University of Alberta

Examination of I*n Vitro* Prostate Cancer Models Supplemented with Lycopene, Vitamin E and Fish Oil

by

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ABSTRACT

This study examined the effects of lycopene, Vitamin E and fish oil on growth, prostate serum antigen (PSA) production and integrin expression ($\alpha_2\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_3$ vitronectin) using *in vitro* models.

Lycopene had no effect on growth. Vitamin E increased cell growth in all malignant cell lines, and fish oil decreased growth in all cell lines. All of the nutrients decreased the expression of $\alpha_v\beta_3$ and VR in PC-3 cells, and there were significant changes in PSA production and $\alpha_2\beta_1$ expression with addition of nutrients.

Decreased growth with fish oil suggests it may have anti-proliferative effects. The increase of growth with Vitamin E supplementation, does not lend support to supplementation during the progression of PCa. Decreased $\alpha_v\beta_3$ expression with these nutrients may correspond to decreased invasiveness of the cells as vitronectin is a ligand of these integrins and is expressed in mature bone, where PCa cells preferentially metastasize.

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This Work Is Dedicated To:

My Grandparents

Norma & Eric Dodd

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LIST OF ABBREVIATIONS

α	Alpha
AA	Arachidonic Acid
AFHE	Agriculture Forestry and Home Economics
AR	Androgen Receptor
ATCC	American Type Culture Collection
AUA	American Urological Association
β	Beta
BPH	Benign hyperplasia
°C	Degree Celsius
cPSA	Complexed PSA
DHA	Docosahexanoic Acid
DOB	Date of Birth
DNA	Deoxyribonucleic Acid
DRE	Digital Rectal Examination
DU-145	Dura-145
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunoassay
EPA	Ecosapentanoic Acid
FITC	Fluoroscein isothiocyanate
FCS	Fetal Calf Serum
fPSA	Fractional PSA
γ	Gamma
γ-CEHC	2,7,8-trimethyl-2-(β-carboxy-ethyl)-6-hydroxychroman
HBS	HEPES-Buffered Saline
HNRU	Human Nutrition Research Unit
HPV-18	Human papillomavirus 18
HRPC	Hormone Refractory Prostate Cancer
HS	Human Serum

IGF-1	Insulin Growth Factor
IL-6	Interleukin 6
KSFM	Keratinocyte-SFM
L	Liter
LA	Linoleic Acid
LD_{50}	Lethal Dose (50%)
LDH	Lactate Dehydrogenase
LNA	α-Linolenic Acid
LNCaP	Lymph Node Carcinoma of the Prostate
MEM	Minimum Essential Medium
mg	Milligram
µg	Microgram
ml	Milliliter
µl	Microliter
µM	Micromolar
MTT	3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyl-tetrazolium bromide
MVD	Microvessel Density
NRUA	Neutral Red Uptake Assay
PC	Phosphatidylcholine
PCa	Prostate Cancer
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PC-3	Prostate Cancer 3
PFA	Paraformaldehyde
PMSA	Prostate-Specific Membrane Antigen
PS	phosphatidylserine
PSA	Prostate Serum Antigen
PSAD	PSA density
QOL	Quality of Life
RPMI 164	Roswell Park Memorial Institute 1640 Medium
PBS	Phosphate Buffered Saline

PFA	Paraformaldahyde
PUFA	Polyunsaturated Fatty Acids
SFA	Saturated Fatty Acids
THF	Tetrahydrofuran
tPSA	Total PSA
TRUS	Trans-rectal Ultrasound
TV	Tumor Volume
Tween 40	Polyoxyethylene Sorbitan Monopalmitate
Tween 80	Polyoxyethylene Sorbitan Monooleate
ULN	Upper Limit of Normal
VES	Vitamin E Succinate
VN	Vitronectin
VR	Vitronectin Receptor

1. LITERATURE REVIEW

1.1 PROSTATE CANCER OVERVIEW

Prostate cancer (PCa) is the number one diagnosed cancer in men and the ninth most common cancer in the world. The highest rates of PCa are in North America, Europe and Australia, whereas the lowest rates are reported in Asia (World Cancer Research Fund, 1997). The dramatic differences in occurrence between geographical areas of the world suggest a potential influence of the environment as a risk factor for the disease (Chan, 2002). Hereditary factors account for a relatively small percentage (<10%) of prostate cancers and are generally associated with early onset disease (reviewed by Abate & Shen, 2000). In Canada, the demographics are shifting towards an increasingly older population, which is at risk for PCa (Canadian Cancer Statistics, 2001). An estimated 20,700 men will be diagnosed with and 4,200 will die of PCa in Canada in 2006 (Canadian Cancer Society, 2006). The incidence of PCa and deaths from it are increasing with age. Age adjusted incidence rates of PCa have been rising by approximately 3% worldwide every year for the past several decades, partly due to increased screening (Boyle *et al.*, 1995).

1.2 PROSTATE BIOLOGY

The prostate gland is found exclusively in mammals. The prostate surrounds the urethra at the base of the bladder and functions by contributing secretory proteins to the seminal fluid. The prostate is divided into three morphological regions: the peripheral zone, the transition zone and the central zone (McNeal, 1969). Benign hyperplasia (BPH) occurs mainly in the transition zone whereas prostate carcinoma arises primarily in the peripheral zone. Within the prostatic epithelium there are three cell types that can be distinguished by their morphological characteristics, functional significance and relevance for carcinogenesis. These cell types as reviewed by Abate and Shen (2000) include the predominant cell type: secretory luminal cell, which is a differentiated androgen-dependent cell that produces prostatic secretory proteins. Basal cells are the second type of epithelial cells that are found between the luminal cells and the basement membrane forming a continuous layer. These cells do not produce prostatic secretory

proteins. The last major cell type are the neuroendocrine cells, which are androgen independent and are dispersed throughout the basal layer (reviewed by Abate and Shen, 2000).

1.3 PROSTATE CANCER AND METASTASIS

It is currently thought that PCa progresses from a normal to a cancerous phenotype with retention of androgen responsiveness, but later progresses to a stage of androgen independent growth. This shift is accompanied by metastasis (McNeal, 1969). When this occurs, the standard treatment options are limited and consist of palliative radiotherapy or chemotherapy (Chi *et al.*, 2001) with a median survival time of twelve months (Cheville *et al.*, 2002). Little is known about the early events and signals that cause tumor cells to become hormone refractive and gain metastatic potential The progression of cancer likely involves dysregulation in the transcription and translation of many genes, some of which are regulated by the androgen receptor (Stewart *et al.*, 2004)..

Metastasis is a multi-step process involving growth in a primary organ, neoangiogenesis, intravasation and survival in circulation, followed by attachment and extravastion at a target organ and growth of a secondary neoplasm (Tantivejkul *et al.*, 2004). There are two main theories explaining metastasis. The "seed and soil" theory of Paget (1889) proposes that there is something specific about metastatic sites that promote growth of secondary neoplasms, whereas Ewing's theory suggests that cancer cells are directed to specific metastatic sites due to the direction of blood flow and lymphatics (1928). Both theories have merit as Ewing's theory accounts for cancer growth in the draining lymph nodes and liver, and Paget's theory describes distal metastases that are organ specific, like those that primarily occur in PCa (Tantivejkul *et al.*, 2004). Fidler (2002) redefined Paget's theory of metastasis to include three principles. Firstly, cancerous tissues contain heterogeneous subpopulations of cells with different angiogenic, invasive and metastatic properties. Secondly the metastatic process is selective for cells that survived the journey to a distal organ and lastly the success of the metastatic cells depended on the ability of those cells to adapt to and utilize their new microenvironment.

According to Bubendorf *et al.* (2000), 90% of all PCa metastases occur in the bone. Several factors are thought to account for the high frequency of bone metastases. Blood flow is high in areas of red marrow, and tumor cells produce adhesive molecules that bind them to marrow stromal cells and bone matrix (Roodman, 2004). Bone is also a large repository for immobilized growth factors (Kahn *et al.*, 1994) which provide fertile ground in which tumor cells can grow (Roodman, 2004). Once tumors metastasize to the bone they are not curable (Roodman, 2004). Bone metastases are classified as osteolytic or osteoblastic (Roodman, 2004). Osteolytic metastases result in the dissolution of bone and osteoblastic metastases result in increased bone production (Evan *et al.*, 2004). The consequences of osteolytic bone metastasis can include severe pain, pathologic fractures, life-threatening hypercalcemia and spinal cord compression whereas osteoblastic metastases include bone pain and pathologic fractures due to the poor quality of bone produced by osteoblasts (Roodman, 2004). Due to the consequences of metastasis, early detection of cells that are likely to metastasize would be beneficial with regards to earlier intervention and prevention.

1.4 SCREENING AND DIAGNOSTIC TOOLS FOR PROSTATE CANCER

Presently there is no screening method that is completely accurate in the screening and detection of PCa, although several different screening tools are used, each with limitations. The three most commonly used screening tools are the digital rectal examination (DRE), trans-rectal ultrasound (TRUS) and assessment of prostate specific antigen (PSA) levels in blood. General screening for PCa is of potential use as there are usually no early symptoms of the disease, and treatment usually is not sought until the disease has progressed. When the disease is localized to the prostate, more treatment options exist, therefore early detection is important. Although general screening would be of use with respect to the above reasons, there are many drawbacks to testing including false positives and infection, and the fact that early screening cannot change the outcome of the course of the disease as there is currently no cure.

1.4.1 DIGITAL RECTAL EXAMINATION

The digital rectal examination (DRE) is performed by physicians and is a subjective method that requires experience and continuous training. Detection by this method is limited because the finger can only palpate the posterior and lateral aspects of

the prostate gland. It is suggested that between 20-35% of tumors occur in portions of the prostate that cannot be examined with the DRE (Schersten *et al.*, 1999), therefore this method should not be the only screening/detection method used. The positive predictive values obtained using the DRE was found to be 28% in a randomized study by Varenhorst *et al.* (1999) and reported positive predictive values have ranged from 5 to 69% depending on the reference (Schersten *et al.*, 1999). Regardless of criticism against the DRE's ability to screen for PCa, case-control studies have shown that it is associated with a reduced risk of death due to PCa (Weinmann *et al.*, 2005). Today, standard indications for biopsy are still either an abnormal DRE or a total PSA (tPSA) ≥4.0ng/mL, but most agree that it is the combination of screening tools that provide the best insight as to whether or not one has PCa.

1.4.2 TRANSRECTAL ULTRASOUND

Transrectal ultrasound (TRUS) is performed by inserting an ultrasound transducer into the rectum. TRUS gives an image of the gland's contour inner architecture and adjacent structures, and indicates prostate volume, which can be used in assessing PSA density (Schersten *et al.*, 1999). TRUS is resource intensive and requires extensive training, with high equipment costs (Schersten *et al.*, 1999). TRUS is not usually able to detect cancers in the transitional zone, which is the primary location for 20-30% of prostate cancers that are not detected by TRUS (McNeal *et al.*, 1986). There has also been variation reported with regards to the sensitivity of this test, but has been reported at 89% (Gustafsson *et al.*, 1992). TRUS cannot distinguish between benign and malignant nodules, making the positive predictive value low, and the size is often underestimated with this method (Schersten *et al.*, 1999). There are also undesirable effects of frequent biopsies including the psychological stress that may arise from false positives (Gustafsson *et al.*, 1990) and an infection rate between 4.1 and 6.2% (Gustafsson *et al.*, 1992; Aus *et al.*, 1993). Use of TRUS as the primary instrument in mass screening would be costly and there is no direct evidence to suggest that this would improve disease specific survival rate therefore the main application of TRUS is in secondary diagnostics in combination with histopathology (Schersten *et al.*, 1999).

1.4.3 PROSTATE SPECIFIC ANTIGEN

Prostate specific antigen (PSA) is a 33kD serine protease, synthesized by normal and malignant epithelial cells of the human prostate, but is expressed and released into the serum at higher levels by malignant cells (Kim and Coetzee, 2004). PSA is secreted by the prostatic epithelium and epithelial lining of the periurethral glands, and involved in the liquefaction of the seminal coagulum to allow the release of spermatozoa (Lilja, 1985). PSA is released in low concentrations in the blood and circulates at approximately 80-90% in complex with the enzyme inhibitor antichymotrypsin (Schersten *et al.*, 1999).

PSA is a tissue specific marker; however it is not specific for cancer as it is elevated in blood in other benign prostate conditions (Schersten *et al.*, 1999). In spite of this, elevated serum levels of PSA are currently used to diagnose and monitor PCa. Increases in serum PSA following local and systemic treatments are highly correlated with tumor recurrence and progression (Kim and Coetzee, 2004). These associations have helped establish PSA as a clinically important biomarker. The introduction of routine PSA-based screening has led to an increase in the rate of PCa detection and a subsequent shift to earlier staging at the time of diagnosis (Hernandez and Thompson, 2004). The initial use of PSA as a screening tool came about because of its higher sensitivity in detecting localized disease as compared to prostatic acid phosphatase, the previous marker for prostate carcinoma (Seamonds *et al.*, 1986; Stamey *et al.*, 1987). Early observations that have had important clinical applications have included: a decrease in PSA with hormonal therapy (Ferro *et al.*, 1989); increased PSA after treatment appeared to precede disease recurrence; and disease recurrence occurs if PSA is detectable after radical prostatectomy (Hudson *et al.*, 1989). The introduction of PSA screening into clinical practice has resulted in a high rate of disease detection, due to the slightly higher sensitivity of this test over the use of the DRE (Hernandez and Thompson, 2004). Although PSA screening has a slightly higher sensitivity than the DRE, at a positive predictive value just above 32% it is not substantially higher than for screening with DRE alone (Schersten *et al.*, 1999).

The current upper limit of normal (ULN) for PSA levels is accepted to be 4.0ng/mL, and at this level 80% of tumors can be detected by current diagnostic technologies (Schersten *et al.*, 1999). It has been suggested that lowering the value to 2-

3ng/ml would aid with earlier detection, but this would also increase the number of false positives and would result in an increased use of biopsies and TRUS. Oesterling *et al.* (1993) reported a correlation between patient age and prostatic volume, Gustafsson *et al.* (1998) found a significant correlation between PSA and prostate volume, PSA and age as well as between prostate volume and age. Oesterling *et al.* (1993) and Gustafsson *et al.* (1998) suggest that it is more appropriate to have age-specific reference ranges for PSA. Age-specific reference ranges have the potential to make serum PSA a more discriminating tumor marker for detecting clinically significant cancers in older men (increasing specificity) and to find more potentially curable cancers in younger men (increasing sensitivity) (Oesterling *et al.*, 1993).

1.4.4 PSA MODIFICATIONS

Several modifications to improve PSA screening have been suggested in addition to age adjusted PSA levels. Among the other factors that may be considered are: the effect of ethnicity on PSA levels; PSA velocity (measuring PSA in the blood over time, based on the assumption that PCa patients should have rapidly increasing PSA levels); PSA density (relating PSA level in blood to gland volume as measured by ultrasound), as PSA levels have been noted to be higher in men with larger prostate, leading to the suggestion that PSA level should be corrected for prostate size; PSA isoforms, (that is bound versus free PSA, have been reported to be related to the risk of prostate carcinoma detection (Hernandez and Thompson, 2004).

Ethnicity

In comparison to other ethnicities, African Americans have the highest incidence of PCa in the world. African Americans also have higher PSA levels at a younger age (de Koning and Shroder, 1998). Thus, decreasing the normal upper limit may increase PSA test sensitivity in African American men (Hernandez and Thompson, 2004). The importance of considering age and ethnicity before having PSA levels taken has been realized by the American Urological Association (AUA). The AUA has developed age-specific PSA reference ranges in ten-year intervals starting from 40 years of age until 80 years of age for Caucasians, African Americans and Asians (AUA, 2000).

Fractional PSA

Protein bound and unbound portions of PSA, also referred to as fractional PSA, includes free, complexed and total PSA (fPSA, cPSA and tPSA, respectively) in the blood, therefore tPSA=fPSA + cPSA (Hugosson *et al.*, 2003). These PSA fractions are made visible by immunohistochemistry (Barqawi *et al.*, 2005). A total of 60% to 95% of PSA is complexed to alpha-antichymotrypsin (cPSA), fPSA comprises 5% to 40% and is usually found in men with benign prostatic hyperplasia (BPH), and only 1% to 2.5% is bound to a macroglobulin, which has less pathological significance (Barqawi *et al.*, 2005). The value of cPSA in early diagnosis of PCa is still actively debated, some have concluded that it is more specific for the early detection of PCa (Christensson *et al.*, 1993), whereas others such as Finne *et al.* (2002) have disputed that a low percentage of fPSA is a stronger risk factor and is associated with increased risk of advancing disease. For example, they found that men with a PSA of 3-4ng/mL have an average risk of 15-20%, but among these, those with a low percentage of fPSA have a much higher risk.

Some studies have gone as far as using f/tPSA ratios to decrease the number of benign biopsies required. For example, one study has shown that specificity of PSA screening can be increased beyond that of the standard minimum of 4ng/mL, if lowered to a PSA minimum of 3ng/mL and combined with f/tPSA ratio of less than or equal to 18% (Hugosson *et al.*, 2003). Excluding men with >18% f/tPSA would save 136/262 (51%) with benign biopsies from further examination, however, 18 cancers would have been missed with this algorithm, therefore uncertainty remains.

PSA Velocity

PSA velocity has demonstrated great potential for increasing PSA screening specificity in many ways. Firstly, monitoring changes in PSA levels despite a low absolute level (ex. >4.0ng/mL), may be a indicator of PCa. Secondly, evidence now exists to suggest that the level of PSA change during the year prior to diagnosis is possibly associated with disease-specific mortality (D'Amico, 2005). For example, one study concluded that an annual PSA velocity of > 2.0ng/mL before the year of diagnosis of PCa was significantly associated with a high risk of death from PCa despite undergoing radical prostatectomy (D'Amico *et al.*, 2004). Some have suggested that a mere increase of 0.75ng/mL per year should prompt a biopsy, regardless of the PSA level (Hernandez & Thompson, 2004). Thirdly, several studies have shown that rate of rise of

PSA before diagnosis of PCa can help predict tumor stage, grade and perhaps even time to disease recurrence for those undergoing a radical prostatectomy. D'Amico *et al.* (2004) found that an annual PSA velocity of 2.0ng/mL or greater was frequently associated with more advanced tumor characteristics such as metastasis to the lymph nodes, a stage of T2, and a higher grade (i.e. Gleason score of 8,9 or 10), which also predicted the time to death from PCa.

Tumor Volume

Controversy exists as to whether or not there is any value in measuring tumor volume (TV) as an independent prognostic factor in screening for PCa. Prostate volume increases naturally as a man ages. It has also been observed that the amount of PSA found in serum is proportional to the mass of the prostatic cells that produce PSA, and that small tumors are less likely to produce increased PSA than larger tumors (Datta *et al.*, 2005). This is both confusing and concerning when it comes to using PSA serum levels to test for PCa, since PSA level may increase and decrease with the size of both malignant and benign prostate tumors. For example, Stamey *et al.* (2004) showed in 1987 that excision of large nodules of BPH decreased serum PSA dramatically. African Americans who, despite showing low screening PSA levels, had similar tumor sizes to those of Caucasian men with much higher serum PSA values, therefore TV alone does not clearly reflect the progression of PCa, since it may increase as one ages (i.e. is benign), or depending on one's ethnicity, therefore not accurately reflecting the progression of PCa.

Those arguing for TV as an independent prognostic factor believe pathologists should measure this on a regular basis, which in turn requires tedious serial sectioning of the specimen and detailed, planimetric measurement of cancer area in each section (Kukuchi *et al.*, 2004). Most pathologists and urologists however, agree with studies such as the one of Kukuchi *et al.* (2004), that while TV is clearly associated with Gleason grade and pathological stage, it provides no independent prognostic information on its own.

PSA Density (PSAD)

Prostate volume has proven to be of extreme importance in screening for PCa when combined with other methods, such as serum PSA. Knowing the prostate volume increases the diagnostic accuracy of serum PSA by allowing for the calculation of PSA density (PSAD). The PSAD is obtained by dividing the PSA value with the prostate

volume (Finne *et al.*, 2002). Men with a score above 0.15 are more likely to have cancer than men with lower values (Gretzer & Partin, 2003). A multivariate analysis revealed that the inclusion of prostate volume improved prediction of biopsy findings and provided a significant reduction of unnecessary biopsies as compared to either the percentage of fPSA or PSAD alone (Finne *et al,* 2002). Therefore, correcting PSA for prostate size may improve the test specificity in men with larger glands as well as the sensitivity in men with smaller glands (Hernandez & Thompson, 2004). Overall, the utility of PSA transformations, including PSAD, PV, age-specificity, ethno-specificity, fPSA and cPSA, and PSA velocity continue to be debated based on relative sensitivities and specificities in screening for PCa (Svetec & Thompson, 1998).

The fact that PSA is not a cancer specific marker leads to the question of whether or not PSA screening has improved health outcomes (i.e. decreased mortality and morbidity). There have been studies supporting and refuting improved health outcomes with PSA screening, and further research is needed due to the lack of long-term randomized studies assessing mortality rates. Weinmann *et al.* (2005) attempted to examine the relationship between both DREs and PSAs with mortality. Unfortunately, due to limitations of the available data they were only able to conclude that DREs but not PSAs were associated with a decreased mortality rate. However, they also concluded that because the PSA is a more sensitive screening test for PCa than DRE, it is plausible that PSA screening is associated with a mortality reduction at least as large as that of DRE. Isola *et al.* (2001) reported a decrease in mortality with PSA screening, by analyzing several different markers of aggressiveness such as tumor grade, tumor proliferation rate, p53 over-expression and aneuploidy. Perron *et al.* (2005) divided a population of men 50 years into 15 birth cohorts, and found most birth cohorts showed an increase in PCa incidence and a subsequent decrease in mortality, the sizes of these changes were not inversely correlated, and therefore PSA screening could not explain the decline in PCa mortality. Stamey et al (2004) through the examination of cancer volume, palpable nodules, Gleason scoring and other histological parameters over the course of twenty years, determined that serum PSA was related to PCa twenty years ago, but in the last five years serum PSA has only been related to benign prostatic hyperplasia. These studies only further exemplify the need for serum markers that accurately reflect PCa.

1.4.5 OTHER POTENTIAL CANCER MARKERS

Prostate-specific membrane antigen (PMSA) has been evaluated as a new diagnostic and prostatic marker for PCa (Carroll and Grossfeld, 2002). PMSA is a 1,000,000kDa transmembrane glycoprotein with intra and extracellular domains, the function of which is currently unclear (Carroll and Grossfeld, 2002). PMSA shows differential staining between benign and malignant prostate tissue, but is not prostate tissue specific. Development of new antibodies to PSMA will aid in determining value and efficacy of this tumor marker in the diagnosis of PCa (Carroll and Grossfeld, 2002).

There are other markers that are currently under investigation in attempts to produce more accurate predictions of tumor aggressiveness and response to therapy. Included among these markers is the Ki-67 labeling index which is a marker for cellular proliferation and has been reported to correlate with Gleason grade (Bubendorf *et al.*, 1998). Increased p53 staining by immunohistochemistry has also been associated with high Gleason grade and decreased survival in men with PCa (Grossfeld *et al.*, 1998). Prostatic microvessel density (MVD) had also been evaluated as a prognostic sign for PCa, as studies have found a positive correlation between MVD and pathologic stage in radical prostatectomy specimens (Rogatsch *et al.*, 1997).

1.5 CLINICAL INFORMATION

1.5.1 STAGING

Treatment options for PCa depend strongly on both the stage and the grade of the tumor, as well as the patient's age and preference. A Gleason score is used to assess the grade of the tumor, and is calculated by a pathologist following a positive biopsy and PSA test. The score of the tumor can range from 2-10, with 10 being the worst case scenario. The score is calculated by identifying the two most commonly occurring patterns (grades) of tumor in the biopsy tissue and adding them together as a sum (Calabrese, 2004). The higher the Gleason score, the more aggressive and less differentiated the cancer, and therefore, more harmful the cancerous cells.

1.5.2 GRADING

The PCa stage is determined by the TNM staging system. The T refers to the primary tumor, and can range from being hardly evident to invading adjacent anatomy. The N relates to regional lymph node involvement, whereas the M refers to distant metastases,

and is the most advanced stage (AJCC Cancer Staging Manual, 2002). It is evident that the stage and grade provide crucial information as to the cancer's progression, which in turn helps the patient decide which form of treatment is best suited for them.

1.5.3 TREATMENT OPTIONS

Each stage of PCa whether localized, locally advanced, metastatic or recurring, has a corresponding set of treatment options. Firstly, however it is imperative for the patient and their physician to explore all possible treatment routes before coming to a decision. Unfortunately, all current treatment options have accompanying side effects, therefore proper intervention is needed to prevent or minimize the negative effects that a particular treatment may have on the patient's quality of life (QOL). Clinical intervention trials have been designed to address the physical and psychosocial concerns of men treated for PCa. Johnson (1996) aimed to evaluate the effect of clinical care education for men receiving radiation treatment for PCa. They concluded that improving men's understanding of treatment was the intervention's most important function. Therefore, perhaps informing the patient as to the uncertainties of their treatment route is the key to improving QOL during treatment.

Options for localized cancer vary greatly from surgery and radiation therapy, to an observational route known as watchful waiting. Watchful waiting is defined as initial surveillance followed by active treatment if and when progression of the prostate tumor produces bothersome symptoms (Adolfsson, 1995). Watchful waiting is an option for older men with well-differentiated low-volume PCa and a life expectancy of less than ten years (Wallace et al., 2004). Routine screening, such as that of PSA serum testing serves as a method of surveying the advancement of the cancer. The rationale for watchful waiting is that due to the slow growing nature of PCa, and the rising number of organ confined cancers detected by PSA testing, more people are dying with PCa than from it.

A recent 20 year long study following conservative management of clinically localized PCa does not support aggressive treatment such as surgery for low-grade cancers (Albertson et al., 2005). Within a 20-year follow up period an extremely low risk of dying (6 deaths per 1000 people) was reported with low-grade (i.e. Gleason score 2-4) PCa. They also concluded that men with a Gleason score of 5 or 6 have an intermediate risk of PCa death, and that men with high-grade (i.e. Gleason score 8-10) tumors have a

high probability of dying within 10 years of diagnosis. Therefore, perhaps more aggressive treatment such as surgery is only needed for patients with an intermediate to high-grade tumor.

Siston et al. (2003) followed patients undergoing surgery or radiation before and after treatment or watchful waiting. Patients receiving surgery and radiation had significant disease-specific QOL changes over 12 months post treatment from sexual and urinary dysfunction, where as the watchful waiting group reported declines in urinary and sexual functioning problems prior to treatment. Other negative side effects of watchful waiting include stress and anxiety due to the uncertainty that accompanies this observational route and living with cancer. In one large randomized trial, comparing the survival rates of people who have had radical prostatectomies versus watchful waiting, Holmberg et al. (2002) found that there was no significant difference between surgery and watchful waiting in terms of overall survival. Three years later, Holmberg et al. concluded that radical prostatectomy reduces disease-specific mortality, overall mortality, and the risks of metastasis and local progression.

Locally advanced cancers differ in their treatment options due to increased aggressiveness of the cancer, often resulting in a need for a combination of several treatments, such as both surgery and radiation. Metastatic PCa treatment options include hormone therapy and castration, which have their share of negatively impact QOL. Recurring cancers are detected by rising PSA levels following a primary treatment, and treatment options therefore vary according to the treatment (Calabrese, 2004).

1.6 CELL CULTURE AND PROSTATE CANCER

Cell culture is prominently used for the study of human PCa. Animal models are also available for the study of this disease, but due to dissimilarities in anatomy and morphology of rodent and human prostates, along with the absence of spontaneous PCa in rodents (Abate and Shen, 2000), cell culture becomes an attractive experimental model. Due to difficulties associated with primary culture of prostate cells, several cell lines have been immortalized from various sources. Cell lines related to different progressive and metastastic stages of PCa, ranging from a normal prostate epithelial cell phenotype to brain and bone metastases, are commercially available (Table 1.1).

1.7 INTEGRINS

Integrins have been implicated in cell migration, survival, growth, differentiation, gene expression, and modulation of signaling pathways (Moro *et al.*, 2004), making them a marker of interest with respect to cancer progression and metastasis. Integrins are heterodimeric, transmembrane receptors that are composed of an α and β subunit (Slack-Davis and Parsons, 2004). The type of integrins expressed determines the extracellular matrix proteins that the cell will interact with and is dependent upon cell type (Zheng *et al.*, 1999). To date there are 18 known variants of the α subunit and 8 of the β subunit (Zheng *et al.*, 1999). The different combinations of the α and β subunits dictate the specificity of integrins for extracellular ligands (Zheng *et al.*, 1999). Each subunit contains a large extracellular domain, a membrane spanning segment and a cytoplasmic tail (Fornaro *et al.*, 2001). The presence of integrins in the cell membrane and their ability to interact with molecules both externally and internally make them candidates for involvement in PCa progression. Integrins regulate growth and migratory pathways, possible sites of intervention in the treatment of PCa (Fornaro *et al.*, 2001).

Table 1-2 lists the integrins that have been investigated in relation to PCa and their reported expression on cell models from the literature. Several biological properties have been attributed to integrins including angiogenesis, cell adhesion, migration and motility as well as cleavage of basement membranes (Stewart *et al.*, 2004; Cooper *et al.*, 2000; Kostenuik *et al.*, 1997; Edlund *et al.*, 2001; Cooper *et al.*, 2000). The conflicting reports in the literature make it important to determine the range of expression of integrins in a variety of PCa cell lines to link it to a mechanism of a progression to more aggressive cancer types. Conflicting evidence also exists for the role of integrins at various stages of PCa (Stewart *et al.*, 2004; Murant *et al.*, 1997; Rabinovitz *et al.*, 1995; Brooks *et al.*, 1995). Integrins are a current focus of targeted anti-invasive therapies, thus appear to be an important marker to characterize and determine if its expression can be modulated by nutrients. Integrin antagonists are an area of therapeutic interest in the treatment of other diseases including breast cancer which also metastasizes primarily to the brain (Brooks *et al.*, 1995). It has been stated that the integrin superfamily is the best opportunity to target small-molecule antagonists for therapeutic and diagnostic utility, in diseases lacking treatment options (Mousa, 2002). In this study we examined the $\alpha_v\beta_3$, vitronectin receptor (VR) and $\alpha_2\beta_1$ integrins.

As a novel target of anti tumor therapies, surprisingly little is known about how they are regulated in PCa. Whether integrin expression is regulated by nuclear events related to the androgen receptor through downstream signals within the prostate cell have not been identified. It is known however, that there are changes in the types and amounts of specific integrins as cells become hormone refractory and metastatic. Thus, this may offer a novel marker for early diagnosis and risk of cancer progression. While mechanistic evidence is still emerging in the study of PCa, integrins are known to be associated with changes in growth and invasiveness thus linked to a potential mechanism of cancer cell growth. It is important to determine their expression in cell lines and in human prostate samples to identify the range of integrin expression to assess the usefulness and relevance of these potential markers as an adjunct to the use of PSA. The relationship(s) of integrins to one or more of the clinical criteria of PCa diagnosis have not been determined, although their relationship to invasiveness supports the possibility for their use as a diagnostic tool. It is known that some cell surface molecules are shed from the cell surface upon activation and/or engagement and can thus be detected in the blood; however, the extent to which integrins could be detected in the blood has not been characterized. Metastases, which occurs to the bone in 90% of cases (Tantivejkul *et al.*, 2004), is a multi step process, which includes the growth and escape of the cancer cells from the prostate, subsequent survival of the cells in circulation, and the growth of the tumor in the bone (Cooper *et al.*, 2000). Many steps in metastasis involve changes in cell adhesion to adjacent cells and the extracellular matrix (ECM). Changes in integrin signaling and expression have been implicated in the multi-step process of metastasis including involvement in cell survival, migration, and invasion (Fornaro *et al.*, 2001). It is not known how engagement of the androgen receptor by testosterone (dihydroxytestosterone) or other co-activators causes an up-regulation of integrins associated with more aggressive types.

1.7.1 $\alpha_v\beta_3$ AND $\alpha_v\beta_3$ VITRONECTIN RECEPTOR

Edlund *et al.* (2001) reported that immunoprecipitation with the antibody to the $\alpha_v\beta_3$ heterodimer revealed nearly undetectable levels of $\alpha_v\beta_3$ in the LNCaP cell line. The use of the $\alpha_v\beta_3$ heterodimer does not appear to be important for laminin attachment in the LNCaP cells, as LNCaP cells attach to laminin primarily with $\alpha_6\beta_4$, whereas cells in the

more invasive C4-2 subline attach with $\alpha_3\beta_1$ and $\alpha_v\beta_3$ (Edlund *et al.,* 2001). Edlund et al. (2001) also report that in the LNCaP model system, $\alpha_v\beta_3$ was similar to $\alpha_3\beta_1$ in that its individual subunits were expressed in all LNCaP models, but the assembled, functional heterodimers were only detectable in the more metastatic cell lines C4, C4-2, and C4-2B (Edlund *et al.,* 2001). This is the first study to reveal shifts in integrin use between cells with a common genetic background but different *in vivo* metastatic potentials.

Cooper *et al.* (2003) characterized $\alpha_v\beta_3$ expression in a variety of cell lines and determined that PC-3 cells expressed the greatest amount of this particular integrin compared to the LNCaP and DU-145 cell lines. Zheng *et al.* (1999) also report that the PC-3 cell line expressed the $\alpha_v\beta_3$ integrin and that the LNCaP cell line did not express this particular integrin. Zheng *et al. (*1999) found that PC-3 cells adhered to and migrated on vitronectin (VN), which is an $\alpha_v\beta_3$ ligand expressed in mature bone where PCa cells preferentially metastasize and LNCaP cells did not adhere to or migrate on VN. $\alpha_v\beta_3$ involvement in epithelial cells is unusual as it is usually expressed in lymphocytes and other migratory cell types, but is common to a number of bone metastases (Stewart et *al.*, 2004). Zheng *et al.* (1999) examined primary human PCa cells isolated from 16 surgical specimens and showed that these cells expressed $\alpha_v\beta_3$, whereas normal prostate epithelial cells did not.

1.7.2 $\alpha_2\beta_1$

Antibody function blocking studies revealed that PC-3 cells utilize $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins to adhere to collagen I (Kiefer and Farach-Carson, 2001). PC-3 cells that were plated on collagen I exhibited increased rates of proliferation over cells plated on fibronectin or tissue culture plastic (Kiefer and Farach-Carson, 2001). This is of importance as collagen I is a major component of mineralized bone matrix (Stewart *et al.,* 2004).

1.7.3 ANTI-INTEGRIN THERAPY

Integrin $\alpha_5\beta_1$ is involved in the direct regulation of angiogenesis, and therefore antagonists of this integrin may be useful in the inhibition of angiogenesis, which is important with regards to the growth and spread of human tumors (Mousa, 2002). Srivatsa *et al.* (1997) showed that selective $\alpha_v\beta_3$ blockade is an effective anti-restenosis strategy that can limit neointimal growth and lumen stenosis following deep arterial

injury. Brooks *et al.* (1995) used a SCID mouse model to investigate whether antagonists of the $\alpha_v\beta_3$ integrin may be an effective anti-angigogenic treatment for breast cancer. Transplanted full thickness human skin containing $\alpha_v\beta_3$ -negative human breast tumor cells were implanted and treated with LM609 (monoclonal antibody). Mice treated with antibody exhibited significantly fewer human blood vessels and the tumors in these animals appeared to be less invasive than tumors in control animals (Brooks *et al.*, 1995). $\alpha_4\beta_1$ antagonists have been reported to inhibit airway eosinophil infiltration in a murine model of allergic pulmonary inflammation, and therefore may be useful in the treatment of this condition (Kudlacz *et al.*, 2002). $\alpha_4\beta_1$ antagonists are also being explored for treatment of chronic inflammatory diseases, and the platelet $\alpha IIb\beta_3$ integrin has been shown to have therapeutic and diagnostic applications in thromboembolic disorders (Mousa, 2002). These examples demonstrate the importance of assessing the changes in integrin expression over the course of PCa.

1.8 THE EFFECT OF NUTRIENTS ON PROSTATE CANCER DEVELOPMENT AND PROGRESSION

1.8.1 ECOSAPENTAENOIC AND DOCOSAHEXANOIC ACID

Mammals are unable to synthesize *n-3* and *n-6* polyunsaturated fatty acids, therefore they must be obtained through dietary sources (Rose and Conolly, 1999). N-3 and n-6 fatty acids are crucial for many cellular processes, including cell proliferation and differentiation (Karmali, 1987), are important components of all cell membranes, and are incorporated into the phospholipids of cell membranes of many cell types, including tumor cells following the inclusion in diet or cell culture media (Karmali *et al.,* 1989; Simopoulos, 1991; Hardman, 2002). Once obtained through diet, linoleic acid (C18:2n-6, LA) α-linolenic acid (C18:3n-3, LNA), are converted to arachidonic acid (C20:4n-6, AA) or eicosapentaenoic acid (C20:5n-3, EPA) via elongase and desaturase enzymes (Rose and Connolly, 1999). Dietary sources of n-3 fatty acids are fish and fish oil (docosahexanoic acid (DHA) and EPA) and oils such as soybean, canola and flaxseed (LNA) (Rose and Connolly, 1999). The composition of the western diet has changed over time, from approximately equal amounts of n-6 and n-3 fatty acids to a preponderance of n-6 fatty acids with a relative deficit of n-3 fatty acids, such that the ratio of n-6:n-3 fatty acids in the diet is between 10:1 and 20-25:1 (Karmali, 1987), and

may be one factor contributing to the increased incidence of chronic disease such as cancer.

Animal and cell culture evidence suggests that fatty acids of the n-6 series promote and n-3 fatty acids inhibit the growth of tumor cells, mediated by alterations in eicosanoid synthesis (reviewed in Cave, 1991; Rose, 1997a; Rose 1997b). Eicosanoids derived from n-6 fatty acids have been shown to enhance tumor growth, and possibly metastasis (Kelavka *et al.,* 2000; Ghosh and Myers, 1998; Ghosh and Myers, 1997; Spindler *et al.,* 1997) and angiogenesis (Nie *et al.,* 2000). LA (18:2n-6) does not appear to be a causal agent of PCa but rather promotes growth of established tumors. Previous work has shown a significant inhibition of growth of tumors in the presence of n-3 fatty acids (Robinson *et al.,* 2002; Robinson *et al.*, 2001).

The experimental evidence providing a mechanistic rationale for targeting eicosanoid biosynthesis is a reduction in eicosanoid synthesis. The anticancer effects of n-3 fatty acids are the result of competition with n-6 fatty acids for enzymes shared in chain elongation, desaturation and eicosanoid biosynthesis (Rose, 1991). N-3 fatty acids are a likely candidate for studies on tumor growth and nutrition as there are numerous reports of their effectiveness in reducing tumor cell growth in culture, animal models and in humans, although the mechanisms remain unclear at this time. With specific relevance to PCa, dietary n-3 fatty acids have been reported to reduce testosterone and 5-α reductase activity (Liang and Liao, 1992). Eicosanoids derived from n-3 fatty acids also display opposite or reduced bioactivity relative to their n-6 counterparts (Rose, 1997a).

N-3 fatty acids have been shown to reduce the incidence, increase the latency of tumor development, and decrease tumor load (Karmali, 1987) *in vitro* or when transplanted into animals (Karmali *et al.*, 1987; Rose and Cohen, 1988). This suggests that n-3 fatty acids affect all stages of carcinogenesis and has a protective effect in delaying or preventing the conversion of pre-neoplastic cells to the tumor phenotype. Karmali *et al.* (1987) show that nude mice transplanted with DU-145 human prostatic cells and fed a fish oil diet (low n-6 fatty acids) relative to a corn oil diet (n-3 fatty acid deficient) had reduced tumor growth, smaller tumors, and a reduction in tumor prostaglandin E2 (PGE_2). The role of eicosanoids in modulating tumor growth has also been demonstrated in parallel using eicosanoid inhibitors. Inhibition of eicosanoid

synthesis is correlated with reduced tumor growth (Rose and Connolly, 1991; Chaudry *et al.*, 1994). Chung *et al.* (2001) reported that DHA and EPA had inhibitory effects on the androgen receptor (AR) mediated actions in the LNCaP cell line. DHA supplementation decreased PSA secretion in the LNCaP cells, as did EPA although higher concentrations of EPA were required for significant inhibition. A study by Pandalai *et al.* (1996) showed promotion of cell growth in culture at low concentrations (1ng/ml) of EPA, although at higher concentrations, EPA inhibited prostate cell growth. Rose and Connolly (1991) found that DHA and EPA inhibited PC-3 cell growth in a dose-dependent manner, with both being equally effective with ~65% reduction in growth, at 2.0μg/ml (2mg/L). It has also been shown i*n vitro* that the growth of PC-3 cells is stimulated in the presence of linoleic acid (Connolly *et al.*, 1997).

While these reports would suggest a definitive role for fatty acids in tumor inhibition, the role of n-3 and n-6 fatty acids when fed within the ranges of balance and quantity consumed by humans is far less clear. Case-control and cohort studies linking diet to cancer show a positive relationship between animal fat and PCa but no strong evidence for a relationship with either linoleic acid or long chain n-3 fatty acids (Willett, 1997; Zock and Katan, 1998). The strongest epidemiological evidence that diet may play a role in PCa comes from migration studies. Men who migrated from Japan and Poland to the United States had higher PCa rates relative to men from their native countries (Willett, 1997). Epidemiological support for a protective influence of n-3 fatty acids against PCa has been reported (Karmali *et al.*, 1987), as well as epidemiological studies that show diets high in n-6 fatty acids stimulate PCa development (Rose and Connolly, 1992). A review of the literature concluded that clinical intervention trials designed to reduce total fat intake and increase the ratio of n-3 to n-6 fatty acids in the diet should be targeted at high risk groups for PCa, and surgically treated cancer patients to prevent disease recurrence (Rose, 1997). Norrish *et al.* (1999) reported a reduced risk of PCa among men who had higher erythrocyte phosphatidylcholine levels of EPA and DHA, a reflection of dietary intake.

Other human studies have not been as conclusive with mixed reports of n-3 fatty acids having some effect (Bairati *et al.,* 1998; Gann *et al.,* 1994; Giovannucci *et al.*, 1993; Yang *et al.,* 1999) and no effect (Godley *et al.*, 1996) on cancer incidence, thus,

studies remain inconclusive with regards to fatty acid intake. Clearly there are discrepancies between the strong experimental data from *in vitro* studies and animal studies and weak epidemiological evidence. These differences are likely due to the long-recognized limitations on the sensitivity to which human studies can detect differences in dietary intake (Norrish *et al.,* 1999). Food frequency questionnaires rely on recall by the subject, which is understandably variable and to some degree biased towards projecting a more healthy diet (Marks *et al.*, 2006).

Nutrient analysis is reliant upon the accuracy of food databases, which may be limited in scope of foods from different cultures, or simply due to the multitude of commercial products developed each year. Further, the difficulty in interpreting case-control, retrospective and prospective studies is the lack of evidence demonstrating causation and mechanism. For example, the association of saturated fat or red meat with PCa has been observed in several studies, but carcinogens formed from cooking animal meat may be the key factor (Gann *et al.,* 1994; Giovannucci *et al.*, 1993). Identification of specific groups of subjects and cancer types may also be warranted. Willet (1997) suggests that there are 2 prevailing forms of PCa, (1) a latent form (probably genetic in origin) and of low variability among different populations and, (2) an aggressive form (environmental in origin). There are several limitations inherent to human studies involving nutrient intake, due to the lack of mechanistic data exists to support any conclusions one could draw. Biomarkers against which to measure PCa incidence and risk may be helpful to further clarify the role of nutrients in the inhibition of PCa progression. The inherent limitations of interpreting the human data lies in the low sensitivity of epidemiological studies reliant upon food questionnaires (see above), thus, the use of serum fatty acid levels to indicate dietary intake have been explored. Tumor invasiveness has been reported to relate to the fatty acid composition of both malignant tissue and host plasma. Yang *et al.* (1999) analyzed serum fatty acids from 24 patients with benign prostatic hyperplasia, 19 patients with prostatic cancer and 21 age matched controls and showed that serum levels of n-3 fatty acids were reduced in patients with PCa. Similarly, Godley *et al.* (1996) analyzed erythrocyte membranes and subcutaneous adipose fat collected from 89 cases and 38 controls. Linoleic acid in both erythrocyte and adipose fat were positively correlated with PCa, but failed to show a protective effect of

n-3 fatty acids. More compelling evidence comes from biopsies of prostate adenocarcinomas. Significant reductions in polyunsaturated fatty acids (PUFA) with a 2-4 fold lower n-3 to n-6 ratios along with higher levels of saturated fatty acids (SFA) have been reported in malignant tissue (Chaudry *et al.,* 1994; Freeman *et al.,* 2000). The ratio of n-3/n-6 fatty acids also may have an important association with the benign and malignant status of prostatic disease. Decreased 20:4n-6 concentrations reported in malignant tissue may be due to increased metabolism of 20:4n-6 via the lipoxygenase and cyclooxygenase pathways to produce higher concentrations of eicosanoids and active metabolites (Freeman *et al.,* 2000) which have been linked to cancer progression.

1.8.2 LYCOPENE

Lycopene is a red-orange carotenoid with potent anti-oxidant activity. It is found primarily in tomatoes and tomato-derived products as well as other red fruits and vegetables (Klein, 2005). Lycopene ($C_{40}H_{56}$) is a highly unsaturated acyclic isomer of β-carotene with eleven linearly arranged double bonds, and therefore has the potential to exist in 2048 different isomers (Hadley *et al.*, 2002). Due to its highly conjugated nature, lycopene is subject to oxidative degradation and isomerization, and like other carotenoids, is vulnerable to chemical and physical factors such as light exposure, oxygen, high temperature, and pH extremes. Lycopene exists primarily as an all-trans-isomer in food, but the majority of lycopene exists in the body as a variety of cis-isomers, which may be involved in different biological reactions (Wu *et al.,* 2003). It is currently believed that the conversion of the all-trans to cis forms of lycopene in the human body occurs during digestion (Boileau *et al.*, 2002).The normal levels of lycopene in human plasma are between 0.2–0.3μM but may increase depending on foods consumed and the absorptive capacity of an individual (Wu *et al.,* 2003; Hantz *et al.,* 2005).

Lycopene has been identified as having potential anticancer properties (reviewed in Giovannucci, 2002; Rao *et al.,* 1999)). It is currently believed that there are several mechanisms by which lycopene may affect the prostate. One possible mechanism is an association between lycopene and insulin growth factor (IGF-1). High levels of IGF-1 have been linked to increased PCa risk, and high lycopene consumption has been found to be inversely related to IGF-1 levels (Wertz *et al.,* 2004). Another possible mechanism involves the inhibition of tumor growth and increased differentiation of normal cells by

increasing gap-junction communication in healthy prostate cells (Wertz *et al.,* 2004). Currently the most widely accepted mechanism involves lycopene acting as a scavenger for singlet oxygen molecules, which are thought to damage DNA and cause cancer. Large amounts of lycopene are found naturally in the prostate, as compared to other tissues (Kucuk *et al.,* 2004).

Tang *et al.* (2005) also examined the inhibitory effect of lycopene on the growth rate of DU-145 tumor xenografts in BALB/c male nude mice. Tumor growth rate in mice treated with 100 and 300 mg/kg lycopene was inhibited by 55.6 and 75.8%, respectively. Tumors did not occur when DU-145 cells were pretreated with 20μmol/L lycopene, prior to injecting them into the mice (Tang *et al.*, 2005). Herzog *et al.* (2004) studied the effect of lycopene in normal prostate tissue in rats to examine how lycopene may contribute to PCa prevention. Young rats were supplemented with 200ppm lycopene for up to 8 weeks, and uptake was measured in the individual prostate lobes. Herzog *et al.* (2004) found that lycopene accumulated in all four prostate lobes, with higher content occurring in the lateral lobe. Lycopene did not interfere with normal prostate growth in the rats. Reduced gene expression of androgen metabolizing enzymes and androgen targets were reported: decreased IGF-I expression in the lateral lobe, and transcript levels of pro-inflammatory cytokines, immunoglobulins, and immunoglobulin receptors were decreased in the lateral lobe with lycopene supplementation (Herzog *et al.*, 2004).

Hantz *et al.* (2005) examined the effect of lycopene at physiologically attainable concentrations on apoptosis, cellular proliferation, and necrosis in the LNCaP cell line. Lycopene did not affect cellular proliferation or promote necrosis but altered mitochondrial function and induced apoptosis in LNCaP cells. Lycopene was found to inhibit the growth of DU-145 and PC-3 (androgen independent) cells more than the androgen-dependent LNCaP cell line by Tang *et al.* (2005). The 50% inhibitory concentration of lycopene for these cell lines was 26.6μmol/L, 40.3μmol/L and 168.5μmol/L respectively (concentrations that are much higher than physiological levels as noted above). Lycopene treatment caused DU-145 cells to accumulate in the G_0/G_1 phase as well as undergo apoptosis in a dose-dependent manner. This rate of apoptosis was up to 42.4% lower in the DU-145 cells treated with 32μmol/L lycopene when compared with the untreated control cells (Tang *et al.*, 2005). Tang *et al.* (2005) suggest

that their experiments provide evidence that lycopene may specifically inhibit the growth of androgen-independent prostate cancers.

A review of epidemiological evidence concluded that evidence is scant and inconclusive, with regards to lycopene (Kristal and Cohen, 2002). A principal problem highlighted by the authors was the lack of sensitive instruments to detect and quantify dietary intake of lycopene and suggest the enhancement of nutrient databases to reflect foods rich in lycopene. Another recommendation was the recognition of the bioavailability of lycopene from different food sources which is affected by heat processing and consumption with fat. Not unlike the epidemiological problems encountered with the measure of dietary fatty acid intake from dietary recall, a similar situation is also evident for the determination of lycopene. Freeman *et al.* (2000) report that plasma levels of tocopherols and carotenoids are better biomarkers than self-reported dietary intakes.

The potential of lycopene to reduce cancer risk was first reported in a food frequency cohort study of 47,894 men (reviewed in Giovannucci, 2002) however, in subsequent follow-up studies this finding has not been clearly reproducible. Ford *et al.* (2002) showed significant variations in serum carotenoid concentrations, including lycopene among US children and adolescents. Optimal doses of lycopene as a dietary supplement have yet to be determined, although epidemiologic studies have suggested that 6 mg/day is beneficial, an amount that is obtainable through the diet (Everson and McQueen, 2004). Commercial supplements contain between 5 and 15 mg of lycopene per capsule, although the *trans* and *cis* content of these products isn't specified (Everson and McQueen, 2004). The bioavailability of lycopene in food has not been measured, but appears to be readily absorbed, and some dietary fat is required for its absorption (Everson and McQueen, 2004). Lycopene is considered to be safe, and no adverse effects are presently known. The only known interaction with other nutrients is enhanced absorption in the presence of β-carotene (Everson and McQueen, 2004).

Kucuk *et al.* (2002) reported post intervention levels of plasma lycopene to be 23.5ug/dl in the intervention group and 17.5ug/dl in the control group in men receiving 30mg of lycopene per day for 3 weeks before radical prostatectomy. This difference is not significant due to the great variability in plasma lycopene levels and the small

numbers of subjects in each group; however it does demonstrate a dose-dependent response. Case-control and cohort studies have been conducted to determine whether dietary intake of lycopene and other carotenoids had an etiological association with PCa. Findings indicate that a decreased risk was proportional to an increased consumption (Giovannucci *et al.*, 1995) in a dose–response relationship (Jian *et al.*, 2005).

Ansari and Gupta (2004), in a prospective study investigated the efficacy of lycopene as a treatment for patients with hormone refractory PCa (HRPC), who were previously unresponsive to anti-androgen therapies. The patients had evidence of progression of disease, as measured by an increase in PSA levels of more than twice the normal PSA value. 10 mg of lycopene was administered daily to the 20 subjects over a period of 3 months. One of the patients had a complete response, which was defined as the normalization of PSA (<4ng/mL) and the disappearance of signs of the disease for at least 8 weeks. Partial response was achieved in 6 of the subjects, as defined by a 50% decrease in PSA in association with improvement for at least 8 weeks or no decline. Ten of the patients remained stable (50% decrease or 25% increase in PSA associated with no worsening or bone pain for at least 8 weeks), and the disease progressed in 3 subjects. The results of Ansari and Gupta (2004) show that lycopene supplementation appears to be an effective and safe adjuvant treatment for HRPC.

Matlaga *et al.* (2001) followed a 62-year-old white male who supplemented with lycopene and saw palmetto, as an alternative therapy for HRPC. Prior to supplementation his PSA was 365.0ng/ml. PSA decreased to 139.6ng/ml within one month and future decreased to 8.1ng/ml in the subsequent month, and remained between 3 to 8ng/ml for the next 18 months. A repeat bone scan demonstrated improvement of bony metastases. He continued to take 10 mg lycopene orally once daily and 300 mg saw palmetto orally 3 times daily (capsules); at the time of this publication his last follow-up had been asymptomatic (Matlaga *et al.*, 2001).

Chen *et al.* (2001) examined the effects of consumption of tomato sauce-based pasta dishes on lycopene uptake, oxidative DNA damage, and PSA levels in patients with PCa (localized adenocarcinoma). Patients consumed 30 mg of lycopene/day in the form of pasta sauce, prior to radical prostatectomy. Following this dietary intervention, serum and prostate lycopene concentrations were statistically significantly increased, leukocyte

oxidative DNA damage, and prostate tissue oxidative DNA damage was also statistically significantly lower in men who had the intervention with lycopene. Serum PSA levels decreased after the intervention, from 10.9ng/mL to 8.7ng/mL.

1.8.3 VITAMIN E

Vitamin E is found in vegetable oils, nuts, egg yolk, margarine, parmesan and cheddar cheeses, chick peas, soya beans, wheat germ, oatmeal, avocado, olives, carrots, green leafy vegetables, sweet potatoes, tomatoes and watercress (Willis and Wians, 2003). It has been suggested that certain forms of vitamin E may have a potential role as an anticancer agents (Jiang *et al.*, 2004). The precise mechanisms for vitamin E's benefits are largely unknown, however it is know to scavenge free radicals, inhibit peroxidation of lipid membranes and reduce carcinogen-induced DNA damage (reviewed by, Fleshner and Kucuk, 2001).

Vitamin E is a fat-soluble vitamin that consists of a group of tocols and tocotrienols that are hydrophobic in nature, but they possess a hydroxyl substituent that confers amphipathic character properties (Wang and Quinn, 2000). Vitamin E has eight different forms: α β γ and δ-tocopherols and the α β γ and δ-tocotrienols (Lippman *et al.*, 2005). All forms of vitamin E are absorbed, although the tocopherols are of biological importance with α-tocopherol possessing the most potency (Wang and Quinn, 2000). α-tocopherol has the ability to bind highly reactive and genotoxic electrophiles and prevent the propagation of these free radicals and the damage induced by them in cellular DNA and biological membranes (Willis and Wians, 2003). Only certain stereoisomers of α-tocopherol are maintained in the human plasma, therefore only α-tocopherol is considered to contribute towards the recommended daily allowance for vitamin E and is also the most common form of vitamin E found in supplements (Lippman *et al.*, 2005).

Due to the fat solubility of these compounds, and their relative in solubility in water, they are primarily found in lipid containing membranes (Willis and Wians, 2003), where its acts as a lipid antioxidant, protecting polyunsaturated membrane lipids against free radical attack (Wang and Quinn, 2000). Vitamin E is able to scavenge free radicals by donating its phenolic hydrogen atom to fatty acyl or oxygen (O_2^-) (Willis and Wians, 2003). It is also thought that Vitamin E acts as a membrane stabilizer through the action of forming complexes with the products of membrane lipid hydrolysis (Wang and Quinn,

2000). The average daily intake in the US of α-tocopherol is 10 mg for men and 7 mg for women, which is lower than the recommended 15 mg, according to the Institute of Medicine Food and Nutrition Board (Lippman *et al.*, 2005). The tolerable upper limit of vitamin E supplementation is 1000 mg/day in adults and the LD_{50} (lethal dose to kill half of tested animals) is 2000 mg/day in rats, mice and rabbits (Lippman *et al.*, 2005).

Recent studies indicate that γ-tocopherol may be important to human health and that it possesses unique features distinguishing it from α-tocopherol. It appears to be a more effective trap for lipophilic electrophiles and is well absorbed and accumulates to a significant degree in some human tissues. γ-tocopherol and 2,7,8-trimethyl-2-(β-carboxy-ethyl)-6-hydroxychroman (γ-CEHC) inhibit cyclooxygenase activity and therefore possess antiinflammatory properties. The metabolite of γ-tocopherol, γ-CEHC has natriuretic activity that the metabolite of α-tocopherol does not possess (Jiang *et al.*, 2001). Jiang *et al.* (2001) suggest that the properties of γ-tocopherol should be further evaluated because high doses of α-tocopherol deplete plasma and tissue γ-tocopherol where as γ-tocopherol supplementation increases levels of both. NO_x species are trapped more effectively by γ-tocopherol than by α-tocopherol, which may make this an important *in vivo* mechanism for trapping nitrogen oxide and preventing the cellular damage that can occur from the formation of reactive nitrogen and nitric oxide free radicals (Willis and Wians, 2003).

Siler *et al.* (2004) showed in the MatLyLu Dunning model of PCa that vitamin E (supplementation) accumulated in the tumor tissue, and that it was capable of regulating local gene expression. Vitamin E reduced androgen signaling without affecting androgen metabolism in this study. Siler *et al.* (2004) indicate that their findings may suggest that vitamin E contributes to the reduction of PCa by interfering with internal autocrine or paracrine loops of sex steroid hormone, growth factor activation/synthesis and signaling in the prostate.

The results of Fleshner *et al.* (1999) suggests that the mechanism of dietary fat induced growth of human PCa cells (LNCaP) in nude mice is mediated by oxidative stress, which raises the possibility of a therapeutic benefit of vitamin E in preventing PCa, due to decreased tumor growth in mice fed high fat diets that were supplemented with vitamin E, as compared to mice fed high fat diets alone.

The RRR-α-tocopheryl succinate derivative of vitamin E (VES – Vitamin E succinate), inhibits the proliferation of the metastatic human prostatic cancer cell lines, LNCaP, PC-3, and DU-145 (Fleshner *et al.,* 1999). According to Israel *et al.* (1995), DNA synthesis in the three cell lines is inhibited at 5, 10, and 20 micrograms/ml in a dose-dependent manner. Contrary to the results of Israel *et al.* (1995), Jiang *et al.* (2004) report that γ-tocopherol, but not α-tocopherol inhibits proliferation of PC-3 and LNCaP PCa cells. Jiang *et al.* (2004) also report that γ-tocopherol has no effect on normal prostate epithelial cells (PrEC), and the combination of γ-tocopherol and α-tocopherol exhibit additive or synergistic inhibitory effects, Jiang *et al.* (2004) also emphasize other immunomodulatory effects of γ-tocopherol. Another study by Israel *et al.* (2004), found that VES triggers apoptosis in human prostate carcinoma cells (LNCaP and PC-3) but not normal prostate cells (PrEC), as well as modulating Fas signaling. The SELECT trial in humans did not use VES because although it is very active *in vitro*, it is efficiently hydrolyzed before absorption and has no biologic activity with oral administration in humans (Lippman *et al.,*2005).

Epidemiological studies have associated high intakes of vitamin E with a reduced PCa risk (Siler *et al.,* 2004). In a study conducted by Eichholzer *et al.* (1996) low vitamin E levels in smokers were related to an increased risk for PCa, a finding supported in a cohort study conducted by Chan *et al.* (1999), which found that vitamin E supplementation reduced the risk of PCa in smokers, but was not generally associated with PCa risk. The outcomes from the Selenium and Vitamin E Cancer Prevention Trial (SELECT) (a research study to determine if selenium and vitamin E can help prevent PCa) are currently being awaited (SELECT, 2005).

The current literature demonstrates a strong role for the involvement of nutrients in cancer progression and development. Although it is currently difficult to assess the efficacy of nutritional supplementation as there are no known biomarkers, by which to do this. Therefore studies linking nutrients to a mechanism that can inhibit cancer progression are needed.

1.9 LITERATURE CITED

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Table 1-1 Androgen Receptor (AR), Prostate Specific Antigen (PSA) and androgen response properties and origins of RWPE-1, 22Rv1, LNCaP, DU-145 and PC-3 prostate cancer cell lines.

Cell Line	*RWPE-1*	*22Rv1*	*LNCaP*	*DU-145*	*PC-3*
Type	Normal	Tumor	Tumor	Tumor	Tumor
Origin	Normal Human Prostate Cells	Xenograft from Mice	Human Lymph Node Metastasis	Human Brain Metastasis	Human Bone Metastasis
Androgen Receptor	Yes	Yes	Yes	Weak Expression	Weak Expression
Androgen Responsiveness	Yes	Yes	Yes	No	No
PSA Producing	Yes	Yes	Yes	No	No

Table 1- 2 Integrins of interest and their potential involvement in prostate cancer progression

Integrin	*Involvement*	*Reference*
$\alpha_v\beta_3$	Bone Metastasis	Cooper *et al.*, 2002
$\alpha_3\beta_1$	Bone Metastasis	Kostenuik *et al.*, 1997
$\alpha_2\beta_1$	Cell Adhesion	Stewart *et al.*, 2004
$\alpha_6\beta_1$	Invasion	Edlund *et al.*, 2001
$\alpha_6\beta_4$	Expression Lost in Carcinomas	Knox *et al.*, 1994

2. STUDY RATIONALE

2.1 RATIONALE

It is widely accepted that diet plays an important role in cancer development; however, there is a lack of information relating food components to mechanisms associated with the development and growth of malignant cells. At the present time, there is limited information on the efficacy of specific dietary nutrients on prostate cancer (Wynder *et al.,* 1994). Current practice is to advise patients to follow a "prudent" diet; however, there is no empirical basis on which to define what nutritional recommendations that may encompass. The demographics of Western Countries such as Canada is shifting towards an increasingly older population, which is at risk for prostate cancer (Canadian Cancer Statistics, 2001). Prostate cancer is the most frequently diagnosed cancer in men over the age of 50 and currently there is no efficacious treatment, exemplifying the need for identification of potential targets of anti-tumor therapies.

Cell culture is prominently used for the study of human prostate cancer (PCa). Animal models are also available for the study of this disease, but due to dissimilarities in anatomy and morphology of rodent and human prostates, along with the absence of spontaneous prostate cancer in rodents (Abate and Shen, 2000), cell culture becomes an attractive experimental model. Due to difficulties associated with primary culture of prostate cells, several cell lines have been immortalized from various sources. Cell lines related to different progressive and metastastic stages of PCa, ranging from a normal prostate epithelial cell phenotype to brain and bone metastases, are available but have never been studied simultaneously for markers that may correlate with greater malignant potential.

Almost all reports in the literature investigating prostate cancer using cell culture models supplement the culture media with fetal calf serum (FCS). Interpretations from these studies are erroneous because nutritionally deficient media is used which affects how cells grow and develop. An absence or imbalance of nutrients such as the essential fatty acids impacts on cellular signaling and gene expression. FCS is also devoid of testosterone and other nutritional components found normally in human serum. To better

reflect the development of prostate cancer in humans, testosterone and other nutritive components in culture media is required.

Most of the cell lines available for PCa research have been isolated from metastatic lesions rather than primary tumors (Abate and Shen, 2000; Table 1-1). Numerous phenotypic differences distinguish primary cultures of prostatic epithelial cells from established prostate cancer cell lines. These differences are assumed to reflect features of normal versus malignant biology, when perhaps they reflect different culture conditions, or differences between short-term culture and immortalized cell lines (Peehl, 2004). This raises concerns about how well cell lines represent primary adenocarcinomas of the prostate, as well as questions regarding how long term cell culture conditions can alter the biological properties of cell lines (Peehl, 2004). For these reasons and due to the appropriateness of using human cells for studies of human cancers, it is important to have established methods of primary culture for malignant prostate cancer cells.

The evidence to date demonstrating a role for various bioactive nutrients in the prevention and treatment of prostate cancer is qualitative and suggests that a relationship exists, but the underlying mechanisms involved are poorly understood. Therefore, the objectives of this work are to identify biomarkers expressed in human prostate cancer that relate to clinical pathology and to evaluate changes in the expression of these biomarkers induced by specific nutrients. Target proteins affected by specific nutrients need to be defined to direct future investigations in this area of research. It is clear that a greater understanding linking nutrients, specific cellular and molecular changes in the growth of androgen dependent and independent prostate cell types is necessary. Understanding the mechanism of how bioactive nutrients modulate the growth of prostate cancer cells will aid in providing rationale for dietary recommendations, with respect to these nutrients, for patients at risk and with prostate cancer.

2.2 OBJECTIVES AND HYPOTHESES

The overall objective of this thesis research is to examine the effects of lycopene, Vitamin E and fish oil at physiologically relevant levels on growth, integrin expression, PSA production in a range of prostate cancer cell lines, when these cell lines are cultured in media containing HS.

1. Comparison of PCa Cell Lines Cultured in Media Supplemented with HS or FCS

Objective: To establish growth of cell cultures in HS and assess changing the supplementation of the media from FCS to HS will affect the growth rate, PSA production and expression of the $\alpha_v\beta_3$, VR, $\alpha_2\beta_1$ integrins in a series of cell lines representing increasing invasiveness (RWPE-1, 22Rv1, LNCaP, DU-145 and PC-3).

Hypothesis: It is hypothesized that:

i. Growth rates of the cell lines cultured in media supplemented with HS will be greater than the growth rates of the same cell lines cultured in media supplemented with FCS.

ii. PSA levels of the cell lines cultured in media supplemented with HS will be different than the PSA levels of the same cell lines cultured in media supplemented with FCS. Specifically, PSA levels will be higher in HS than in FCS, due to the fact that FCS is devoid of testosterone, and due to the relationship of PSA production to androgen production.

iii. The difference in fatty acid composition of the supplement (HS or FCS) will affect proteins that reside in cell membranes, therefore the expression of integrins (transmembrane proteins) will differ between the two culture conditions.

iv. The expression of the $\alpha_v\beta_3$, VR integrins will be highest in the PC-3 cell line, as this cell line is derived from a bone metastasis (Kaighn *et al.*, 1979), and these integrins migrate to VN which is expressed in mature bone, the preferential site of prostate cancer metastasis (Zheng *et al.*, 1999).

2. Culturing Primary Prostate Epithelial Cultures

Objective: To establish a method for the isolation and culture of epithelial cells from human prostates obtained through radical prostatectomy, and obtain preliminary growth and integrin data on these samples.

Hypothesis: It is hypothesized that:

i. A method for the isolation and culture of epithelial cells from human prostates will be established, by optimizing previous methods outlined in the literature.

ii. More than $1x10^6$ cells per every ~2.5 gram sample of tissue from radical prostatectomies will be obtained.

iii. Expression of the $\alpha_v\beta_3$, VR and $\alpha_2\beta_1$ integrins will be similar to those found in the more invasive cell lines, as the human samples will be derived from patients diagnosed with prostate cancer.

3. The Effects of Lycopene, Vitamin E and Fish Oil on Growth and Integrin Expression in Cell Lines Cultured in HS.

Objective: To examine the effects of physiologically relevant concentrations of lycopene, vitamin E or fish oil on growth rates and $\alpha_v\beta_3$, VR, $\alpha_2\beta_1$ integrin expression of RWPE-1, 22Rv1, LNCaP, and PC-3 cell lines.

Hypothesis: It is hypothesized that:

i. Physiologically relevant concentrations of lycopene, vitamin E or fish oil will result in decreased growth rates of all the cell lines, due to multiple reports in the literature that claim these nutrients have protective effects against prostate cancer development and anti-tumor properties.

ii. Integrin expression will decrease in all cell lines with the addition of lycopene, Vitamin E or fish oil. Adhesion and extravastion are characteristic properties of malignant cells, thus changes in malignant potential by these nutrients will be reflected in integrin levels.

2.3 CHAPTER LAYOUT

The hypotheses posed were tested in a sequence of experiments. These experiments are organized as thesis chapters.

Chapter 3 characterizes the differences in growth, PSA levels and integrin expression of cell lines grown in media supplemented with FCS compared to HS. It establishes

standard culture model for human prostate cell lines, and confirms that this model is representative of the human condition. **(Hypothesis 1: i, ii, iii, iv)**. This chapter is entitled "**Comparison of prostate cancer cell line characteristics cultured in media containing human serum or fetal calf serum"** has been in part presented at Experimental Biology 2006, San Francisco, CA, April 2006, and published in part in abstract form. (Hurdle, H.L., Mazurak, V.C., Clandinin, M. T. Characteristics of *in vitro* prostate cancer models grown in human serum. FASEB J. LB467)

Chapter 4 consists of *in vitro* primary cell culture work that isolated and cultured epithelial cells from human prostate samples obtained from, as well as presenting preliminary integrin data on these samples **(Hypothesis 2: i, ii, iii)**. This chapter is entitled "**Culturing primary prostate cancer epithelial cultures from human prostate samples."**

Chapter 5 Examines the effects of lycopene, Vitamin E and fish oil on the growth, PSA, and $\alpha_v\beta_3$, VR, $\alpha_2\beta_1$ integrin expression of RWPE-1, 22Rv1, LNCaP, and PC-3 cell lines **(Hypothesis 3: i, ii)**. This chapter is entitled "**Cell line growth and integrin expression with the addition of physiologically relevant levels of lycopene, vitamin E and fish oil to culture media."**

Chapter 6 is an overall general summary and discussion of primary cell culture, the culturing of cell lines in HS, integrin expression and growth rates of the cell lines and the relationship to the nutrients examined in this thesis.

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3. COMPARISON OF PROSTATE CANCER CELL LINE CHARACTERISTICS CULTURED IN MEDIA CONTAINING HUMAN SERUM OR FETAL CALF SERUM[1]

3.1 INTRODUCTION

Cell Culture and Prostate Cancer Cell Lines

Cell culture is prominently used for the study of human PCa. Animal models are also available for the study of this disease, but due to dissimilarities in anatomy and morphology of rodent and human prostates, along with the absence of spontaneous PCa in rodents (Abate and Shen, 2000), cell culture becomes an attractive experimental model. Due to difficulties associated with primary culture of prostate cells, several cell lines have been immortalized from various sources. Cell lines related to different progressive and metastastic stages of PCa, ranging from a normal prostate epithelial cell phenotype to brain and bone metastases, were selected for our studies (Table 1-1).

The RWPE-1 cell line is derived from histologically normal epithelial cells from a Caucasian adult human prostate, initially isolated and transfected with a plasmid carrying one copy of the human papillomavirus 18 (HPV-18) genome (Bello *et al.,* 1997). The 22Rv1 cell line is derived from a human prostatic carcinoma xenograft, CWR22R (Sramkoski *et al.*, 1999). The Lymph Node Carcinoma of the Prostate (LNCaP) cell line was isolated from a needle aspiration biopsy of the left supraclavicular lymph node of a Caucasian male with metastatic PCa (Horoszewicz *et al.*, 1983). The Dura-145 (DU-145) cell line was isolated from a lesion in the brain of a Caucasian male with metastatic PCa (Stone *et al.*, 1978). Prostate Cancer 3 (PC-3) cell line was established from a bone metastasis of a grade IV prostatic adenocarcinoma Caucasian male (Kaighn *et al.*, 1979). The RWPE-1, 22Rv1, and LNCaP cell lines express the androgen receptor, are androgen responsive and secrete PSA (Bello *et al.,* 1997; Sramkoski *et al.*, 1999; Horoszewicz *et al.*, 1983; American Type Culture Collection). The DU-145 and PC-3 cell lines are not androgen responsive and do not produce PSA (Stone *et al.*, 1978; Kaighn *et al.*, 1979;

[1] This work was presented in part at Experimental Biology 2006, San Francisco, CA, April 2006, and published in part in abstract form. (Hurdle, H.L., Mazurak, V.C., Clandinin, M. T. Characteristics of *in vitro* prostate cancer models grown in human serum. FASEB J. LB467)

American Type Cell Culture, Manassas, VA, USA), thereby representing more advanced clinical disease.

Human Serum versus Fetal Calf Serum

All reports in the literature investigating PCa using cell culture models supplement the culture media with fetal calf serum (FCS). Interpretations from these studies are erroneous because nutritionally deficient media is used which affects how cells grow and develop. An absence or imbalance of nutrients such as the essential fatty acids impacts on cellular signaling and gene expression. For example Figure 3-1 and Table 3-3 depict the large differences in composition of n-3 fatty acids, the n6:n3 fatty acid ratio, an the polyunsaturated: saturated fatty acid ratio between HS and FCS. FCS is also devoid of testosterone, a requirement for growth of many cell lines, and other nutritional components found normally in human serum. In humans, testosterone circulates as part of sex hormone binding protein and such a preparation is not currently commercially available. In order to better reflect the development of PCa in humans, testosterone and other nutritive components in culture media is required. Therefore, early work focused on optimizing culture conditions to better reflect human PCa.

PSA and Integrins

There is need for a cancer specific marker for the detection of PCa, due to the controversy that surrounds PSA (Chapter 1, Section 1.4.4). We measured the levels of PSA in the cell lines that are known to produce PSA (RWPE-1, 22Rv1 and LNCaP), to determine if there was any difference in these levels when cells were cultured in HS or FCS.

Integrins have been implicated in cell migration, survival, growth, differentiation, gene expression, and modulation of signaling pathways (Moro *et al.*, 2004), making them a marker of interest with respect to cancer progression and metastasis (Chapter 1, Section 1.7). We determined integrin expression in cell lines and in human prostate samples to identify the range of integrin expression to assess the usefulness and relevance of these potential markers as an adjunct to the use of PSA.

Serum derived from healthy human males was used to better reflect the development of PCa in humans. Growth, morphology, PSA and integrin expression, were compared for cells cultured in HS and FCS. We hypothesized that there would be

differences in growth, morphology, PSA and integrin expression by the cell lines when the media contained HS compared to FCS.

We conclude that growth of all cell lines, with the exception of RWPE-1 was significantly lower when media was supplemented with HS. Confluency of the monolayers differed for all cell lines depending on whether the media was supplemented with HS or FCS. There were no significant differences between PSA production when cells are grown in HS or FCS. There were significant differences in integrin expression in certain cell lines when media contained HS or FCS.

3.2 MATERIALS AND METHODS

Human Serum

Ethical approval was provided by the Faculty of Agriculture Forestry and Home Economics (AFHE) Health Research Ethics Board (Biomedical Panel) to obtain human serum from healthy males subjects between the ages of 18-35. Subjects had to have no history of cancer, autoimmune disease or other disease. Subjects using steroidal medications or hormone therapies, or having had surgery within the last three months were excluded.

Cell Culture Technique

RWPE-1, 22Rv1, LNCaP, DU-145 and PC-3 cell lines were obtained from the American Type Culture Collection (ATCC Manassas, VA, USA) and maintained in $75cm^2$ culture flasks (Fisher Scientific, Edmonton, Alberta, Canada). All media and media components were obtained from Gibco Invitrogen Corporation (Burlington, Ontario, Canada), unless otherwise stated. All media types contained L-glutamine and were supplemented with 1% v/v antibiotic/antimycotic solution (1×10^5 U/L penicillin, 100 mug/L streptomycin, and 25 mg/L amphotericin B).

HS was obtained from human subjects in the Human Nutrition Research Unit (HNRU) by a phlebotomist from Dynacare Kasper. Blood (50ml) was drawn from the subcubital vein and spun immediately to obtain the serum fraction. The serum was pooled and added to media (1% v/v) based on fatty acid concentrations in HS versus FCS. Cultures that were not supplemented with HS were supplemented with the standard 10% v/v FCS used in the literature.

All cell lines were maintained in the culture media recommended by ATCC (Manassas, VA, USA). RWPE-1 cells were cultured in Keratinocyte-SFM (KSFM) media supplemented with bovine pituitary extract (193ul/100ml) and epidermal growth factor (0.591ul/100ml). 22Rv1 and LNCaP were cultured in Roswell Park Memorial Institute medium 1640 (RPMI 1640) containing sodium bicarbonate (2.0 g/L), 1M HEPES (1% v/v) and 100mM sodium pyruvate (1% v/v; Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada). The DU-145 cell line was cultured in Minimum Essential Medium (MEM) containing Earle's salts, non-essential amino acids, sodium bicarbonate (2.2.g/L) and 100mM sodium pyruvate (1% v/v) (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada), and the PC-3 cell line was cultured in F-12 Nutrient Mixture. The cell cultures were grown at 37°C in 5% CO_2 at 98% relative humidity. The culture media was changed every 2-3 days, and cells passaged every 7 days, when confluency was attained, as determined using light microscopy. The media in the flasks at the time of passaging was collected and frozen immediately at -20°C for use in Prostate Specific Antigen (PSA) and interleukin 6 (IL-6) assays. 0.25% Trypsin-0.03% EDTA was added to the 75cm^2 flasks at the time of passaging to detach the cells from the flasks. The flasks were then rinsed with 4 ml of their respective media types and collected in 15ml conical centrifuge tubes (Fisher Scientific, Edmonton, Alberta, Canada). The cells were pelleted and re-suspended in media and counted using trypan blue exclusion (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada), cell viability of greater than 95% was obtained. Cell counts were recorded at every passage and $1x10^6$ cells were seeded into a total volume of 7ml containing either 10% FCS (v/v) or 1% HS (v/v). Means and standard deviations were calculated for all cell lines ($n \geq 15$) and the Student's unpaired t-tests were conducted to determine statistical significance between HS and FCS for each cell line.

PSA Measurement

Supernatants were collected at time of passage (as outlined above). The samples were kept at -20°C and thawed immediately prior to PSA measurement. PSA was measured using a total PSA Enzyme Linked Immunoassay (ELISA, Hope Laboratories Belmont, CA, USA). This assay had a sensitivity ranging from 0–100ng/ml PSA. Means and standard deviations were calculated for all cell lines and the Student's unpaired t-

tests were conducted to determine statistical significance between HS and FCS for the PSA values of each cell line.

Integrins

Passaging of the cell lines was followed as outlined above, but after obtaining the cell counts for the respective samples, cells were added to a 96 well plate that had been coated with Phosphate Buffered Saline: 4% FCS (Gibco Invitrogen Corporation Burlington, Ontario, Canada) solution for 20 minutes. The cells were added to their respective wells with a minimum of $5x10^4$ cells per well, and centrifuged for 2 minutes at 1000rpm. After centrifugation the supernatant was removed from each well and the plate was lightly vortexed to displace cells from the bottom of the wells. The appropriate integrin antibody $\alpha_v\beta_3$, VR, $\alpha_2\beta_1$ (10ug/ml) or Fluoroscein isothiocyanate (FITC) (20ug/ml) was added to the appropriate wells (Chemicon Int., Cedarlane Laboratories Ltd. Hornby, Ontario, Canada), and PBS: 4% FCS was added to control wells. The plate was incubated in the dark at 4°C for 20-30 minutes. After the incubation, cells were rinsed and vortexed three times. FITC (20ug/ml) (Chemicon Int., Cedarlane Laboratories Ltd. Hornby, Ontario, Canada) was added to all of the wells except the control wells to which 10ul PBS: 4% FCS was added. The plate was incubated in the dark at 4°C for 20-30 minutes. After the incubation the cells were washed and vortexed as above, and fixed in 300ul of PBS: 0.5% Paraformaldahyde (PFA) fixative solution. Integrin expression was analyzed using the FACScalibur machine and CellQuest Software (BDBiosciences, Bedford, MA, USA). Means and standard deviations were calculated for all cell lines and the Student's unpaired t-tests were conducted to determine statistical significance between HS and FCS for the integrin expression of each cell line.

Fatty Acid Analysis

n-3 fatty acid content of HS and FCS (in μg/ml serum) was compared and the ratios of n-6 to n-3 fatty acids and polyunsaturated to saturated fat ratios were calculated for each. An aliquot of HS (1mL) and FCS (1mL) was used for fatty acid analysis. Total lipids were extracted using Folch (Folch *et al.*, 1957) and individual PL separated by thin layer chromatography, as previously described (Pratt *et al.*, 2002). Bands corresponding to phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS) were be visualized on the silica plates by spraying with 8-

anilino-1-naphthalene-sulfonic acid (0.1% w/v) and identified using the appropriate standards. Samples were methylated immediately using 14% (w/w) BF_3/methanol and separated by gas liquid chromatography (Vista 6010, Varian Instruments, Georgetown, ON, Canada) on a fused silica BP20 capillary column (25 m x0.25 mm internal diameter, Varian Instruments, Georgetown, ON, Canada), as previously described (Pratt *et al.*, 2001).

3.3 RESULTS

Cell Growth

All cell lines grew significantly slower in HS than in FCS with the exception of RWPE-1 (Figure 3-2, Table 3-1). Confluency of the monolayers after 7 days in culture was observed using light microscopy. The confluency of all cell lines after the same number of days in passage, with the same number of cells initially seeded, was always greater for those cultured in FCS, with the confluency in the FCS being about double of that for all cell lines in comparison to those cultured in HS (Figures 3-6 through 3-10)

PSA

Total PSA was measured for the cell line supernatants at the time of passaging using a commercially available ELISA kit (HOPE Laboratories, Belmont, CA, USA). Total PSA was measured for RWPE-1, 22Rv1 and LNCaP cell lines as they produce PSA (Bello *et al.,* 1997; Sramkoski *et al.*, 1999; Horoszewicz *et al.*, 1983; ATCC Manassas, VA, USA). No significant difference was found in PSA production for all 3 of the cell lines when cells were cultured in HS and FCS (Figure 3-3).

Integrins

$\alpha_v\beta_3$, $\alpha_v\beta_3$ VR, $\alpha_2\beta_1$ levels were measured in all cell lines. There were significant differences found between the $\alpha_v\beta_3$ integrin in the RWPE-1 and LNCaP cell lines (Table 3-3), VR integrin in DU-145 (Figure 3-5), and $\alpha_2\beta_1$ in 22Rv1 and PC-3 cell lines (Figure 3-4). The expression of the $\alpha_v\beta_3$, $\alpha_v\beta_3$ VR, $\alpha_2\beta_1$ integrins were all greater than in HS for all significant differences reported (Figure 3-4, Figure 3-5 and Table 3-3).

3.4 DISCUSSION

The differences in growth we observed between the cancerous cell lines cultured in HS versus FCS, brings into question the exclusive use of FCS in the literature. A major difference between HS and FCS is the fatty acid composition, therefore culturing

the cell lines in HS better reflects the environment to which a cancer cell may be exposed, essentially more closely resembling *in vivo* growth conditions. This enables a more accurate identification of biomarkers that may be of diagnostic or prognostic importance in men with PCa. Some cell lines grow in the absence of hormones, whereas some cell lines respond to hormones (Table 1-1) and thus it is necessary to add a source of testosterone to the media, in order to be certain that the effects of the nutrients that we are observing on these cell lines is not due to the lack of testosterone. Adding human serum to the cultures enables us to make judgments about the growth of cancer based on the presence of malignant markers when cultured with and without testosterone.

RWPE-1 represents normal human prostate epithelial cells and is not a cancerous phenotype, whereas all the other cell lines utilized represent varying degrees of cancerous phenotypes. It is of interest that this was the only cell line that did not grow more slowely (Figure 3-2, Table 3-3). The LNCaP cell line exhibited the most rapid growth in FCS and the second greatest growth rate in HS (Figure 3-2, Table 3-3). However, growth was highly variable. The cell line informatics (ATCC Manassas, VA, USA) state that these cells "grow very slowly and do not become confluent" which is in conflict with our results, in fact, LNCaP became more confluent than all other cell lines. Additionally ATCC (Manassas, VA, USA) states that "the cells do not produce a uniform monolayer, but grow in clusters which should be broken apart by repeated pipetting when subcultures are prepared." This is in accordance with what was observed and may account for the large standard deviations that were obtained in the growth data for this cell line (Figure 3-2, Table 3-3).

Several modifications to improve PSA screening have been suggested, among these are: the effect of ethnicity on PSA levels; PSA velocity (measuring PSA in the blood over time, based on the assumption that PCa patients should have rapidly increasing PSA levels); PSA density (relating PSA level in blood to gland volume as measured by ultrasound, as PSA levels have been noted to be higher in men with larger prostates and it has been suggested that PSA level should be corrected for prostate size); PSA isoforms, (that is PSA not in the free form, have been reported to be related to the risk of prostate carcinoma detection) (Hernandez and Thompson, 2004). The PSA measured in this study is most similar to PSA density as we corrected for number of cells.

After correcting PSA to cell count we found that there was no significant difference between the PSA values for the cells cultured in HS and FCS, for the PSA producing cell lines (Figure 3-2). This is in spite of a significant decrease in growth of cell lines when they were grown in HS as compared to FCS (Figure 3-2, Table 3-3), emphasizing the need for a cancer specific marker.

The relationship(s) of integrins to one or more of the clinical criteria of PCa diagnosis have not been determined, although their relationship to invasiveness supports the possibility for their use as a diagnostic tool. Changes in integrin signaling and expression have been implicated in the multi-step process of metastasis including involvement in cell survival, migration, and invasion (Fornaro *et al.*, 2001). In support of this the $\alpha_v\beta_3$ went from being expressed in less than 1% of the cells in the RWPE-1, 22Rv1 and LNCaP cell lines, to being expressed in almost 10% of the cells in the PC-3 cell line with intermediate expression in the DU-145 cell line. The RWPE-1, 22Rv1 and LNCaP cell lines also exhibited very low expression ($\leq$1.6%) of the VR integrin whereas expression of this integrin occurred in over 35% of the cells in the DU-145 and PC-3 cell lines (Figure 3-5, Table 3-3). These changes demonstrate an increased expression of these integrins that corresponds to an increasing invasiveness of the cell types.

Edlund *et al.* (2001) found that immunoprecipitation with antibody to the $\alpha_v\beta_3$ heterodimer revealed nearly undetectable levels of $\alpha_v\beta_3$ in the LNCaP cell line, which is in agreement with our findings, and those of Zheng *et al.* (2001). Edlund *et al.* (2001) concluded that the use of the $\alpha_v\beta_3$ heterodimer does not appear to be important for laminin attachment in the LNCaP cells, and that LNCaP cells attached to laminin primarily with $\alpha_6\beta_4$. Characterization $\alpha_v\beta_3$ expression in a variety of cell lines by Cooper *et al.* (2003), determined that PC-3 cells expressed the greatest amount of this particular integrin in comparison to the LNCaP and DU-145 cell lines. Zheng *et al.* (2001) also reported that PC-3 expressed the $\alpha_v\beta_3$ integrin. The findings of Cooper *et al.* (2003) and Zheng *et al.* (2001) are in agreement with our results. Zheng et al. (2001) found that PC-3 cells adhered to and migrated on vitronectin (VN), which is an $\alpha_v\beta_3$ ligand expressed in mature bone where PCa cells preferentially metastasize.

LNCaP cells did not adhere to or migrate on VN (Zheng *et al.*, 2001), also in agreement with our findings (Figure 3-5, Table 3-3). $\alpha_v\beta_3$ expression is unusual in

epithelial cells, as it is usually expressed in lymphocytes and other migratory cell types, but is common to a number of bone metastases (Stewart *et al.* 2004), therefore the lack of expression of $\alpha_v\beta_3$ in RWPE-1, 22Rv1 and LNCaP cell lines is consistent with the expression of $\alpha_v\beta_3$ in epithelial cells, as none of these cell lines represent bone metastases. The DU-145 cell line expresses the $\alpha_v\beta_3$ and VR in similar levels to those of PC-3. Although the DU-145 cell line is not a bone metastases it represents a highly invasive phenotype, which may explain why it expresses integrins not normally found in epithelial cells.

Antibody function blocking studies revealed that PC-3 cells utilize $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins to adhere to collagen I (Kiefer *et al.*, 2001), which would correlate with our results due to the high levels of $\alpha_2\beta_1$ expressed by the PC-3 cell line. The large percentage of the $\alpha_2\beta_1$ integrin expressed in all of the other cell lines may indicate that these cell lines also use $\alpha_2\beta_1$ to adhere to collagen I. There is a marked decrease in expression of $\alpha_2\beta_1$ in the 22Rv1 and LNCaP cell lines in comparison to the RWPE-1 (normal epithelial phenotype) and DU-145 and PC-3 (most invasive phenotype), which is of interest because it may represent a shift in the ability of the cancer cells to adhere to collagen I over the progression of the disease. Adhesion of cells to collagen I is also of importance as it is a major component of mineralized bone matrix (Stewart *et al.*, 2004), and metastasis to the bone occurs in 90% of PCa cases (Tantivejkul *et al.*, 2004). Therefore it is appropriate that the PC-3 cells express $\alpha_2\beta_1$ at levels greater than 85%, as this cell line was originally derived from a bone metastases.

In conclusion, the differences seen in cell growth, integrin expression, and cell morphology when the cell lines are grown in HS as compared to FCS support the validity of growing prostate cell lines in HS rather than FCS. The changes demonstrate that further investigation is warranted with regards to other properties of these cell lines when the form of supplementation is changed. The fact that there were no significant differences in PSA production with different supplementation of the culture media only furthers the controversy that surrounds the use of PSA as a marker for PCa, and emphasizes the need to find a cancer specific marker for this disease. Integrins relate to invasive potential and may thereby serve as a biomarker upon which to test efficacy of nutritional interventions.

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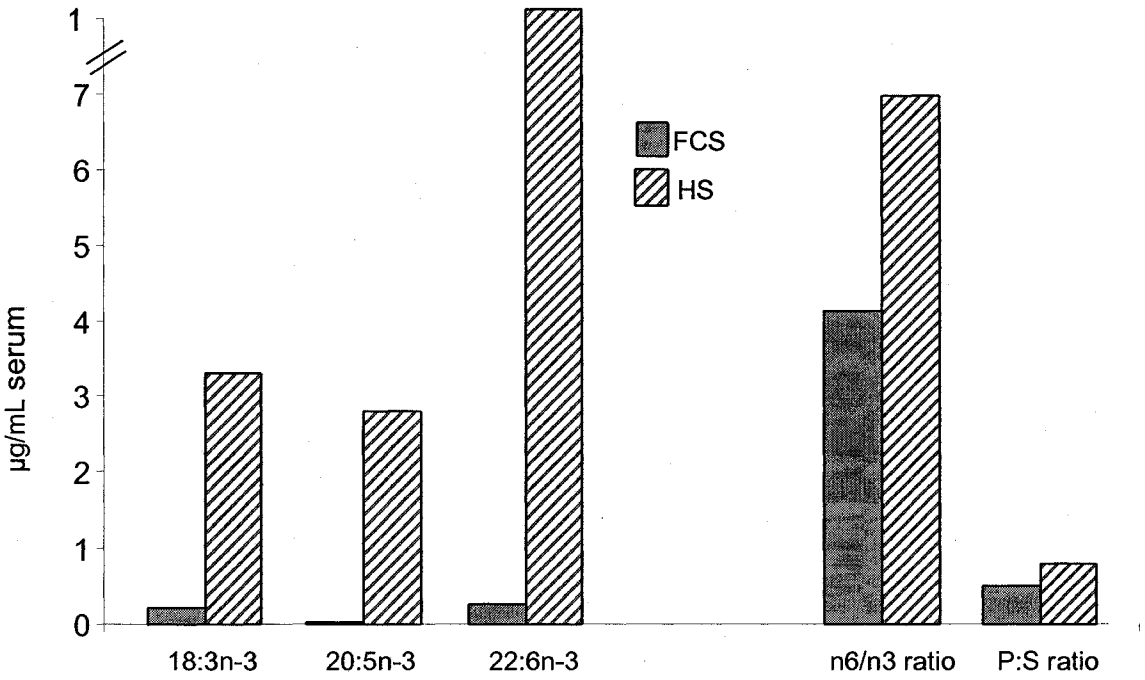


Figure 3-1 Comparison of n-3 fatty acid content in HS and FCS (in µg/ml serum): Ratios of n-6 to n-3 fatty acids and polyunsaturated to saturated fat ratios for each. Samples were analyzed using 1 ml of each serum type using gas liquid chromatography.

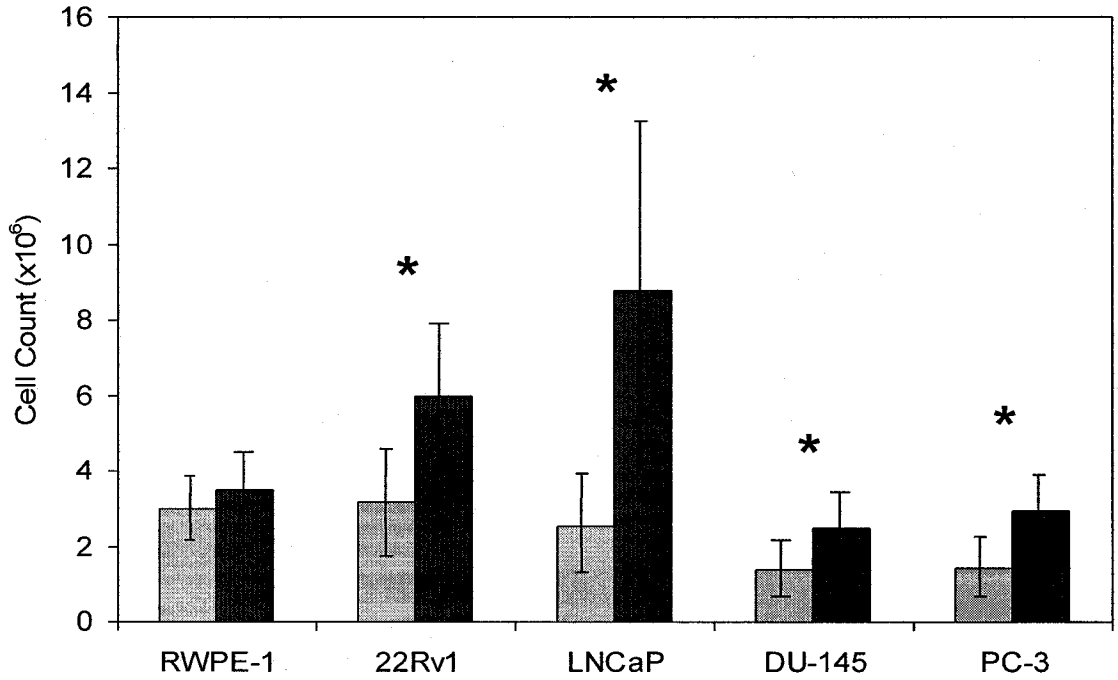


Figure 3-2 Comparison of cell growth for RWPE-1, 22Rv1, LNCaP, DU-145 and PC-3 cell lines cultured in HS and FCS. Viable cells were counted to determine cell population growth after 7 days in culture, as described in "Materials and Methods". (Bars represent the means $\pm$ SE of 15 to 22 counts; dark bars represent FCS; * signify statistical significance between HS and FCS for the cell line; $p<0.01$).

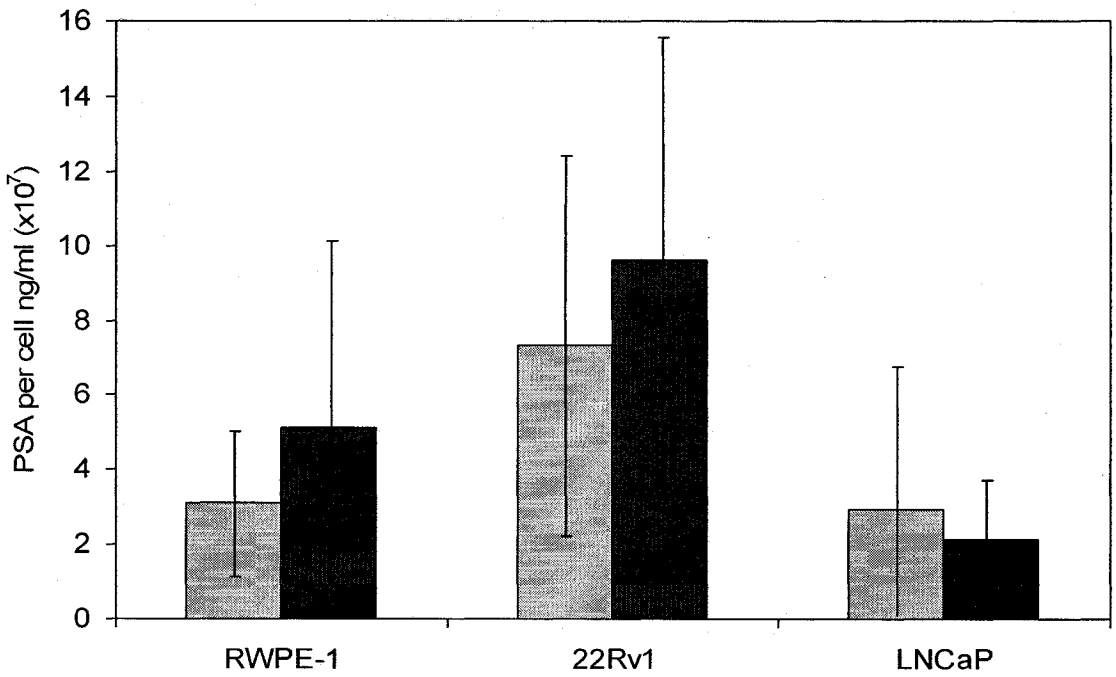


Figure 3-3 Comparison of PSA levels in RWPE-1, 22Rv1 and LNCaP cell lines cultured in HS and FCS. Supernatants from cell culture were analyzed using a colometric PSA ELISA assay from HOPE Laboratories as described in "Material and Methods". (Bars represent the means $\pm$ SE; n = 3-15; dark bars represent FCS; * signify statistical significance between HS and FCS for the cell line; $p<0.05$).

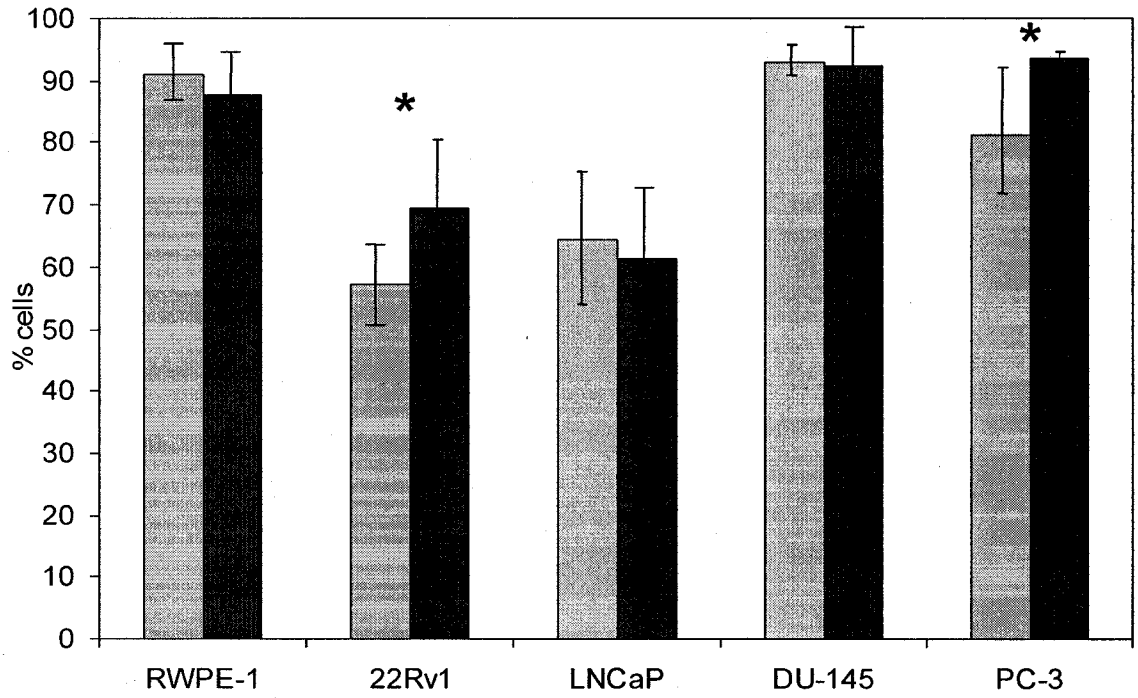


Figure 3-4 $\alpha_2\beta_1$ integrin expression in RWPE, 22Rv1, LNCaP, DU145 and PC3 cell lines grown in human serum (HS) and fetal calf serum (FCS) (Bars represent the means $\pm$ SE; $n \geq 10$; dark bars represent FCS; * signify statistical significance between HS and FCS for the cell line; $p<0.05$).

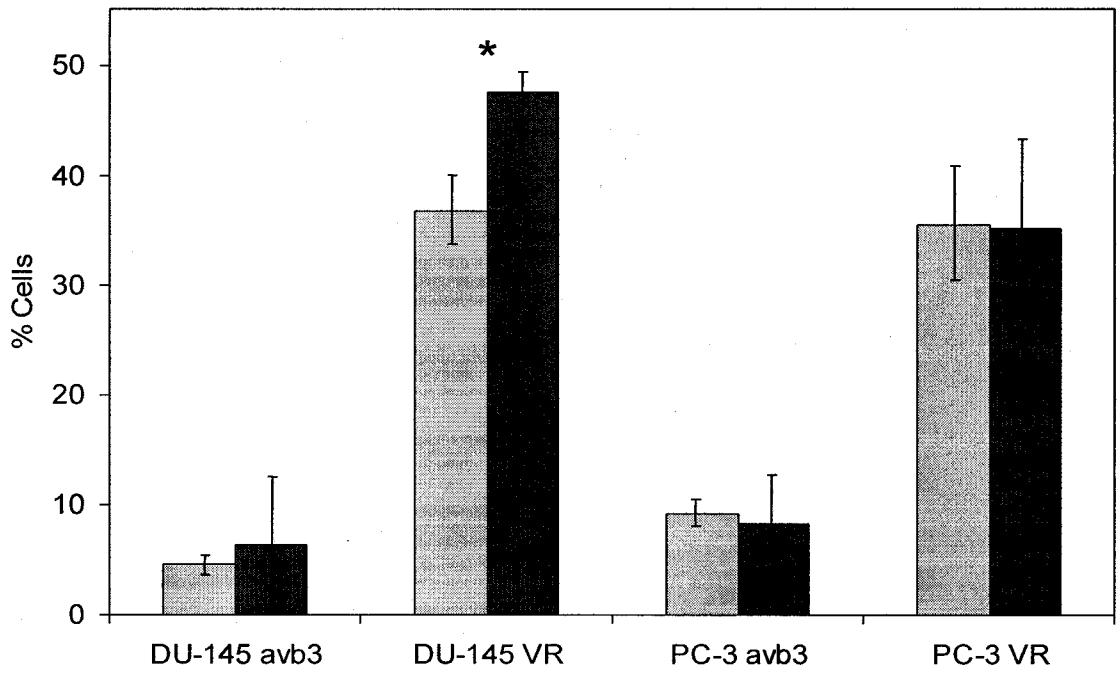
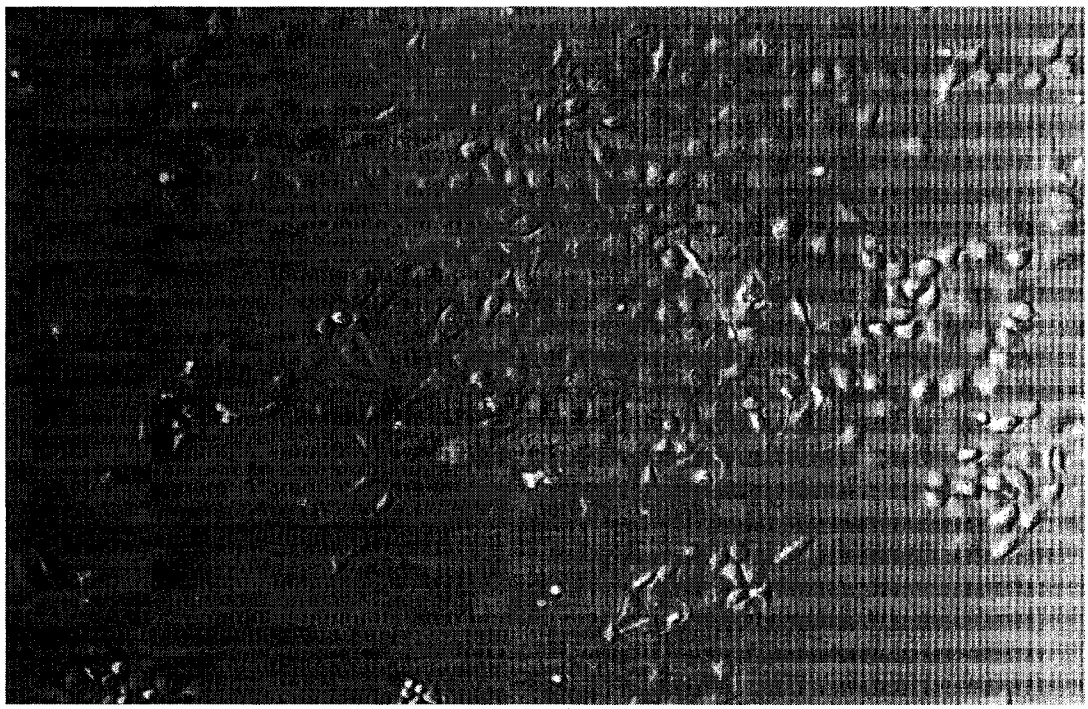


Figure 3-5 $\alpha_v\beta_3$ and $\alpha_v\beta_3$ VR integrin expression in DU-145 and PC-3 cell lines grown in human serum (HS) and fetal calf serum (FCS) (Bars represent the means $\pm$ SE; $n \geq 10$; dark bars represent FCS; * signify statistical significance between HS and FCS for the cell line; $p<0.05$).



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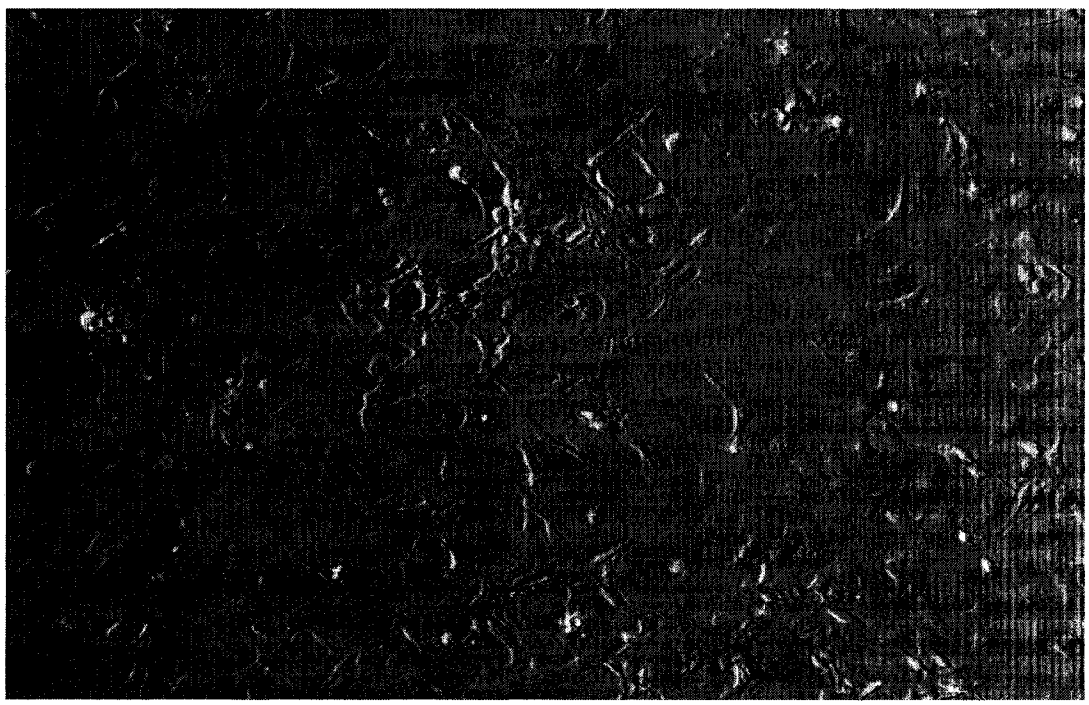

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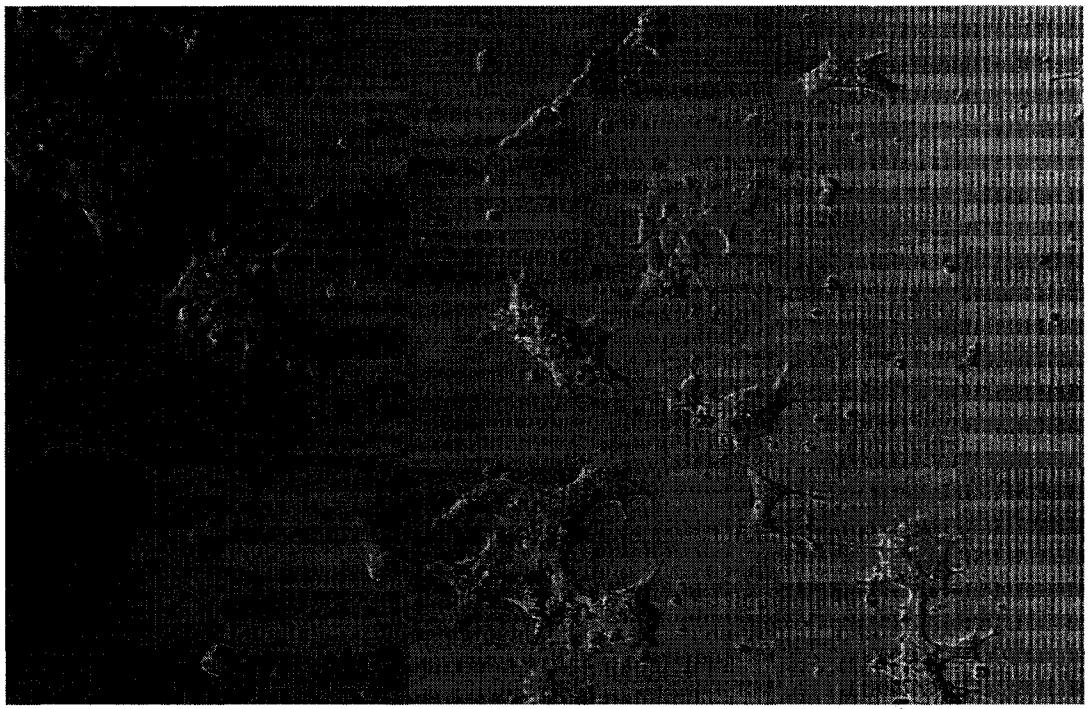
Figure 3-6 Light microscopy photographs of the RWPE-1 in 1% HS[1] and 10% FCS[2]; days after $1x10^6$ cells were seeded in $75cm^2$ flasks (Passage 7, February 13, 2006).



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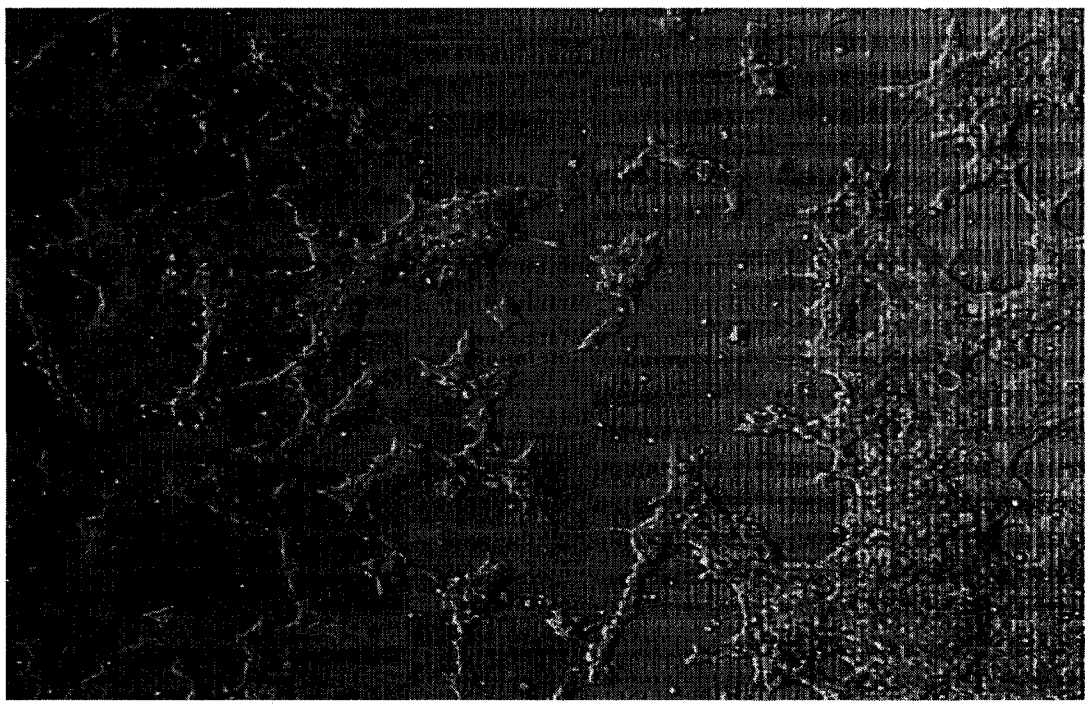
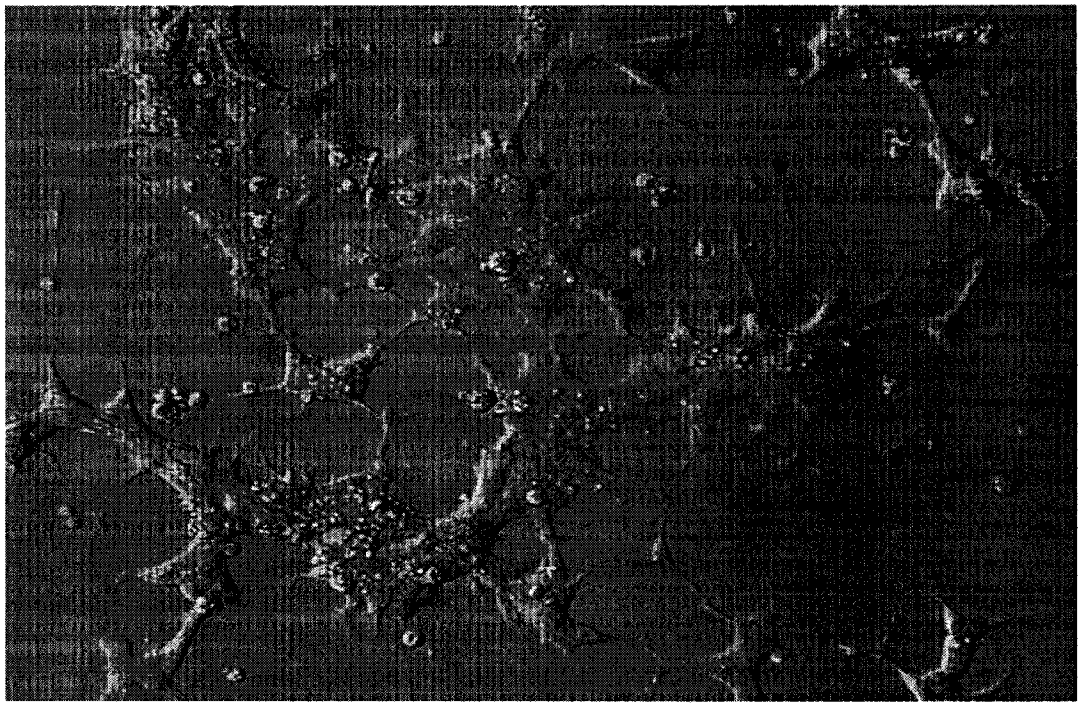


2

Figure 3-7 Light microscopy photographs of the 22Rv1 in 1% HS[1] and 10% FCS[2]; 7 days after $1x10^6$ cells were seeded in $75cm^2$ flasks (Passage 26, February 13, 2006).



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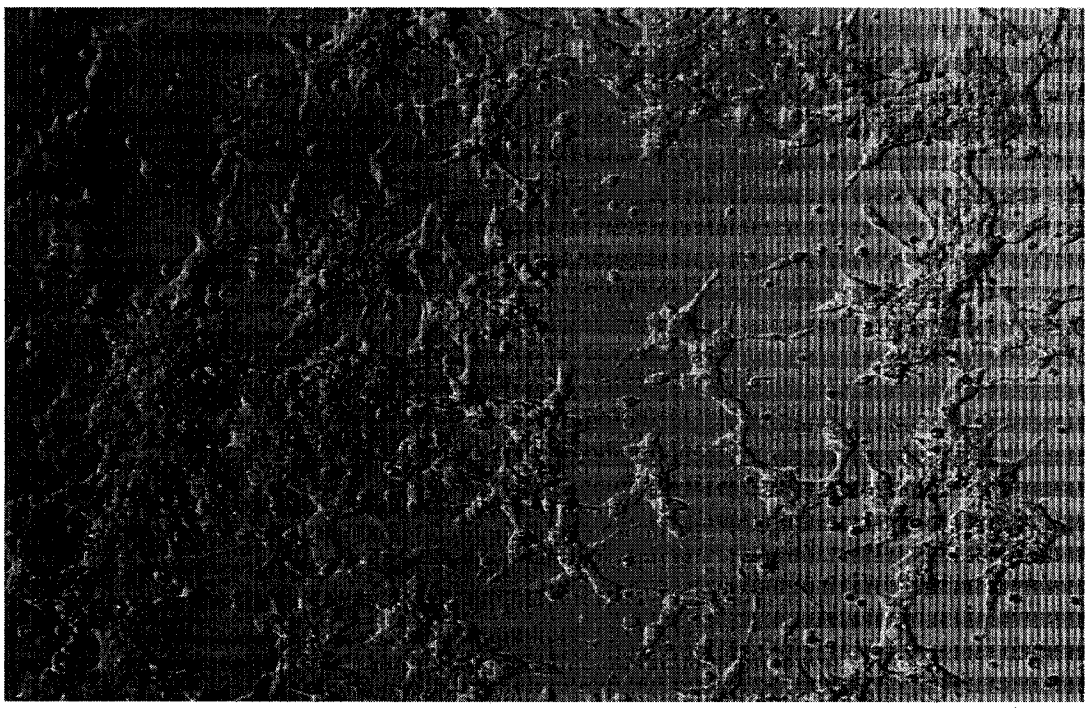
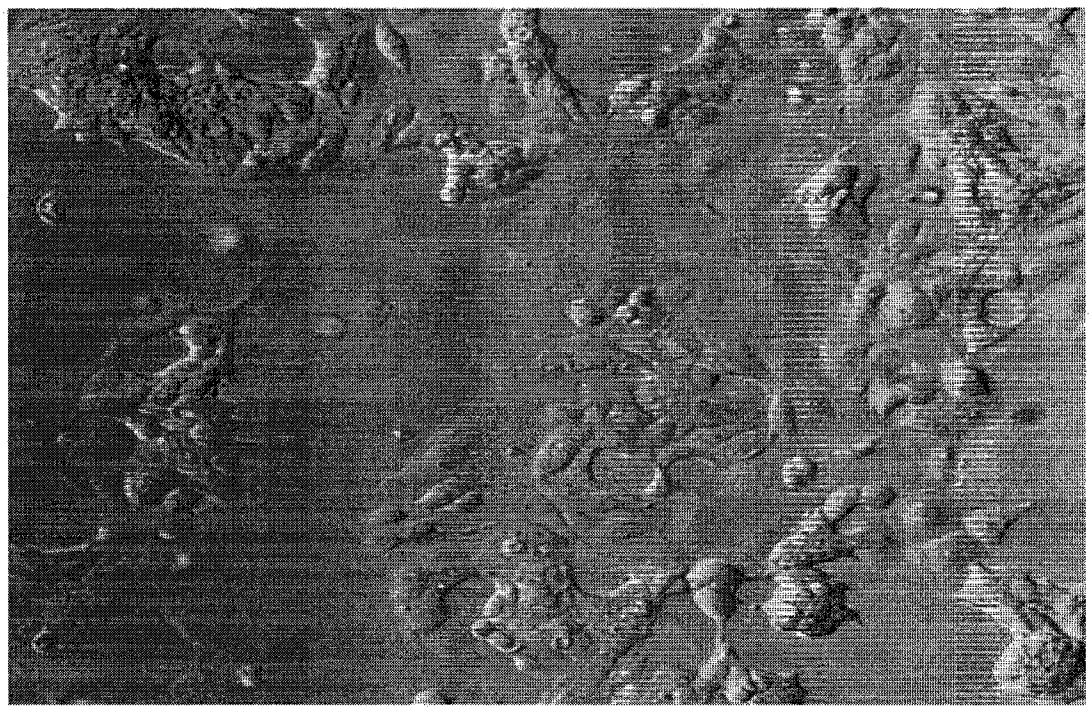


2

Figure 3-8 Light microscopy photograph of the LNCaP in 1% HS[1] and 10% FCS[2]; 7 days after $1x10^6$ cells were seeded in $75cm^2$ flasks (Passage 25, January 24, 2006).



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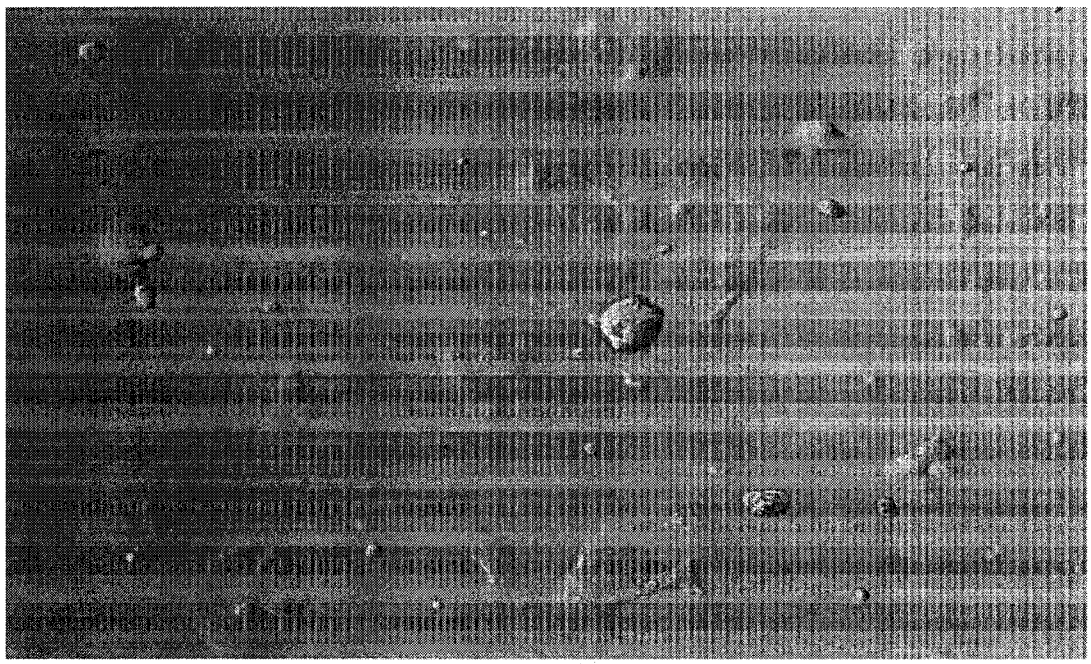
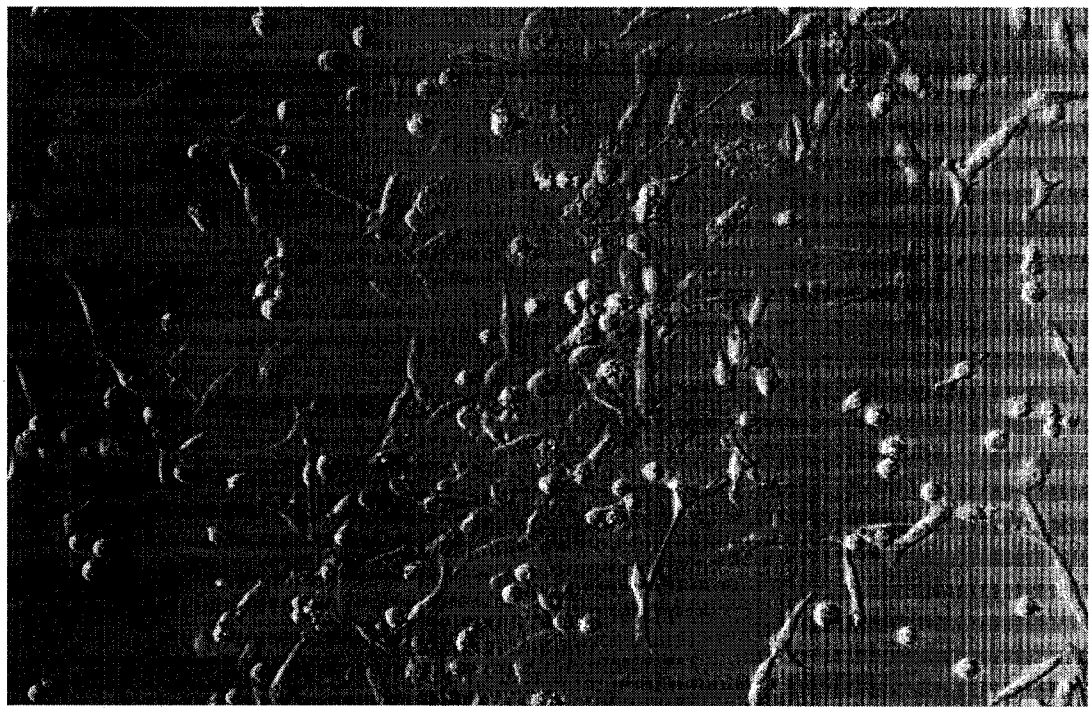


2

Figure 3-9 Light microscopy photograph of the DU-145 in 1% HS[1] and 10% FCS[2]; 7 days after $1x10^6$ cells were seeded in $75cm^2$ flasks (Passage 14, June 26, 2006).



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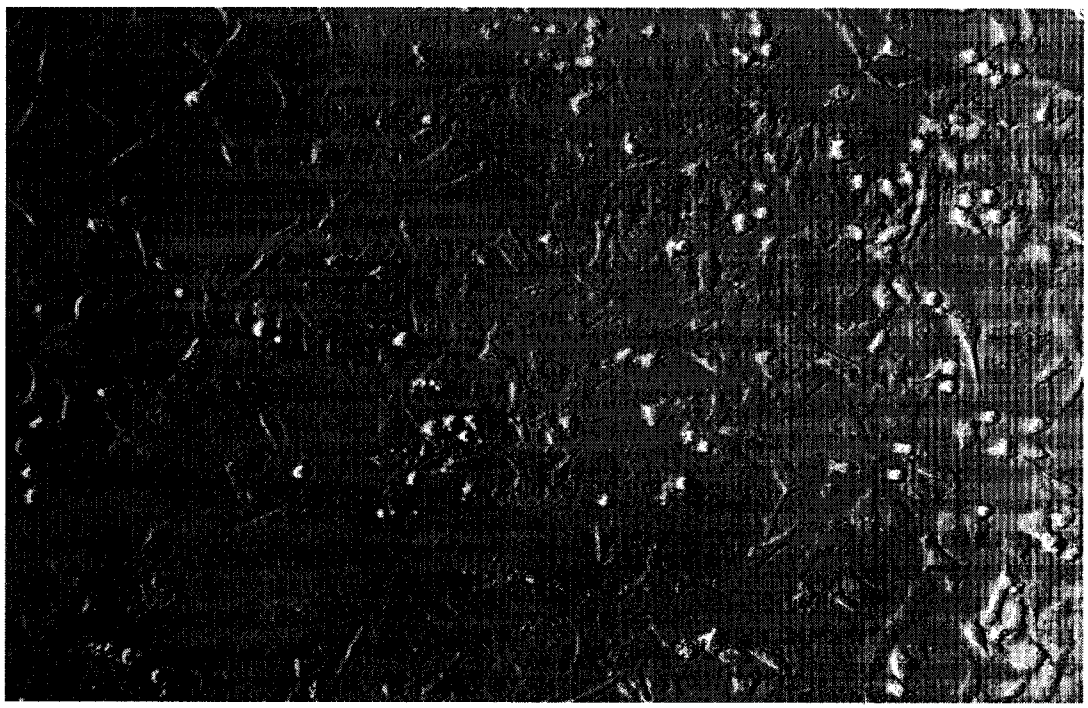

2

Figure 3-10 Light microscopy photograph of the PC-3 in 1% HS[1] and 10% FCS[2]; 7 days after $1x10^6$ cells were seeded in $75cm^2$ flasks (Passage 8, February 13, 2006).

Table 3-1 Comparison of serum fatty acid content of fetal calf serum (FCS), healthy adults and breast fed babies. Values determined using liquid gas chromatography, all values in µg/ml.

Fatty Acid	*Fetal Calf Serum*[a]	*Healthy Adults*[b]	*Breast Fed Babies*[c]
Total PL	12.1	664.4	501.9
Total SFA	5.4	313.3	230.9
Total MUFA	4.0	84.9	86.2
Total PUFA	2.7	266.2	184.8
Total n-6	2.1	242.6	161.6
Total n-3	0.5	26.6	23.2
p:s ratio	0.7	0.9	0.8
n-6:n-3 ratio	4.1	10.3	7.0

[a:] 1ml aliquot used in liquid gas chromatography
[b:] Pratt *et al.* (2002)
[c:] Clandinin *et al.* (1999)

Table 3-2 Comparison of serum fatty acid content of fetal calf serum (FCS) and human serum (HS). Values determined using liquid gas chromatography, all values in µg.

Fatty Acid	PL		TAG		***CE***	
	FCS	*HS*	*FCS*	*HS*	*FCS*	*HS*
16:0	2.2	13.4	93.7	275.4	0.3	4.8
18:0	1.7	5.8	17.4	49.8	0.7	3.2
18:2n-6	0.14	7.13	24.1	170.0	0.0	97.6
18:3n-3	0.2	0.1	3.0	14.2	0.4	0.5
20:4n-6	0.2	3.1	25.31	6.1	0.02	10.1
20:5n-3	0.0	0.2	2.1	1.6	0.0	1.5
22:6n-3	0.3	1.2	4.3	2.8	0.0	0.8
∑ SFA	4.6	20.7	133.9	355.1	3.2	33.7
∑MUFA	2.7	5.4	112.1	521.9	2.2	45.9
∑PUFA	1.3	13.3	71.03	220.5	0.8	112.9
n-6/n-3 ratio	1.3	1.0	5.4	5.6	0.3	34.6
Total µg	8.4	39.5	317.1	1097.4	6.29	192.6

Table 3-3 Comparison of cell growth, integrin and PSA data for RWPE-1, 22Rv1, LNCaP, DU-145 and PC-3 cell lines cultured in human serum (HS) or fetal calf serum (FCS); $n \geq 9$.

Cell Line	*RWPE-1*		*22Rv1*		*LNCaP*		*DU-145*		*PC-3*	
Serum Type	HS	FCS	HS	FCS	HS	FCS	HS	FCS	HS	FCS
Average Count ± SE (10^6) n = 9-24	3.0 ± 0.9	3.5 ± 1.0	3.2 ± 1.5	6.0 ± 2.0	2.6 ± 1.4	8.8 ± 4.6	1.4 ± 0.6	2.5 ± 1.2	1.4 ± 0.8	3.0 ± 1.0
p value	0.11		2.1×10^{-5}		6.9×10^{-6}		0.003		1.2×10^{-5}	
$\alpha_v\beta_3$ % ± SE n = 10-21	0.1 ± 0.3	1.0 ± 0.7	0.7 ± 1.2	0.3 ± 1.6	0.1 ± 0.3	0.8 ± 0.9	4.6± 0.9	6.4 5.9	9.3± 1.3	8.3± 4.3
p value	0.002		0.40		0.005		0.84		0.43	
VR % ± SE n = 9-20	1.0 ± 1.1	1.8 ± 1.2	0.4 ± 0.6	1.6 ± 1.5	0.4 ± 0.7	0.9 ± 1.4	36.7 ± 3.2	47.4 ± 2.1	35.5 ± 5.2	35.1 ± 8.2
p value	0.12		0.053		0.30		1.8×10^{-7}		0.91	
$\alpha_2\beta_1$ % ± SE n = 10-46	91.0 ± 5.0	87.6± 7.4	57.0 ± 6.5	69.5± 11.2	64.5 ± 11.7	61.6± 10.8	93.1 ± 2.5	92.5 ± 6.9	81.3± 10.6	93.4 ± 1.0
p value	0.19		9.6×10^{-5}		0.26		0.64		0.005	
PSA (ng ml^{-1} $cell^{-1}$) ± SE (10^{-7}) n = 3-15	3.1 ± 1.8	5.1 ± 4.7	7.3 ± 4.8	9.6 ± 6.3	3.0 ± 3.5	2.1 ± 1.5				
p value	0.50		0.35		0.45					

4. CULTURING PRIMARY PROSTATE CANCER EPITHELIAL CULTURES FROM HUMAN PROSTATE SAMPLES

4.1 INTRODUCTION

Most of the cell lines available for PCa research have been isolated from metastatic lesions rather than primary tumors (Abate and Shen, 2000; Table 1-1). Numerous phenotypic differences distinguish primary cultures of prostatic epithelial cells from established PCa cell lines. These differences are assumed to reflect features of normal versus malignant biology, when perhaps they reflect different culture conditions, or differences between short-term culture and immortalized cell lines (Peehl, 2004). This raises concerns about how well cell lines represent primary adenocarcinomas of the prostate, as well as questions regarding how long term cell culture alters the biological properties of cell lines (Peehl, 2004). For these reasons and due to the appropriateness of using human cells for studies of human cancers, it is important to have established methods of primary culture for malignant PCa cells.

Various methods to obtain primary cultures from human prostates have been attempted since 1917 when Burrows *et al.* first reported the cultivation of bladder and prostatic tumors outside the body. Most primary cultures have been derived from radical prostatectomy samples and cadavers (a few hours old at most), although other methods include needle biopsies, and bone marrow aspirates (Peehl, 2005). Various methods were attempted including mechanical separation and use of Type I collagenase (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada), to culture epithelial cells from human prostates (Edmonton, AB, Canada). The method that yielded the most cells per prostate sample was adapted from that of Peehl *et al.*, as published by Russell *et al.*, (2003).

4.2 MATERIALS AND METHODS

Isolation of Epithelial Cells from Human Prostates

The protocol for the isolation of epithelial cells from human prostates was modified from a protocol found in "Prostate Cancer Methods and Protocols" (Russell *et al.*, 2003). Human prostate samples were obtained from radical prostatectomies performed (by Dr. J. B. Metcalfe at the University of Alberta Hospital, Edmonton, AB,

Canada), with informed patient consent. Pertinent clinical information such as date of birth (DOB), Gleason score and pre-operative PSA values were recorded for all samples obtained. All materials used in this protocol were obtained from Fisher Scientific (Edmonton, Alberta, Canada), unless otherwise noted. Complete F-12 Nutrient Mixture contained L-glutamine and was supplemented with 1% v/v antibiotic/antimycotic solution (1 x 10^5 U/L penicillin, 100 mug/L streptomycin, and 25 mg/L amphotericin B), both of which were obtained from Gibco Invitrogen Corporation (Burlington, Ontario, Canada). This complete media also contained 1% human serum (HS). All procedures were performed under sterile conditions.

The tissue sample collected at time of surgery was stored at 4°C in 5 to 10ml HEPES-Buffered Saline (HBS) for up to a maximum of 4 hours or harvested immediately (preferable). The tissue sample was transferred to a Petri dish and washed two times with HBS (2 x 5ml). Tissue was minced into small pieces (~1mm^3) using scissors and tweezers, transferred to a 15 ml falcon tube and pelleted (1000 rpm for 5 minutes). The supernatant was removed and the pelleted tissue was re-suspended in complete F-12 Nutrient Mixture containing 40U/mL type I collagenase (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada). Type I collagenase was prepared and filter sterilized (0.22um filter Millipore Corporation, Bedford, MA, USA), immediately prior to use. The tissue was incubated and shaken at 37°C for 24 hours. After the 24 hour incubation, the digested tissue was pipetted vigorously to break up remaining clumps, and centrifuged at 1000rpm for 5 minutes. The pellet was rinsed twice with 5 ml HBS, before repeating the vigorous pipetting and centrifugation. The pellet was re-suspended in 5ml complete F-12 Nutrient Mixture, counted using trypan blue exclusion (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada), with 90% viability. The cells were transferred to a T-25 Collagen PRO coat Cultureware culture flask. The primary cell cultures were grown at 37°C in 5% CO_2 at 98% relative humidity, and fed with F-12 Nutrient Mixture every 3-4 days.

Passaging Primary Human Prostatic Epithelial Cell Cultures

Supernatants were frozen for use in other assays. The flask was rinsed once with 5ml HBS, and 0.25% Trypsin- 0.03% was added, the cells were incubated at 37°C until they detached from flask. Cells were added to a centrifuge tube, and the flask was rinsed once

more with 5 ml complete F-12 Nutrient Mixture, and added to the centrifuge tube. Cells were pelleted and re-suspended in 5 ml complete F-12 Nutrient Mixture. Cells were counted using trypan blue exclusion, and seeded into T-25 Collagen PRO coat Cultureware culture flasks, incubated at 37°C in 5% CO_2 at 98% relative humidity, and fed with F-12 Nutrient Mixture every 3-4 days, until confluency was reached.

Preliminary Integrin Runs

The $\alpha_v\beta_3$, VR, $\alpha_2\beta_1$ integrins were measured on the primary human prostate epithelial cultures using the *Integrins* procedure (Chapter 3) and the *Isolation of Epithelial Cells from Human Prostates,* or *Passaging Primary Human Prostatic Epithelial Cell Cultures* as outlined above.

4.3 RESULTS

Isolation and Culture of Epithelial Cells from Human Prostates

The method for the isolation and culturing of epithelial cells from human prostates as outlined in "Materials and Methods" gave the greatest number of viable cells per tissue sample, and allowed for successful passaging of the isolated cells. An average of $4x10^6$ cells were obtained per tissue sample (~2-3g) using this method. At the time of passaging (7-14 days after initial seeding), cell count remained constant, although there was no proliferation of the cell culture. The number of viable cells remained constant for up to 30 days, as counted using trypan blue exclusion (same as initial number of cells isolated) at the time of passaging. Using other culturing methods (adapted from Cooke and Littleton, 1985; Oishi *et al.*, 1981), on average $5x10^5$ to $1x10^6$ cells per tissue sample, were obtained. Figures 4-1 through 4-4 are light microscopy images of the primary cultures of human epithelial prostate cells.

Preliminary Integrin Data

Integrin data was obtained for the $\alpha_v\beta_3$, $\alpha_v\beta_3$ VR and $\alpha_2\beta_1$ integrins on three of the prostate samples that were cultured (Table 4-1). This data is preliminary as it was collected on different samples only once, after varying amounts of time in cell culture. Collection of this data is to be continued further in order for complete analysis of this data.

4.4 DISCUSSION

Isolation and Culture of Epithelial Cells from Human Prostates

Several different protocols for the isolation and culture of epithelial cells from human prostates were attempted, with the greatest success occurring with the method outlined in "Materials and Methods". The use of other culturing methods adapted from Cooke and Littleton, 1985; Oishi *et al.*, 1981, were used in the isolation of prostate epithelial cells from Sprague Dawley rats and patients with benign prostatic hyperplasia (BPH), respectively, whereas the method adapted from Peehl *et al.* had been successfully used to isolate malignant prostate cells.

Collagenases are enzymes capable of dissolving fibrous collagen by peptide bond cleavage under physiological pH and temperature. This is of importance as collagen makes up 33% of the total protein in mammalian organisms (Webber, 1979). Webber (1979) found that the addition of collagenase facilitated isolation of prostatic acini, and determined that collagenase is not toxic to epithelial cells, does not damage their membranes, and helped eliminate fibroblasts from the cultures. Webber (1979) was able to incubate the prostate tissue at 37°C in 400U/ml collagenase for 24 to 48 hours or in 200U/ml collagenase for 60 hours. Among Webber's (1979) other observations were that tissue from younger donors (< 21 yrs) did not require as long a period of digestion with collagenase as did specimens from older donors (≥ 40 yr); very necrotic tumor samples also required longer digestion periods. Webber's (1979) observations are in agreement with what we observed with regards to digestion with collagenase. Other enzymes such as trypsin are not effective in dissociating tissues rich in collagen, such as prostate tissue (Webber, 1979). Trypsin can also cause cell membrane damage, and is therefore not appropriate for the isolation of intact prostatic epithelial cells (Webber, 1979).

Peehl and Stamey (1986) determined that cultures from normal and BPH tissues were similar in appearance to those from PCa, and were composed of monolayers of cuboidal cells, and that morphology could be manipulated by culture conditions (Peehl *et al.*, 1996). Differences in growth media for established cell lines to those used in primary culture make it difficult to directly compare the morphology of cell lines to primary cultures. Peehl (2004) notes that no consistent morphological differences have been noted between primary cultures derived from normal or BPH tissues versus

adenocarcinomas when grown in monolayer culture, and primary cultures from adenocarcinomas do not morphologically resemble established cancer cell lines. We agree that the primary cultures did not resemble any of the established cell lines (Figures 4-1 to 4-4; Figures 3-6 to 3-10 in Chapter 3). Cultures derived from primary adenocarcinomas of the prostate rarely become immortal (Peehl, 2004). Generally cells derived from normal and BPH tissues as well as from cancers undergo an average of 30 population doublings (5-6 passages) before becoming senescent (Peehl *et al.*, 1991, Schwarze *et al.*, 2001). The cells we isolated here did not seem to undergo any doublings but remained viable for 5-6 passages, which may indicate that our cells were senescent before 30 population doublings. According to Peehl (2004), distinctive features of primary cultures derived from cancers compared to primary cultures from normal tissues have been much more difficult to identify, in contrast to the differences seen between cell lines and primary epithelial cultures, which is problematic due to the lack of a method or specific cellular marker to determine which cells are normal epithelial cells and which are malignant in nature (Peehl, 2004).

Preliminary Integrin Analysis

Zheng *et al.* (1999) showed that epithelial cells from prostate carcinoma showed a strong migratory response on vitronectin (VN), whereas epithelial cells from normal prostate tissue did not. Primary cancer cells also expressed high levels of the $\alpha_v\beta_3$ integrin, which was significantly different from normal prostate cells (Zheng *et al.*, 1999). This observation leads to the hypothesis that the increased $\alpha_v\beta_3$ mediated migration of PCa cells is likely to generate a metastaic phenotype *in vivo* (Zheng *et al.*, 1999). This hypothesis holds merit due to the migratory response the prostate carcinoma cells showed on VN, because this ligand is found in mature bone tissue where PCa cells preferentially metastasize (Zheng *et al.*, 1999). Table 5-1 shows that sample 3, which was in culture for 35 days expressed high levels of $\alpha_v\beta_3$ VR, whereas samples 1 and 2 which were in culture for less than 10 days show little expression of this integrin. Zheng *et al.* (1999) used the isolated epithelial cells immediately in integrin assays and found high levels of $\alpha_v\beta_3$ VR which may suggest that the time in culture should not have an effect on the expression of this integrin. Perhaps the varying degrees of expression of the $\alpha_v\beta_3$ VR integrin in our samples were due to a mixture of normal and cancerous cells in

culture, or perhaps they could be attributed to different staging of the cancer in our subjects, as Zheng *et al.* (1999) limited the surgical specimens collected to those from patients with localized tumors lacking metastatic lesions. Due to our small sample size, further samples need to be collected and processed in order to make strong conclusions.

Adhesion to type IV collagen, type I collagen and laminin I is mediated by $\alpha_2\beta_1$and is restricted to the basal cells of the prostate (Knox *et al.*, 1994). Collins *et al.* (2001) showed higher surface expression of the α_2 integrin subunit on human prostate epithelium correlates with colony forming ability and the potential to regenerate a fully differentiated prostate epithelium *in vivo*. The results in Table 5-1 showed high expression of the $\alpha_2\beta_1$ integrin in sample 3 but not in the other two samples, which may suggest that this sample has a greater potential to regenerate a fully differentiated prostate epithelium *in vivo*. If there was correlation between the integrin expression seen in the PCa cell lines (Chapter 3) and epithelial cultures derived from cancer samples, it could be suggested that Sample 3 may have a more invasive phenotype due to its greater expression of $\alpha_v\beta_3$ VR and $\alpha_2\beta_1$, which would correlate with the expression of those integrins in the DU-145 and PC-3 cell lines (Figure 3-4, Table 3-3).

In conclusion, we have been able to successfully isolate and culture epithelial cells from human prostate samples obtained during radical prostatectomies. We have also obtained preliminary integrin data on these samples, and by examining more primary epithelial cultures we may be able to correlate integrin expression to the invasiveness and degree of the cancer in the patients in question.

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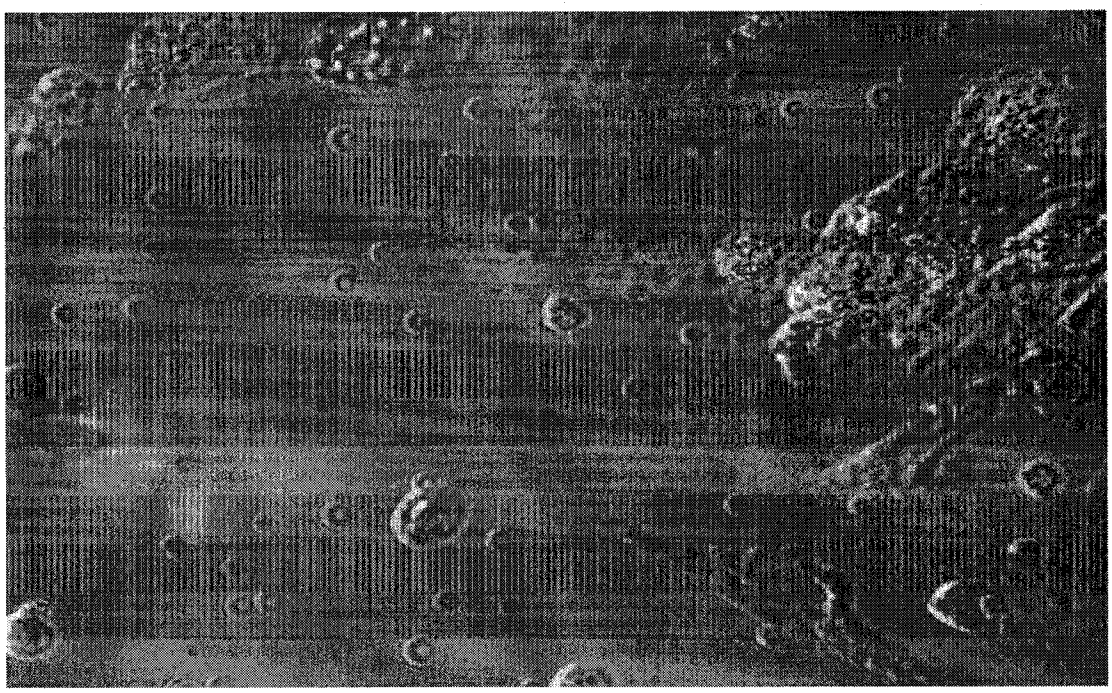

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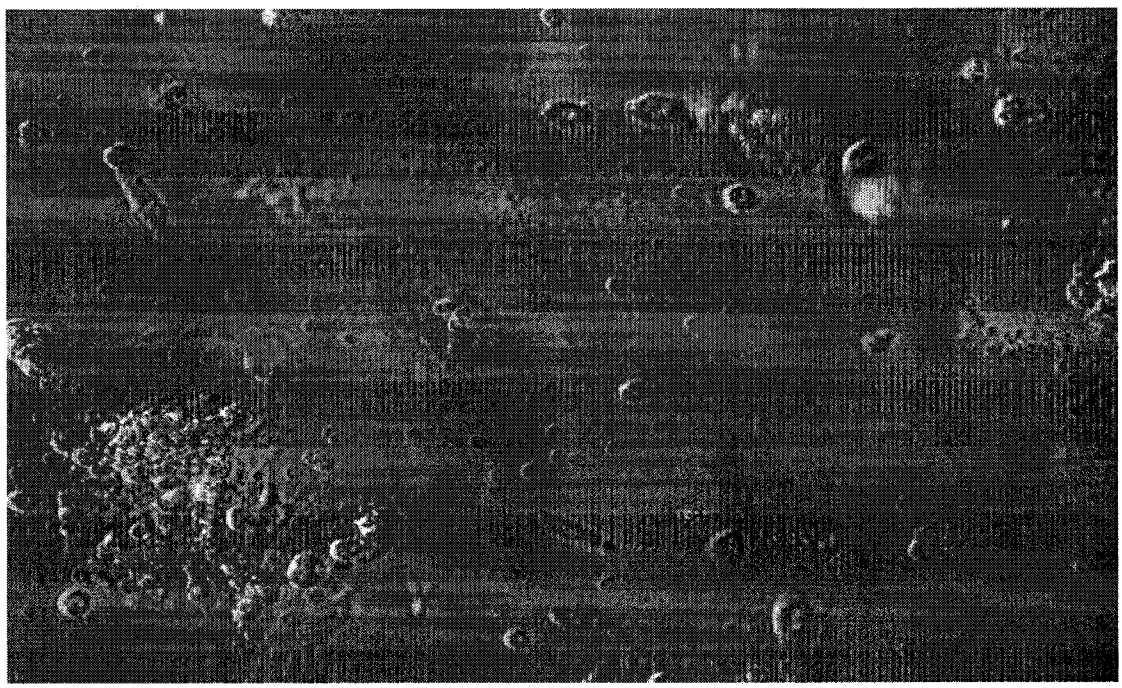

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Figure 4-1 Primary prostate cancer epithelial cell culture in F12 (Hams) media containing 1% HS, $2.5x10^6$ seeded on Feb 7, 2006 (PSA = 3.5; Gleason: 3+3=6; Feb 9, 2006; 1.10X 2. 40X).

1

2

Figure 4-2 Primary prostate cancer epithelial cell culture in F12K Hams media containing 1% HS, 2.5x10e6 seeded on Feb 7/06 (PSA = 3.5; Gleason: 3+3=6; Feb 15, 2006; 1.10X 2. 40X).

Table 4-1 Preliminary integrin results from primary epithelial cultures cultured from human prostate samples. Integrins were run on sample 1 after 10 days in culture, on sample 2 after 7 days in culture and on sample 3 after 35 days in culture.

Measurement		Sample				
		1	2		3	
Laboratory Parameters	Cell Count	$1.8x10^6$	$5.25x10^6$		$5x10^6$	
	$\alpha_v\beta_3$	1.6	3.3	3.1	9.0	
	$\alpha_v\beta_3$ VR	5.7	5.8	5.3	61.2	
	$\alpha_2\beta_1$	31.9	4.5	4.6	61.8	59.7
Clinical Parameters	PSA	22	15		3.5	
	Gleason Score	3+3=6	3+4=7		3+3=6	

5. CELL LINE GROWTH AND INTEGRIN EXPRESSION WITH THE ADDITION OF PHYSIOLOGICALLY RELEVANT LEVELS OF LYCOPENE, VITAMIN E AND FISH OIL TO CULTURE MEDIA.

5.1 INTRODUCTION

Nutrients

Epidemiological studies have associated lycopene (Giovannucci *et al.*, 1995), n-3 fatty acids (Karmali *et al.*, 1987), and high intakes of vitamin E with reduced PCa risk (Siler *et al.*, 2004). The majority of research relating nutrients to PCa has been epidemiological in nature and has largely focused on prevention of the disease (see Chapter 1). While more information about how to prevent PCa is required, further research into how nutrients affect the progression and disease after the diagnosis of PCa is needed. It is widely accepted that diet plays an important role in cancer development. However, there is a lack of information relating food components to mechanisms associated with the development and growth of malignant cells. The evidence to date demonstrating a role for various bioactive nutrients in the prevention and treatment of PCa is qualitative and suggests that a relationship exists, but the underlying mechanisms involved are poorly understood. Current literature focuses on one or two cell lines and typically one nutrient or class of nutrients. Furthermore, the cell culture work is performed in nutrient deficient media and often with concentrations of nutrients that have no relevance to human plasma levels or dietary intakes.

Lycopene is a red-orange carotenoid with potent anti-oxidant activity. Several potential mechanisms by which it affects the prostate have been suggested (see Chapter 1). Vitamin E is a fat-soluble vitamin (Willis and Wians, 2003), also known for its antioxidant acitivty. Only certain stereoisomers of α-tocopherol are maintained in the human plasma, therefore only α-tocopherol is considered to contribute towards the recommended daily allowance for Vitamin E (Lippman *et al.*, 2005). It has been suggested that certain forms of vitamin E may have a potential role as anticancer agents (Jiang *et al.*, 2004), although the precise mechanisms for vitamin E's benefits are largely unknown (reviewed by Fleshner and Kucuk, 2001). Mammals are unable to synthesize n-*3* and n-6 polyunsaturated fatty acids; therefore they must be obtained through the diet (Rose and Connolly, 1999). N-3 fatty acids are crucial for many cellular processes,

including cell proliferation and differentiation (Karmali, 1987). All cell membranes are comprised of n-3 fatty acids as they are incorporated into phospholipids of cell membranes of many cell types, including tumor cells following the inclusion of n-3 fatty acids in diet or cell culture media (Karmali *et al.,* 1989; Simopoulos, 1991; Hardman, 2002). Long-chain n-3 fatty acids (DHA and EPA) have been shown to inhibit *in vitro* and *in vivo* cell growth of PCa cells (Karmali *et al.*, 1987; Rose and Connolly, 1991). The wealth of epidemiological studies suggest eating fish as an effective way to prevent cancer (Chapter 1, Section 1.8.1).

The objective of this study was to examine the effects of physiological levels of lycopene, Vitamin E and fish oil (Table 5-1) on cell growth and integrin expression in RWPE-1, 22Rv1, LNCaP and PC-3 cell lines. It was hypothesized the addition of these nutrients to the cell culture media would result in decreased growth, due to the associations reported in previous studies, as well as due to the potential hypothesized actions of these nutrients on cancer cells (Chapter 1). It was hypothesized that $\alpha_v\beta_3$, VR, and $\alpha_2\beta_1$ integrins would serve as a biomarker of malignant potential by which to test the efficacy of the nutrients proposed. Their expression would be decreased on the cell lines as an indicator of the anti-cancer potential of the nutrients.

Overall, only fish oil decreased cell growth, whereas Vitamin E increased cell growth and lycopene showed no significant effect. Changes in $\alpha_v\beta_3$ and VR expression were seen in the most invasive of cell lines with the addition of nutrients. Lycopene and fish oil changed $\alpha_2\beta_1$ expression for all cell lines, whereas expression only differed for 22Rv1 with the addition of Vitamin E.

5.2 MATERIALS AND METHODS

All cell lines were cultured in the media requirements outlined in Chapter 3. All media contained 1% HS (v/v), as outlined in Chapter 3. All materials used in these experiments were those used and supplied by the same companies as outlined in Chapter 3 unless otherwise noted. Vitamin E ($\pm$ α-tocopherol 95% HPLC), lycopene ($C_{40}H_{56}$) and Tween 40 were supplied by Sigma-Aldrich (Canada Ltd., Oakville, Ontario, Canada). The concentrations of Vitamin E and lycopene used are within physiological ranges and were determined to be non toxic to the cell lines using lactate dehydrogenase (LDH) assays.

Addition of Vitamin E and Lycopene to Cell Culture

There have been many different methods used to add tocopherols and β-carotenes to cell culture, due to the difficulty involved in solubilizing these lipophilic compounds. Organic solvents tetrahydrofuran (THF), dimethylsulfoxide, ethanol and n-hexane have been used as delivery vehicles for carotenoids, but pose problems due to the cytotoxicity of the solvents used, the instability and insolubility of the carotenoids, and the ability of these nutrients to complex with certain delivery vehicles (e.g. methyl-β- cyclodetrin), muddling the effect of the actual nutrient on *in-vitro* experiments (Sullivan *et al.,* 2004).

Sullivan *et al.* (2004) compared the use of polyoxyethylene sorbitan monopalmitate (Tween 40) and polyoxyethylene sorbitan monooleate (Tween 80) to THF, as delivery vehicles for a mixture of carotenoids and tocopherols to colonic adenocarcinoma cells (CaCo-2). Using this method, lycopene was detected in the cells with Tween 40 and 80 but not with the use of THF, as determined using high performance liquid chromatography (HPLC). They determined that Tween 40 was slightly better at delivering lycopene to the cells than Tween 80, which Sullivan *et al.* (2004) suggest may be due to Tween 40's fatty acid side chain being palmitic acid while that of Tween 80 is oleic acid, and thus possessing a more favorable hydrophilic-lipophilic balance value. No toxic effects to membranes or energy metabolism of CaCo-2 cells were observed, nor did Tween disrupt the antioxidant potential of the cells. No cytotoxic effects were found with the use of Tween 40 and 80 as determined using Neutral Red uptake assay (NRUA), 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay, and the lactate dehydrogenase release assay (LDH). The stability of the phytochemicals was consistent over the incubation (24, 48 and 72 hours) and the compounds were taken up by the cells within 6 to 12 hours. When the test media containing the phytochemicals was removed and replaced with regular media, the compounds remained stable in the cells for up to 24 hours afterwards (Sullivan *et al.,* 2004). Due to the lack of cytotoxicity, relative stability and the ability of Tween 40 to deliver the phytochemicals to the cells, we adopted the used of Tween 40 as the vehicle for adding lycopene and Vitamin E to cell culture.

Nutrient Media Preparation

Control media was prepared in the same manner as the nutrient media, with the addition of Tween 40 or 95% ethanol to the standard media in the same quantities used in the nutrient media preparation. Nutrient media was freshly prepared for each experiment. All nutrients were stored in the dark, and light exposure was kept to a minimum during media preparation, addition and incubation, to prevent degradation due to light.

Lycopene Media Preparation

Stock lycopene solution (10µl) was suspended in 100µl of Tween 40 and 9.2ml of complete culture media was added to the mixture, vortexed and filtered using a 0.22µm filter (Millipore Corporation, Bedford, MA, USA). The filtered stock solution was then serially diluted to a working concentration of $1x10^{-8}$M in the appropriate volume for the experiment and added to the 6 well plates or the $75cm^2$ flasks.

Vitamin E Media Preparation

Vitamin E was weighed using an analytical balance and re-suspended in 100µl of Tween 40, and vortexed. The initial concentration of Vitamin E was determined using molar mass (430.72g/mol). The stock solution was then diluted to a concentration of 5 $x10^{-3}$M using the appropriate media, and filter sterilized using a 0.22µm filter (Millipore Corporation, Bedford, MA, USA). This solution was then serially diluted to a working concentration of $5x10^{-6}$M in the appropriate volume for the experiment and then added to the 6 well plates or the $75cm^2$ flasks.

Fish Oil Media Preparation

A stock solution of 10mg/ml was prepared in 95% ethanol and stored at -80°C, under N_2(g). The amount of stock solution to make a concentration of $1x10^{-6}$M in the final amount of media required for the experiment was calculated, aliquoted and dried down using N_2(g). The fish oil was re-suspended in 1% HS of the final volume of the medium being prepared and incubated in a water bath for 1 hour at 37°C with vortexing every 10 minutes. The media was then adjusted to the final volume and used immediately in the 6 well plates or $75cm^2$ flasks. Preparation of fish oil supplemented media was adapted from the procedure used by Schley (2005).

Cell Line Growth Data

Cells were cultured, passaged and counted as described in Chapter 3 and $1x10^5$ cells were seeded into 6 well plates, with each well containing 3ml of the appropriate media. The cells were incubated as described in Chapter 3 for 48 hours (to allow for cell adherence as outlined in the cell characteristics of cell lines by ATCC, Manassas, VA, USA), at which time the media was removed and new media containing the nutrients was added. The cells were incubated for 48 hours in 3ml of media containing lycopene ($1x10^{-8}$M), Vitamin E ($5x10^{-6}$M), fish oil ($1x10^{-6}$M), their respective control vehicle media or complete growth media, which served as overall controls for the experiments, and to ensure no toxicity of the nutrient vehicles. Cells were detached from their respective wells after the 48 hour nutrient incubation, by the addition of 1ml of 0.25% Trypsin-0.03% EDTA to each well. The wells were rinsed using the appropriate media and pellets were obtained for counting using trypan blue exclusion. Means and standard deviations were calculated for all cell lines and the Student's unpaired t-tests were conducted to determine statistical significance between the control vehicles and nutrient supplements for each cell line.

Integrins

Cells were cultured as described in Chapter 3, along with the modifications noted here. Cells ($1x10^6$) were seeded into flasks and allowed to adhere for 48 hours at which time the nutrient media in the concentrations outlined above were added. After 48 hours, they were passaged using the protocol outlined in Chapter 3, and supernatants were removed and frozen immediately for use in IL-6 and PSA assays. $\alpha_v\beta_3$, VR, $\alpha_2\beta_1$ integrin expression were assessed as described in Chapter 3. Means and standard deviations were calculated for all integrins and the Student's unpaired t-tests were conducted to determine statistical significance ($p<0.05$) between the control vehicles and nutrient supplements for each cell line.

PSA

PSA was measured in RWPE-1, 22Rv1 and LNCaP cell lines as outlined in Chapter 3. Means and standard deviations were calculated and the Student's unpaired t-tests were conducted to determine statistical significance ($p<0.05$) between the control vehicles and nutrient supplements for each cell line.

5.3 RESULTS

Cytotoxicity of Nutrient Delivery Vehicles

The lactate dehydrogenase (LDH) assay confirmed that the delivery vehicles were not cytotoxic. Trypan blue exclusion determined that cell viability in these experiments was greater than 99%, also demonstrating that the delivery vehicles were not cytotoxic to the cells, and no marked changes in cell morphology were observed with the lycopene, Vitamin E or fish oil supplementation of the media.

Lycopene

There was no effect of lycopene ($1x10^{-8}M$) on growth of any of the cell lines (Figure 5-1, Table 5-2). Supplementation of the cell lines with lycopene resulted in a significant increase in expression of $\alpha_2\beta_1$ in RWPE-1, and a significant decrease of $\alpha_2\beta_1$ expression in 22Rv1, LNCaP and PC-3 cell lines (Figure 5-4, Table 5-3). Lycopene decreased the expression of $\alpha v\beta_3$ and VR in the PC-3 cell line (Figure 5-7, Figure 5-8, Table 5-3). PSA was significantly higher with lycopene supplementation in 22Rv1 and LNCaP (Table 5-4).

Vitamin E

A significant increase in cell growth was observed in all cell lines (Figure 5-2), except RWPE-1. Supplementation with Vitamin E ($5x10^{-6}M$) resulted in a significant decrease in $\alpha_2\beta_1$ expression in 22Rv1 (Figure 5-5, Table 5-3), and a decrease in the expression of $\alpha v\beta_3$ and VR in the PC-3 cell line (Figure 5-7, Figure 5-8, Table 5-3). PSA was significantly lower in 22Rv1 and significantly higher in LNCaP with Vitamin E supplementation (Table 5-4).

Fish Oil

A significant decrease in cell growth was seen with fish oil ($1x10^{-6}M$) supplementation in all cell lines (Figure 5-3, Table 5-2). Fish oil significantly decreased the expression of $\alpha v\beta_3$ and VR in the PC-3 cell line (Figure 5-7, Table 5-3). There was a significant increase in $\alpha_2\beta_1$ expression in RWPE-1 and 22Rv1 cell lines whereas the LNCaP cell line exhibited a significant decrease in $\alpha_2\beta_1$ compared to the control media (Figure 5-6, Table 5-3). PSA was significantly higher in 22Rv1 and LNCaP with fish oil supplementation (Table 5-4).

5.4 DISCUSSION

Lycopene

Adding lycopene to the cell cultures did not affect cell growth. A lycopene concentration of $1x10^{-8}$M was used, which was not toxic to the cells using the LDH assay and trypan blue exclusion. Kim *et al.* (2002) reported a dose-dependent effect of lycopene on the growth of LNCaP after various times of incubation, with only $1x10^{-7}$M, $1x10^{-5}$M and $1x10^{-4}$M of lycopene producing a statistically significant decrease after 48 and 72 hours of incubation. Cell growth was completely inhibited with $1x10^{-3}$M lycopene. This level of lycopene far exceeds physiological levels even in people who consume high amounts of lycopene in their diet. Richards *et al.* (2003) also found no significant decrease in growth with $1x10^{-6}$M lycopene but found a significant difference when $1x10^{-5}$M lycopene was used. Stahl and Sies (1996) report an upper limit of approximately $1x10^{-6}$M for the physiological concentration of lycopene in the blood, therefore the inhibitory effect observed by Kim *et al.* (2002) at $1x10^{-5}$M and $1x10^{-4}$M may not be feasible by dietary intake. The data in our cell lines is consistent with the lack of significant decrease in growth that Kim *et al.* (2002) reported a concentration $1x10^{-8}$M of lycopene. Stahl and Sies (1996), report that lycopene is present in human blood at levels of approximately $5x10^{-7}$M in human plasma. The levels used in the present studies would be considered as low physiological levels, but perhaps more relevant to the general population. Therefore in future experiments increasing the concentration of lycopene to higher physiological concentrations may produce a significant decrease in growth, although methods to prevent cytotoxicity would have to be explored.

$\alpha_2\beta_1$ mediates cell adhesion to type I and type IV collagen and laminin I, which are components of the extracellular matrix restricted to the basal cells of the prostate (Knox *et al.*, 1994). A significant increase in expression of this integrin in RWPE-1 was observed when lycopene was added to the media. Expression of $\alpha_2\beta_1$ was reduced in the 22Rv1, LNCaP and PC-3 cell lines. It is believed that there are several mechanisms by which lycopene may affect the prostate (Chapter 1), one of these involves inhibition of tumor growth and increased differentiation of normal cells by increasing gap-junction communication in healthy prostate cells (Wertz *et al.*, 2004). This is of interest as gap junctions are involved in the regulation of cell-cell signaling (Nelson and Cox, 2002), as

is the $\alpha_2\beta_1$ integrin. Therefore the increase in $\alpha_2\beta_1$ observed in the RWPE-1 cells (Figure 5-4, Table 5-3) with the addition of lycopene to the culture media may also indicate increased cell to cell communication in these phenotypically normal cells. Whatever the effect, it appears to be lost in cells with cancerous phenotypes. $\alpha_v\beta_3$ and VR integrin expression was significantly decreased in the PC-3 cell line with the addition of lycopene, which would perhaps suggest a decrease in the invasiveness of these cells and their ability to migrate to bone (see Chapter 1).

In spite of not affecting growth, the production of PSA was increased by at least 2 fold in the 22Rv1 and LNCaP cell lines. This was an unexpected finding that requires further investigation. Richards *et al.* (2003) found that PSA decreased in the LNCaP cell line corresponding to a decrease in growth using lycopene concentrations of 10uM and 1uM, although decrease in growth at the lower concentration was not significant.

Vitamin E

Jiang *et al.* (2004) report no inhibition of PC-3 and LNCaP PCa cells at a concentration of $1.16x10^{-2}$M (5mg/ml) of α-tocopherol. This finding is in conflict with the findings of Richards *et al.* (2003), who report a significant decrease in growth of LNCaP cells using $2x10^{-8}$M and $2x10^{-7}$M Vitamin E. Our findings are in conflict with both outcomes, as a concentration of $5x10^{-6}$M Vitamin E significantly increased cell growth in all cell lines except for RWPE-1. Physiological serum levels range from ~$1.16 x10^{-6}$M to $1.16 x10^{-4}$M (Lagua and Claudio, 1996), therefore the amount of Vitamin E used by Richards *et al.* (2003) falls below this range and the concentration used by Jiang *et al.* (2004) is much higher than this range. The concentration of Vitamin E used in our studies are physiological. The lack of significant decrease or increase of growth in the RWPE-1, which represents phenotypically normal prostate cells, neither supports or refutes the epidemiological evidence that suggests Vitamin E may have protective effect in the prevention of PCa. Our data also suggest that Vitamin E may aid in the proliferation of cancer cells, due to the increased growth rates observed in all cell lines with cancerous phenotypes. While our data cannot support or refute the cancer prevention properties touted for Vitamin E, it questions the validity of supplementing once one has been diagnosed.

A significant decrease in expression of $\alpha_2\beta_1$ was observed for 22Rv1 only. Vitamin E is primarily found in lipid containing membranes, as it is fat soluble (Willis and Wians, 2003). $\alpha_2\beta_1$ is a transmembrane protein that may be upregulated by incorporation of Vitamin E into cell membrane, due to its involvement with components of the ECM. $\alpha_v\beta_3$ and VR integrin expression decreased significantly in the PC-3 cell line with the addition of Vitamin E, which would suggest a decrease in the invasiveness characteristics of these cells despite an increase in cell growth. PSA levels decreased in 22Rv1 but increased in LNCaP with the addition of Vitamin E, which only further questions the use of PSA as a PCa marker, as Vitamin E increased growth in both of these cell lines.

Fish Oil

N-3 fatty acids have been shown to reduce the incidence, increase the latency of tumor development, and decrease tumor load (Karmali, 1987) *in vitro* or when transplanted into animals (Karmali *et al.*, 1987; Rose and Cohen, 1988). This suggests that n-3 fatty acids affect all stages of carcinogenesis and have a protective effect in delaying or preventing the conversion of pre-neoplastic cells to the tumor phenotype. Previous work has shown a significant inhibition of growth of tumors in the presence of n-3 fatty acids, using similar methods (Robinson *et al.*, 2001; Robinson *et al.*, 2001). The growth data obtained here supports that of others, and the suggestion that n-3 fatty acids affect all stages of carcinogenesis, as growth decreased in phenotypically different PCa cell lines.

Rose and Connolly (1991), report that DHA and EPA inhibited PC-3 cell growth in a dose-dependent manner, with both being equally effective (~65% decrease) at 2.0μg/ml ($1x10^{-3}$M). We observed an ~53% decrease in growth in this cell line using $1x10^{-7}$M fish oil, which contains both EPA and DHA. The comparable decrease in growth that we observed with a much lower concentration of fish oil is encouraging as it shows that growth of these cancerous cells can be decreased using an amount of fish oil that is perhaps more easily attained through diet. A study by Pandalai et al. (1996) showed promotion of cell growth in culture (PC-3 and LNCaP) at low concentrations ($1x10^{-6}$M) of EPA, although at higher concentrations, EPA inhibited prostate cell growth. This finding is in contradiction of our findings, using a very similar concentration. This

result is also in contradiction with previous findings of others who used similar methods to the present work (Robinson *et al.,* 2001; Robinson *et al.*, 2001) as well as those of Rose and Connolly (1991).

The addition of fish oil to cell culture resulted in significant changes in $\alpha_2\beta_1$ expression in all cell lines. The increase in $\alpha_2\beta_1$ expression by the RWPE-1 and significant decrease in expression by the LNCaP and PC-3 cell lines corresponds to what was observed with lycopene in these same cell lines. These differences may correspond to a decrease in cell growth, which occurred in both of these cell lines with the addition of fish oil and lycopene and may suggest a membrane associated mechanism. The increase in $\alpha_2\beta_1$ expression by the 22Rv1 cell line is the opposite of what was observed with the other two nutrients. The decrease in growth with lycopene was not significant, but may have impacted on markers due to the general trend in the decrease of growth observed. $\alpha_v\beta_3$ and VR integrin expression decreased significantly in the PC-3 cell line which corresponds to the lower growth rate with fish oil. Lower $\alpha_v\beta_3$ and VR expression in cells by addition of fish oil to the culture media could be of interest in decreasing metastases to the bone, as cells that express the $\alpha_v\beta_3$ integrins adhere to and migrate on vitronectin (VN), an $\alpha_v\beta_3$ ligand expressed in mature bone where PCa cells preferentially metastasize (Zheng *et al.*, 2001).

PSA production in RWPE-1 was undetectable for lycopene and Vitamin E, and there was no significant difference in production for fish oil. PSA production increased significantly in the 22Rv1 and LNCaP cell lines with fish oil supplementation. Cell growth decreased significantly in both these cell lines with the addition of fish oil. This is of interest because an increase in PSA is theoretically supposed to indicate cancerous growth, although Stamey *et al.* (2004) suggest that this may no longer be the case, which would agree with the findings here, due to the inhibition in growth but an increase in PSA production. Further examination of PSA in these cell lines is required, although these cell lines only produce levels within the normal human range and should perhaps be questioned as a viable biomarker, due to a lack of large increase in PSA in the more aggressive phenotypes.

In conclusion, we found that there was no significant decrease in growth with the concentration of lycopene used, therefore higher concentrations need to be tested for

growth inhibitory effects and cytotoxicity. Contrary to reports in the literature we found no inhibitory effect on phenotypically normal PCa cells (RWPE-1) with the addition of Vitamin E, and growth rate was increased in the cell lines representing cancerous phenotypes with this nutrient. Fish oil showed growth inhibitory effects on all of the cell lines tested, giving further credibility to its protective effects in cancerous cells. Decrease in levels of the $\alpha v\beta_3$ and VR in the most invasive cell line with the addition of these nutrients, is a crucial finding because the primary site of metastasis in PCa is bone, and these integrins are involved in migration to VN which is expressed in mature bone. This finding is especially interesting with the addition of fish oil due to the corresponding decrease in cell growth. The results fuel the controversy regarding PSA as a biomarker because it showed little relationship to growth results and integrin expression in the cell lines examined.

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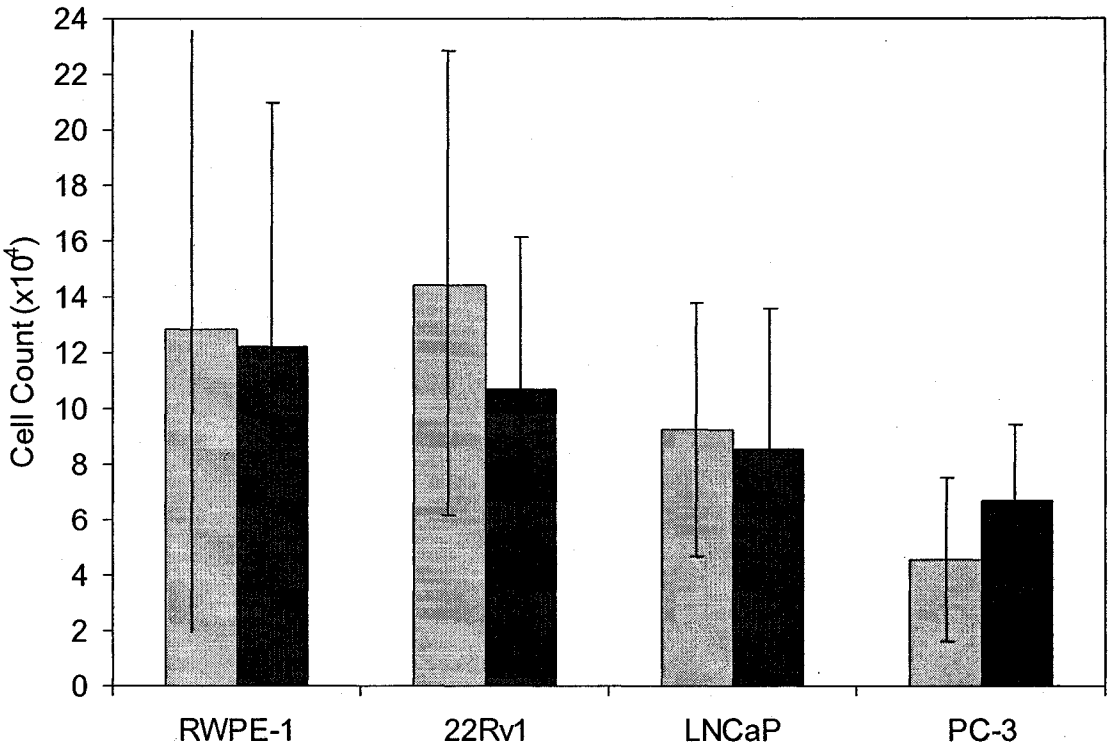

Figure 5-1 Comparison of cell growth for RWPE-1, 22Rv1, LNCaP, and PC-3 cell lines when media contained lycopene ($1x10^{-8}$M). Cells were cultured in 6 well plates, as described in "Materials and Methods". (Bars represent the means $\pm$ SE; n = 11-24 (PC-3 lycopene n =5); dark bars represent fish oil treatment and * signify statistical significance between lycopene and the vehicle control for the cell line; $p<0.05$).

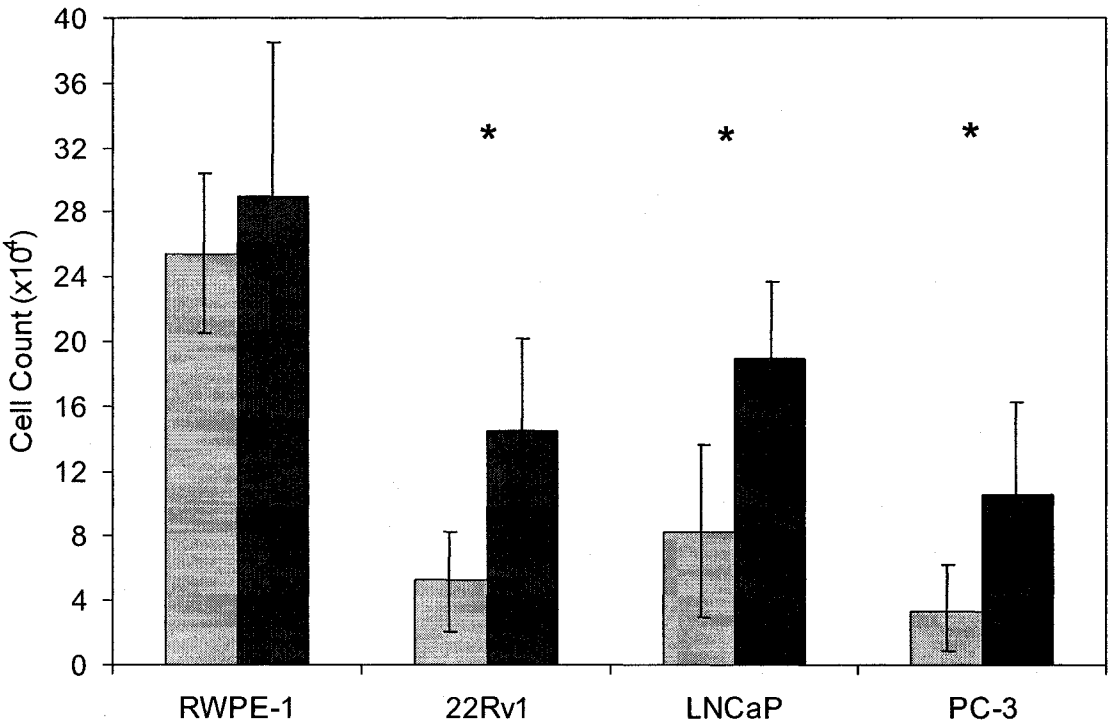

Figure 5-2 Comparison of cell growth for RWPE-1, 22Rv1, LNCaP, and PC-3 cell lines when media contained physiological levels of Vitamin E ($5x10^{-6}$M). Cells were cultured in 6 well plates, as described in "Materials and Methods". (Bars represent the means $\pm$ SE; n = 9-22; dark bars represent fish oil treatment and * signify statistical significance between Vitamin E and the vehicle control for the cell line; $p<0.05$).

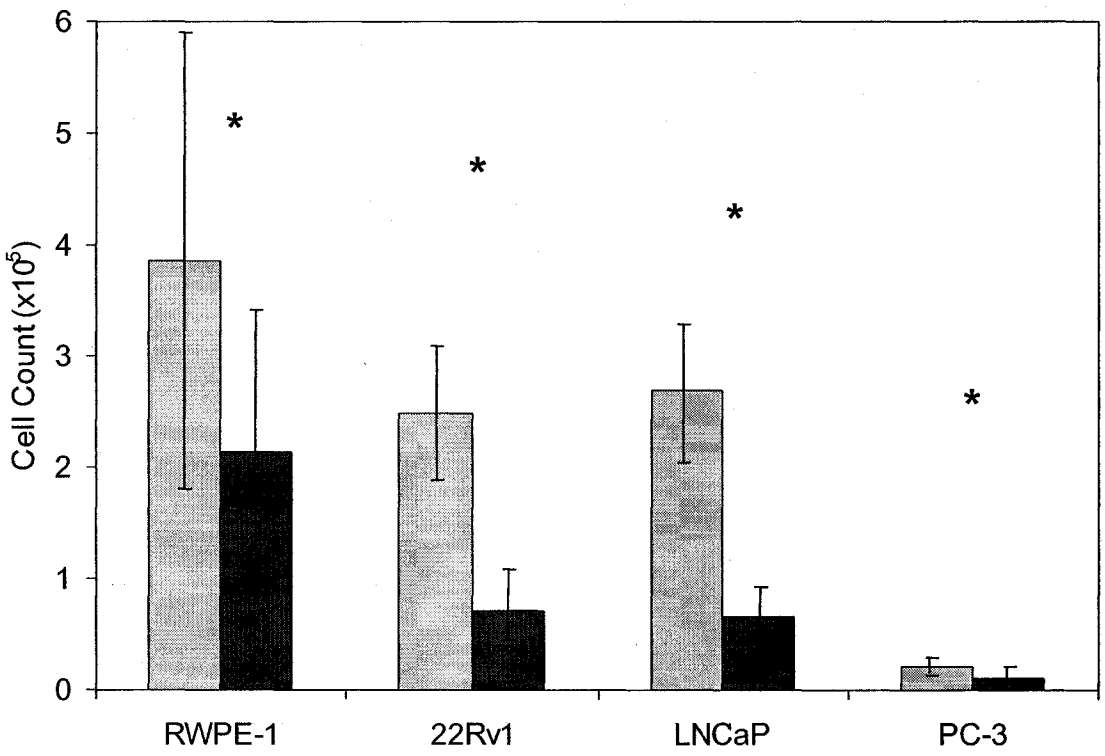

Figure 5-3 Comparison of cell growth for RWPE-1, 22Rv1, LNCaP, and PC-3 cell lines when media contained physiological levels of fish oil ($1x10^{-6}$M). Cells were cultured in 6 well plates, as described in "Materials and Methods". (Bars represent the means $\pm$ SE; n = 11-23; dark bars represent fish oil treatment and * signify statistical significance between fish oil and the vehicle control for the cell line; $p<0.05$).

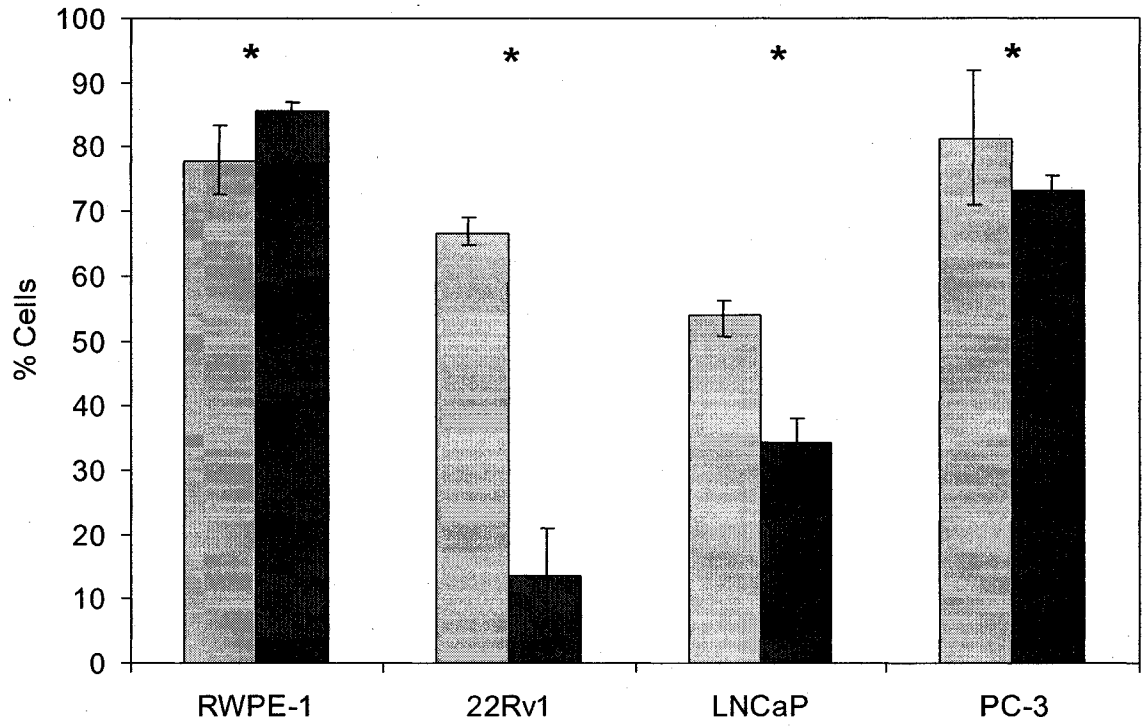

Figure 5-4 Comparison of $\alpha_2\beta_1$ integrin expression for RWPE-1, 22Rv1, LNCaP and PC-3 cell lines when media contained physiological levels of lycopene ($1x10^{-8}$M). Cells were cultured, as described in "Materials and Methods". (Bars represent the mean % $\pm$ SE; n = 9-20 (except LNCaP vehicle control n = 5); dark bars represent lycopene treatment and * signify statistical significance between lycopene and the vehicle control for the cell line; $p<0.05$).

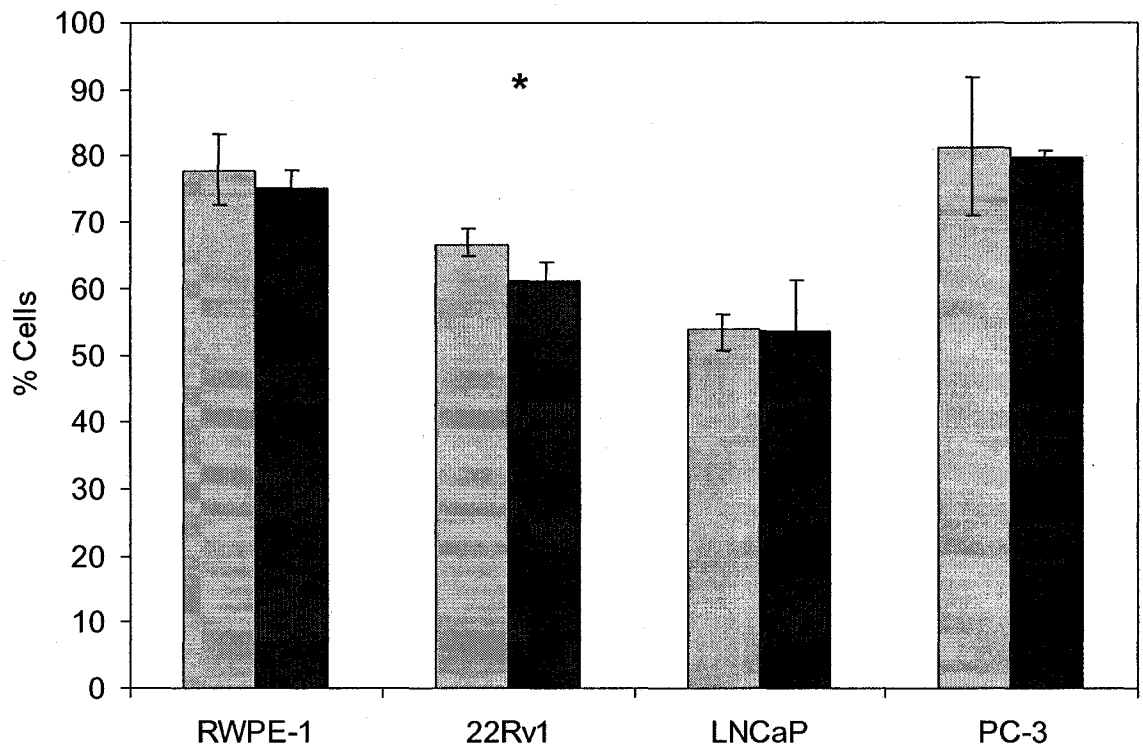

Figure 5-5 Comparison of $\alpha_2\beta_1$ integrin expression for RWPE-1, 22Rv1, LNCaP and PC-3 when media contained physiological levels of Vitamin E ($5x10^{-6}$M). Cells were cultured, as described in "Materials and Methods". (Bars represent the mean % $\pm$ SE; n = 8-20 (except LNCaP vehicle control n = 5; dark bars represent Vitamin E treatment and * signify statistical significance between Vitamin E and the vehicle control for the cell line; $p<0.05$).

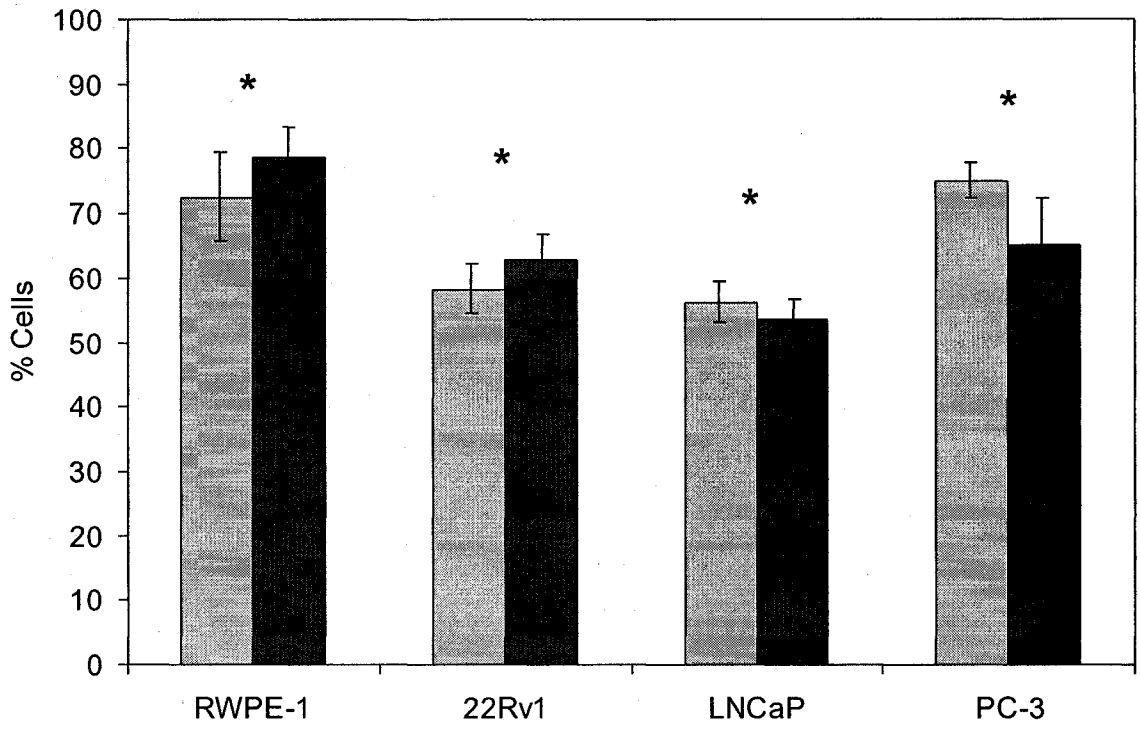

Figure 5-6 Comparison of $\alpha_2\beta_1$ integrin expression for RWPE-1, 22Rv1, LNCaP and PC-3 cell lines when media contained physiological levels of fish oil ($1x10^{-6}$M). Cells were cultured, as described in "Materials and Methods". (Bars represent the mean % $\pm$ SE; n = 10-21; dark bars represent fish oil treatment and * signify statistical significance between fish oil and the vehicle control for the cell line; $p<0.05$).

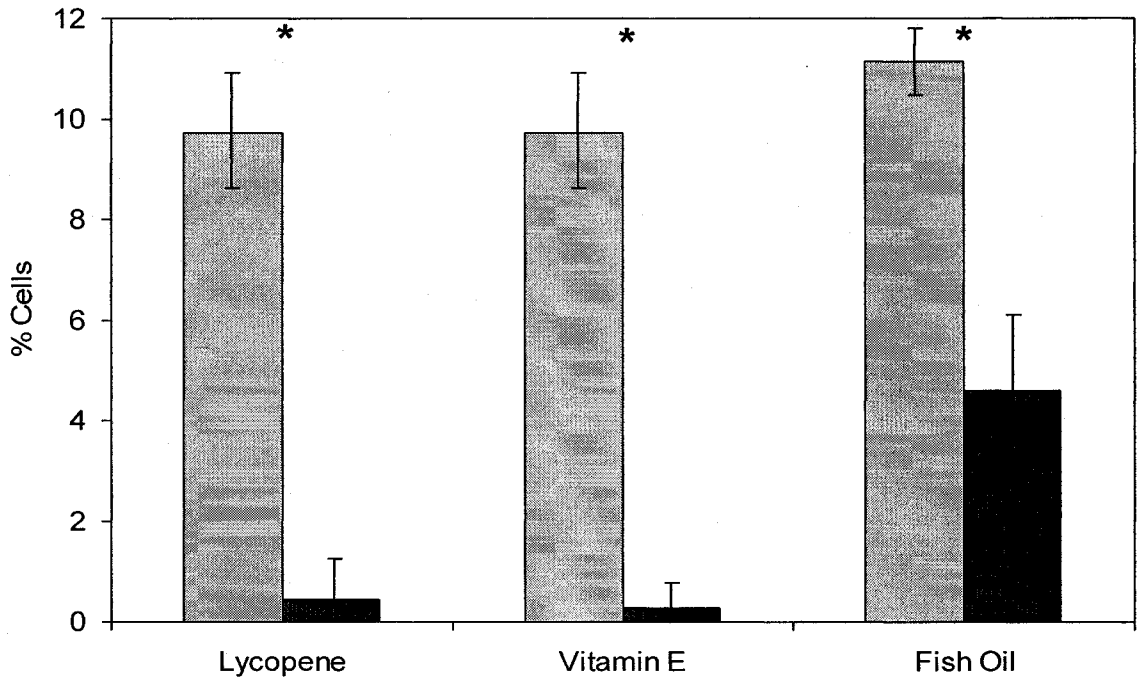

Figure 5-7 Comparison of $\alpha_v\beta_3$ integrin expression for the PC-3 cell line when media contained physiological levels of lycopene, vitamin E and fish oil ($1x10^{-8}$M, $5x10^{-6}$M, $1x10^{-6}$M respectively). Cells were cultured, as described in "Materials and Methods". (Bars represent the mean% $\pm$ SE of n = 5-11; dark bars represent fish oil treatment and * signify statistical significance between fish oil and the vehicle control; $p<0.05$).

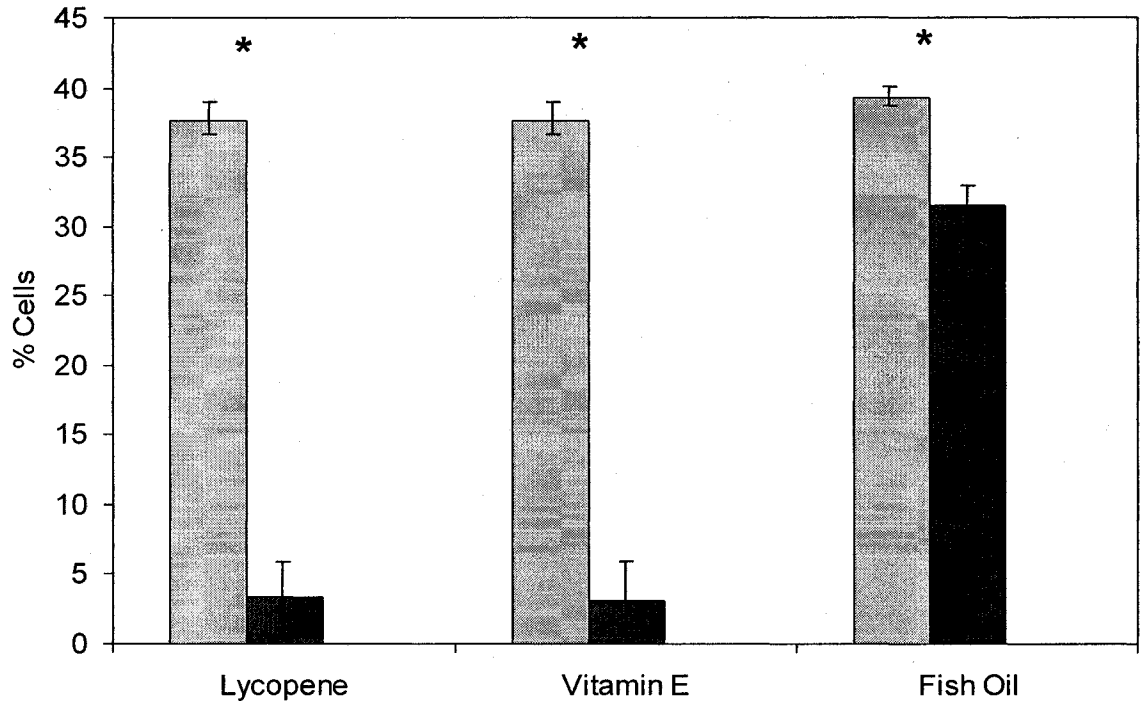

Figure 5-8 Comparison of VR integrin expression for the PC-3 cell line when media contained physiological levels of lycopene, vitamin E and fish oil ($1x10^{-8}$M, $5x10^{-6}$M, $1x10^{-6}$M respectively). Cells were cultured, as described in "Materials and Methods". (Bars represent the mean% $\pm$ SE of n = 11; dark bars represent fish oil treatment and * signify statistical significance between fish oil and the vehicle control; $p<0.05$).

Table 5-1 Physiological concentrations (means and ranges) of lycopene, Vitamin E and fish oil found in current literature with corresponding experimental concentrations that did not induce cytoxicity.

Nutrient	*Human Concentrations*	*Reference(s)*	*Experimental Concentration*
Lycopene	Mean: ~ 0.5μM Range: 0.22μM – 1.06μM	Stahl and Sies (1996)	0.01μM
Vitamin E	Range: ~1.16μM - 116μM 18μM – 25.6μM*	Lagua and Claudio (1996) Cao *et al.* (1998)	5μM
Fish Oil	0.1μM	Pratt *et al.* (2001)	0.1μM

*subjects eating 5 servings of fruit and vegetables per day at baseline (Cao *et al.*, 1998)

Table 5-2 Comparison of cell growth in cell lines with the addition of lycopene, Vitamin E and fish oil ($1x10^{-8}$M, $5x10^{-6}$M and $1x10^{-6}$M respectively). Cells were cultured as described in "Materials and Methods". (Values expressed as mean ($x10^4$) ± SE with n = 9-24 for each nutrient (except PC-3 lycopene n = 5).

Cell Line	*Lycopene*		*Vitamin E*		Fish Oil	
	Vehicle Control	Nutrient	Vehicle Control	Nutrient	Vehicle Control	Nutrient
RWPE-1	12.8 ± 10.7	12.2 ± 8.7	25.4 ± 5.0	29.0 ± 9.8	38.6 ± 20.6	21.3 ± 12.5
p-value	0.85		0.19		0.004	
22Rv1	14.4 ± 8.2	10.7 ± 5.3	6.0 ± 3.5	14.5 ± 5.7	24.80 6.10	7.1 ± 3.2
p-value	0.15		$2x10^{-4}$		$4.5x10^{-10}$	
LNCaP	9.2 ± 4.5	8.6 ± 4.9	8.3 ± 5.3	19.0 ± 4.6	26.9 ± 6.3	6.6 ± 3.0
p-value	0.70		$8x10^{-4}$		$5.7x10^{-8}$	
PC-3	4.5 ± 2.9	6.7 ± 2.7	3.4 ± 2.7	10.6 ± 5.8	2.1 ± 0.5	1.1 ± 0.5
p-value	0.16		0.0001		0.0001	

Table 5-3 Comparison of integrin expression in cell lines with the addition of lycopene, Vitamin E and fish oil ($1x10^{-8}$M, $5x10^{-6}$M and $1x10^{-6}$M respectively). Cells were cultured as described in "Materials and Methods". (Values expressed as mean % ± SE with n =5-21 for each nutrient.

Cell Line	*Integrin*	*Lycopene*		*Vitamin E*		*Fish Oil*	
		Vehicle Control	Nutrient	Vehicle Control	Nutrient	Vehicle Control	Nutrient
RWPE-1	$\alpha_2\beta_1$	77.8 ± 5.5	85.7 ± 1.4	77.8 ± 5.5	75.33 ± 2.41	72.4 ± 6.8	78.8 ± 4.3
	p-value	$2.6x10^{-6}$		0.10		0.02	
22Rv1	$\alpha_2\beta_1$	66.5 ± 2.1	13.44 ± 7.37	66.5 ± 2.1	61.2 ± 2.8	58.2 ± 3.8	62.9 ± 3.9
	p-value	$5.6x10^{-7}$		$9.5x10^{-5}$		$4.4x10^{-4}$	
LNCaP	$\alpha_2\beta_1$	53.8 ±2.9	34.20 ± 3.92	53.8 ± 2.9	53.74 ± 8.01	56.2 ± 3.1	53.5 ± 3.1
	p-value	$4.1x10^{-7}$		1.00		0.01	
PC-3	$\alpha_2\beta_1$	81.3 ± 10.6	73.1 ± 2.1	81.3 ± 10.6	79.8 ± 2.1	74.8 ± 2.7	65.2 ± 6.8
	p-value	0.04		0.68		0.001	
PC-3	$\alpha_v\beta_3$	9.7 ± 1.2	0.6 ±0.7	9.7 ±1.2	0.4 ± 0.4	11.1 ± 0.7	4.6 ±1.5
	p-value	$2.65x10^{-10}$		$6.75x10^{-11}$		$2.2x10^{-9}$	
PC-3	VR	37.7 ± 1.2	3.4 ± 2.4	37.7 ± 1.2	3.1 ± 3.1	39.3 ± 1.5	31.5 ± 2.4
	p-value	$3.44x10^{-10}$		$1.17x10^{-8}$		$6.3x10^{-8}$	

Table 5-4 Comparison of PSA levels in RWPE-1, 22Rv1 and LNCaP cell lines with the addition of lycopene, Vitamin E and fish oil ($1x10^{-8}$M, $5x10^{-6}$M and $1x10^{-6}$M respectively). PSA measured as described in "Materials and Methods". (Values expressed as mean % ± SE with n = 12 (except for RWPE fish oil); all PSA values (ng/ml) corrected for cell count ($x10^{-7}$).

Cell Line	*Lycopene*		*Vitamin E*		*Fish Oil*	
	Control	Nutrient	Control	Nutrient	Control	Nutrient
RWPE-1	n/a	n/a	n/a	n/a	2.3 ± 0.3	3.5 ± 0.03
p-value					0.20	
22Rv1	7.3 ± 1.0	22.7 ± 3.7	7.3 ± 1.0	5.68 ± 0.4	7.21 ± 0.98	9.77 ± 4.35
p-value	$9.4x10^{-5}$		$7.6x10^{-5}$		$8.6x10^{-6}$	
LNCaP	5.0 ± 0.7	10.7 ± 1.7	5.0 ± 0.7	10.5 ± 0.7	6.79 ± 0.92	11.0 ± 4.9
p-value	$2.6x10^{-8}$		$2.6x10^{-15}$		0.01	

n/a = not enough PSA produced to measure

6. GENERAL SUMMARY AND DISCUSSION

6.1 SUMMARY OF RESULTS

The objectives of this research were to test the following hypotheses:

1. **There will be differences in growth rate, PSA production and expression of the $\alpha_v\beta_3$, vitronectin receptor (VR), $\alpha_2\beta_1$ integrins in the RWPE-1, 22Rv1, LNCaP, DU-145 and PC-3 cell lines when culture media is supplemented with FCS to HS.**

This hypothesis was supported or rejected by the results reported in chapter 3. Specifically, it was hypothesized that:

i. **Cell lines cultured in media supplemented with HS will have a greater growth rate than same cell lines cultured in media supplemented with FCS, due to large differences in fatty acid composition between HS and FCS.**

This hypothesis was rejected. The results reported in Chapter 3 show that cell growth was significantly slower in HS for all cell lines except RWPE-1.

ii. **PSA levels of the cell lines cultured in media supplemented with HS will be higher than those cultured in FCS, due to the fact that FCS is devoid of testosterone, due to the relationship between PSA levels and androgen production.**

This hypothesis was rejected. The results in Chapter 3 show no significant difference between PSA levels in cells cultured in media supplemented with HS or FCS.

iii. **Integrin expression of the cell lines cultured in media supplemented with HS will be different than the integrin expression of the same cell lines cultured in media supplemented with FCS.**

This hypothesis was supported by the results in chapter 3 as there was a significant difference in the expression of VR in the DU-145 cell line, with expression being significantly higher in the cells grown in FCS. Significant differences in $\alpha_2\beta_1$ expression occurred in the 22Rv1 and PC-3 cell lines, with expression being significantly higher in the cells grown in FCS for both cell lines.

iv. **The expression of the $\alpha_v\beta_3$, VR integrins will be the highest in the PC-3 cell line.**

This hypothesis was supported by there results in chapter 3. The results in chapter 3 also showed that the DU-145 cell line expresses very similar amounts of $\alpha_v\beta_3$ and VR to the PC-3 cell line.

2. **We will successfully establish a method for the isolation and culturing of epithelial cells from human prostates, and obtain preliminary growth and integrin data on these cultures.**

This was supported by the results reported in chapter 4. Specifically that:

i. **We will successfully establish a method for the isolation and culture of epithelial cells from human prostates by examining the previous methods outlined in the literature and optimizing them for our use.**

This was supported by the adaptation of a protocol developed by Peehl *et al.* for use in our laboratory.

ii. **We will obtain more than $1x10^6$ cells per every ~2.5 gram sample of tissue obtained from the radical prostatectomies.**

This was supported by the results in Chapter 4, as we obtained an average of $4x10^6$ cells for every sample of tissue obtained using our final method for the isolation and culture of prostatic epithelial cells.

iii. **The expression of the $\alpha_v\beta_3$, VR and $\alpha_2\beta_1$ integrins will be similar to those found in the more invasive cell lines.**

Integrin expression in the preliminary data varied with the sample tested, as reported in Chapter 4. Sample 3 had expression that was similar to that found in the more invasive cell lines (Du-145, PC-3), although more samples need to be collected for conclusive results.

3. **Lycopene, vitamin E and fish oil will decrease growth and change integrin expression in cell lines cultured in HS.**

This hypothesis was supported or rejected by the results reported in chapter 5. Specifically, it was hypothesized that:

i. **Physiological relevant concentrations of lycopene, vitamin E and fish oil will result in decreased growth rates for all cell lines.**

This hypothesis was both supported and rejected dependent upon the nutrient used, as reported in the results in chapter 5. No significant change in growth rates of any of the cell lines was observed with the addition of lycopene. A significant increase in growth was seen with the addition of Vitamin E to all cell lines except RWPE-1. A significant decrease in growth was observed in all cell lines with the addition of fish oil.

ii. Integrin expression will decrease in all cell lines with the addition of lycopene, Vitamin E or fish oil.

This hypothesis was both supported and rejected as reported in the results in chapter 5. Significant decrease in $\alpha_v\beta_3$ and VR expression was seen in the PC-3 cell line when cells were supplemented with fish oil. Significantly lower expression of $\alpha_2\beta_1$ expression was observed in 22Rv1 with lycopene and Vitamin E. This cell line exhibited higher expression of $\alpha_2\beta_1$ when cultured with fish oil. Fish oil was the only nutrient that produced a decrease in cell growth in this cell line. $\alpha_2\beta_1$ expression was lower in LNCaP cells with the addition of lycopene or fish oil whereas $\alpha_2\beta_1$ expression was higher in RWPE-1 when lycopene or fish oil was added to the media.

6.2 GENERAL DISCUSSION

The first issue this study addressed was the matter of making the cell line model of PCa more relevant to the human condition by culturing cell lines in HS rather than FCS. There was a significant decrease in cell growth when compared to FCS, thereby asserting the validity of growing the cell lines in HS. The higher growth rate of cell lines in FCS was perhaps due to the increased amount of linoleic acid (LA, 18:2n-6) found in FCS compared to HS. LA does not appear to be a causal agent of PCa but rather promotes growth of established tumors. It could be suggested that increasing the percentage of HS in culture would result in increased growth. Initially, several concentrations of HS ranging from 1 to 10% (v/v) were used, with no significant differences in growth observed between the percentages of HS used. There are large differences in fatty acid composition and quantity of the two media supplements, including n-3 fatty acids, the n6:n3 fatty acid and the polyunsaturated: saturated fatty acid ratio between HS and FCS. It was determined that for direct comparison of cell line growth, 1% HS would correspond to 10% FCS, with regards to amounts of major fatty acids.

Fatty acid analysis on patients with varying stages of disease compared to healthy human serum would also be beneficial to see if there are any marked changes over time with the progression of this disease. It would be interesting to culture cell lines in human serum from patients who have malignancy that the specific cell line most represents.

More human samples would be required to most appropriately match clinical specimens to each of our cell lines.

There were no significant differences seen in PSA levels when cell lines capable of producing it were cultured in HS or FCS. This serves to further increase the controversy over this particular marker due to the different composition of HS and FCS, and the fact that HS contains testosterone, an androgen that has been reported to up regulate PSA expression in RWPE-1, 22Rv1 and LNCaP cell lines (ATCC, Manassas, VA, USA). We did not examine free versus total PSA in this study, due to the lack of significant difference observed between cells cultured in media supplemented with HS versus FCS.

Preliminary work on obtaining epithelial cells from human prostates has been completed in our lab. Comparative studies of growth responses of prostatic cells derived from normal central zone, normal peripheral zone, BPH nodules and adenocarcinomas did not reveal any qualitative differences (Peehl and Stamey, 1986). Characterization of the epithelial cells obtained from human subjects will be useful to determine the amount of the cellular population that has cancerous phenotypes versus normal epithelial phenotypes in culture. Peehl *et al.* (1988) report that the colony-forming efficiency of a cell population derived from cancer tissue was not significantly different from those populations derived from normal tissues, also supporting characterization of the epithelial cells isolated from the prostate samples.

It would be of use to have a pathologist characterize the area of the prostate from which samples are obtained in order to be assured that the area received contains primarily adenocarcinoma cells rather than normal or BPH cells. Peehl *et al.* (2005) note that there are difficulties with gross dissection of malignant tumors from tissues, and that tissues comprised of >90% cancer are only obtainable about 50% of the time. Peehl *et al.* (2005) have observed a decrease in the mean volume of primary adenocarcinomas of the prostate diagnosed at Stanford from a mean volume of 5.3cc (20 years ago) to 2.4 cc presently, decreasing the capacity to obtain grossly dissected tissues that are mostly cancer. This observation furthers the need for a surface marker that can reliably sort cancer from non-malignant cells. Webber (1979) determined that epithelial cell growth was enhanced when 50U/ml Type I collagenase was incorporated into the culture media. This may be worth exploring with primary culture of prostate epithelial cells, due to a

lack of proliferation seen in our cultures, although it should be noted that no other groups have added Type I collagenase to the culture media. Culturing prostatic epithelial cells from ultrasound-guided needle biopsies may be worth exploration for future studies, as Peehl *et al.* (1991) have successfully obtained prostate epithelial cells free of fibroblast, with maintained expression of PSA *in vitro*.

Five cell lines relating to progressive and metastatic stages of PCa were characterized in this study. This is a unique approach as the majority of studies reported in the literature use only one or two cell lines, usually representing only invasive cell phenotypes. A large part of the integrin research reports on individual subunits, rather than heterodimers, which is more relevant because that is how these proteins are found in the body, thus there is a need for further research examining the heterodimers.

Zheng *et al.* (1999) demonstrated that the focal adhesion kinase (FAK) pathway modulated prostate epithelial cell migration on VN. Expression of the $\alpha_v\beta_3$ integrin and its engagement by VN in PCa cells generate a migratory phenotype that correlates with a specific increase in FAK tyrosine phosphorylation, suggesting a role for FAK-signaling pathways in prostate epithelial cell migration on VN since FAK-related non kinase (FRNK), a negative regulator of FAK, blocks migration of the cancer cells, suggesting that perhaps activation of FAK will modulate *in vivo* migration and invasion of PCa cells via $\alpha_v\beta_3$ (Zheng *et al.*, 1999). This study demonstrates that the pathways involved in the changes in integrin expression over the progression of PCa need further investigation.

The only nutrient which inhibited growth of the cell lines was fish oil, confirming other epidemiological and experimental data. Dietary intake of products that contain fish oil may be a potential recommendation for those with PCa, as well as for preventative use. Due to the significant inhibition in growth and change in integrin expression observed with fish oil, it would be worthwhile to examine whether EPA and or DHA is responsible for these effects. Examination of phospholipid composition of the cell lines before and after supplementation with fish oil would also be worth examining. The increase in cell growth that was seen in the cell lines with Vitamin E supplementation may indicate that Vitamin E supplementation should not be recommended to patients with PCa, at this time. The lack of significant change in growth in the RWPE-1 cell line with Vitamin E supplementation neither supports nor refutes the preventative properties

that have been touted for this nutrient. Lycopene reduced cell growth (although this was not significant), and resulted in significant changes in integrin expression. Therefore it would be useful to determine whether or not higher concentrations of lycopene do have a significant effect on cell growth. Overall, further experimental research on the possible mechanisms by which these nutrients exert their effects is warranted.

The results of this study demonstrate that cell culture conditions that resemble human physiological conditions as closely as possible are of importance when using *in vitro* research tools, and that the three nutrients examined in this study at the respective concentrations tested do indeed have an effect on cell growth and integrin expression. The inhibition of cell growth with the addition of fish oil to cell culture is of importance as it provides support for dietary supplementation with fish oil prior to and during the progression of PCa.

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