines most rapidly and to the greatest extent. EPA also effectively inhibited cancer cell growth, though more gradually than DHA. PC3 cells treated with EPA reached nearly the same endpoint as DHA-treated cells, ultimately exhibiting a similar magnitude of inhibition. ALA inhibited the cells more slowly and to a lesser extent than both DHA and EPA. While the same inhibitory trends were observed in both androgen independent and dependent cell lines, the more protracted response in LNCaP cells may be attributed to their slower rate of cell division. Viability is plotted as treatment normalized to corresponding BSA vehicle control treatments at each time point. Values are shown as the mean of replicates from four independent experiments (n=4-6) with error bars indicating standard error.
2.4.2 Transcriptional Modulation of Cell Cycle Regulation and Apoptotic Genes

To elucidate the possible mechanism(s) for the difference between DHA, EPA compared to ALA, transcript levels of several genes involving cell cycle regulation and apoptosis were examined to determine the effects of treatment with omega-3 fatty acids (Table 2-1). Cell cycle gene mRNAs including cyclin A2, cyclins D1 and D2, CDK4, p21 (CDKN1A) and p57 (CDKN1C) were examined after 24-hour treatment with omega-3 fatty acids. None of the fatty acids had a significant effect on cyclins D1 and D2, CDK4 or p57 transcript levels. All fatty acids had an effect on cyclin A2 mRNA; ALA slightly increased cyclin A2 expression while both DHA and EPA decreased its transcript levels. Expression of p21, a cyclin dependent kinase inhibitor, was significantly increased by DHA but not EPA or ALA.

Growth inhibition of prostate cancer cell lines by omega-3 fatty acids could also be the result of apoptotic induction. ATF-3, Bcl-2, Bcl-6 and NF-κB mRNA expression levels were examined 24 hours after treatment with omega-3 fatty acids (Table 2-1). The greatest change in gene expression was seen in DHA treated cells, which exhibited a large increase in ATF-3 mRNA. EPA had a significant 5-fold induction of ATF-3, which peaked at 12 hours (data not shown). DHA treated cells also had a small but significant increase in Bcl-6 gene expression with no significant effect on Bcl-2 gene expression. All fatty acids lowered Bcl-2 transcript levels, although only EPA treatment resulted in a statistically significant decrease. None of the fatty acids significantly altered NF-κB mRNA expression (Table 2-1), nor did the fatty acids affect NF-κB activity using a NF-κB reporter assay (data not shown). Graphs of these PCR data may be found in Appendix C.
Table 2-1: Gene regulation by omega-3 fatty acids

RNA was isolated from PC3 cells treated with 100 µM PUFA for 24 hours. Real-time PCR was used to quantitate the indicated gene. Levels were normalized to the housekeeping gene RPL13a and fold change is given. Statistical significance is indicated by shading and was determined with all groups using one-way ANOVA followed by Tukey’s post-test. * represents p < 0.05.

<table>
<thead>
<tr>
<th>Apoptosis</th>
<th>Gene</th>
<th>ALA</th>
<th>DHA</th>
<th>EPA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATF-3</td>
<td>1.23 ± 0.11</td>
<td>11.14 ± 1.95*</td>
<td>2.82 ± 0.29</td>
</tr>
<tr>
<td></td>
<td>Bcl-2</td>
<td>0.73 ± 0.10</td>
<td>0.58 ± 0.13</td>
<td>0.33 ± 0.06*</td>
</tr>
<tr>
<td></td>
<td>Bcl-6</td>
<td>0.86 ± 0.09</td>
<td>1.82 ± 0.14*</td>
<td>1.04 ± 0.05</td>
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<tr>
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<td>NFκB</td>
<td>0.79 ± 0.10</td>
<td>0.70 ± 0.17</td>
<td>0.63 ± 0.08</td>
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<td>Cell Cycle</td>
<td>Cyclin A2</td>
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<td>0.20 ± 0.02*</td>
<td>0.18 ± 0.02*</td>
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<td>Cyclin D2</td>
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<td>CDK4</td>
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<td>p21</td>
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<td>IL-1β</td>
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<td></td>
<td>IL-6</td>
<td>1.44 ± 0.24</td>
<td>13.66 ± 1.33*</td>
<td>14.16 ± 1.44*</td>
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<tr>
<td></td>
<td>IL-8</td>
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<td>1.43 ± 0.08</td>
<td>1.47 ± 0.25</td>
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<td>IL-10</td>
<td>0.75 ± 0.09</td>
<td>5.29 ± 0.98*</td>
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<tr>
<td></td>
<td>MCP-1</td>
<td>0.43 ± 0.04*</td>
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<td></td>
<td>TGF-β1</td>
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<td>Metabolism</td>
<td>FASN</td>
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<td>LDLR</td>
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<td>SREBP-1</td>
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<td>SREBP-2</td>
<td>0.88 ± 0.28</td>
<td>1.40 ± 0.46</td>
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2.4.3 Fish Oil Omega-3 Fatty Acids Induce Apoptosis

Adherent PC3 cells were pooled with dead cells from the supernatant in each dish for staining and flow cytometry analysis. Throughout the three days, DHA treated cells exhibited the highest rates of apoptosis, as revealed by positive staining with Annexin V. Prostate cancer cells treated with EPA showed intermediate rates of apoptosis throughout the time course, while ALA cells exhibited no apoptosis above baseline levels. Subsequent comparison of results from flow cytometry and proliferation studies revealed that rates of apoptosis and death for each fatty acid complemented the live cell counts (Table 2-2, Figure 2-3, Appendix D). Based on these comparisons, apoptosis induction was determined to be the primary mechanism of proliferation inhibition in DHA treated samples. Although ALA did not induce cells to undergo apoptosis, it did induce cell death. Data collected on day 3 of ALA treatment reveal that, while apoptosis in ALA treated cells never exceeds baseline rates, the percentage of dead cells is significantly elevated compared to vehicle control-treated samples. Similarly, while apoptosis is observed in EPA treated cells, the percentages of dead cells exceed the percentage of apoptotic cells throughout the experiment, indicating that all deaths following EPA treatment cannot be attributed to apoptosis (Table 2-2). This finding prompted us to examine other mechanisms that could be responsible for PC3 growth inhibition by omega-3 fatty acids.
Table 2-2: Cellular demise induced by omega-3 fatty acids

PC3 cells were treated with 100 μM PUFA for 3 days. Some cells were cultured in 96 well plates and were analyzed by Cell-Titer-Glo Assay. Cell Titer Glo values are compared to control (BSA) treated cells. Other cells were grown on 60 mm dishes and analyzed by Flow Cytometry following annexin staining. Percent apoptotic and percent dead cells represent percent of total cells collected. Statistical significance is indicated by shading and was determined with all groups using one-way ANOVA followed by Tukey’s post-test, where * represents p < 0.01.

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment</th>
<th>Cell Titer Glo (% control)</th>
<th>% Apoptotic Cells</th>
<th>% Dead Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BSA</td>
<td>100.00 ± 11.43</td>
<td>2.63 ± 1.01</td>
<td>2.11 ± 0.48</td>
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<tr>
<td></td>
<td>ALA</td>
<td>103.75 ± 14.25</td>
<td>3.50 ± 0.82</td>
<td>8.29 ± 1.93*</td>
</tr>
<tr>
<td></td>
<td>DHA</td>
<td>53.67 ± 24.08*</td>
<td>12.10 ± 0.89*</td>
<td>11.44 ± 0.16*</td>
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<tr>
<td></td>
<td>EPA</td>
<td>97.02 ± 10.32</td>
<td>5.06 ± 1.34</td>
<td>15.80 ± 1.76*</td>
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<tr>
<td>2</td>
<td>BSA</td>
<td>100.00 ± 24.71</td>
<td>4.42 ± 1.97</td>
<td>8.34 ± 1.75</td>
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<tr>
<td></td>
<td>ALA</td>
<td>85.06 ± 14.25</td>
<td>4.01 ± 2.13</td>
<td>10.80 ± 4.05</td>
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<tr>
<td></td>
<td>DHA</td>
<td>19.86 ± 5.40*</td>
<td>23.94 ± 4.63*</td>
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<td></td>
<td>EPA</td>
<td>63.17 ± 4.08*</td>
<td>9.68 ± 1.60</td>
<td>19.90 ± 2.05*</td>
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<tr>
<td>3</td>
<td>BSA</td>
<td>100.00 ± 26.77</td>
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<tr>
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<td>ALA</td>
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<td>DHA</td>
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<td>EPA</td>
<td>26.85 ± 13.46*</td>
<td>24.29 ± 1.65*</td>
<td>39.77 ± 6.20*</td>
</tr>
</tbody>
</table>
Figure 2-2: The “fish oil” omega-3 fatty acids induce apoptosis

Trends of apoptotic gene expression were confirmed by flow cytometric analysis of samples following treatment with omega-3 fatty acids. PC3 cells were treated with 100 µM ALA, DHA, or EPA conjugated to BSA following overnight serum starvation. Cells and supernatants were collected every 24 hours for 3 days and analyzed for apoptosis (annexin staining) and cell death (AAD-7 staining) by Flow Cytometry. The apoptosis induction trends correlated with what was expected. DHA induced apoptosis to significant levels within 24 hours and had the greatest overall induction as well. EPA significantly induced apoptosis but later than DHA and not to the same extent. ALA did not induce apoptosis above BSA control levels. Significance was assessed using the one-way ANOVA test with *p<0.01. Values are shown as the mean of replicates from three independent experiments with error bars indicating standard error.
2.4.4 Transcriptional Modulation of Inflammatory Genes

Elevated expression levels of pro-inflammatory cytokines are associated with poor prognosis in many tumor types including prostate cancer. Transcript levels of COX-2, IL-1β, IL-6, IL-8, IL-10, MCP-1, TGF-β1 and TNF-α were examined following a 24-hour treatment with 100 µM fatty acids (Table 2-1). None of the fatty acids had a significant effect on IL-1β, IL-8, TGF-β1 or TNF-α. All of the fatty acids significantly lowered the expression of MCP-1 in a pattern similar to growth inhibition. In both assays, DHA was the most effective, followed by EPA and then ALA. Only DHA significantly increased the mRNA levels of the pro-inflammatory gene COX-2 and the anti-inflammatory gene IL-10. Surprisingly, both DHA and EPA increased the RNA for IL-6.

2.4.5 Omega-3 Fatty Acids Regulate Fatty Acid Metabolizing Genes

Fatty acid synthase (FASN) has been suggested to be a prostate cancer oncogene (80). Both the RNA transcript and the protein are increased in prostate cancer Gleason’s grade 4 and 5 tumor samples compared to normal prostate tissue (81) as well as in Figure 3A by real time PCR and confirmed by IHC (Figure 2-3 B versus 2-3 C). It is important to note that normal prostate tissue from this study was derived from tumor margins, although it was not classified as hyperplastic. FASN mRNA expression was decreased by 50% in PC3 cells following treatment with omega-3 fatty acids (Table 2-1) with no significant difference between the fatty acids. Inhibition of FASN and SREBP-1 mRNA following fatty acid treatments indicated a potential mechanism for prostate cancer cell proliferation inhibition by omega fatty acids, but does not account for the differences in efficacy and potency between the fatty acids. FASN is regulated by sterol response element binding protein 1c (SREBP-1c), which is also increased in tumor samples (Figure 2-3D). In addition, IHC reveals a
translocation of SREBP-1 to the nucleus in prostate cancer tumor cells (Figure 2-3 F) as compared to normal prostate tissue (Figure 2-3 E) where SREBP-1 is predominately located in the cytoplasm. SREBP-1c mRNA was decreased to a comparable extent by all omega-3 fatty acid treatments (Table 2-1). The expression of caveolin-1 (Cav-1), a gene negatively regulated by SREBP-1c, was examined to validate the downstream effects of SREBP-1c. As predicted, the RNA expression pattern of Cav-1 inversely follows the pattern of SREBP-1c levels in both prostate and cell lines (data not shown). None of the fatty acids had an effect on Low Density Lipoprotein Receptor (LDLR) or SREBP-2.
Figure 2-3: FASN and its regulator SREBP-1c are both upregulated in clinical prostate cancer isolates

Normal prostate tissue and tumors isolated from patients at prostatectomy were analyzed and compared by real time PCR gene expression and immunohistochemistry (IHC). RNA was isolated from the clinical samples or PC3 cells treated with n-3 PUFA and gene expression was assessed through quantitative real time PCR. Clinical isolates showed elevated transcript levels of both (A) fatty acid synthase (FASN) and (D) its regulator sterol response element binding protein 1c (SREBP-1c) when compared to normal prostate tissue. These trends were immunohistochemically confirmed. Normal prostate tissue reveals low levels of expression of FASN (B) and SREBP-1c (E) at 200x compared to Gleason’s Grade 5 tumor revealing FASN in the cytoplasm (C) and nuclear staining of SREBP-1c (F) 400x. For clinical isolates (A and D), statistical significance was assessed using the paired t test with *p<0.05.
Figure 2-4: Cellular pathways involved in fatty acid metabolism, inflammation, cell cycle regulation, and apoptosis are differentially modulated by omega-3 fatty acids

Figure courtesy of Dr. Jerry Thompson. Expression of all genes was quantified in PC3 cells using quantitative real time PCR. ALA, DHA, and EPA all inhibited fatty acid synthase and its regulator sterol response element binding protein 1c to 50% of control levels. All three n-3 PUFA also inhibited inflammatory protein and autocrine prostate cancer growth factor MCP-1, although the extent of inhibition varied among them. DHA produced the most effective inhibition, with MCP-1 levels dropping to 10% control levels in treated PC3 cells, while EPA inhibited MCP-1 transcript levels by 30%, and ALA reduced them to 50% of control. DHA and EPA reduced transcript levels of cyclin A2 to 20% of control levels to reduce cell proliferation. Only DHA activated ATF3, leading to inhibition of anti-apoptotic factors downstream of NF-κB and thereby inducing apoptosis in PC3 cells.
2.5 DISCUSSION

All omega-3 fatty acids effectively inhibit proliferation of both androgen dependent (LNCaP) and independent (PC3) prostate cancer cell lines. The androgen dependent LNCaP cell line demonstrated a delay in response that may be attributed to the slower rate of cell division observed in this cell line when compared to PC3. Based on the fact that DHA leads to a more rapid and extensive effect on cell proliferation and gene expression data, there appears to be different mechanisms of inhibition by the n-3 PUFA. DHA was the most efficient and potent inhibitor of PC3 proliferation, followed by EPA and then ALA. To test whether the differences observed in the viability assay were a result of mechanistic differences, we examined pathways involved in fatty acid metabolism, cell cycle regulation, and apoptosis. N-3 and n-6 responses appear to be cell specific, although trends are conserved, because different trends of inhibition were observed when different cancer cell lines were treated with PUFA for 72 hours (Appendix E).

All omega-3 PUFA examined modulated fatty acid synthesis by decreasing accumulation of SREBP-1c mRNA. In addition, others have shown that omega-3 fatty acids inhibit the cleavage of inactive SREBP-1c to active SREBP-1c. In the prostate cancer tissue, the majority of the SREBP-1 staining is nuclear compared to cytosolic in the normal prostate tissue indicating the activation state of this transcription factor. Inhibition of SREBP-1c, the major regulator of fatty acid synthase, leads to accumulation of PUFA-containing cholesteryl esters within the cell, which results in cell cycle arrest in colon cancer cells (82). Since fatty acid metabolism is an important source of energy for prostate cancer cells, the inhibition of SREBP-1c and its target gene FASN is likely to have contributed to the decrease in PC3 and LNCaP viability observed following treatment with omega-3 fatty acids.
The low-density lipoprotein receptor pathway is important in providing cells with essential fatty acids, especially those for prostaglandin synthesis. None of the n-3 fatty acids altered transcription of LDLR or SREBP-2, a potent regulator of the LDLR promoter. While all omega-3 fatty acids examined resulted in equal inhibition of SREBP-1c and FASN (approximately 50% reduction), they exhibited different efficiencies in inhibition of proliferation. This implied that alternative apoptotic or anti-proliferative mechanisms needed to be invoked to bring about the more substantial inhibitory trends observed following DHA and EPA treatment of PC3 and LNCaP cells.

Differences in PC3 proliferation and cell viability over time may be attributed to differential regulation by the omega-3 fatty acids of several target genes involved in cell cycle, inflammation and apoptosis. Of particular importance to the androgen independent prostate cancer model studied was inhibition of MCP-1 by all the omega-3 fatty acids with DHA and EPA exhibiting inhibition of about 95% and 80% decrease from control, respectively. MCP-1 not only acts as an autocrine growth factor for prostate cancer, it is also involved in the hypoxic response and angiogenesis of primary tumors (66). In prostate cancer that has metastasized to bone, MCP-1 is implicated in paracrine modulation of osteoblast and osteoclast activity, leading to osteoclastogenesis and alteration of the bone matrix (64). Differential modulation of MCP-1 in prostate cancer cells results in both growth inhibition and decrease of metastatic capability and success of androgen independent prostate cancer.

A seemingly contradictory finding, considering the known role of omega-3 fatty acids as anti-inflammatory substances, was a 13-fold induction of IL-6 in DHA and EPA-treated samples and the greater than 3-fold induction of COX-2. Since chronic expression of IL-6
and COX-2 has been correlated with development and progression of prostate cancer and multi-drug resistance (63, 68, 70), we expected lowered expression of these genes following fish oil treatment. Although IL-6 is induced at the early 24-hour time point, in DHA- and EPA- treated PC3 cells, its expression rapidly drops off to half the original induction at 48 hours (data not shown). Therefore, induction of IL-6 by these omega-3 fatty acids does not seem to cause the chronic inflammatory response that promotes carcinogenesis and growth. While COX-2 is often implicated in production of inflammatory prostaglandins from AA, it is also the enzyme responsible for conversion of n-3 PUFA to anti-inflammatory eicosanoids, such as the 1-series and 3-series prostaglandins, which counteract cancer progression (Figure 1-1) (14, 15). So in this case, increased levels of COX-2 transcript may factor into the inhibitory role of DHA and EPA against prostate cancer. Both IL-6 and COX-2 can be regulated by NF-κB; however, there were no alterations in the transcript levels for this transcription factor. In addition, none of the fatty acids increased NF-κB activity as determined by an NF-κB reporter assay. Others have reported the involvement of NF-κB in sensitizing prostate cells to growth arrest by DHA (83). However, the cell models between the studies differ and the lack of NF-κB reporter activity in PC3 cells suggests that NF-κB is not involved in the induction of IL-6 and COX-2 in DHA and EPA treated cells.

DHA also increased genes involved in induction of apoptosis to a greater extent than the other fatty acid treatments. The induction of apoptosis is confirmed by the annexin staining of PC3 cells treated with n-3 fatty acids. DHA induced annexin staining sooner and to a greater extent than DHA while ALA treated cells showed no staining above background. ATF-3 is the most modulated pro-apoptotic gene identified. Transfection of PC3 cells with an ATF-3 expression construct resulted in apoptosis. Additionally, these authors show that
activation of KLF-6, which activates ATF-3 in PC3 and LNCaP cells, resulted in apoptotic death of the cells due to induced apoptosis by inhibiting NF-κB activity (75). While our data indicate that NF-κB pathways are not involved, these results clearly indicate that DHA activates an ATF-3-dependent apoptotic pathway in androgen independent prostate cancer cells.

DHA consistently inhibited gene expression of cell cycle promoting genes and affecting genes involved in induction of apoptosis to a greater extent than the other fatty acid treatments. DHA was the most effective fatty acid examined at decreasing prostate cancer cell viability because it targeted both anti-inflammatory/anti-proliferative and pro-apoptotic genes and induced more substantial changes than the other omega-3 fatty acids tested. Taken together, these results may indicate that the different efficacies of fatty acids tested in decreasing cell viability may be due not only to modification of different pathways within androgen independent prostate cancer cells, but different magnitudes of response. Consideration could be made for encouraging patients to alter their diets in favor of fish products or adding omega-3 fatty acid supplements rich in DHA to their diets. The omega-3-acid ethyl esters contained in the hypertriglyceridemia medication Lovaza™ could also be considered in prostate cancer treatment or prevention strategies.
Chapter 3

ADIPOCYTES MODULATE THE INHIBITORY EFFECTS OF DIETARY FATTY ACIDS ON PROSTATE CANCER CELLS IN VITRO
3.1 ABSTRACT

Obesity in men, particularly an abundance of abdominal fat deposits, has been correlated with increased incidence and poor prognosis of prostate cancer. Fat may also play a significant role in the therapeutic targeting of prostate cancer cells that metastasize to bone, as the bones of older individuals are composed predominantly of fatty tissue. Omega-3 polyunsaturated fatty acids (PUFA) are well characterized as effective agents in cancer prevention and therapy. Previous studies show an inhibitory effect of omega-3 PUFA on PC3 prostate cancer cell proliferation. Additionally, we now demonstrate that other fatty acids, including certain omega-6 PUFA, and both 10-trans, 12-cis (10e12z) and 9-cis,11-trans (9z11e) isomers of conjugated linoleic acid (CLA), also inhibit proliferation of PC3 prostate cancer cells. To mimic fat cells in the body, and determine whether the presence of these fat cells would affect the inhibitory actions of fatty acids, we used a two-step model of media preconditioning and treatment. Culture media containing inhibitory concentrations of fatty acids was first conditioned on 3T3-L1 adipocytes, and then administered to PC3 cells. Here, we show that 3T3-L1 adipocytes modulate the effects of fatty acid treatment, generally rendering the fatty acids less effective in proliferative inhibition of PC3 prostate cancer cells. The mechanism of inhibition of the PC3 cells’ response to fatty acids in the presence of fat cells may be significant to understanding the correlation between obesity and resistance to prostate cancer therapy. A similar effect is observed in adipocyte modification of the proliferation inhibiting activity of chemotherapeutic drugs. These findings may be combined with clinical data to evaluate the consequences of an overabundance of adipocytes in the tumor microenvironment for prostate cancer and normal prostate tissue gene expression.
3.2 INTRODUCTION

Prostate cancer has been linked to obesity and nutrition both in incidence and mortality (48). An increase in dietary omega-3 polyunsaturated fatty acids has been linked to good prostate health (47). Omega-3 fatty acids include the plant-based alpha-linolenic acid (ALA), and fish oils docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). The effects of other fatty acids on prostate cancer are not as well characterized (79). To better understand the effects of different classes fatty acids on prostate cancer growth, we tested ALA, DHA, and EPA, as well as omega-6 PUFA arachidonic acid (AA) and linolenic acid (LA) and two isomers of conjugated linoleic acid (CLA) on the human prostate cancer cell line PC3. The 9-cis, 11-trans (9z11e) isomer of CLA has previously been associated with anti-inflammatory responses (25). Conversely, the 10-trans, 12-cis (10e12z) isomer of CLA has been implicated in perpetuating inflammatory pathways in different cell types (27). Inflammatory factors in the tumor microenvironment promote proliferation of prostate cancer cells, so the CLA isomers are expected to have distinct direct and indirect effects on PC3 cell proliferation (63).

Obesity is correlated with increased incidence of prostate cancer, poor prognosis, and decreased responsiveness to therapy (84). We therefore hypothesize that presence of adipocytes will diminish the inhibitory effect of fatty acids on prostate cancer cells. The objective of these studies was to understand how adipocytes affect the interaction between dietary fatty acids and prostate cancer cells, and whether the effects observed are fatty acid-specific. Adipocytes are highly metabolic cells, and may diminish the effects of fatty acids through simple uptake and storage, making the fatty acids unavailable to the prostate cancer cells. Alternatively, adipocytes may metabolize fatty acids into inflammatory prostaglandins.
and secrete factors to activate alternative proliferative pathways in prostate cancer cells, thereby counteracting fatty acid inhibition of proliferation by activating opposing pro-inflammatory pathways. Obesity results in greater adiposity in the area surrounding a tumor, which is known to promote inflammation and tumor progression in a variety of cancers, including prostate cancer (32). An inflammatory microenvironment perpetuated by increased adiposity may be responsible for mitigating the antiproliferative effects of dietary fatty acids or other therapeutic agents on prostate cancer cells.
3.3 MATERIALS AND METHODS

3.3.1 3T3-L1 Adipocyte Differentiation

3T3-L1 fibroblasts from ATCC were cultured and passaged in High Glucose Dulbecco’s Modified Eagles Medium (HG DMEM) (Sigma) supplemented with 10% bovine calf serum. Fibroblasts were plated for differentiation in 12-well clear cell culture plates (Invitrogen) at a density of 50,000 cells per well and allowed four days to reach confluence. Four days after plating, the 3T3-L1 cells were induced to differentiate in HG DMEM supplemented with 10% fetal bovine serum (FBS) containing 1 µg/mL insulin, 0.5 mM isobutylmethylxanthine (IBMX), and 1 µM dexamethasone. The day on which adipocytes were treated with this differentiation cocktail and induced to differentiate was termed Day -2. After differentiation induction, the 3T3-L1 cells were fed every other day in differentiation medium (HG DMEM/10% FBS containing 1 µg/mL insulin) for ten days until they were fully differentiated into adipocytes. In some cases, differentiation medium was supplemented with 2 µM rosiglitazone on Day 0. Day 10 adipocytes were used in all studies.

3.3.2 3T3-L1 Adipocyte-PC3 Coculture Conditioning Studies

Differentiated 3T3-L1 adipocytes were treated with HG DMEM/10% FBS containing 100 µM treatments of omega fatty acids for 48 hours. A treatment dose of 100 µM was chosen for clinical relevance, as this level of fatty acid is attainable in serum (85). Following this 48-hour conditioning period on 3T3-L1 adipocytes or undifferentiated confluent 3T3-L1 fibroblasts as a conditioning control, media was removed, centrifuged for five minutes at 1000 RPM, and used to treat PC3 cells for a viability assay (protocol described in Chapter 2).
3.3.3 Statistical Analysis

Figures are displayed as the mean of replicates ($n=6$-$18$, see figure legends) with error bars representing population SEM. Statistical significance was determined with all groups using one-way ANOVA with the Tukey post-test using GraphPad Prism software.
3.4 RESULTS

3.4.1 PUFA inhibit prostate cancer cell proliferation at different rates

As noted in Chapter 2, the three n-3 fatty acids show marked differences in their abilities to inhibit of PC3 cell proliferation. Rather than clustering together in their inhibition of PC3 proliferation, each n-3 fatty acid exhibited different rates and magnitudes of proliferative inhibition. Analogous differences are observed when comparing the inhibition of PC3 proliferation following treatment with n-6 fatty acids and conjugated linoleic acid isomers: each fatty acid exhibits a different efficacy and potency when inhibiting prostate cancer cell proliferation. As observed previously, DHA is the most effective n-3 fatty acid, both in terms of rate and overall magnitude of proliferative inhibition (Figure 3-1 A). Of the n-6 fatty acids, AA exhibits the most pronounced inhibitory trend, producing a proliferation curve identical to the one observed following DHA treatment (Figure 3-1 B). The n-3 fatty acid EPA clusters similarly with the n-6 fatty acid LA in its magnitude and inhibition of PC3 proliferative inhibition (Figure 3-1 A and 3-1 B). ALA inhibits PC3 proliferation slightly, though not to the extent of the other n-3 PUFA (Figure 3-1 A). The omega-9 monounsaturated fatty acid OA did not inhibit PC3 proliferation, and consistently showed a slight induction of proliferation after 72 hours of treatment (Figure 3-1 B).

Unlike the n-3 and n-6 PUFA, the CLA isomers did not exhibit a marked difference in their effects on PC3 cell proliferation. Both isomers inhibited PC3 proliferation at a comparable rate, and to a comparable extent. A 50/50 mixture of the two isomers showed an identical inhibitory trend to that observed following treatment with 10e12z CLA, indicating that the two isomers do not interact to produce additive or antagonistic effects (Figure 3-1 C).
PC3 cells (1000 cells/well) were seeded on white, 96-well cell culture plates (Greiner Bio-One, Frickenhausen, Germany) in Dulbecco’s Modified Eagle’s Medium/Ham’s F-12 medium (DMEM/F-12; Sigma, Saint Louis, MO) supplemented with 10 % FBS (Hyclone, Logan, UT). The following day, the cell media was changed to OPTI-MEM supplemented with 0.1 % FBS. The following day the media removed and fresh media containing 1 % FBS and 100 µM conjugated fatty acid/BSA conjugate was added (n ≤ 4). Cell viability or growth was measured using the Cell Titer-Glo Luminescent Cell Viability Assay (Promega, Madison, WI). Results represent the mean of three independent experiments, shown as cell viability relative to vehicle control BSA. PC3 inhibition trends were not conserved within fatty acid families. Rather certain fatty acids of each class appeared to inhibit proliferation. Of the omega-3 PUFA, DHA was the most efficacious and potent inhibitor of PC3 proliferation. EPA and ALA both caused some inhibition of proliferation, but neither was as effective as DHA. Omega-6 PUFA AA was also a potent inhibitor of proliferation. LA also inhibited PC3 proliferation, but more slowly and to a lesser extent. Both 9z11e and 10e12z isomers of CLA rapidly and significantly inhibit PC3 cell proliferation.
3.4.2 3T3-L1 adipocytes counteract the inhibitory effect of fatty acids on prostate cancer cells, and may promote prostate cancer cell proliferation

Preconditioning omega-3 fatty acids on adipocytes or fibroblasts inhibits their anti-proliferative effect on PC3 cells (Figure 3-2 A). ALA and DHA action was significantly counteracted, though to comparable extents, following preconditioning of fatty acid-containing medium adipocytes or fibroblasts. Adipocyte and fibroblast conditioning did not have a significant effect on the inhibitory action of EPA on PC3 prostate cancer cells. Preconditioning of omega-6 fatty acids on adipocytes or fibroblasts showed comparable results to what was observed in omega-3 fatty acids (Figure 3-2 B). In the case of the omega-6 fatty acids, however, there was a more marked difference between the curves for adipocyte-conditioned and fibroblast-conditioned medium containing AA or LA. This indicates a more adipocyte-specific effect at play for omega-6 fatty acids. Finally, the post-conditioning pro-proliferative action of either 9z11e CLA or 10e12z CLA, or a combination of the two isomers, is adipocyte specific: fibroblast conditioning does not affect the inhibitory action of the fatty acid treatments.

When the proliferation data are analyzed at the 72-hour time point, several fatty acids inhibit proliferation when used directly to treat adipocytes (Figure 3-3). For omega-3 and omega-6 fatty acids treatments, this inhibitory effect, where it exists, is eliminated following conditioning on both adipocytes and fibroblasts. On the other hand, for CLA treatments, fibroblast conditioning does not significantly alter the inhibitory effect of the fatty acids. Conditioning 9z11e CLA on adipocytes significantly induces PC3 proliferation to approximately two-fold of control levels following 72 hours of treatment. A similar trend of increased PC3 proliferation is observed following conditioning of the 10e12z CLA treatment on adipocytes, although the induction is not statistical at the 72 hour time point (Figure 3-3).
Figure 3-2: Preconditioning PUFA treatments on 3T3-L1 fibroblasts or adipocytes differentiated with rosiglitazone alters their inhibitory activity

3T3-L1 fibroblasts were differentiated into adipocytes. Undifferentiated 3T3-L1 fibroblasts were plated at confluency and used as a non-adipocyte conditioning control. 100 µM treatments were prepared as before and conditioned for 48 hours on adipocytes, fibroblasts, or in the incubator (unconditioned media, UCM). Media was then harvested and centrifuged at 1000 rpm for 5 minutes. Supernatants were used to treat serum starved PC3 cells. The proliferation assays were carried out as described. Results represent the mean of three independent experiments (n ≤ 4). PUFA exhibited distinct inhibition patterns after adipocyte conditioning, but none of them retained their inhibitory capacity following conditioning.
Figure 3-3: Effects of preconditioning on adipocytes or fibroblasts on PUFA activity

The 72-hour time point was used to compare inhibition trends of each PUFA under different preconditioning treatments. Data were analyzed using one-way ANOVA. Treatment conditions that caused a statistically significant deviation from BSA vehicle control according to Tukey’s post-test were noted. * Indicates results reported as significant with $p \leq 0.01$. 
3.4.3 Differentiation induction using rosiglitazone alters properties of adipocytes, influencing their effects in conditioning studies

Adipocyte differentiation conditions affected the result of adipocyte conditioning on PC3 cells differentiated with omega-6 fatty acids. Adipocytes differentiated with rosiglitazone eliminated the AA- or LA-mediated inhibition of PC3 cell proliferation, but did not elicit a pro-inflammatory response in the adipocytes. In contrast, adipocytes differentiated without rosiglitazone-induced proliferation in PC3 cells after treatment with omega-6 fatty acids. Proliferation curves from omega-6 treatments conditioned on adipocytes differentiated without rosiglitazone induced proliferation of 3-fold (in the case of AA) to 6-fold (for LA) above levels observed after conditioning on rosiglitazone-treated adipocytes. Treatment of adipocytes differentiated without rosiglitazone with omega-9 fatty acid oleic acid also resulted in a 6-fold increase in PC3 proliferation above conditioning on adipocytes differentiated without rosiglitazone.

Adipocyte differentiation conditions do not appear to affect the PC3 proliferation outcome of CLA conditioning studies. Treatment of PC3 cells with either adipocyte conditioned 9z11e CLA or adipocyte conditioned 10e12z CLA induced cell proliferation, regardless of whether the adipocytes had been differentiated in the presence or absence of rosiglitazone.
Figure 3-4: Omega-6 and omega-9 fatty acid conditioning studies on adipocytes differentiated with and without rosiglitazone

PC3 proliferation following treatments with adipocyte-conditioned media was measured as described previously (n=6). Conditioning omega-6 fatty acids on adipocytes differentiated with rosiglitazone did not result in significant induction of PC3 proliferation above control levels. Treatment of PC3 cells with omega-6 fatty acids that had been conditioned on adipocytes differentiated without rosiglitazone induced significant proliferation of PC3 cells above control levels.
Figure 3-5: CLA conditioning studies on adipocytes differentiated with and without rosiglitazone
PC3 proliferation following treatments with adipocyte-conditioned media was measured as described previously (n=6). CLA treatments conditioned on adipocytes induced similar proliferation patterns in PC3 cells, independently of whether the adipocytes had been differentiated with or without rosiglitazone.
3.5 DISCUSSION

Different PUFA tested exhibited different efficacies in inhibiting PC3 proliferation. DHA, AA, both isomers of CLA, and to a lesser extent EPA and LA, inhibited PC3 cell proliferation when used to directly treat the cells. Inhibition of proliferation by the n-3 PUFA was to be expected since previous studies have demonstrated the inhibitory effects of DHA and EPA on prostate cancer (1, 2). However, the inhibitory effects observed following treatment of PC3 cells with the n-6 PUFA AA and LA were unexpected, since much of the literature points to a contradictory role for n-6 PUFA with regard to cancer (3). AA and 10e12z CLA have both been characterized as pro-inflammatory fatty acids since both activate the arachidonic acid pathway, ultimately leading to production of cyclooxygenase-2 (COX-2) and other inflammatory prostaglandins (18). The inhibition of PC3 cell proliferation following treatment with AA and 10e12z CLA indicates that these fatty acids are also capable of eliciting other anti-proliferative responses in PC3 cells that prevent or overcome the effects of inflammatory prostaglandins produced in the arachidonic acid pathway.

Two dominant inhibitory patterns emerged among the PUFA tested. In the first trend, observed following treatment with 9z11e and 10e12z CLA, the fatty acids directly inhibited PC3 proliferation, but resulted in increased proliferation when working through adipocytes. This effect was observed to be adipocyte-specific, as both isomers of CLA retained their inhibitory potential following fibroblast preconditioning. The second trend was observed in the omega-3 and omega-6 PUFA. These fatty acids also directly inhibited PC3 proliferation, but lost their inhibitory capacity following preconditioning. Preconditioning did not induce proliferation of PC3 cells, and the effect observed was not adipocyte-specific, as it was also
seen after fibroblast conditioning. The two trends observed may represent the difference between uptake and metabolism of fatty acids into inflammatory or pro-proliferative factors by adipocytes (Trend 1), versus uptake and storage of fatty acids by adipocytes or pre-adipocytes (Trend 2). In Trend 2, fatty acids appear to be simply removed from the medium by adipocytes or fibroblasts, so that they become unavailable to inhibit PC3 cell proliferation. In the case of Trend 1, pro-proliferative metabolites of fatty acids released from adipocytes may be inducing PC3 proliferation. This kind of mechanism has previously been observed in breast cancer cell lines following preconditioning of 10e12z containing medium on 3T3-L1 adipocytes (26). Alternatively, Trend 1 could be brought about by induction of other pro-inflammatory signals in adipocytes, which would lead to secretion of interleukins, adipokines, or other factors from these cells, ultimately resulting in increased PC3 proliferation. The class of factors responsible for PC3 proliferation following adipocyte conditioning may be identified in the future through lipid extractions or heat inactivation of conditioned medium prior to PC3 treatment.

In some cases, moderate inhibition of PC3 proliferation could still be observed following fibroblast conditioning with omega-3 and omega-6 fatty acids. This supports the hypothesis that the adipocytes’ role in counteracting fatty acid-mediated inhibition of PC3 proliferation was mainly a function of uptake: adipocytes are significantly more metabolically active cells than confluent, non-dividing fibroblasts, and would therefore be expected to remove more factors from media than would the less metabolically active precursor fibroblasts.

Adipocytes differentiated with rosiglitazone appear to have a different effect on PC3 cell proliferation when used to condition n-6 PUFA treatments than adipocytes differentiated
without rosiglitazone. While n-6 treatments conform to Trend 2 when conditioned on adipocytes that had been induced to differentiate with rosiglitazone, the PC3 proliferation pattern observed after conditioning on adipocytes that did not receive rosiglitazone is much more similar to Trend 1. This indicates a difference between adipocytes differentiated with or without rosiglitazone as far as their response to n-6 PUFA treatments.

Adipocytes induced to differentiate with rosiglitazone are only treated with rosiglitazone once (at day zero). Ten days elapse and the medium on the adipocytes is changed five times without subsequent addition of rosiglitazone. Given the amount of time between treatment with rosiglitazone and treatment of cells, compounded with the high metabolic rate of adipocytes, it is unlikely that residual rosiglitazone elicits the anti-inflammatory effect observed in adipocytes differentiated with rosiglitazone. Rather, it is likely that the rosiglitazone alters the differentiation pathway, resulting in a different population of adipocytes, which respond differently to treatment with n-6 fatty acids. For example, inducing 3T3-L1 cells to differentiate with rosiglitazone may produce a population of adipocytes that are less prone to secrete pro-inflammatory signals, even following stimulation.

Although differences are observed in conditioned media responses of PC3 cells treated with omega-6 fatty acids depending on whether the adipocytes were differentiated with rosiglitazone, CLA treatments showed nearly identical PC3 proliferation patterns, independently of whether they had been conditioned on adipocytes that had or had not received rosiglitazone. However, the magnitude of the proliferative response induced by 9z11e and 10e12z CLA isomers was not greater than the proliferative response induced by n-6 PUFA conditioned on adipocytes that had not been exposed to rosiglitazone. This indicates
that different mechanisms may be at work to promote PC3 cell proliferation following adipocyte conditioning of fatty acid treatments: the pro-proliferative pathway induced by the CLA isomers is active independently on the population of adipocytes, while the pro-proliferative response induced by n-6 PUFA is dependent on the adipocyte population used for conditioning.
Chapter 4

CONCLUSIONS AND FUTURE DIRECTIONS
4.1 CONCLUSIONS

4.1.1 Omega-3 fatty acids inhibit prostate cancer cell proliferation through different mechanisms

Omega-3 PUFAs inhibit prostate cancer cell proliferation at different rates due to induction of different inhibitory mechanisms. ALA, DHA, and EPA all inhibit mRNA and protein levels of fatty acid metabolizing enzyme FASN and its regulator SREBP-1c. Inhibition of fatty acid metabolizing enzymes may result in inhibition of prostate cancer cell proliferation due to the reliance of prostate cancer cells on fatty acids as an energy source. All three n-3 PUFAs also decrease transcript levels of MCP-1, an important autocrine growth factor in prostate cancer, to varying levels. Differences in the extent of MCP-1 inhibition by n-3 PUFAs may be partially responsible for the differences observed between the inhibitory rates of different n-3 PUFA. Finally, the fish oils DHA and EPA induce apoptosis in PC3 cells. DHA significantly upregulates pro-apoptotic transcription factor transcript ATF3 and induces apoptosis in 50% of PC3 cells. EPA does not significantly upregulate ATF3 mRNA, and induces apoptosis in 25% of PC3 cells, showing that EPA may induce apoptosis through a partially ATF3-independent mechanism. Plant-based ALA does not induce apoptosis in PC3 cells, although it slightly inhibits PC3 proliferation through other mechanisms.

The inhibition of PC3 proliferation by DHA and EPA but not ALA is likely due to the fish oils’ ability to modulate expression of a range of different genes with diverse functions. DHA and EPA also give way to a number of bioactive metabolites, which are the effectors of many of the observed biological effects (16). In addition to their regular range of bioactive metabolites, both DHA and EPA give rise to compounds called resolvins, which have been characterized as having anti-inflammatory and anti-cancer properties, when COX enzymes
are aspirinated and inhibited (86, 87). ALA does not give rise to this range of activated metabolites, and therefore does not effect the same magnitude of PC3 inhibition.

Modulation of genes involved in energy homeostasis, SREBP-1c and FASN, by all n-3 PUFAs has potentially important implications for inhibition of cancer progression at the metabolic level. The Warburg Hypothesis proposes a metabolic origin for cancer, whereby mitochondrial instability results in carcinogenesis by causing genomic instability, cellular dysfunction, and production of genotoxic metabolites (88). The theory is supported by the fact that all transformed cells exhibit a shift from oxidative phosphorylation to glycolysis as a means of energy production — among other metabolic alterations (89). Here, n-3 PUFA are shown to disrupt a novel metabolic pathway established by cancer cells. This presents a powerful mechanism for targeted inhibition of cancer cells, as altered metabolic homeostasis is vital to cancer development and progression (90, 91). Inhibition of cancer cell-specific metabolic pathways by n-3 PUFAs may be used as a selective treatment against cancer cells.

Taken together, these findings relating to the relative potency and mechanisms of action of n-3 PUFAs have significant therapeutic implications. Prescription grade n-3 PUFAs may be prescribed alongside conventional chemotherapies to potentiate cancer cell-specific cytotoxicity (5, 6). When prescribing n-3 PUFA concurrently with chemotherapy for prostate cancer, it is important to understand the relative potencies of the fatty acids, and the precise mechanisms of cancer cell killing to prescribe the most effective fatty acid. For example, ALA, DHA, or EPA may be used to enhance the effects of a potential chemotherapeutic compound such as Cerulenin that specifically inhibits FASN since all three PUFA modulate transcription of FASN in PC3 cells (92). Conversely, DHA would likely be best candidate to use alongside an apoptosis-inducing chemotherapeutic drug.
4.1.2 Adipocyte preconditioning mitigates the inhibitory effects of fatty acids on PC3 cells

Like n-3 PUFAs, n-6 PUFAs and CLA isomers exhibit different rates of inhibition of proliferation when placed directly on PC3 cells. AA and both 9z11e and 10e12z isomers of CLA were shown to be potent inhibitors of PC3 proliferation. AA has commonly been linked to promotion of cancer progression, primarily due to the pro-proliferative activity of its metabolites produced by cyclooxygenase (COX) and lipooxygenase (LOX) enzymes (93, 94). Production of pro-inflammatory prostaglandins provides the rationale for existing cancer therapies that inhibit the enzymatic activity of COX-2 (95). However, it is important to note that the pro-inflammatory prostaglandins produced by COX-2 may further be enzymatically converted into other prostaglandins, some of which have been shown to specifically induce apoptosis in cancer cells (96, 97). Enzymatic conversion of preliminary inflammatory metabolites into downstream anti-cancer prostaglandins in PC3 cells could account for the inhibitory effect observed following treatment with AA.

Consumption of meat and dairy products constitutes a major dietary source of CLA (98, 99). Clinical epidemiological and observational studies have linked the consumption of diets high in meat and dairy products to increased risk of development of prostate cancer (99, 100). CLA occurs naturally as a mixture of 10e12z and 9z11e isomers in meat and dairy products, although 9z11e CLA is the dominant isomer in these nutritional sources (101, 102). In spite of a correlation between cancer and increased intake of meat and dairy, CLA is often characterized as a protective and non-carcinogenic constituent of these products (98, 99).

The effect of all of the fatty acids tested on PC3 cells was altered by presence of adipocytes. In some cases, as observed with n-3 and n-6 PUFA, adipocytes took away the inhibitory effect observed when PC3 cells were directly treated with the fatty acids.
Alternatively, fatty acids working through adipocytes could elicit a pro-proliferative response in the PC3 cells, as observed for 9z11e and 10e12z CLA treatments. These two trends observed may be attributed to uptake of fatty acids by adipocytes (n-3 and n-6 PUFAs) versus metabolism to pro-proliferative prostaglandins, or activation of other pro-inflammatory responses in adipocytes (CLA isomers).

The cell specificity of the conditioning responses supports this scenario. The inhibitory effect of n-3 and n-6 PUFAs is counteracted by not only adipocytes, but also to a lesser extent by 3T3-L1 fibroblasts. This indicates that the cell type (adipocyte or fibroblast) used in conditioning is removing PUFAs from the medium, making them inaccessible to the PC3 cells. A slight inhibitory effect may still be observed following conditioning of n-3 or n-6 PUFA-containing medium on fibroblasts, which may be attributed to the slower metabolic rate of confluent fibroblasts as compared to adipocytes, resulting in reduced PUFA uptake from medium (leaving residual PUFA to inhibit PC3 cell proliferation). Conversely, fibroblasts have a negligible effect on the activity of 9z11e and 10e12z CLA on PC3 cells, indicating induction of an adipocyte-specific pro-inflammatory mechanism.

Unexpectedly, conditioning PUFA treatments on adipocytes differentiated in the presence versus absence of PPARγ agonist rosiglitazone elicited different outcomes on PC3 proliferation. The pro-proliferative response induced in PC3 cells by treatment with adipocyte-conditioned CLA was independent of whether the adipocytes had been differentiated with or without rosiglitazone. On the other hand, n-6 PUFA conditioned on adipocytes induced to differentiate without rosiglitazone induced a pro-proliferative response in PC3 cells that is not observed after conditioning on adipocytes differentiated with rosiglitazone.
These findings support the hypothesis that the population of adipocytes formed with rosiglitazone is distinct from the population formed in its absence. These two populations may process n-3 and n-6 PUFAs differently to elicit distinct effects on PC3 cells treated with adipocyte-conditioned medium. The function of rosiglitazone in adipocyte differentiation is twofold: to activate its target PPARγ, and to promote insulin signaling (103). However, rosiglitazone has also been found to activate other cellular responses among 3T3-L1 cells, notably, increasing mitochondrial biogenesis and augmenting adiponectin release (104). Increased mitochondrial biogenesis through rosiglitazone treatment increases fatty acid oxidation and overall metabolic rate of treated cells (105). This could result in rapid elimination of n-6 PUFA from adipocytes differentiated with rosiglitazone, thereby diminishing the effects these fatty acids would have on PC3 cells. Adipokines such as adiponectin are known to promote progression of several types of cancers, and implicated in the cancer-obesity link (106). Since adiponectin is released in higher concentrations from adipocytes differentiated with rosiglitazone, it is unlikely that it accounts for the increase in PC3 proliferation following treatment with n-3 PUFA conditioned on adipocytes differentiated without rosiglitazone.

Here, 3T3-L1 adipocytes have been observed to counteract the inhibitory effects of fatty acids on PC3 cells. Although the mechanisms through which adipocytes alter PUFA action have yet to be elucidated, this finding has important implications for fatty acid intake by obese cancer patients. Obesity results in hypertrophy and altered metabolism within fat cells, which contributes pro-inflammatory signals to the tumor microenvironment, and may thus alter availability or effects of PUFA on cancer cells (107). The presence of fat cells has been shown to eliminate or reverse the inhibitory actions of fatty acids on prostate cancer
cells. If these observations hold true in vivo, dietary or supplemented fatty acids might not only lose their ability to inhibit proliferation of prostate cancer cells, they may also work through fat cells to exacerbate prostate cancer. Further studies are needed to establish the mechanisms involved in the interplay between PUFAs, adipose, and cancer, and to devise dietary interventions that are effective when treating obese individuals with prostate cancer.

4.2 FUTURE STUDIES

To follow up the studies into the mechanisms of action of PC3 proliferative inhibition by n-3 PUFA, gene expression and induction of apoptosis studies could be completed for the n-6 PUFA and CLA isomers. This would allow comparison of the inhibitory trends following treatment with the different fatty acids on the basis of mechanisms of inhibition. The adipocyte conditioning studies may be completed in a true coculture system to gain a more complete understanding of the interplay between adipocytes and prostate cancer cells, and determine whether similar effects on prostate cancer cell proliferation are observed in a more complete model of microenvironment. Further adipocyte conditioning studies also need to be conducted to establish the mechanisms involved in alteration of fatty acid action on PC3 cells by adipocytes. These studies would be essential to determine whether fatty acid metabolites or other adipocyte-derived pro-inflammatory factors are responsible for inducing proliferation following conditioning with CLA isomers. Finally, the difference between adipocytes differentiated with or without rosiglitazone has not been previously characterized. Since rosiglitazone is widely used to achieve complete differentiation of adipocytes, it is important to characterize the differences between adipocytes differentiated with or without rosiglitazone to assess whether adipocytes differentiated with rosiglitazone are an acceptable cell culture model for fat tissue.
REFERENCES


Appendix A: PCR Primer Sequences

ATF3: F, TCACTGTCAGCGACAGACCC and R, CTACCTCGGCTTTTGTGATGG;
Bcl-2: F, GCATGCGGCCTCTGTGATTTCT and R,
AGGCATGTGACTCTCATTGTGAGG;
Bcl-6: F, AAGACCCTCATACCGGTAGAAA and R,
GCAGGTTTGCATTGAGGCTT;
COX-2: F, AAGTGCGATTGTACCGGAGC and R, CGGTGTTGAGCAGTTTCTCC;
Cyclin A2: F, GCATGTCACCGTCTCTCTT and R, GTGAACGCAGGCTGTTTACTGT;
Cyclin D1: F, AACCTGAGGAGCCCCAACA and R, GAAGCGTGTGAGGCGGCTAGTA;
Cyclin D2: F, TGGCTCAAGGAGGAGCAGC and R, CAGGCTATTGAGGAGCACC;
FASN: F, AGGAGCAAGGCGTGACCTT, and R, ACAACGAGCGGATGAGCTG;
IL-1β: F, CACGGCCACATTTGGTTCTAA and R, CAGAATGTGGGAGCGAATGAC;
IL-6: F, GCCACTCACCTCTCTAGGAGC and R, CGGTGTTGAGGCTGTTTCTCC;
IL-10: F, GGGAGCCCCTTTGTAGAATTTAA and R, GGGAATCCCCTCCAAGACAT;
MCP-1: F, ATACGAGCCACCTTCATCC and R, TGCACGTAGATCTCTTATTGG;
NF-κB: F, AGGCTATGAGGCTCCAAGAG and R, TGTCACCGCAGTACGAAAAAG;
p21: F, GCAGAGGAAGACCATGTTGTGAC and R, GCGAGGCAAGGAGGTGACAAG;
p57: F, CGGCGATCAAGAAGCTGTC and R, GCTTGGCGAAGAAAATCGGA;
RPL13a: F, CATCGTGGCTAATACAGGTACTG and R, GCACGACCTTGAGGCGACCC;
SREBP-1c: F, CCATCTGAGGAGGCCAGTG and R, GGTGTTGTAGCCAGGCTGTC;
TNFα: F, ATCAATCCGCCGCATTACTTCT and R, TGGATGTTGCTCTCTACAGA;
TGF-β1: F, CTATTGCTTCCAGCTCCACAGGA and R, AGGTCTTTGCGGAAGTCAATG.
Appendix B: Comparison of Cell Titer-Glo and BrdU analysis of cell viability

![Graphs showing comparison of Cell Titer-Glo and BrdU analysis at PC-3 48 hr, 72 hr, and 168 hr.](image)
Appendix C: PCR data by gene

[Graphs showing gene expression data for various genes]
Appendix D: 72-hour Flow Cytometry Apoptosis Assay Results

ALA: Replicate 1
ALA: Replicate 2

Institution: Huck
Protocol: MitoDamage-3.PRO
Listmode Replay: Runtime Protocol
Analysis Date: 11-Nov-2011, 13:46:50
Listmode File: ALA2 00027867 LMD

Run Date: 11-Nov-11,
Sample
User ID: vande
Acquisition Time/Events: 16.5s / 10000 (PRO)
Tube ID:

[Diagrams and tables related to the analysis are shown here, but not transcribed.]
ALA: Replicate 3
BSA (vehicle control): Replicate 1
BSA (vehicle control): Replicate 2
BSA (vehicle control): Replicate 3

Institution: Huck
Protocol: MitoDamage-3.PRO
Listmode Replay: Runtime Protocol
Analysis Date: 11-Nov-2011, 13:44:08
Listmode File: ALA1_00027885.LMD

Run Date: 11-Nov-11,
Sample
Acquisition Time/Events: 15.7s / 10000 (PRC)
Tube ID:

[Graphs and data tables related to flow cytometry analysis]

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DHA: Replicate 1
DHA: Replicate 2
DHA: Replicate 3
EPA: Replicate 1
EPA: Replicate 2

Institution: Huck
Protocol: MitoDamage-3.PRO
Listmode Replay: Runtime Protocol
Analysis Date: 11-Nov-2011, 13:55:57
Listmode File: EPA2 00027873.LMD

Run Date: 11-Nov-11
Sample ID:
User ID: vande
Acquisition Time/Events: 53.6s / 10000 (PROC)
Tube ID:

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[Graphs and tables related to MitoDamage-3.PRO analysis provided here.]
Appendix E: Inhibition of other cancer cell lines by PUFA treatment

PC-3

MCF-7

MiaPaca

HEK293-T
VITA

Pinar Ozden Eser

Education

*The Pennsylvania State University, Schreyer Honors College* (Summer 2008-Spring 2012)

- Expected graduation: May 2012
- M.S. Molecular Medicine
- B.S. Toxicology (with Honors)
- B.S. Immunology and Infectious Disease
- Minor: French and Francophone Studies

*Universidad Autónoma de Madrid, Spain: Department of Biology* (Fall 2008-Spring 2009)

- Courses taken in Spanish:
  - Cellular Origin and Development: 9.3/10
  - Biochemistry (class/lab): 7.7/10
  - Ecology (class/lab): 9.1/10: Highest among 64 students
  - Microbiology (class/lab): 8.2/10

Awards and Honors

- NCI Integrated Cancer Biology Program Summer Fellow at MIT: Summer 2011
- Alpha Epsilon Delta: The Premedical Honor Society: Member, Spring 2011-present
- Pennsylvania State University Summer Discovery Research Grant: Summer 2010
- Gamma Sigma Delta: the Honor Society of Agriculture: Member, Spring 2010-present
- The Honor Society of Phi Kappa Phi: Member, Spring 2010-present
- Schreyer Honors College Academic Excellence Scholarship: Fall 2009-present
- Oswald Scholarship: Fall 2009-Spring 2010
- C. Melville, Jr. and Kenneth Barr Scholarship: Fall 2009-Spring 2010
- Schreyer Honors College Ambassador Grant: Spring 2009

Publications and Posters


