THE EFFECTS OF ELLAGIC ACID ON INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-2 IN HUMAN PROSTATE CANCER CELLS

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ABSTRACT

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Ellagic acid (EA), a dietary phenolic compound found in berries and nuts, elicits important cellular activities such as apoptosis and cell cycle arrest in a variety of cancer cells. Little is known of EA effects on androgen-dependent prostate cancer cells. The purpose of the present study was to determine the in vitro effects of EA on proliferation of human prostate cancer (LNCaP) cells. Additionally, the effect of EA exposure (0.1-10 uM) on concentrations of insulin-like growth factor binding protein-2 (IGFBP-2), a growth factor implicated in prostate cancer, was determined. Cell proliferation was assessed after 1-3 days of exposure to EA (0.03-100 uM), media alone (control), or 0.016% DMSO (vehicle) by counting cells or estimating mitochondrial activity using a cell viability assay (MTS, Promega, Madison, WI). IGFBP-2 secreted into culture media was determined by an enzyme-linked immunosorbant assay (R&D Systems, Minneapolis, MN).

EA significantly inhibited cell proliferation of LNCaP cells in a dose-dependent manner. Cell count and MTS data both demonstrated a significant decrease in cell growth for concentrations of EA greater than 0.1 uM compared to vehicle (p<0.05). Cell counts were 89%, 72%, 68%, 38%, and 29% of control for 0.1, 1, 2, 10, and 100 uM EA exposure, respectively. Little change in cell viability occurred within the first 24 h for cells exposed to 0.03 to 50 uM EA; however, marked reduction in cell viability after 24 h at 100 uM EA was observed. IGFBP-2 concentrations were significantly less for cells exposed to 10 uM EA compared to vehicle (8.7, 5.1 ng/mL/500,000 cells, respectively, p<0.05). IGFBP-2 concentrations were lower for 2 uM and 1 uM EA cells as well (7.3, 8.9 ng/mL/500,000 cells), but not statistically different than controls.
The present study demonstrated dose-dependent effects of EA in decreasing cell proliferation and suppressing secretion of IGFBP-2 in prostate cancer cells. Doses of EA used in the current study were lower than previous reports using other cancer cell lines, and approximate to EA concentrations reported in human serum. The potential benefits of modifying cancer cell proliferation and growth factors, such as IGFBP-2 via dietary bioactive compounds such as EA are promising and warrant further investigation.
This work is dedicated to the only true God, Jehovah.
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Prostate cancer is the second leading cause of cancer deaths among men in the United States. Prostate cancer etiology remains unknown despite the high incidence and mortality rates. Some of the implicated factors include age, diet, race, family history of prostate cancer, hormones (e.g., androgen), and circulating levels of the insulin-like growth factors (IGF).

Nutrition is one of the areas investigated in detail in regard to prostate cancer etiology and prevention. Increased consumption of fruits and vegetables has been linked to reduced risk of several cancers, although their role is less clear in prostate cancer. There is mounting evidence that much of the health benefit of these plant foods may come from phytochemicals and bioactive substances. Examples of such compounds include lycopene from tomatoes, isothiocyanates from cruciferous vegetables and ellagic acid (EA) from fruits and nuts. EA has been widely investigated with respect to its potential chemopreventive effects on different cancer cell types including prostate cancer.

Information gained from extensive study of growth factors, although inconsistent, suggests that increased risk of several cancers, such as prostate cancer, is related to the insulin-like growth factor (IGF) system. The IGF system is involved in many critical aspects of growth, development, reproduction, and aging in health and disease. Insulin-like growth factor-1 and -2 (IGF-1 and -2) have been found to be potent mitogens in many cancer cell lines. The independent activities of insulin-like growth factor binding protein-2 (IGFBP-2), such as cell proliferation, in cell cultures have been reported. IGFBP-2 has been found to be expressed by different tumors and tumor cell lines of several cancers including prostate cancer. It is suggested
that IGFBP-2 is one of the most commonly expressed genes in hormone refractory prostate cancer (1).

Several studies have reported the inhibitory properties of EA on different cancer cells in culture. None however has examined the effects of EA on human androgen dependent prostate cancer (LNCaP) cells in culture, although the gene expression pattern of LNCaP cells exposed to EA in combination with another phenolic compound, resveratrol, has been documented. Furthermore, the influence of EA on secreted IGFBP-2 concentrations in LNCaP cell is yet to be reported.
Statement of Problem

Epidemiological and experimental studies suggest that biologically active compounds in foods may have important impacts on the prevention and treatment of prostate cancer. Ellagic acid, a phytochemical found in berries and nuts, may have potent effects on prostate cancer cells. Growth factors, such as the IGF system, appear to play a pivotal role in the neoplastic process, and may be impacted by environmental factors, such as dietary intake of bioactive compound rich foods. The present study will investigate the effects of ellagic acid on the proliferation and secretion of IGFBP-2 in human prostate cancer cells in culture.

Significance of Problem

Androgen deprivation therapy (ADT) or androgen withdrawal is one of the optimal therapies for patients with advanced stage prostate cancer since most developing prostate tumors are androgen-dependent. However, evidence shows that androgen-independent prostate cancer cells secret greater levels of IGFBP-2, which has been implicated in rapid cell division and growth. The present study will measure the direct effects of ellagic acid on cell viability, but also its ability to suppress IGFBP-2 levels. Prostate cancer incidence is expected to rise with the increasing aging population in the United States, thus making it a public health crisis. The present study will contribute important knowledge regarding possible dietary interventions that may modify the risk or progression of prostate cancer.
Research Questions

(1) Does exposure to ellagic acid affect the proliferation and growth of human prostate cancer cells (LNCaP) in culture?

(2) What effect does ellagic acid have on the concentration of IGFBP-2 secreted by LNCaP cells?
CHAPTER II. LITERATURE REVIEW

Prostate cancer is the most commonly diagnosed male cancer and the second leading cause of cancer deaths among men in the United States. In the year 2006, about 234,460 new cases of prostate cancer will be diagnosed in the United States with 27,350 deaths expected (2). Worldwide, the greatest mortality rate is found among men of African descent in countries, such as the Bahamas, Trinidad, Barbados, and Tobago and least in Asian countries- Japan, South Korea and Philippines (3).

Prostate cancer incidence rates vary both nationwide and worldwide as do mortality rates. The greatest incidence rate in the world is found among African-Americans, followed by Caucasian-Americans; rates are much less in Central America and other South American countries (4). There has been an increase in the incidence rates around the world up to the early 1990s. Between the late 1970s and early 1980s, the incidence rate was elevated in the western countries, and this is attributed to early detection techniques (5). Also, a sudden increase was noted between 1986 and 1992 as a result of increased use of prostate specific antigen (PSA) as a screening tool (6). In the United States, the incidence rate of prostate cancer, which was reduced in the mid-1990s, has gradually risen recently (7). Similarly, prostate cancer incident rates in Asian countries have increased as a result of increased westernization (8).

The incidence rate of prostate cancer is expected to increase as the population ages in the United States. The number of people aged 80 and above, is expected to increase by 135% by the year 2020 (9). The predictable rise in prostate cancer cases will make the disease a major public health crisis. Prostate cancer etiology remains unknown despite the incidence and mortality rates. Some of the implicated risk factors include increasing age, diet, race, family history of prostate cancer, hormones (e.g. androgen) and the insulin-like growth factor (IGF) axis.
Prostate Cancer and Age

Several studies have demonstrated that the incidence of prostate cancer increases with age. More than 80% of prostate cancer in developing countries is diagnosed in men over 65 years old (10). Estimates from the 1996-2000 Surveillance, Epidemiology, and End Results (SEER) Program, show that prostate cancer incidence rates for men who are 65 years and older was 974.7 per 100,000 person-years compared to those who are 65 years old or less with an incidence rate of 56.8 (7). Although the incidence of prostate cancer increases with advancing age, the presence of a molecular relationship between aging and prostate cancer remains unclear. One of the proposed mechanisms is the methylation of estrogen receptor alpha (ESR1). Li and colleagues (11) evaluated the age-dependent methylation of ESR1 in 83 prostate cancer samples from patients aged 49-77 years using the bisulfite genomic sequencing technique. Findings revealed ESR1 promoter methylation in 54 out of 83 prostate samples. There was a remarkable increase in methylation rate from 50.0% in patients aged 60 years and below to 89.7% for patients aged 70 years and above. These findings may provide a possible relationship between aging and prostate cancer.

Another possible mechanism is the role of cyclooxygenase (COX)-2 expression and the concomitant change in prostaglandin E2 (PGE2). The role of COX-2 in aging prostate has not been elucidated, but there is evidence that COX-2 can be associated with, or involved in the aging process. A possible mode of action of COX-2 is by increasing cell resistance to apoptotic activities. This is supported by a study (12) that examined the age-associated changes in the expression pattern of COX-2 and related apoptotic markers in the cancer susceptible area of the prostate of 4- to 100-week old rats. Increase in COX-2 expression (accompanied by up-regulation of PGE2) was linked to a decrease in the pro-apoptotic signaling in the prostate gland.
during aging. This evidence thus points to age as a risk factor in the development of prostate cancer.

Prostate Cancer and Diet

Nutrition is one of the more investigated areas in regard to prostate cancer etiology and prevention. Fat consumption is the most investigated risk factor in association with diet in prostate cancer development. A high fat diet has been linked to prostate cancer, probably through an influence on hormones. The effect of a low-fat diet (<10% of calories) and exercise on sex hormone-binding globulin (SHBG), which binds androgen, was observed in one study (13); insulin, which in part regulates this process, was also examined. Results from the 27 obese men who underwent the three-week diet-and-exercise program showed a decrease in insulin and lipid levels while increasing SHBG. More testosterone (androgenic hormone) would be bound as a result of the increase in SHBG. Thus, androgen, a risk factor of prostate cancer, is less available to bind to prostate receptors. It was suggested that the decrease in insulin might also reduce the mitogenic actions in the prostate. Therefore, a low fat diet and exercise may be protective against prostate cancer as a result of SHBG increase and lowering of insulin levels derived from this regimen.

Many epidemiologic studies (14,15) have examined the role of total, animal and/or saturated fats. Results from these studies, although inconsistent, suggest a possible inverse association with omega-3 fat and a positive association with animal, saturated and monounsaturated fats; findings for polyunsaturated fats are less consistent. Meat intake, especially processed meat and red meat, has also been linked consistently to elevated risk of prostate cancer (16).
Fruit and vegetable intake is associated with a reduced risk of several cancers, although their influence in prostate cancer is not well understood. Current research investigates the advantages of phytochemicals and bioactive components from fruits and vegetables on health. Lycopene found in tomatoes, for example, has been consistently found to reduce the risk of prostate cancer (17). It was suggested that lycopene might act through mechanisms that could potentially reduce normal and cancerous prostate cell growth, improve oxidative stress defense and reduce DNA damage. Other proposed mechanisms relating lycopene to decreased prostate cancer include inhibition of androgen signaling, prostatic insulin-like growth factor (IGF)-1 signaling, and interleukin (IL)-6 expression. Lycopene may also possess the ability to improve gap junctional communication and initiate phase II drug metabolizing enzymes (18). Besides lycopene, isothiocyanates from cruciferous vegetables, such as cabbage, brussels sprouts, broccoli, and cauliflower, has also been shown to be inversely associated with risk of prostate cancer (19). In a case-controlled study (20), the intake of allium vegetables such as chives, garlic and onions, was associated with reduced risk of prostate cancer. The inhibitory activities of organosulfur compounds present in allium vegetables were implicated in this protective role against prostate cancer. Increased consumption of fruits and vegetables may have a beneficial role in reducing the risk of prostate cancer.

Prostate cancer has been linked to dietary calcium from consumption of either dairy foods or nutritional supplements. Calcium regulates the production of vitamin D, and as a result, may down-regulate the antiproliferative effect of vitamin D on prostate cancer (4). A recent prospective cohort study (21) of United States men, suggests that an intake of over 2000 mg of calcium per day may be associated with prostate cancer, thereby, showing a potential risk of high calcium intake in the development of prostate cancer. The epidemiological evidence of a role for
calcium is not well understood, and is complicated by the difficulty in measuring widely varying amounts of intake and differences in assessment of dietary calcium versus circulating level (22).

Selenium and vitamin E supplementations are currently being investigated widely in the chemoprevention of prostate cancer. Molecular data indicate that selenium may prevent spread of tumors by inducing cell cycle arrest, modulating p53 dependent DNA repair mechanisms, and increasing apoptosis (23). Analyses of clinical trial data obtained from a postintervention follow-up study of prostate cancer (and lung cancer) incidence and cause-specific mortality (24), indicate that vitamin E supplementation may help reduce prostate cancer risk. A large-scale study called Selenium and Vitamin E Chemoprevention Trial (SELECT) is under way to examine and define the role of selenium and vitamin E in the prevention of prostate cancer (23). Final data will be available in the year 2013.

Prostate Cancer and Androgen

The role of androgen has been examined extensively in the pathogenesis of prostate cancer. Most developing prostate tumors are androgen dependent, and this is supported by the notion that castrated men are not afflicted with the disease. Many laboratory studies show that androgens promote cell proliferation and prevent prostate cell death both in vivo and in vitro (25,26). Furthermore, the administration of testosterone to rats has been shown to induce prostate cancer in several studies (27). It is suggested that androgen may influence proliferation and differentiation of prostatic stromal cells by regulating the expression of transforming growth factor beta1 and basic fibroblast growth factor. On the other hand, findings from epidemiological data that support the role of androgens were found to be inconclusive since only one of seventeen prospective studies observed a significantly greater risk of prostate cancer among men with greater serum testosterone levels (4).
The IGF system

Several epidemiological studies, although inconsistent, suggest that increased risk of several cancers including prostate cancer may be related to the insulin-like growth factors (IGF) system. The IGF system includes two ligands, insulin-like growth factor-1 and -2 (IGF-1 and IGF-2), two receptors (the IGF-1 receptor [IGF-1R], and the IGF-2 receptor [IGF-2R] with cross-reactivity with the insulin receptor [IR], and six insulin-like growth factor binding proteins (IGFBP-1 to IGFBP-6). The IGF-1 and -2 were first known as “sulfation factors” because of their ability to stimulate sulfation or incorporation of sulfur-containing amino acids into proteins, an important step in cartilage and bone synthesis; they were later named “somatomedins” because of their role in mediating somatic growth during pre- and postnatal life. IGF effects are not separated from growth hormone (GH) and are exerted at the hypothalamic-pituitary axis. As the name implies, IGF and insulin share significant structural homology and biological activities. In addition to acting through the same receptor, IGF exhibit insulin-like growth promoting actions, as well as metabolic activities (28).

Similar to other traditional hormones, IGF are transported in the blood to have biological effects on cells at distant sites. However, several IGF also have autocrine and paracrine functions (i.e., they are produced and have their biological effects locally). IGF affect almost every tissue and organ system in the body. For example, protein synthesis, cell differentiation, mitosis, and apoptosis have all been attributed to the influence of the IGF protein and their receptors (29).

IGF-1

The human IGF-1 (formerly named somatomedin C) is a 70-amino acid residue single-chain polypeptide with structure similar to proinsulin. Circulating IGF-1 is produced in and released from the liver; it serves autocrine and paracrine functions, and is synthesized by most
body tissues (28,30). IGF-1 biosynthesis is regulated by factors, such as growth hormone, nutritional status and insulin (29-31). IGF-1 plays a fundamental role in growth and development. The deletion of the IGF-1 gene in mice led to prenatal and postnatal growth retardation during the peri-pubertal growth spurt (32-34), but certain soft tissue growth is not affected in these animals (34,35), thus showing that IGF-1 plays a role especially in musculoskeletal growth. Several studies show that tissue-specific promoter-driven expression of IGF-1 transgenes may initiate the growth and development of several tissues, including thyroid gland, bone, and skeletal muscle (36-38). The increase in serum IGF-1 could, therefore, become a potential risk for cancer development. Several prospective studies (39,40) show that circulating levels of IGF-1 and IGFBP-3 may be risk factors in the development of prostate cancer, and the relationship of IGF-1 in prostate cancer is stronger in the advanced stage of the disease (40).

*IGF-2*

The human IGF-2 is a 67-amino acid residue single-chain polypeptide. Its structure is similar to that of IGF-1 and insulin. IGF-2 levels, in contrast to IGF-1, are very high in prenatal life (41). The role of IGF-2 has been less studied than IGF-1. Like IGF-1, IGF-2 plays a critical role in growth and development. The role in growth and development was noted when the IGF-2 gene was ablated in mice (33). Proportionate growth retardation from embryonic day 11 onward was observed and was attributed to a decrease in IGF-2 expression and circulating levels after birth. Several preliminary studies of IGF-2 expression have also been reported.

IGF-2 expression in cultured cells is regulated by factors, such as adrenocorticotropic hormone (ACTH) and cAMP in fetal adrenal cells, glucose in pancreatic beta cell lines and glucocorticoid and thyroid hormones in hepatic cells (42). The autocrine/paracrine role of IGF-2 can be seen in skeletal muscle myoblast differentiation *in vitro* (43). It has been reported that
patients with certain types of tumors release “big IGF-2”, a larger precursor form with an extension of 21 amino acid residues (E-peptide). Big IGF may interrupt the normal actions of the IGFBP on neutralizing circulating IGF, resulting in hypoglycemia and thus enabling big IGF-2 to bind with insulin receptors (44). IGF-2 expression observed in cell cultures may play a role in the pathogenesis of several cancers including prostate.

IGFBP

Thus far, six insulin-like growth factor binding proteins (IGFBP-1 to -6) have been identified and described. The IGFBP have high affinity for the IGF, are expressed at tissue levels locally, and are present in the circulation. Although they serve as “transport proteins” for IGF in circulation, they also act as modulators of IGF activity at the local level (45). In the circulatory system, the majority of IGF is attached to a 150 kDa complex that includes IGFBP-3 and an acid-labile subunit (ALS), which protects the IGF from being degraded and extends their half-life in circulation. Other IGFBP are associated with a 50-kDa complex (46) and may also act as carrier protein in transferring the IGF to their target cells.

The IGFBP play various roles at the cellular level. Some IGFBP modulate the effects of IGF, while others act independent of IGF and IGF-1R (47). The IGFBP prevent the IGF from binding to the IGF-1R, thereby inhibiting the mitogenic effect of IGF-1. However, altering the binding affinities of IGFBP can enhance the interaction of IGF with the IGF-1R. Some modifications that are capable of interrupting the binding affinities of IGFBP include adherence to cell surface or extracellular matrix, phosphorylation, and partial proteolysis. Adherence of IGFBP-3 to cell surface, for example, decreases its affinity for IGF; similarly, dephosphorylation of IGFBP-1 reduces its affinity for IGF (48).
Although all the IGFBP have a transport function, several have distinct, unrelated functions. IGF-independent actions of IGFBP-3 have been reported. It was suggested that the action of IGFBP-3 in inhibition of cellular proliferation through the type V TGF-beta was completely independent of IGF (49). The proapoptotic action of IGFBP-3, independent of IGF or p53, has been reported (50). Also, some potential IGF-independent survival effects of IGFBP-4 and IGFBP-5 have been studied, although the mode of actions of these effects is yet to be elucidated. The IGF-independent actions of IGFBP-2 have also been examined and reported. The role it plays especially in the pathogenesis of disease is especially noteworthy and will be discussed later.

As mentioned previously, factors, such as nutrition, pathological states and normal development, have tremendous influence on the effects of IGF and IGFBP. In order to understand the role of IGF in normal growth and development and in some pathological states such as cancer, it is crucially important to understand the interplay between nutrition and the IGF system.

Nutrition and the IGF system

Nutrition is one of the major regulators of IGF. For example, the mechanism by which nutrient deprivation, decreases protein synthesis and growth involves the IGF protein. Studies show that starvation causes a decline in body cell mass and growth arrest, and the mechanism by which this is brought about is not understood. Since IGF-1 has stimulatory effects on cell growth and differentiation, as well as anabolic effects on protein metabolism, its decline in the states of undernutrition observed in humans with catabolic conditions in one study (51) was suggested to be a contributing factor to the observed loss of cell mass and growth arrest. Similarly, Clemmons and colleagues (52) reported that fasting in normal individuals resulted in IGF-1 decline, and the
altered IGF-1 level was found to parallel changes in nitrogen balance. It was suggested that an increase in protein breakdown or decrease in protein synthesis might be mediated by the decline in IGF-1. Conditions, such as kwashiorkor, marasmus, and anorexia nervosa could also result in decreased serum IGF-1 (53). This evidence indicates that changes in the concentration of IGF-1 in nutritional status may have a profound effect on cell mass. The IGF-1 has now been recognized to serve as a marker for nutrient intake adequacy; many researchers have concluded that serum IGF-1 is a very useful index of nutritional status (54,55).

Micronutrients such as zinc, also regulates IGF-1 gene expression and circulating IGF-1 levels, and eventually affects the role of IGF in growth and development. Studies (56,57) show that serum growth hormone and serum IGF-1 levels were decreased as a result of zinc deficiency in rats. These findings suggested that the inhibition of growth as a result of zinc deficiency could be explained by the impaired GH/IGF-activity.

Role of IGF in Cancer

The IGF play important roles in tissue growth and have been reported to be potent mitogens for many different cancer cell lines. Both IGF-1 and IGF-2 are expressed in several tumors, and IGF-2 especially has been shown to be overexpressed in different tumors including, liver, colon and many pediatric cancers (58-60). The increase in expression of these growth factors is said to be related to more aggressive tumors (60). Some mechanisms underlying IGF-1-cancer associations that have been postulated include mitogenic effects and apoptosis.

Mitogenic effects

Extensive data from in vivo and in vitro studies suggest that the mitogenic actions of IGF-1 increase epithelial cell numbers. Findings from a prospective human study suggest that the presence of high IGF-1 or IGF-1/IGFBP-3 would advance any neoplastic lesion very quickly to
clinical presentation (61). The most consistent and strongest associations with mitogenic effects of IGF-1 have been reported for prostate, colon and breast cancer cells (61-63).

*Apoptosis*

Another mechanism that could explain the association between IGF-1 and cancer is apoptosis. Apoptosis is the most important defense against the cancerous cell—the natural mechanism used in the removal of damaged, infected, or inappropriately growing cells from the body. Studies have shown that IGF-1 is a very potent factor for cell survival. IGFBP-3 on the other hand promotes apoptosis, as well as controlling the availability of IGF-1 (64). This points to the importance in the balance between IGF-1 and IGFBP-3, and this balance may regulate the activation of apoptotic activity. Increasing the ratio of IGFBP-3 to IGF-1, as suggested by one study (65), would increase apoptotic activity of cells, thereby reducing cancer risk; whereas, increasing the ratio of IGF-1 to IGFBP-3 would increase cell survival thus increasing the risk of cancer. Therefore, the anti-apoptotic action and mitogenic effects of the IGF-1 can affect the proliferation of abnormal cells, such as in prostate cancer.

**Prostate Cancer and the IGF system**

Several epidemiological studies have examined the roles of IGF and their binding proteins in prostate cancer etiology and have found a positive association between IGF-1 and IGFBP-3 in prostate cancer risk. Some prospective studies suggested that elevated levels of circulating IGF-1 were associated with an increase in prostate cancer risk, and the association was greater in the advanced stages of the disease (39,66). Data from experimental studies (67-69) suggested that prostatic stromal cells and epithelial cells in culture secrete IGFBP, while stromal cells produce IGF-2; both epithelial and stromal cells are responsive to IGF-1 respecting proliferation. An *in vivo* study (70) reported that systemic administration of circulating IGF-1 in
rats led to an increase in prostate growth, thereby supporting the role of IGF activity in prostate growth.

In regard to IGF-2, which is less researched compared to IGF-1, one study investigated its relationship to prostate cancer and found that serum levels of IGF-2 may help inhibit the development of prostate cancer (71). In contrast, Gnanapragasam and coworkers (72) reported that IGF-2 increases the expression of androgen receptors, which is likely to result in prostatic tissue growth. The role of IGFBP-2 in prostate cancer development has recently become one of the focused areas in research. It has been suggested that IGFBP-2 may have both stimulatory and inhibitory roles in prostate cancer cells.

IGFBP-2 and Prostate cancer

IGFBP-2 is the second most abundant IGFBP in the serum, and found in different body fluids and tissues; it inhibits growth by binding and sequestering free IGF, thus decreasing their bioavailability (73,74). Using IGFBP-2 assay levels in experimental and clinical cases, Blum and colleagues (75) suggested that free IGF-2 could be a major regulator of IGFBP-2 levels in circulation since these levels were reportedly elevated in cases where IGF-2 levels were expected to be high. Other factors influencing the regulation of IGFBP-2 include IGF-1, growth hormone (30), estradiol (76), and insulin (77).

Levels of IGFBP-2 are also influenced by other factors, such as disease state. Several epidemiologic studies have reported elevated IGFBP-2 levels in patients with cancers of the lung, colon and central nervous system (78-80). Additionally, IGFBP-2 is secreted and expressed in cancer cell lines obtained from prostate, colon, breast, lung (81-84) and others. IGFBP-2 appears to inhibit IGF activities, especially those of IGF-2, and has the ability to stimulate IGF-1 actions in certain specific cell types (85).
Inhibitory effects of IGFBP-2

An increase in the level of IGFBP-2 has been shown to inhibit cell growth. In a study using human embryonic kidney fibroblast and IGF-responsive colon cancer cell lines, proliferation of these cells were inhibited by conditioned media of IGFBP-2 secreting clones (86). It was suggested that IGFBP-2 might have sequestered the IGF, and thereby prevent mitogenic signals from being transferred. Reeve and coworkers (87) found that soluble IGFBP-2 administered to small cell lung cancer cells, resulted in the inhibition of IGF-dependent DNA synthesis, and IGF were found linked solely to IGFBP-2 rather than the receptors (IGF-1R and IGF-2R), that were in the culture as well. In view of these findings, it was concluded that both membrane-associated and soluble IGFBP-2 prevented the IGF from binding to their receptors, and as a result, the activities of IGF were inhibited.

Stimulatory effects of IGFBP-2

Conversely, several studies have found positive association between cell proliferation and IGFBP-2 levels. In human colon cancer cells (Caco-2), IGFBP-2 levels were correlated to cell proliferation (88). Similar findings were noted in other studies that used human breast cancer cells (89) and bovine kidney cells (90). Of relevance to this research, is a study (91) that examined a model of malignant transformation using prostate cells with different degrees of malignancy, which includes benign prostate cells, P69 cells, two sublines of P69 cells, and SV40 large T antigen- prostate epithelial cells (immortalized). Results show that IGFBP-2 was the predominant IGFBP in the malignant cells as the cancer progressed. Notable, also, is the fact that the cells increasingly became independent of IGF-1 as the malignancy increased. This suggests that IGFBP-2 may be capable of exerting its action(s) independent of the IGF. Findings from the above study have prompted a closer inspection of the role of IGFBP-2 in prostate cancer.
IGFBP-2 is considered the main IGFBP in the prostate. This is supported by the findings that IGFBP-2 was the major IGFBP secreted in both normal and cancerous prostate tumor cells in culture (67,68). Also, IGFBP-2 levels of prostate cancer patients have been shown to correlate with prostatic specific antigen (PSA) levels (92,93). Likewise, greater tissue levels of IGFBP-2 are observed in end-stage hormone refractory prostate cancer but less in primary prostate cancer or benign prostate glands (94). This suggests that the increase in IGFBP-2 levels observed in prostate cancer cells may be a result of androgen deprivation.

Androgen withdrawal has become one of the primary therapies for patients with advanced stage prostate cancer. However the effect of androgen on IGFBP-2 levels in prostate cancer patients is a very confusing matter as many conflicting findings have been reported. One study (1) findings showed greater levels of IGFBP-2 secreted by LNCaP cells that were androgen deprived. Similar findings were reported in rat models of prostate cancer (95). In another experiment (96), androgen treated prostate cancer cells likewise showed increased expression of IGFBP-2 levels. In a recent retrospective study (74), patients who were treated with androgen deprivation therapy (ADT) for three months had significantly increased IGFBP-2 levels compared to patients without the treatment. Surprisingly, the greater levels of IGFBP-2 were associated with better prognosis. Another interesting result noted in the study is that the growth inhibitory properties of IGFBP-2 were maintained in the normal prostatic epithelial control cells examined throughout the study. It was concluded that a switch must have occurred in the role of IGFBP-2 in the transition from normal prostate cells to the malignancy that led to a change in IGFBP-2 from a growth inhibitor into a growth stimulator. It was suggested that androgen might directly affect gene regulation of IGFBP-2 through unknown pathway(s). Along the same line of thought, other studies (1,97) suggest that growth inhibitory effect of IGFBP-2
observed in normal prostate cell growth and stimulatory effects in the abnormal cell growth “assumes” an androgen-independent phenotype. It was further suggested that since IGFBP-2, which is IGF-independent, causes cell proliferation, there is a possibility that the mechanism could be mediated through the androgen receptor system.

Thus far, a large amount of evidence suggests that IGFBP-2 is a tumorgenic factor which may play an important role in the aggressive proliferation of prostate cancer cells, although the exact mechanisms remain unclear. It has been suggested that inhibiting IGFBP-2 secretion may offer one strategy to delay progression of prostate cancer after androgen withdrawal (1). This may be accomplished by using a non-toxic agent, such as ellagic acid.

Ellagic Acid

Ellagic acid (EA), 2, 3, 7, 8-tetrahydroxy [1] benzopyranol[5,4,3-c,d,e][1]benzopyran-5,10-dione, is a natural plant phenol and a product of hydrolyzed plant tannins (98). It can be found in several ellagitannins present in varieties of edible fruits and nuts. The quantity of EA has been determined in various fruits and nuts with the greatest amount obtained from strawberries, raspberries, blackberries, cranberries, walnuts, and pecans (98,99). EA is found as ellagic hydrolyzed tannin, called ellagic tannins, in flowering plants, such as *Fagaria xanassa* Duch., *Rubus L Rubus species*, *Punica Granatum*. Ellagic tannin is a glucose ester which contains hexahydroxydipenic acid that releases ellagic acid as active compound when hydrolyzed (100). The chemical structure of EA is shown in figure 1.
Ellagic acid is a weak acid in nature. It is a very stable compound with a high melting point above 360 degrees Celsius. When recrystallized from pyridine, it produces a cream-colored powder. It is soluble in aqueous alkaline and somewhat in pyridine. Because of its planar centosymmetrical structure, it is not soluble in water and organic solvents. EA can react with organic compounds such as pyridine, to form complexes. It can also produce metal complexes with sodium, calcium, magnesium, copper and iron (102,103).

The biological activities of EA were not recognized until in 1968 when Bhargava and Westfall (104) demonstrated that the use of EA may result in the regression of mammary tumors in mice. Further studies on inhibitory effects of EA, to be discussed later, followed thereafter.

Ellagic Acid Metabolism

The absorption and distribution of EA in different tissues have been studied although consistency of results is yet to be achieved. Poor absorption of EA has been reported in several in vivo studies involving animals. Pharmacokinetics of EA was examined in one study (105) after oral administration of EA (from pomegranate leaf extract) in rats. Approximately an hour after oral administration of extract at a dose of 0.8g/kg, maximum concentration of 213 ng/ml of EA
(≈ 0.6 uM) was assayed in rat plasma after 0.55 h. Although part of EA was absorbed in the stomach, overall, the study concluded that ellagic acid had poor absorption and rapid elimination time. Using male Swiss-Webster mice, another study (106) showed that EA was poorly absorbed into blood, tissues and bile, and almost the entire absorbed dose was found in excreted urine. The bioavailability of EA has also been examined in human studies. Seeram and colleagues (107) were the first to report the bioavailability of EA in the human body. After an hour post consumption of 180 ml of pomegranate juice (containing 25mg EA) by human subjects, a maximum concentration of 31.9 ng/ml of EA (about 0.1 uM) was detected although this amount was excreted by four hours.

A year later, another study (108) examined the absorption of EA by the human gut in 12 healthy adults aged 20 to 60 years. Blood sample analysis revealed that approximately 25% of EA present in the administered raspberry puree was absorbed and excreted without being modified in the urine during the study duration (24 hours). Higher levels of EA were noted in some of the 24-hour urinary output samples, but this was attributed to conversion of tannins to EA in the small intestine or stomach. An elimination half-life of 8.57 hours was achieved even though oral bioavailability of EA from a single dose of raspberries was low. The results suggest that EA is not quickly excreted or metabolized when it is in the blood stream. Protein-binding capacity of ellagic acid (297.3 microgram bovine serum albumin per milligram) reported in one study (109) shows its ability to bind and precipitate proteins in the circulation.

Other evidence demonstrates that the transcellular absorption of EA in human intestinal cells (Caco-2 cells) in culture shows a greater accumulation of EA in the cells; 93% of EA was bound to protein, and as much as five times this amount was bound to cellular DNA (110). It was concluded that EA preferably accumulates in cancerous (intestinal epithelial) cells where its
cancer preventive effects may be manifested. Similarly, an \textit{in vivo} study (111) demonstrated that EA localized with preference in the lung tissues of A/J mice with induced lung tumors. These findings confirm that EA can be absorbed into the cells and inhibit tumorigenesis.

\textit{Proposed mechanism of EA activities and chemoprevention}

Epidemiologic studies demonstrated a correlation between consumption of diets rich in fruits and vegetables and risk of developing cancer (112). Components of these fruits and vegetables have been examined and tested for their effectiveness in inhibiting cancer formation; these include phenols (ellagic acid), coumarines, selenium, aromatic isothiacyanates, retinoids, sterols, ascorbic acid, and others.

Ellagic acid is referred to as a chemopreventive agent because of its significance in antitumor and antiproliferative activities. It is also effective in antimutagenesis assays, and has been demonstrated to inhibit chemically induced cancer in organs, such as the liver, skin, esophagus and lung of rodents (113-115). Chemopreventive agents that have been studied thus far can be classified into: compounds that are capable of preventing the formation of cancers (116), such as phenols, tocopherols, and ascorbic acid; compounds that stop cancer causing agents from reaching the target tissue (117,118), also known as “blocking agents”, e.g. phenols (including ellagic acid), flavones, coumarines and so on; and compounds that exert their effects on cells that have been exposed to carcinogens (suppressing agents), and suppress the formation of neoplasia, e.g., selenium salts, phenols, carotenoids, retinoids; and others (119,120).

Several studies have proposed possible mechanisms of inhibitors that act as “blocking agents”, such as EA. The ability to stop the activation of cancer precursors from being converted to their carcinogenic forms, and increasing and inducing the activity of enzymes whose main function is to detoxify cancerous substances (121) is one of the possible modes of action of EA.
Another postulated mechanism is the ability of EA to protect DNA bases from electrophillic attack. Several studies have been conducted regarding possible mechanism(s) of EA in inhibiting DNA-adduct formation and/or mutagenicity against specific type of carcinogenic compounds.

_Inhibitory effects of EA against polycyclic aromatic hydrocarbons_

Wood and colleagues (122) showed the ability of EA to inhibit the mutagenicity of benzo(a)pyrene-7,8-dihydrodiol-9-10-epoxide (BPDE) in *Salmonella typhimurium* and in Chinese hamster V-79 cells. Findings indicate that 3 nmol of EA inhibited the mutation caused by the diol epoxide by 90%. The binding of BPDE to calf thymus DNA was significantly reduced by 90% by EA in a different study (123). Using strain A mouse lung explants pretreated with EA, the effect of EA was clearly seen in the reduction of benzo[a]pyrene metabolism and binding, and adduct formation of benzo[a]pyrene and benzo[a]pyrene-7,8-dihydrodiol (115). Another study (124) suggested that structural capability of EA may be responsible for rendering BPDE incapable of binding to DNA. Therefore, EA may act as an inhibitory agent.

_Inhibitory effects of EA against N-nitroso Compounds_

N-nitrosamines, such as N-nitrosobenzylmethlyamine (NBMA), N, N-dimethyl nitrosamine (DMN), and N-methly N-nitrosourea, are examples of N-nitroso compounds that have been investigated in several *in vitro* studies of EA activities. Using strain TA of *Salmonella typhimurium*, Dixit and Gold (125) demonstrated that EA inhibits the mutagenicity of DMN. Another study (126) showed that EA treatment in cultured rat esophageal explants led to a 40% inhibition of DNA methylation, which was induced by MBNA. An *in vivo* study investigated the effect of dietary EA on MBNA-induced esophageal cancer in rats; findings show a 60% decline in tumor dividing cells after a EA diet for 25 weeks (127). Similarly, EA inhibited the stages of initiation and promotion/progression of carcinogenesis in N MBA-induced esophageal
tumorigenesis in F344 rats (128). Mechanisms in which EA exerts its inhibitory effects are not clearly known, but some studies suggest that the binding of EA to the DNA of cultured rat tissues and to the DNA of calf thymus (114,129,130) are possible indications that EA may bind to the active site(s) of DNA and protect the site that would have been occupied by (or form adduct with) the carcinogen or mutagen.

**Inhibitory effects of EA against Aflatoxin B1 (AFB-1)**

Aflatoxin has the capability to bind to DNA and form adducts. Mutagenesis and heptocellular neoplasm induced by AFB-1 in rats was significantly reduced after dietary administration of EA (131). Also, EA was shown to inhibit the mutagenesis of Aflatoxin B1 in cultured rat tracheobronchial explants by up to 50%, and microsuspension assays of *Samonella* tester strains TA98 and TA100 (132,133).

**Inhibitory effects of EA against 4-nitroquinoline-1-oxide and N-2-fluorenylacetamide**

*In vivo* studies (134,135) show the inhibitory effects of dietary EA on tongue and liver cancers induced by 4-nitroquinoline-1-oxide and N-2-fluorenylacetamide, respectively in rats. Findings indicate that the rate of neoplasm formation in the liver and tongue were significantly reduced when administered together with the carcinogens.

**The effects of EA on Human cancer cells**

The experimental studies considered thus far have presented promising results respecting the effects of EA on induced carcinogenesis in different organs in animals. It would be worthwhile to examine whether the same positive effects would be realized in studies using human cancer cells. EA activities are not limited to animal tissues and cells. Several studies have clearly demonstrated that the chemopreventive effects of EA could be extended to human cancer cells.
In cultured human bronchus explants, DNA and benzo[a]pyrene binding was inhibited by EA by 26 to 77% (136). Another study (137) demonstrated that EA moderately inhibited DNA adduction induced by mammary carcinogen dibenzo[a]pyrene (DBP) in the human breast cells MCF-7 (138). Additionally, EA inhibitory activity was demonstrated in the human bladder tumor cell lines (T24 and TSGH 8301), and results show that N-acetyltransferase (NAT) activity in the cells was inhibited by EA.

Other activities of EA on human cancer cells include antiproliferative, apoptotic, antioxidant, antiatherosclerotic, and anti-inflammatory. Premalignant lesions often become invasive and metastatic; one way to control this is by preventing the spread of these cells, and/or inducing cell death (apoptosis). This has been shown in several studies. Changes in gene expression pattern of LNCaP cells exposed to EA ($10^{-5}$ M) in combination with resveratrol, a phenolic compound, for 48 hours suggest that several signaling pathways that leads to growth inhibition of LNCaP cells may be involved (139). Treating cultured colon cancer cells (SW 480) with $10^{-5}$ M EA for 48 hours, resulted in a decrease in cell proliferation and apoptotic activity (140). Low concentration of EA (5 micro mol/L), in combination with quercetin in the MOLT-4 human leukemia cell line, caused reduced proliferation and induced apoptotic cell death (141). Additionally, the effect of cell growth inhibition and apoptosis of EA at $10^{-5}$ M was noted in cervical carcinoma (CaSki) cells after 72 hours of treatment (142). The above study findings thus show that EA may be involved in cell cycle regulation and apoptosis. How the activities of EA are regulated on cancer cells remains unclear and open to debate; some research studies have examined and are currently examining the effects of EA on the IGF system.
**Effects of EA on the IGF System**

Evidence points to IGF and IGFBP as potential risk factors for the development and/or progression of prostate cancer. As suggested by many excellent studies, ellagic acid, may act as an anti-cancer agent. However, very few studies have examined activities of EA on the IGF system in the pathogenesis of prostate cancer. Narayanan and colleagues (140) recently demonstrated that EA downregulates IGF-2 in colon cancer cells, possibly via TGF-beta, p53 and p21 pathways. It was suggested that modulating growth factors such as IGF-2 and their binding proteins may induce cell cycle arrest.

Thus far, only one study (139) has examined the influence of ellagic acid in combination with resveratrol on LNCaP cells but focused on the changes of gene expressions of the cells; data from the study suggested the activation of multiple signaling pathways that lead to growth inhibition of LNCaP cells. None, however, has reported the effects of EA on human prostate cancer (LNCaP) cell in culture. Also, the role of EA on IGFBP-2 secretion in LNCaP cells has not been examined. In this research, study aim is to investigate the antiproliferative effect of EA in human prostate cancer cell line, as well as its effect on secreted IGFBP-2 concentrations.
CHAPTER III. MATERIALS AND METHODS

Cell Culture

LNCaP cells (human androgen-dependent prostate cancer cells) were purchased from American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 medium with phenol red, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% HEPES buffer (1M), 1% non-essential amino acids, 1% sodium pyruvate, 1% glutamine, and 1% streptomycin and penicillin. The cultures were maintained in standard conditions in a humidified incubator (37° C, 5% CO2), collected using 0.25% trypsin-EDTA (Sigma-Aldrich Corporation, St. Louis, MO), and passaged routinely at 80% confluence.

Cell Growth, Viability, and EA Treatments

Cell proliferation assays were designed to test the effect of various compounds on the growth of LNCaP cells. Ellagic acid (Sigma-Aldrich Corporation, St. Louis, MO) dissolved in dimethyl sulfoxide (DMSO) was further diluted in culture media and applied to cells in final concentrations of 0.03 to 100 uM. All ellagic acid solutions were protected from light during preparation and storage. When applying ellagic acid treatments to cells, UV lights were turned off. Control (cells grown in medium only), vehicle (cells grown in medium plus 0.016% DMSO, equivalent to the amount of DMSO in the 10 uM EA treatment) and medium with 0 to 100 uM ellagic acid were tested. Cells were maintained at 37° C, 5% CO2 for 1 to 3 days.

The effects of ellagic acid on cell proliferation were measured by cell count of LNCaP cells exposed to EA for 3 days. Cells in 100 mm dishes grown to 50% confluence in regular medium were exposed to ellagic acid (0.1 to 100 uM) or DMSO vehicle for 3 days. Culture medium was aspirated, and cells were collected with 0.25% trypsin-EDTA and counted with a hemocytometer using a light microscope. Cell counts were standardized to cells grown in
complete media (100%). Each assay was done in duplicate, and results from three to five separate experiments were analyzed.

Cell viability, an additional measure of cell growth that estimates mitochondrial activity of viable cells, was determined in cells grown in 96-well plates. LNCaP cells at 5 x 10³ cells/well plus ellagic acid treatments or DMSO vehicle in media were plated in 96-well tissue culture plates in a total volume of 100 uL and grown under standard conditions for up to 3 days. Since ellagic acid influences spectrophotometric readings, parallel plates prepared with equivalent concentrations of EA, without cells added, were exposed to similar conditions and used for background correction. Cell viability was evaluated with the MTS assay (3-(4,5-dimethylthiazol-2-yl-5-(3-carboxymethoxyphenyl)-2-(4-sulfonphenyl)-2H-tetrazolium, inner salt) assay (CellTitre 96-well proliferation assay, Promega, Madison, WI), which estimates mitochondrial activity, following the manufacturer’s instructions. Briefly, 20 uL of MTS reagent was added to the 100 uL of cells and medium in the 96-well plate. After incubation at 37⁰C for 1-4 hours, absorbance was read at OD 490 nm with a microplate reader (BioRad 1680, Hercules, CA) with subtraction of background (medium only or medium plus specific concentrations of EA). Each experiment consisted of four to eight replicates, and data from multiple separate experiments (as indicated in each figure) were included.

The secretion of IGFBP-2 by LNCaP cells was determined in conditioned media. Cells (500,000 per well) were plated in 6-well plates with regular media in a total volume of 2 mL. After 24 h of growth under standard conditions, medium was aspirated and replaced with DMSO or EA treatments. After three days, culture medium was gently aspirated from cells and immediately stored on ice. Cell debris was removed by centrifugation at 1,000 x g for 4 minutes
at 4°C. Protease inhibitor cocktail (Sigma-Aldrich, Inc, St. Louis, MO) was added to the supernatant at 10 uL per mL. Samples were stored at -70°C until assayed for IGFBP2.

IGFBP-2 Determination

An enzyme linked immunosorbent assay (ELISA) development kit (R&D Systems, Minneapolis, MN), containing basic components of a sandwich ELISA, was used to measure human IGFBP-2 in the cell culture supernatants. The ELISA technique is based on the “antibody sandwich principle”, which involves the use of antibodies to detect an analyte or antigen. The manufacturer’s instructions were followed with fresh preparation of buffers and reagent diluents for each assay. All samples were assayed in duplicate or triplicate. A standard curve of five known concentrations of human recombinant IGFBP-2 was generated in each assay. A general outline of the procedure is described below, with more specific description of steps provided in Appendix A.

First, a solid phase was created in which a capture antibody, which is specific to human IGFBP-2, was bound to a microliter plate. The sample or standard was added to the plate, and the immobilized antibody bound any analyte present. The unbound antibody was washed and removed from the plate; a blocking agent was then added to block nonspecific binding of assay reactants to the well surface. The second step involved incubation of the samples, standards, and controls with the solid phase antibody to ensure that the analyte was captured. The unbound analyte was then washed, and a detection antibody was added resulting in the formation of analyte-antibody complex, which completed the sandwich. The plate was washed to remove unbound detection antibody. In the third step, a detection agent was added to the plate to bind the biotin labeled detection antibody and then washed. Finally, a substrate solution was then added producing a blue color proportionate to the amount of bound analyte; addition of an acidic
solution, which turned the solution yellow, terminated the reaction. The optical density of the solution was read at 450 nm with background correction 570 nm using a microtiter plate reader (BioRad 1680 Hercules, CA).

Statistical Analysis

Four to eight replicates within each assay were averaged. Means and standard deviations were calculated using averaged data from three to five independent experiments. Differences among treatment and control groups were tested using analysis of variance (ANOVA). If a significant effect of treatment was detected by ANOVA (p<0.05), Fisher’s protected least significant difference test was subsequently used to evaluate pair wise comparisons between treatments and vehicle. A control (medium only) was used to standardize the cell growth assays across separate experiments. IGFBP-2 data for both vehicle and control groups was provided to demonstrate the effect of the DMSO vehicle.
CHAPTER IV. RESULTS

Ellagic acid (EA) inhibited the growth of LNCaP in a dose-dependent manner. All but one sample (0.1 uM EA) showed significant growth inhibition in response to EA exposure (Fig. 2). The highest concentration (100 uM of EA) suppressed growth by 71 percent and 1 uM EA concentration by 28 percent.

Figure 2. Effect of ellagic acid on the proliferation of LNCaP cells. Cells in 100 mm dishes (50% confluent) were exposed to ellagic acid or 0.016% DMSO vehicle ("0", equivalent to 10 uM EA) for 3 days, collected with 0.25% trypsin-EDTA and counted with a hemocytometer. Cell counts were standardized to cells grown in complete media (100%). Values are means ± SD of 5 separate experiments. * Different than cells grown in 0.016% DMSO (Vehicle), p < 0.05.

EA reduced cell viability in LNCaP cells in a dose-dependent manner (Fig. 3). EA concentrations of 100 uM, 50 uM, and 10 uM decreased mitochondrial activity by 66, 48, and 34 percent, respectively. EA concentrations of 0.1 and 1 uM and vehicle ("0") had no significant effect on cell viability.
Figure 3. Effect of ellagic acid on cell viability of LNCaP cells. Cells (1500 or 5000/well in 96-well plates) were exposed to ellagic acid or 0.016% DMSO vehicle (“0”, equivalent to 10 uM EA) for 3 days. Mitochondrial activity was estimated by the MTS assay (3-(4,5-dimethylthiazol-2-yl-5-(3-carboxymethoxyphenyl)-2-(4-sulfonphenyl)-2H-tetrazolium, inner salt). Cell viability was standardized to cells grown in complete media (100%). Values are means ± SD of 5 separate experiments (4-8 replicates/experiment). * Different than cells grown in 0.016% DMSO, p < 0.05.

A remarkable growth rate decrease (~54 %) by 100 uM EA was observed on day two of treatment; the growth rate reduction was decreased by day three compared to day two (Fig. 4). EA concentrations of 50 uM and 10 uM suppressed the growth rate of LNCaP cells in a similar time frame; a modest reduction (~22%) was observed for both concentrations when compared with the control. The cells treated with EA concentrations lower than 10 uM and the vehicle (“0”) sample showed insignificant decrease in the rate of cell growth.
Figure 4. Cell viability curve of LNCaP cells exposed to EA. Growth curve of LNCaP cells exposed to ellagic acid. Cells (1500 /well in 96-well plates) were treated with ellagic acid or 0.016% DMSO vehicle (“0”, equivalent to 10 uM EA) for 1, 2, or 3 days. Mitochondrial activity was estimated by the MTS assay (3-(4,5-dimethylthiazol-2-yl-5-(3-carboxymethoxyphenyl-2-(4-sulfonphenyl)-2H-tetrazolium, inner salt). Cell viability was standardized to cells grown in complete media (100%). Values are means ± SD of 4 replicates.

IGFBP-2 was secreted by LNCaP cells after three days of cell culture. The IGFBP-2 detected could not be attributed to the media since there was no detectable IGFBP-2 in the regular media prior to the addition of cells (data not shown). IGFBP-2 level was progressively suppressed by increasing concentrations of EA (Fig. 5); the level of IGFBP-2 exposed to 10 uM EA concentration was significantly (p < 0.05) suppressed (by 39%) when compared to the control cells. No significant effect was observed for EA concentrations of 0.1, 1, and 2 uM, and vehicle (“0”).
Figure 5. Effect of EA on IGFBP-2 secreted by LNCaP. IGFBP-2 secreted by LNCaP cells treated with ellagic acid, regular media (Control) or 0.016% DMSO Vehicle (“0”) for 3 days. IGFBP-2 was measured by enzyme-linked immunosorbant assay and expressed as ng/mL per 500,000 cells. Means ± SD of 3 separate experiments (duplicates within each experiment). * Different than control, p < 0.05.

Limitations of the study

The present study was performed in vitro (in cell culture) and can not be directly extended to humans. Pure concentrate of ellagic acid was used and has not been tested in humans. The amount of EA concentration present in prostate tissue has not been measured by any study. It is not known whether the concentration of EA in serum reflects that present in the prostate. In this research, the physiological range of that found in the serum was used. Generalizing that EA inhibit cell proliferation in prostate cancer cells and suppresses IGFBP-2 secreted can not be made since only one cell type, human prostate cancer (LNCaP), was examined.
CHAPTER V. DISCUSSION

Results show that IGFBP-2 is secreted by human prostate cancer (LNCaP) cells. This is consistent with other studies (1,81). Similar results in other human prostatic cancer cell lines, such as DU145, PC-3 cells, human normal and hyperplastic prostatic cells in primary culture have been reported (69,81). Secretion of IGFBP-2 concentrations has been observed in both in vitro (1,143) and in vivo (144,145). It has been suggested that IGFBP-2 may play a role in human prostate cancer progression particularly after the initiation of hormone therapy. Over expression of IGFBP-2 has also been implicated in malignant transformation in prostate epithelium (1,143). The secretion of IGFBP-2 in the culture medium of prostate cancer cells in the present study suggests that locally produced IGFBP-2 may indeed be involved in the pathogenesis of prostate cancer. The exact mechanism of IGFBP-2 has not been elucidated, however, several researchers suggest that IGFBP-2 may be involved in regulating IGF-1/IGF-2 (potent mitogens in normal and malignant cells) action in the prostate (67,143).

On the other hand, it has been suggested that IGF and their binding proteins are not involved in the etiology of prostate cancer. For example, in a case control study (146), the relationship between baseline plasma level of IGFBP-2 and other variables of the IGF axis and prostate cancer risk was assessed in prostate cancer cases over a 9-year follow-up period. Results indicated that IGF axis variables are not long term predictors of the occurrence of prostate cancer. Also, examining changes in binding proteins including IGFBP-2 in serial postoperative serum samples from prostate cancer patients with and without relapse, investigators (145) concluded that serum levels of IGFBP-2 do not have a predictive values in prostate cancer prognosis but pointed that IGFBP-2 may play a role in the progression of prostate cancer. Interestingly, one retrospective study (74) found that increased serum IGFBP-2 levels were
actually associated with better survival in prostate cancer patients who received neoadjuvant androgen deprivation therapy (ADT), but worse in those without the treatment. It was suggested that a dramatic switch must have occurred in the function of IGFBP-2 dependant on the absence or presence of androgens.

It appears that the implication of locally produced IGFBP-2 in the prostate and that in serum may be different with respect to prostate cancer etiology. IGFBP-2 secreted in the prostate may be more reflective of the disease state than that in the serum; further studies may be needed to determine if locally produced IGFBP-2 in the prostate or that in the serum is more reflective of the disease state. Nonetheless, a great amount of evidence (some already considered) shows that IGFBP-2 is involved in, or is affected by the neoplastic process in prostate cancer.

In the present study, data obtained from the cell proliferation and cell viability assays showed cell growth of LNCaP cells in the untreated medium (control). The secreted IGFBP-2 by LNCaP cells may have contributed to the cell growth observed. A similar observation was made in another study (1) in which changes in IGFBP-2 in the LNCaP tumor model after androgen withdrawal was evaluated. Results from the study show a 2-3-fold increase of IGFBP-2 mRNA and protein levels in LNCaP cells in vitro in LNCaP tumors during androgen independent progression in vivo. Similar observations have been reported with LAPC-4 and DU145 prostate cancer cells (97). Additionally, the effect of IGFBP-2 on prostate cancer cell growth was clearly demonstrated by Chatterjee and coworkers (147) using DU145 prostate cancer cell in culture. By exogenously administering IGFBP-2, cell growth was significantly stimulated, but was inhibited by the addition of IGFBP-2 antibody.

In connection with the aim of the study, ellagic acid was used to suppress IGFBP-2 concentration secreted in the culture medium. The data obtained could not be compared directly
with other studies since the present study appears to be the first quantitative report of IGFBP-2 secretion by LNCaP cells. Other studies have demonstrated the qualitative presence of IGFBP-2 in conditioned media by western blot (69,147). No other study has examined the effects of ellagic acid on the synthesis and secretion of IGFBP-2 in prostate cancer cells.

Data from the cell proliferation and cell viability assays and the three-day growth curve show that EA clearly decreased the growth of LNCaP cells in a dose-dependent manner. The present study is the first report of the effects of ellagic acid on LNCaP cells. However, the present findings are supported by observation of the effects of EA on other cancer cell lines. Using EA concentration of 78.5 uM +/- 0.24, another study reported decreased cell viability, cell growth inhibition, and induced cell death in a dose-dependent manner in several malignant cell lines including human prostate (PC-3), human breast cancer (MCF-7), human osteosarcoma (HOS-1) and others (148). Similarly, 5 and 10 umol/L EA in MOLT-4 human leukemia cells resulted in a significant reduction in cell number at 48 h but not 24 h (141). EA concentrations used in the above studies and results obtained are in line with the observations from the present study.

Several studies previously considered clearly demonstrated that EA is capable of inhibiting certain carcinogen-induced cancers by acting as antiproliferative agent in animals and in many human cancer cell lines. However, the exact mode of action of EA is not clearly understood. One study (142) reported that EA within the concentration range of 10^{-9} and 10^{-5} M induced G_0/G_1 arrest within two days, inhibited overall cell growth, induced cell cycle arrest, and decreased DNA synthesis in cervical carcinoma (CaSki) cells in culture after three days of treatment. The major findings from the study indicate that a mode of action of EA in cell proliferation inhibition may be through p53/p21-mediated G1 arrest and cell death. Also, the
inhibition of cell growth by EA in colon cancer (SW 480) and CaSki cells in culture treated with 
10^{-5} M EA for two days in two studies (139,140) suggest that EA may have been mediated by 
signaling pathways that triggers p53, which in turn leads to activation of p21, mediates DNA 
damage, and also disrupts growth factor expression, leading to insulin-like growth factor-2 (IGF-2) down regulation associated cell cyle arrest. The EA concentration of 10^{-6} M used in the 
present study is within the range that has been tested in several studies, and the results obtained 
indicate the possibility that EA may have been involved independently or dependently in G_0/G_1 
arrest which led to reduction in LNCaP cell numbers in the media culture.

At this point, it is difficult to determine the exact mode of action of EA specifically on 
IGFBP-2 which led to growth inhibition in LNCaP cells in the present study. On the other hand, 
a potential signaling pathway has been proposed (140)- the idea that EA may modulate growth 
factors, such as IGF-2 and their binding proteins, which may trigger p53 signaling pathways that 
could mediate cell growth arrest by inducing cell death. In the present study, the suppression of 
IGFBP-2 levels by EA in the LNCaP cell media culture was clearly demonstrated. It has been 
suggested that IGFBP-2 may be the major binding protein of IGF-2 (75). It is possible that in the 
present study, EA may have been involved in modulating IGF-2 (not examined) and IGFBP-2, 
and triggered a signaling pathway which may have led to the inhibition of cell growth in the 
LNCaP cell culture as suggested in one study (140). It is also possible that other factors, such as 
growth factors (IGF-1 or IGF-2) may have been involved in antiproliferative action of EA as 
well as in the suppression IGFBP-2 in the present study. Further investigation in this area may 
prove beneficial.

The main finding in the present study is that the effect of EA in inhibiting cell 
proliferation in LNCaP cells and suppressing IGFBP-2, a possible risk factor of prostate cancer
pathogenesis and/or progression. The significance of the antiproliferative activity of EA in the present study lies in the potential that environmental factors such as dietary intake, may impact growth factors, such as the IGF system, which appear to play an important role in neoplastic process. It has been demonstrated that edible berries such as black raspberries, which consist almost 2 mg of EA per gram dry weight (149), and other fruits, have substances which contribute to the prevention of cancer (150). A recent study (151) examined the pharmacokinetics of EA in healthy volunteers fed freeze-dried black raspberries daily for seven days. Results show that daily consumption of 45 g of freeze-dried black raspberry powder (ground berry seeds) for seven days were well tolerated, and that ellagic acid can be quantified in plasma and urine. Similarly, Seeram and coworkers (107) reported the bioavailability of EA in human plasma after consumption of pomegranate juice (180 ml) containing EA (25 mg) and hydrolysable ellagitannins (318 mg punicalagins) in healthy human subjects. EA was detected and quantified in plasma samples at a maximum concentration 31.9 ng/ml, which is roughly about 0.1 uM, after one hour post ingestion; after four hours, EA was no longer detected. The amount of EA (~0.1 uM) detected in the human plasma from the above study was used in the present study but had little influence on cell proliferation and viability and IGFBP-2 suppresion (see Fig. 2, 3 and 5).

The concentration of EA in prostate tissue has not been measured and it is unknown whether the concentration of EA in serum reflects the concentration in prostate tissue. However, the results from the pharmakinetics study of EA in human subjects previously considered, indicate that EA may be retained in the human body long enough to exhibit its activities. Furthermore, it has been reported that EA has strong affinity for proteins, although it is not well absorbed in small animals (107).
Diet rich in ellagic acid sources, such as strawberries, raspberries, cranberries, walnuts and pecans, may prove beneficial for the general populations and more with respect to patients suffering from different types of cancers, including prostate cancer, because of its many health benefits.

In the advanced stages of prostate cancer, androgen deprivation therapy (ADT) is considered a standard therapy. However, data from several studies already considered in addition to the present study indicate that androgen-independent prostate cancers secrete IGFBP-2 which has been implicated in aggressive cell division and growth. These findings indicate that dietary intervention with EA may be used to alter rapid cancer cell growth, and therefore, illustrate the possibility of using EA therapy for prostate cancer in patients with ADT.

Patients with hormone refractory prostate cancer have been shown to experience negative side effects while on standard chemotherapy. Interestingly, a recent study (100) has reported that the use of EA as a support therapy may reduce chemotherapy induced toxicity, especially neutropenia, although the authors acknowledge the need for more studies to confirm this. Thus in addition to the antiproliferative effect of EA in prostate cancer cells, EA may also have the potential to counteract one or two negative effects of the standard chemotherapy.
Conclusion/Application

The Research Questions were answered in the following manner.

(1) Does exposure to ellagic acid affect the proliferation and growth of human prostate cancer cells (LNCaP) in culture? Yes, growth is significantly decreased by increasing doses of ellagic acid, in a dose-dependent manner.

(2) What effect does ellagic acid have on the concentration of IGFBP-2 secreted by LNCaP cells? Yes, increasing doses of ellagic acid ($> 10$ uM) significantly decrease the amount of IGFBP-2 secreted by LNCaP cells.

Growth retardation of LNCaP cells by EA in the present study indicates that EA may act a potential anticarcinogenic agent. Data from the present study shows that LNCaP cells are capable of producing IGBFP-2. The suppression of IGFBP-2 by EA suggests that the IGF axis may be involved in the control of prostate growth. The relative importance of nutrition is made clear in the present study, as well as other studies. Thus a diet high in sources of EA, such as berries and nuts, may prove beneficial to those who consume it, but especially to those with prostate cancer and in the advanced stage of the disease. Although EA supplements such as freeze dried black raspberry powder may also prove useful, the use of whole foods sources will decrease the risk of toxicity from large doses of supplement. The present study lays the groundwork for further investigation on IGFBP-2 in human prostate cancer (LNCaP), and other prostate cancer cells utilizing EA as a possible anticancer agent.

Recommendations

It is recommended that future studies conduct further experiments to confirm the results obtained from the present study, perhaps using additional prostate cancer cells such as DU145,
PC-3 in addition to the human prostate cancer (LNCaP) cells used. The mode of action of ellagic acid in growth inhibition of LNCaP cells and IGFBP-2 suppression warrants further investigation. Examining the effects of IGFBP-2 on IGF-1 and IGF-2 may be worthwhile since these growth factors have been shown to be potent mitogens in several cancer cell lines.

The possible effect of having higher or lower IGFBP-2 concentrations on prostate cancer cells may lend support to its role in the pathogenesis of prostate cancer. Future studies could examine the synergistic effects (if any) of ellagic acid and another phytochemical, such as lycopene or isothiocyanates in prostate cancer cell lines. Finally, it is recommended that future research examine possible benefit of incorporating ellagic acid in the diet of patients with androgen deprivation therapy using IGFBP-2 as a possible biomarker.
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carcinogenesis by the naturally occurring plant phenolics caffeic, ellagic, chlorogenic and


APPENDIX A

EXPERIMENTAL MATERIALS/PROCEDURE FOR IGFBP-2 ELISA

Solutions prepared for the procedure

Phosphate Buffered Saline solution (PBS)- 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$, pH 7.2-7.4, 0.2 mM filtered.

Wash Buffer- 0.05% Tween 20 in PBS, pH 7.2-7.4 (R&D Systems Catalog #WA126).

Block Buffer- 5% Tween 20 in PBS with 0.05% NaN$_3$.

Reagent Diluent- 5% Tween 20 in PBS, pH 7.2-7.4, 0.2 mM filtered.

Substrate Solution- 1.1 mixture of Color Reagent A (H$_2$O$_2$) and Color Reagent B (Tetramethylbenzidine) (R&D Systems Catalog # DY999).

Stop Solution- 2 N H$_2$SO$_4$ (R&D Systems Catalog # DY994).

Materials provided by the manufacturer

Capture Antibody- 360 ug/mL of mouse anti-human IGFBP-2 reconstituted with 1.0 mL of PBS.

Detection Antibody- 18 ug/mL of biotinylated goat anti-human IGFBP-2 reconstituted with 1.0 mL of PBS.

Standard- 95 ng/ML of recombinant human IGFBP-2 reconstituted with 0.5 mL of Reagent Diluent.

Streptavidin-HRP- 1.0 mL of streptavidin conjugated to horseradish-peroxidase.
Procedure provided by the manufacturer (R&D Systems, Minneapolis, MN) was followed as outlined below.

Plate preparation

1. The capture antibody was diluted to a working concentration of 2.0 microgram/mL in PBS without carrier protein. The 96-well microplate was immediately coated with 100 microliter of the diluted capture antibody in each well. The plate was sealed and left to incubate overnight at room temperature.

2. Each well was aspirated and washed with wash buffer. This process was repeated two times with a total of three washes. The wash was done by filling each well with 400 uL of wash buffer using a squirt bottle. Complete removal of liquid from each well was ensured in order to obtain good performance. After the last wash, the remaining last wash buffer was removed by inverting the plate and blotting it against paper towels, followed by aspiration.

3. Blocking buffer (300 uL) was added to each well to block the plate, and left to incubate for a minimum of one hour.

4. The aspiration and wash steps (#2) were repeated. The plate was now ready for addition of sample.

Assay Procedure

1. 100 uL of sample and standards in Reagent Diluent was added to each well. Plate was covered with adhesive strip and incubated for 2 hours at room temperature.

2. Aspiration/wash process in step 2 of Plate Preparation was repeated.
3. Detection antibody was diluted to 100 ng in reagent diluent. 100 uL of the diluted detection antibody was added to each well. Plate was covered with a new adhesive and incubated for 2 hours at room temperature.

4. Aspiration/wash step (#2) in Plate Preparation was repeated.

5. 100 microliter of a 1:200 dilution of Streptavidin-HRP was added to each well. Plate was covered to avoid direct light and incubated for 20 minutes at room temperature.

6. Aspiration/wash step was repeated.

7. Substrate Solution (100 uL) was added to each well and left to incubate at room temperature for 20 minutes. Plate was covered to avoid direct sunlight.

8. 50 uL of stop solution was added to the each well to stop the reaction. The plate was gently tapped to ensure thorough mixing.

9. Absorbance was determined immediately at 450 nm with correction at 570 nm in a microplate spectrophotometer (BioRad 1680). The standard curve and calculations of unknown concentrations were determined using a 4-PL curve.