PECTIN INDUCES APOPTOSIS IN HUMAN PROSTATE CANCER CELLS:

STRUCTURE FUNCTION RELATIONSHIP

by

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(Under the Direction of Debra Mohnen, Ph.D.)

ABSTRACT

Prostate cancer is the most common malignancy and second leading cause of death from cancer in men in the United States. The goal of many cancer therapies, such as androgen deprivation therapy (ADT), is to induce apoptosis in tumor cells. Androgen deprivation therapy in prostate cancer is rarely curative because the metastatic cancer is heterogenous, consisting of both androgen-dependent and androgen-independent prostate cancer cells. Therefore, it is critical to identify agents that induce the death of both androgen-responsive and androgen-insensitive cells. Here it is demonstrated that a product of plant cell walls, pectin, is capable of inducing apoptosis in both androgen-responsive (LNCaP) and androgen-independent (LNCaP C4-2) human prostate cancer cells. Fractionated pectin powder (FPP) induced significant apoptosis (approximately 40-fold above non-treated cells) in both LNCaP and LNCaP C4-2 human prostate cancer cells as determined by the Apoptosense assay and activation of caspase-3 and its substrate, PARP. Citrus pectin and pH-modified pectin, PectaSol, had little or no apoptotic activity, suggesting a structure-dependence to pectin's apoptotic activity. Glycosyl residue composition and linkage analyses revealed no significant differences between the pectins. Mild base treatment to remove ester linkages destroyed FPP's apoptotic activity and yielded HGA oligosaccharides. Treatment of FPP with pectinmethylesterase to remove galacturonosyl carboxymethylesters and/or with endopolygalacturonase to cleave non-methylesterified homogalacturonan caused no major reduction in apoptotic activity, implicating the requirement for a base-sensitive linkage other than the carboxymethylester. For the first time, specific structural features of pectin responsible for inducing apoptosis are identified. These findings provide the foundation for future research on the mechanism by which specific pectic structures induce apoptosis in cancer cells and provide a basis for the rational development of pectin-based pharmaceuticals, nutraceuticals, or recommended diet changes aimed at reducing or inhibiting the occurrence and progression of cancer.

INDEX WORDS: pectin, prostate cancer, nutrition, apoptosis

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DEDICATION

I dedicate this thesis to those women making a permanent mark in the world by their

contribution to science and the betterment of mankind.

ACKNOWLEDGEMENTS

God first and foremost.

My parents for your continued support.

Derrick for your patience and unconditional love.

Kahtonna for your true friendship.

Dr. Debra Mohnen, whom I greatly admire, for your vision and contribution to science.

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Scope of Study

Approximately 230 American men in every 100,000 will develop prostate cancer each year, making it the most common malignancy in men in the United States (*Surveillance, Epidemiology, and End Results Program (SEER),* 1975-2000, Division of Cancer Control and Population Sciences, National Cancer Institute, 2003). Prostate cancer is the second leading cause of death from cancer in American men (American Cancer Society, 2004). The goal of many cancer therapies, such as antihormone therapy and chemotherapy, is to induce apoptosis in tumor cells. In hormonally responsive prostate cancer, androgen deprivation therapies induce cell death in androgen-sensitive cells (Colombel and Buttyan, 1995; Buttyan *et al..*, 1997; Perlman *et al..*, 1999; Bruckheimer *et al..*, 1999), while androgen-insensitive cells remain unaffected (Kozlowski and Grayhack, 1991; Santen, 1992; Kreis W, 1995). However, androgen-insensitive cells are capable of undergoing apoptosis. Thus, the identification of novel methods to induce apoptosis in prostate cancer cells, irrespective of their androgen response, has great therapeutic value.

Although the etiology of prostate cancer is poorly understood, according to the American Institute of Cancer Research, 30-40% of all cancer cases are preventable by dietary means. The impact of dietary components on prostatic carcinoma appears to depend on a multitude of genetic and epigenetic factors that have recently been unveiled through molecular tools like expression profiling. One specific dietary constituent exhibiting anticancer capabilities is the plant polysaccharide pectin. Currently, pectin has at least three definable and reproducible effects on health: oral administration of pectin inhibits cancer growth and metastasis (Platt and Raz, 1992;

Pienta *et al.*., 1995; Nangia-Makker *et al.*. 2002), reduces serum glucose levels (Jenkins *et al.*, 1977) and reduces cholesterol levels (Kay and Truswell, 1977). In this paper we demonstrate that pectin, a plant polysaccharide fiber, induces apoptosis in both androgen-responsive and androgen-independent human prostate cancer cells. Furthermore, for the first time, we have identified specific structural features of pectin responsible for inducing apoptosis. These findings provide the foundation for future research targeted at identifying the mechanism by which specific pectic structures induce apoptosis in cancer cells and provide a basis for the rational development of pectin-based pharmaceuticals, neutraceuticals, or recommended diet changes aimed at reducing/inhibiting the occurrence and progression of cancer across populations.

Cancer: Microevolutionary process of cancer

The cells of a multicellular organism are a part of an interdependent network. As such, they collaborate to ensure optimum viability of the organism. In all adult systems, except for the nervous system and muscle, there is regular cellular replication throughout life (Cooper 2000). Occasionally cells will proliferate at an atypical rate, forming a tumor/neoplasm, a localized mass of growing cells (Ewing 1921). As long as this neoplasm is kept clustered together in a single mass, the tumor is said to be benign. It is not until the rapidly-proliferating and abnormal cells invade surrounding tissues that they become malignant or cancerous (Ptot-1986). Benign cells are generally uniformly sized, while malignant cells are abnormally shaped and usually large with irregularly shaped nuclei.

As mentioned above, when cells evade normal growth regulatory mechanisms and begin to divide without constraint, cancer occurs. Different patterns of cancer occur in children than in adults because more cells replicate at earlier ages. As a child, the brain, nervous system, bones,

muscles and connective tissue are rapidly growing and cancer is more common in these tissues in children than they are in the same tissues in adults (WCRF, AICR). Adult tumors are typically derived from epithelial linings. Cancers arising from epithelial cells are known as carcinomas, cancers that have developed from connective tissue or muscle cells are sarcomas, and those cancers originating from hemopoietic cells or the nervous system fall in to the category of leukemias (Fritz *et al.*, 2000). Because cellular proliferation occurs mostly in epithelial cells and because these tissues are more frequently exposed to carcinogens than other tissues, approximately 90% of cancers are carcinomas. Found on the surface of the skin, cavity linings and as the inner lining of lymph and blood vessels (endothelia), epithelial cells are organized as stratified squamous cells (Alberts *et al.*, 2002). The apical surface of epithelial cells corresponds to the side that is exposed to the external environment, such as the air in the skin epidermis, or to the lumen of an organ. The surface that is exposed to the internal environment and is attached to a layer of carbohydrate and protein-rich extracellular matrix called the basal lamina is known as the basolateral surface (Alberts *et al.*, 2002). Anchorage to this extracellular matrix by way of transmembrane glycoproteins called integrins is critical for the survival of most vertebrate cells (Hynes 1992). In fact, this anchorage is lost in cancerous cells, allowing them to invade surrounding tissues. Under normal circumstances, proliferation in the stratified epithelia occurs only in the basal layer (McNeal *et al.*, 1995; Kyprianou *et al.*, 1996). Newly generated cells move outward towards the surface and terminally differentiate into flattened, keratin-rich nondividing cells before dying and being sloughed off, a process known as cellular senescence. In cancer, this organization is lost, and intraepithelial neoplasia occurs (Alberts *et al.*, 2002). In high-grade or moderate to severe intraepithelial neoplasia, cells in all epithelial layers are undifferentiated and dividing and are usually abnormally sized and shaped. The secretion of

serine proteases allow the abnormal cells to pass through the basement membrane to enter the extracellular matrix, traverse through the blood stream, and extravasate to a target organ, hence, facilitating metastasis throughout the body (Fig 1.1) (Kohn, 1993; Liotta *et al.*, 1991).
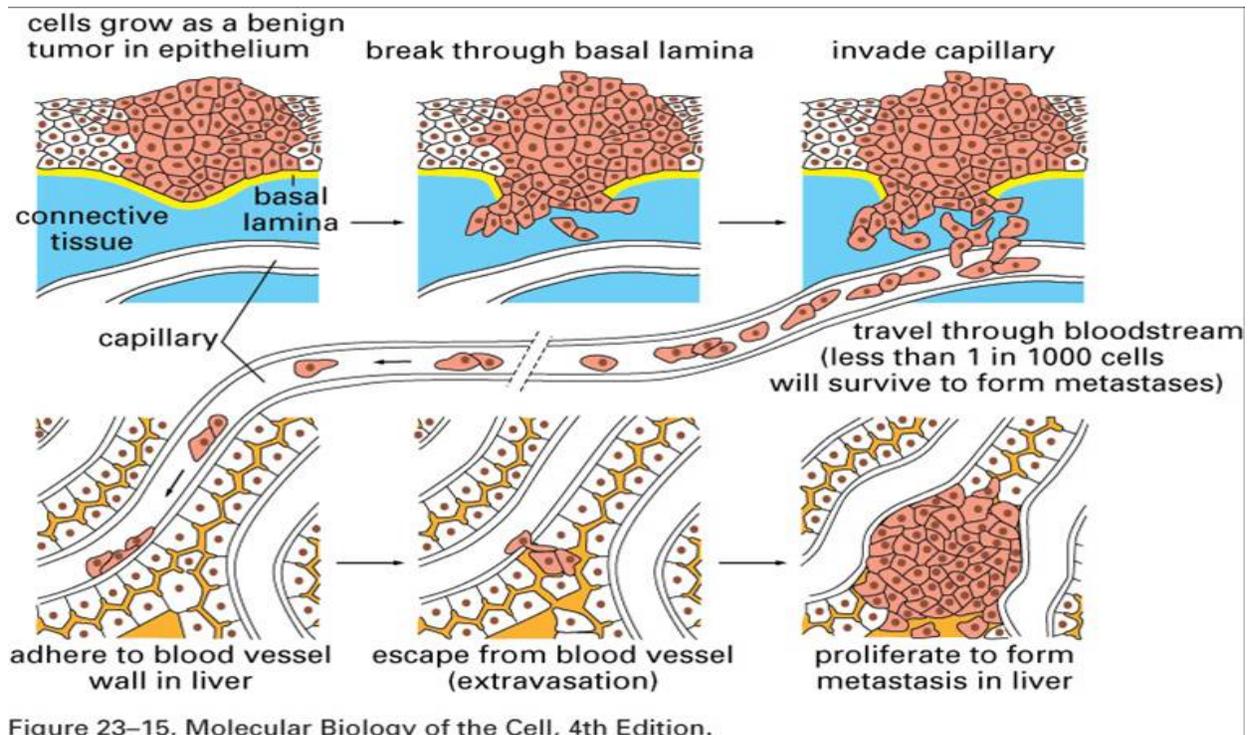
Figure 23–15. Molecular Biology of the Cell, 4th Edition.

Figure 1.1. Steps in the metastatic process. The secretion of serine proteases allow the abnormal cells to 1) pass through the basal lamina and enter the extracellular matrix, 2) traverse through the blood stream, 3) adhere to the blood vessel wall in a secondary site, 4) extravasate to a target organ, and 5) proliferate to form metastasis. (Molecular Biology of the Cell, Fourth Edition, Alberts B *et al.*, 2002)

Cancer cells are a product of genetic mutation or epigenetic change, meaning that they have heritable abnormalities with or without a change in the actual DNA sequence of daughter cells (Cairns 1975; Lengauer 1998; Cahill 1999; Vogelstein and Kinzler, 1993). This property gives cancer cells a competitive advantage over noncancerous cells, allowing them to thrive in the harsh nutrient-deficient, low-oxygen environment of a tumor. Under the same conditions, normal cells would undergo apoptosis or programmed cell death (which will be discussed later). The immortality of cancer cells is in part attributed to their ability to be "naturally selected" in

environmentally unfavorable conditions (Nowell 1976; Cairns 1975). In order to evolve by natural selection, there must be heritable variations in the population that influence the rates of cell survival and reproduction. Being a population of heterogeneous mutant clonal cells (Fey and Tobler, 1996) that escape the mechanisms of cell cycle inhibition and can suppress apoptosis, cancer cells have a competitive advantage over normal cells and thus, are prime candidates for evolution by natural selection.

The tissue origin of each cancer determines its behavior, including the degree of its invasiveness or tendency to migrate throughout the body. For example, carcinomas derived from pigment cells will continue to exhibit the migratory behavior of normal pigment-cells, giving rise to numerous metastases if not eradicated early. Cancer is the result of the accumulation of multiple mutations in a single cell. Metastasized cancerous cells can be traced to a single primary tumor that began from the division of a single aberrant cell (Nowell 1976). Many cancers have a higher incidence rate with increasing age, suggesting that an increased exposure to mutagens over a lifetime results in a higher likelihood of developing cancer with age. (Alberts *et al.*, 2002)

Cancer: Preventable causes of cancer

Carcinogenic agents including chemical carcinogens, ionizing radiations and viruses can create mutations in the DNA sequence of a cell. Some people are more prone to developing certain cancers because they possess a genetic defect in their DNA repair mechanisms which magnifies the rate at which they accumulate chromosomal mutations. Contact with chemical carcinogens such as aromatic amines, intermediates used in dye synthesis and as antioxidants in the rubber and lubricating oil industry, and polycyclic aromatic hydrocarbons such as soot, coal, tar and shale, lead to increased skin, lung and urinary bladder cancer. Surprisingly, these carcinogens

usually enter the body in a chemically inert form and only become "activated" (damaging) after metabolic processing in the body by enzymes known as cytochrome-P-450 oxidases which normally convert ingested toxins into harmless compounds that are excreted from the body (Luch 2005).

Not all cancer-causing substances are direct mutagens. Some chemicals increase the likelihood that a person will develop cancer. So-called tumor initiators act in this manner by causing cellular damage that does not show consequences until the subject is repeatedly exposed to the same or a different carcinogen, thus predisposing cells for cancer formation. Following contact with tumor initiators, exposure to substances known as tumor promoters release previously stated cellular growth restraints and induce the expression of previously mutated growth-controlling genes. Such is the case with phorbol esters that act as tumor promoters by activating the phosphatidylinositol intracellular signaling pathway through protein kinase C, leading to cancer formation (Hofmann 2004).

Cancer: Cancer critical genes

Two classes of genes that are major contributors to cancer formation have been identified, oncogenes and tumor suppressor genes. Tumor suppressors are loss of function mutations while oncogenes are gain of function mutations.

In genetically altered mouse models, malignancy can be induced by the addition of a single gene. Due to their hyperactivity in promoting cellular growth, such genes were given the label oncogene. The widely known *Ras* oncogene was discovered in this manner when a mutated *Ras* gene was found to be the tumor-causing gene in a cancer-causing retrovirus (Alberts *et al.*, 2002). Normally, *Ras* proteins, GTPases, regulate cell growth by transmitting signals from

growth factor receptors on the cell surface (Satoh and Kaziro, 1992; Khosravi-Far and Der, 1994). In mutated *Ras*, this signal is continuously transmitted, and incessant cellular proliferation occurs (Bos, 1989; Clark and Der, 1993). Furthermore, oncogenes can be made overactive by 1) deletions or point mutations that produce a hyperactive product, 2) gene amplification of a protein normally produced at low or regulated levels, or 3) chromosomal rearrangement. (Alberts *et al.*, 2002)

By knowing the location of chromosomal deletions associated with the onset of certain cancers, tumor suppressors were identified. Tumor suppressors are genes whose products inhibit cell division if conditions required for entry into the cell cycle, e.g. the presence of certain growth factors, are not met. One example is the retinoblastoma gene (*Rb*). The retinoblastoma gene encodes a protein that is found in every cell in the human body (p*Rb*). The phosphorylation state of this protein, which is localized in the nucleus, determines whether or not a cell will replicate its DNA and enter the S-phase of the cell division cycle. Cell cycle progression is stimulated when *Rb* is phosphorylated. In mice, knocking out the *Rb* genes causes loss of cell cycle regulation by *Rb* and results in rapid cellular proliferation (Weinberg, 1995). While cell division and thus tumor suppression continues when one allele of the *Rb* gene is mutated, the cell becomes cancer prone. It is not until a subsequent somatic mutation of the remaining normal allele occurs that cancer actually develops. Thus loss of both *Rb* gene copies leads to a loss in p*Rb*'s ability to suppress tumor formation. (Alberts *et al.*, 2002)

Cancer: Cellular repercussions of cancer-critical genes

The generation of transgenic mice that carry an oncogene(s) in all of their cells and of genetic knockout mice, in which certain genes are inactivated, have helped to establish how mutations in oncogenes and in tumor suppressor genes affect the whole organism on a molecular level. As

previously mentioned, deletion of one or both copies of the tumor suppressor gene *Rb* can lead to unremitting cell division in an aberrant cell. In normal cells that contain both *Rb* gene copies, cell cycle progression is stimulated when *Rb* is phosphorylated. In the presence of the *p16* protein, which is produced when cells are stressed, *Rb* is not phosphorylated and the cell is not allowed to enter the S-phase (DNA-replication) of the cell cycle. (Schmitt *et al.*, 2002) (Fig 1.2)
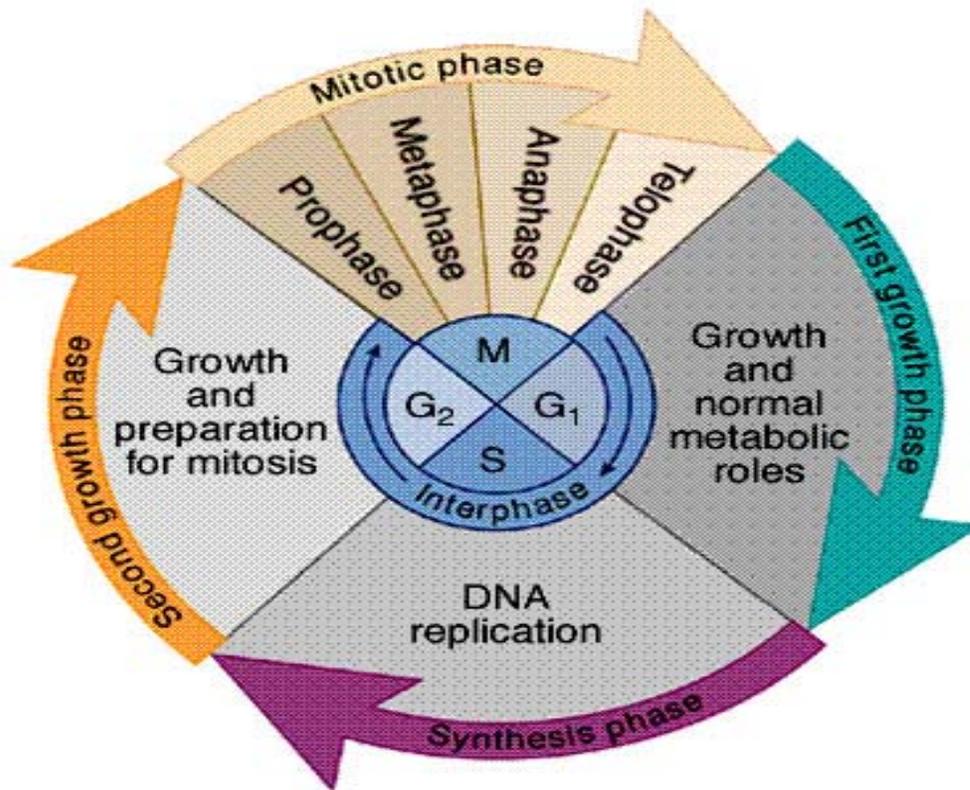
Figure 1.2. Eukaryotic Cell Cycle. In the eukaryotic cell cycle, two cell division events need to be controlled: 1) entry into the S-phase when DNA is replicated and 2) entry into the M-phase when mitosis occurs. Phosphorylation of the tumor suppressor gene *Rb* (retinoblastoma) promotes progression into the S-phase in the cancer process. (http://www.bmb.psu.edu/courses/biotc489/notes/cycle.jpg)

Malignant cells also characteristically evade apoptosis, programmed cell death, which normally occurs when a cell senses disorder or damage. An example of this evasion occurs when the *p53* tumor suppressor gene is mutated (Sherr and McCormick, 2002). Under normal

conditions, *p53* is present in low amounts. When DNA is damaged from factors such as insufficient oxygen, ultraviolet light, etc., *p53* is not degraded as rapidly and is present at high levels, thereby either preventing the cell from dividing as long as the damage is unrepaired, or leading the damaged cell into apoptosis (Hickman *et al.*, 2002). *p53* is mutated in about half of all human cancers, and because it is involved in cell-cycle control, apoptosis initiation, and maintenance of genetic stability, genetically altered *p53* is a triple threat of an organism to cancer. The mutation or absence of *p53* can increase the frequency and size of carcinomas by allowing cells marked for cellular senescence, because of shortened telomeres, to continue dividing (Hayflick and Moorhead, 1961; Harley *et al.*, 1990; Shay and Wright, 2001). Because most human cells lack the enzyme telomerase, telomeres (repetitive DNA sequences that cap the ends of chromosomes) shrink as normal cells divide. Eventually, the shortened telomere acts as a "danger" signal in the cell and growth is arrested by cell-cycle checkpoint genes like *p53*. When *p53* regulation is defective, as in cancerous cells, highly mutated cells continue to survive and divide.

Prostate Cancer: Statistics

Relatively little is known about prostate cancer compared to other cancers, in part because it is difficult to perform long term studies and because the many variants of the disease make it difficult to understand the mechanism of growth and metastasis of prostate cancer. As the most commonly diagnosed nonskin cancer, and the third most diagnosed cancer in males in Western industrialized countries (Parkin, 1999), a man is 33% more likely to develop prostate cancer than a woman is to develop breast cancer. There are generally four disease states associated with prostate cancer progression: 1) localized disease, in which cancer is confined to the prostate, 2)

recurrent disease, when, even after local therapy, there are indications such as high PSA (prostate specific antigen) levels, that the cancer is still present, though the disease cannot be detected in bone scans or by other tests, 3) metastatic disease which occurs when the cancer has invaded tissues outside of the prostate gland and, 4) hormone refractory disease, when cancer continues to grow and persists after androgen ablation therapy (www.prostatecancerfoundation.org). Metastatic prostate cancer that has spread to the lymph nodes or bone is usually incurable (Cheville *et al.*, 2002) (Fig 1.3).
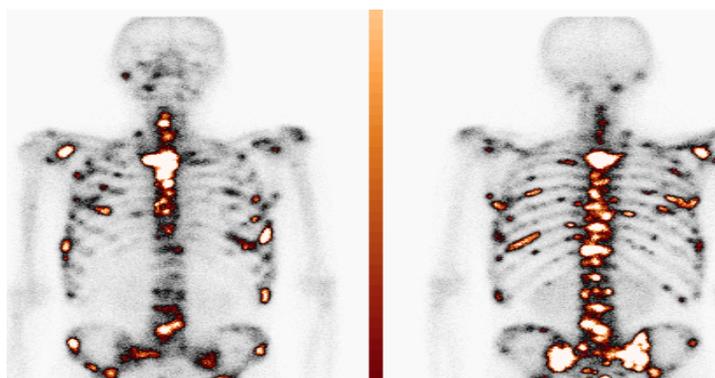
Figure 1.3. Metastasis of prostate cancer to the bone. Radioactive substance injected into the blood stream show areas of rapid bone growth associated with cancer (hot spots). **[www.med.unifi.it/.../ oncologiamed/prostata.htm](www.med.unifi.it)**

Hormone therapy can cause the prostate cancer to shrink temporarily in up to 90% of patients, with a duration of benefit ranging from 6 months to 10 years or more (www.prostatecancerfoundation.org). On average, however, this improvement does not extend past 2 years (Kozlowski and Grayhack, 1996), and of those patients who do experience a relapse, half will die within the first year (Kozlowski and Grayhack, 1996). Prostate cancer is the second leading cause of death from cancer in American men (Greenlee *et al.,* 2000). Generally, risk factors include age, race, diet (Spitz *et al.,* 2000), polymorphic repeats in the androgen receptor gene (Stanford, 1997) and high circulating concentrations of androgens (Spitz *et al.,* 2000). A

connection has also been made between cultural environment and increased cancer incidence. For example, 1) migrants often adopt patterns of cancer incidence seen in the host country, 2) different cancers are more present in certain cultures, and 3) a country as a whole may have increased rates of a specific cancer that are not paralleled by a subpopulation of that country who exemplify a different lifestyle (Muir *et al.,* 1987). In particular, many studies have found that the Western diet is high in consumption of foods posing a health hazard such as animal fat, dairy, and red meat, and substantially low in a number of protective elements against cancer including, selenium, folate, the antioxidant lycopene, and phytoestrogens (Giovannucci, 1999; Schmitz-Drager *et al.*, 2001; Greenwald, 2002; Jankevicius *et al.*, 2002).

Prostate Cancer: Genetic abnormalities of prostate cancer

Because the cells of the prostate require androgens to grow, usually one of first steps in prostate cancer treatment is androgen depletion therapy (ADT). While this method is often initially successful in lowering PSA levels, most often the cancer progresses. In fact, in prostate cells subjected to androgen withdrawal, only the secretory epithelial cells are forced to undergo apoptosis (Kyprianou *et al.,* 1991). Many recent studies have shown that genetic implications may be pertinent to the persistence of prostate cancer (Eder *et al.,* 2001; Feldman and Feldman 2001). When the promoter of the gene that encodes the Androgen Receptor (AR) is hypermethylated, the gene is silenced and the receptor is unexpressed in carcinoma tissues (Jarrard *et al.*, 1998). In addition, *p53,* the tumor suppressor gene and regulator of cell cycle control, genetic stability and repair of DNA, represses AR function when expressed at high levels (Shenk *et al.,* 2001). Other polymorphisms in genes related to hormone response and metabolism, cell protection, DNA repair and nucleotide metabolism have been specifically

associated with prostate cancer. One such polymorphic gene encodes an enzyme responsible for the conversion of testosterone to the more active dihydrotestosterone (DHT). This polymorphism is often present in prostatic carcinomas of African American men (Makridakis *et al.,* 1999). Mutations in the DNA repair breast cancer susceptibility genes BRCA1 and BRCA2 also seem to correlate with an increased predisposition to develop prostate cancer (Rosen *et al.,* 2001; Edwards *et al.*, 2003). Altered expression of several proto-oncogenes is also now linked to prostate cancer development and progression. One such mutation common in metastatic prostate carcinomas leads to overexpression of the growth factor-dependent transcriptional factor MYC and the antiapoptotic protein BCL2—which are not expressed in normal epithelial cells (Shulz *et al.*, 2003; McDonnell *et al.*, 1992). MYC, which is often overexpressed as a result of chromosomal amplification (Jenkins *et al.*, 1997; Nupponen *et al.*, 1998), propagates a cell growth signal that is regulated by pro-apoptotic *p53* and BAX. When MYC is deregulated and the BCL2/BAX ratio is modified, aggressive prostate cancer growth occurs (Shulz *et al*., 2003). New light has been shed on the molecular biology of prostate cancer as tools such as transcript expression profiling, high-throughput genotyping, and proteomics, have emerged in the past decade that allow an analysis of the molecular nature of cancer. This new research has revealed and confirmed that models from other cancers cannot be used to explain the nature of prostate cancer. Instead, each cancer should be uniquely considered (Shulz *et al.,* 2003).

Prostate Cancer: Anatomy of the prostate

The prostate, a collection of glands (derived from epithelial cells) encased as one organ and made up of about 30% muscular tissue, is located at the base of the urinary bladder in front of the rectum and surrounding the urethra (Fig 1.4).
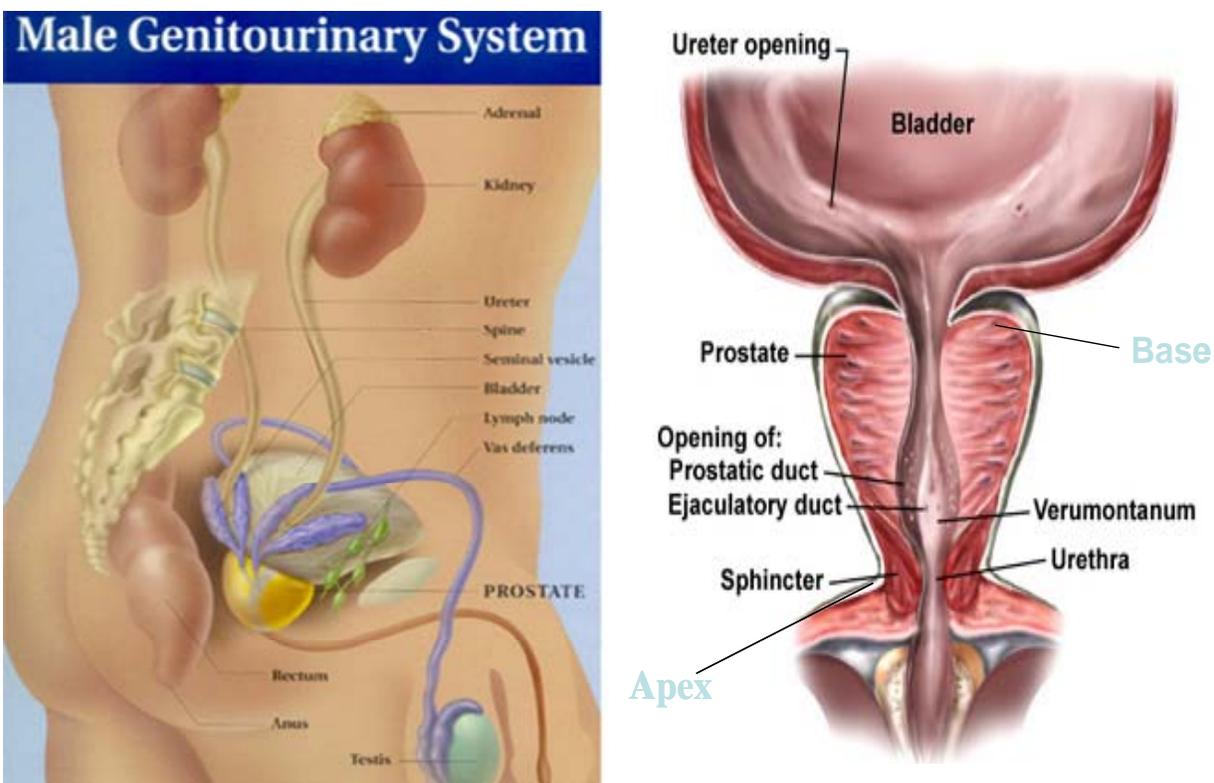
Figure 1.4. Anatomy of the prostate. The prostate is located at the base of the urinary bladder and surrounds the urethra. Sperm travel from the testes to the prostate through the vas deferens and seminal vesicles. In the prostate, nutrient-rich prostatic fluid mixes with seminal fluid to form semen before being ejaculated through the urethra via the ejaculatory ducts. **www.malereproduction.com/ 15_prostatitis.html**

The androgen testosterone promotes prostate epithelial budding from the urogenital sinus of the fetus, producing growth factors that activate the underlying mesenchyme (Cunha, 1994; Podlasek *et al.*, 1999). Androgen receptors are expressed highly in this layer in early development and do not become apparent in the secretory cell layer (McNeal, 1981) until epithelial cell growth and gland morphogenesis is triggered by paracrine growth factors from the mesenchyme. The adult prostate is usually 3-4 cm long and 3-5 cm wide. After maturation in the epididymis, sperm travel from the testes to the prostate through the vas deferens and seminal vesicles. In the prostate, nutrient-rich prostatic fluid mixes with seminal fluid to form semen before being ejaculated through the urethra via the ejaculatory ducts. The prostate is divided into

a fibromuscular anterior lobe, a cone-shaped median lobe that is located between the two ejaculatory ducts and the urethra, lateral lobes which make up the right and left side of the prostate, and the posterior lobe which is the prostatic site that is palpated during a digital rectal exam (Fig 1.5). The prostate is further divided into three different zones (McNeal, 1981) according to function. These zones are the central zone (CZ-5-8%) where the seminal vesicles are connected to the prostate, a peripheral zone (PZ-75%) that is the site where most (70%) prostate cancers begin, and the transitional zone (TZ-20%) which is the site of benign prostatic hypertrophy development (Fig 1.6). The latter two zones originate from the urogenital sinus while the glandular central zone originates from the Wolffian duct (Schulz *et al.*, 2003).

Figure 1.5. The lobes of the prostate. The prostate is divided into a fibromuscular lobe, a median lobe and a posterior lobe that is adjacent to the rectal wall. ***www.malecare.org/ prostate-cancer_42.htm***
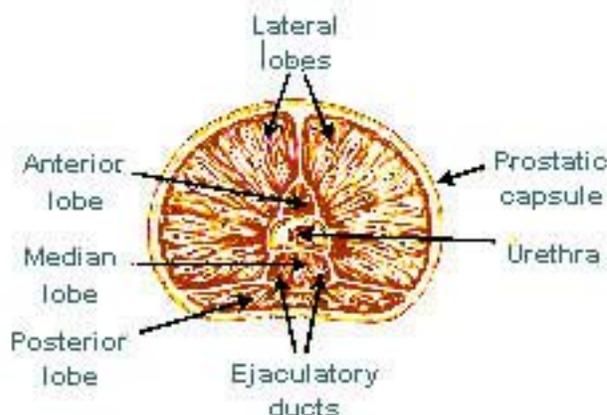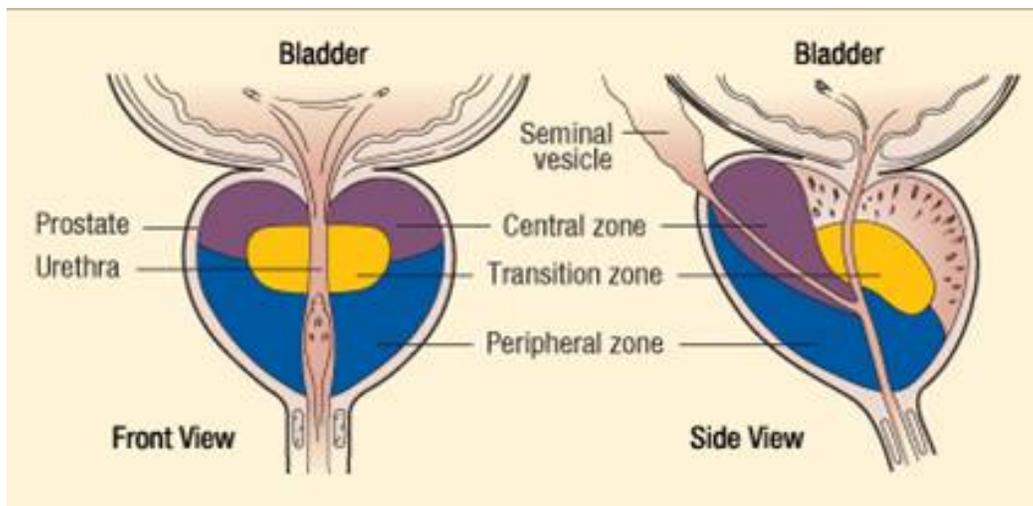
Figure 1.6. The zones of the prostate. The prostate is further divided into three different zones (McNeal 1981) according to function, and include, the central zone (CZ-5-8%) where the seminal vesicles are connected to the prostate, a peripheral zone (PZ-75%) which is the site where most (70%) prostate cancers begin, and the transitional zone (TZ-20%) that is the site of benign prostatic hypertrophy development. (http://www.prostate-research.org.uk/index.htm?aboutprostate/aboutprostate.htm~main)

Prostate Cancer: Benign prostatic hypertrophy

Around the age of 25, a slow noncancerous growth of the prostate known as benign prostatic hypertrophy (BPH) often occurs in men. During BPH the thin fibrous tissue that surrounds the prostate reaches a point of maximum distension causing the prostate glands to generate pressure on the urethra. This leads to complications of frequent and painful urination. BPH may be attributed to higher levels of estrogen than active testosterone in the blood as men age and/or to accumulation of dihydroxytestosterone (DHT), a highly active testosterone derivative (Steers 2001). Alpha-blockers**,** high blood pressure medications, relax the muscles found in the walls of blood vessels and the muscles of the prostate and bladder base. A drug known as Proscar blocks the formation of DHT, but can also mask the presence of cancer (www.proscar.com). Other common therapies of this benign growth of the prostate include laser surgery to remove the prostate tissue causing blockage and surgical resection of the prostate, a surgical procedure

which removes prostatic tissue that obstructs the urethra leaving only the intact capsule that surrounds the prostate.

Prostate Cancer: The nature of prostate cancer

Most prostate cancers begin in the periphery zone, as indicated earlier, and because prostatic cancers retain some glandular structure, they are classified as adenocarcinomas (Schultz *et al.*, 2003). A third of all prostate cancers become locally invasive, spreading to the lymph nodes and metastasizing to the bone, liver and lungs. Metastatic cells usually express the androgen receptor (AR), but some cells will inevitably grow without being affected by androgens. Because it is difficult to separate live cancer from the nonmalignant cells in prostatic tumors and to maintain the fully differentiated secretory epithelial cell phenotype, there are relatively few well-characterized, immortialized cell lines for cancers of the prostate (Peehl, 2005). Furthermore, because the testosterone levels of fetal calf serum (FCS) are lower than those found in normal males (0.2 ng/ml as opposed to 5-12 ng/ml), and the accepted concentration of FCS used in cell culture is 5-20%, the levels of testosterone available to cells in *in vitro* models is less than 1% of the physiological level (Kozlowski and Sensibar 1999).

While there are few early warning signs for prostate cancer, back, rib, hip or shoulder pain could denote metastasis to the bone (Bubendorf *et al.*, 2000; Rubin *et al.*, 2000). Prostate cancer that has spread to the bone can cause either destruction of bone integrity ("osteolytic" metastases) or inappropriate overgrowth of bone and cancerous tumors within the bone ("osteoblastic" metastases). Two common methods are used to detect prostate cancer. The levels of the prostate specific antigen (PSA) can be measured. PSA is a protein produced by the cells of the prostate that is present in the blood in high levels during prostate abnormalities (Balk *et al.*,

2003). A digital rectal exam (DRE), a technique where the back wall of the prostate is palpated through the rectum, is also used to detect prostate cancer (Roehrborn *et al.*, 1996; Wians and Roehrborn, 1996). The PSA test, however, is controversial because false readings can occur from noncancerous ailments. In addition to the PSA and DRE techniques, a more sound method of prostate cancer detection is the use of an ultrasound to highlight areas where biopsies should be taken and to analyze the condition of the prostatic cells. The specific methods used to test for prostate cancer metastases utilize scientific imaging instruments to visualize locations of metastasis (www.postatecancerfoundation.org) . In a bone scan, a radioactive substance is injected into the blood stream to show areas of rapid bone growth associated with so-called cancer hot spots. Where a bone is regenerating, as in a fracture repair or in the presence of cancer, the radioactive substance is absorbed more quickly than in normal bone cells. Undetected microscopic deposits of prostate cancers can exist in the bone, leading to a false negative result in a bone scan. Likewise, arthritis and infections can appear as hot spots, thus leading to false positive results. Soft tissue is visualized via a protascint scan which involves the use of a radiolabeled antibody that detects the presence of prostate cells throughout the body. Computer axial tomography (CAT/ CT) scans can be used to produce high resolution pictures of internal organs by subjecting molecules in the organs to intense magnetic waves and translating the emitted energy into computerized x-ray pictures and magnetic resonance images (MRI).

The Gleason grading system is currently the most common and accurate way to grade the stages of prostate cancer (Gleason, 1977). Using this method, a histological grade is assigned by observing the differentiation of the tumor cells and the glands formed by the tumor cells under a microscope. Five Gleason grades are recognized (Grades 1-5) (Fig 1.7). Grade 5 is representative of the most poorly-differentiated cancer which grows rapidly, while Grade 1 describes the most

well-differentiated cancer that closely resembles the cells and glands of the prostate (Bostwick and Brawer, 1987). To better predict the aggressiveness of the cancer, pathologists assign each tumor with a primary grade and a secondary grade. The primary grade assignment is indicative of the most common pattern of cancer (1=most well-differentiated to 5=most poorly-differentiated), that is seen in needle biopsies. The secondary grade is the next most common pattern seen and must account for at least 5% of the cancer. The lowest possible Gleason score is 1+1=2, when both the primary and secondary patterns are well-differentiated (Grade 1). Likewise, the highest possible Gleason score is 5+5=10, when primary and secondary patterns are both poorly-differentiated (Grade 5). Since approximately 50% of prostate cancers contain more than one Gleason histologic pattern, by adding together primary and secondary patterns, the Gleason grading scale allows for assignment of a variety of phenotypes including lymphatic and skeletal metastases (Kozlowski and Grayhack, 1996; Catalona, 1984; Gleason, 1977). Based on combined primary and secondary grade assignments, tumors are categorically separated into well-differentiated (2-4), moderately-differentiated (5-7) and poorly-differentiated (8-10) stages (Fig 1.8). Well differentiated cancers closely resemble normal prostate glands and are less aggressive and slow-growing than moderately- or poorly-differentiated cancers. Poorly differentiated cancers do not form recognizable glands, grow rapidly and are often fatal. (Kozlowski and Grayhack 1996; Elder and Catalona, 1984; Gleason, 1977).

Benign (Normal tissue)   **High Grade PIN**   **Gleason Pattern 2**   **Gleason Pattern 3**   **Gleason Pattern 4**   **Gleason Pattern 5**
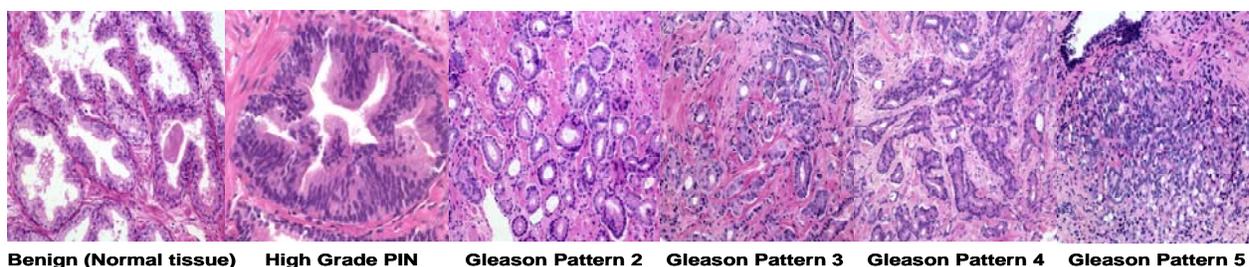
Figure 1.7 Gleason grading system for prostate cancer. <u>Benign Glands</u>: Intermediate to large size glands with irregular branching lumens. There is no enlargement of nuclei or nucleoli. <u>High-grade Prostatic Intraepithelial Neoplasia (HGPIN)</u>: High-grade PIN is the most likely precursor of prostate cancer. It architecturally resembles benign glands but posses the cellular features of cancer (enlarged nucleoli/nucleus, rapid glandular division). <u>Gleason grade 2</u>: The cancer focus is not circumscribed. <u>Gleason grade 3</u>: This is the most commonly seen pattern. There is considerable variation in size, shape, and spacing of the glands. <u>Gleason grade 4</u>: The most distinguishing feature of this grade is fusion of glands. <u>Gleason grade 5</u>: The tumor in Gleason grade 5 grows in solid sheets (as seen here) without forming any discernible glands. (http://www.prostate-help.org/cagleas.htm)
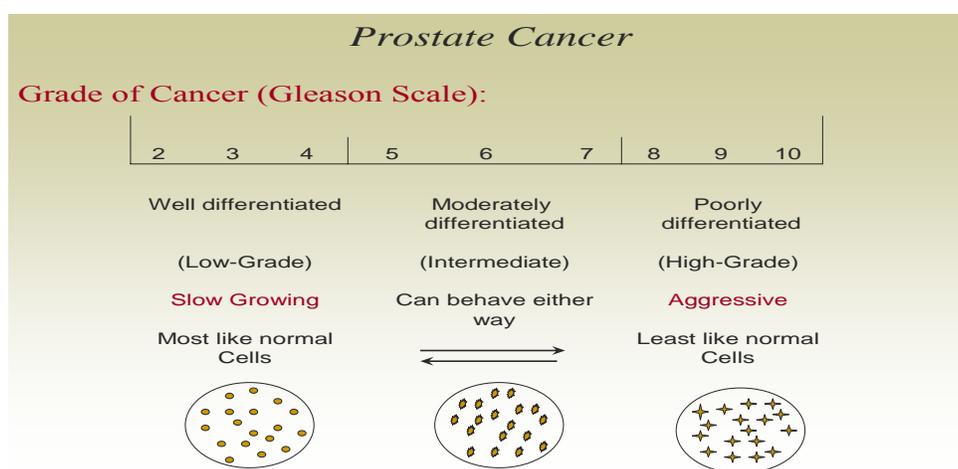
Figure 1.8. The Gleason scale is currently the most accurate and popular way to grade the stages of prostate cancer (Gleason, 1977). Using this method, tumors are categorically separated into well-differentiated (2-4), moderately-differentiated (5-7) and poorly-differentiated (8-10) stages.

Prostate Cancer: Treatment of prostate cancer

There are primarily five categories of treatment options for prostate cancer (www.prostatecancerfoundation.org) . For localized cancer there are three common treatments methods. The first approach is to perform a physical examination and to conduct a PSA test (Balk *et al.* 2003). This may be followed by radical prostatectomy which entails the removal of the prostate through an incision made in the abdominal wall (retropubic prostatectomy) or

between the scrotum and anus (perineal prostatectomy). A third treatment option is radiation therapy, in which high energy x-rays (external beam radiation) or radioactive seeds are applied near the cancer in an effort to kill the cancer cells. After a radical prostatectomy, PSA levels are monitored to ensure that they remain at undetectable levels or less than 0.2 ng/ mL of blood. After radiation therapy, prognosis can often be predicted by how low the "PSA nadir," or the "rock-bottom" PSA level, is after therapy.

For metastasized cancer, liquid nitrogen can be used to freeze prostate cancer cells, which subsequently rupture upon thawing (cryosurgery). Surgical or medical androgen ablation therapy can be used to deplete the supply of androgens to the prostate cells. The testes secrete testosterone by way of Leydig cells. Most of this testosterone is bound to globulin or albumin in blood plasma but some enters the prostate by diffusion across membranes. Testosterone is converted into dihydroxytestosterone (DHT) in the prostate epithelial cells by 5-α-reductase (Aquilina *et al.*, 1997). Both DHT and testosterone can bind the Androgen Receptor (AR), however, DHT binds the AR with 4- to 5-fold higher affinity than testosterone. The activated AR–DHT complex translocates into the nucleus and binds to androgen-responsive elements of target genes involved in prostate cell proliferation (Galbraith and Duchesne, 1997). Hormone therapies reduce the effect of testosterone on the receptor or they reduce the production of testosterone by the testes. If the cancer has spread to the bones, several treatment options can be employed to alleviate pain and, in some cases, to delay progression of the metastases (www.prostatecancerfoundation.org) . One treatment is the intravenous administration of radiopharmaceuticals, eg. samarium 153 and strontium 89. The various radiopharmaceuticals often differ in the number of times they can be administered without having a negative effect on the bone marrow cells. Bisphosphonates such as Zoledronic acid (Zometa) may actually retard

the growth of bone metastases in addition to serving as a pain relief aid. There are currently only three chemotherapy drugs that are approved by the U.S. Food and Drug Administration (USFDA), Taxotere (docetaxel), Novantrone (mitoxantrone hydrochloride) and Emcyt (estramustine sodium phosphate). These drugs fight cancer by killing rapidly dividing cells, including some non-cancerous cells. Clinical trials have shown that Taxotere (docetaxel) is effective in prolonging the life of patients who have hormone-resistant prostate cancer (Gilligan and Kantoff 2002). Because prostate cancer is slow-growing, chemotherapeutic agents are not as effective as they are in more aggressive cancers, unless they are administered to late-stage prostate cancer patients.

Nutrition and Cancer

In the last decade, the role of nutrients and dietary components in the retardation of carcinogenesis has been increasingly accepted. According to both the National Institute of Health (NIH) and the American Institute of Cancer Research (AICR), 30-40% of cancers are preventable by dietary means (Go *et al.*, 2001). Diets rich in fruits and vegetables protect against cancers of the mouth, pharynx, esophagus, stomach, colon, rectum, pancreas, lung, larynx, breast and bladder (AICR). Certain diets affect the carcinogenic process by promoting or inducing apoptosis in a variety of organ sites—indicating that these effects are not always tissue specific. For example, isoflavones, like Genistein, are known to inhibit tumor formation in animal models and induce apoptosis in breast, prostate, bladder and lung (Barnes, 1997; Fritz *et al.*, 1998; Lian *et al.*, 1999; and Zhou *et al.*, 1999; Peterson and Barnes, 1993). Isoflavones are phytoestrogens or plant chemicals that bind to estrogen receptors in mammals. Specifically, low levels of genistein have been shown to promote cancer cell growth, while high levels inhibit cancer

growth. Soy products are rich in isoflavones. Asian men, who have higher soy content in their diet than Western men (Fournier *et al.,* 1998) (Barnes *et al.,* 1998) have a lower incidence of prostate cancer (Spitz *et al.,* 2000). There have also been significant reductions in the occurrence of prostate cancer reported when men received a combination Zinc, Vitamin C, and Vitamin E in their diet (Kristal *et al.*, 1999). A higher consumption of cooked or processed tomato products, which contain the potent antioxidant carotenoid, lycopene, have been associated with a decrease risk of cancers of the gastrointestinal tract (La Vecchia 1998) and prostate (Giovannucci *et al.*, 1995; Giovannucci 1999; Gan *et al.*, 1999). Another major component of fruits and vegetables that provides beneficial effects on human health is the family of plant cell wall carbohydrates known as pectin. Pectin has at least three definable and reproducible effects on health: it can inhibit cancer growth and metastasis (Platt and Raz, 1992; Pienta *et al.*, 1995; Nangia-Makker *et al.*, 2002), reduce serum glucose levels (Jenkins *et al.*, 1977) and reduce cholesterol levels (Kay and Truswell 1977). When ingested, pectin is partially hydrolyzed by gastric acid (pH 2-4) in the stomach, subjected to B-elimination in the intestines (pH 5-6), and fermentated by the microflora of the caecum and colon (pH 6-8) (Dongowski and Anger 1996). Unsaturated oligomers (oligogalacturonides, described later), which could potentially be absorbed into different tissues in the body, are subsequently formed as metabolites during fermentation (Gray *et al.*, 1993). In this thesis, I demonstrate that pectin induces apoptosis in both androgen-responsive and androgen-independent prostate cancer cells. Hopefully, one day the findings reported here will form the basis for the development of pectin-based pharmaceuticals, neutraceuticals, or recommended diet changes aimed at reducing/inhibiting the occurrence and progression of cancer.

Plants: Plant cell wall

All plant cells are contained in a rigid extracellular layer known as the plant cell wall (Fig 1.9). The morphology of the cell wall changes throughout plant growth and development. The plant cell wall is a polysaccharide-rich matrix that is not only a source of structural and mechanical strength for plants, but is also a dynamic entity that regulates the diffusion of water, minerals and small nutrients into plant cells (Carpita and McCann 2000). Like the extracellular matrix of animal cells, the plant cell wall is a reservoir of signaling molecules that alert the organism to the presence of a pathogen or environmental stress, and is therefore a communication channel for cell-cell interactions (Ridley *et al.*, 2001).
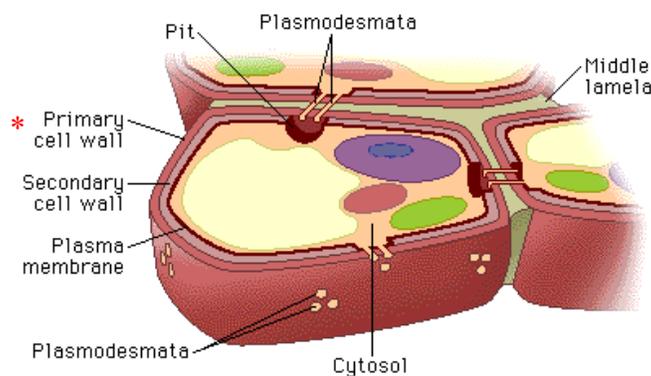
Figure 1.9. The plant cell. All plant cells are surrounded by a primary cell wall* that is important in plant growth, development and disease resistance. (www.ualr.edu/ ~botany/cellwall.gif)

The primary cell wall is the first wall made by plant cells when it is laid down during the first cellular division between two daughter cells as the middle lamella. The primary cell wall is expandable and permits cellular growth. As some cells differentiate and cease growing, however, a cellulose-rich secondary wall may also be produced (Carpita and McCann 2000). The primary wall is composed predominantly (80-90%) of complex polysaccharides, but also contains structural glycoproteins (10-20%) (e.g. hydroxyproline-rich extensions), ionically and covalently bound minerals (e.g. calcium and boron), enzymes, and in some plants, phenolic esters (ferulic and coumaric acids). The three classes of polysaccharides that make up the primary cell wall are cellulose microfibrils (1,4-linked β-D-glucose residues), hemicelluloses (polysaccharides that H-bond to cellulose), and the structurally complex pectins (also known as matrix polysaccharides) (Fig 1.10). Pectin makes up ~30% of the primary wall in all higher plants except for the grass family, where it comprises ~10% of the wall. All pectin contains 4-linked α−D-galacturonic acid residues (O'Neill *et al.*, 1990). Pectins are largely responsible for the texture of plant-derived foods and are a major component of human diets.
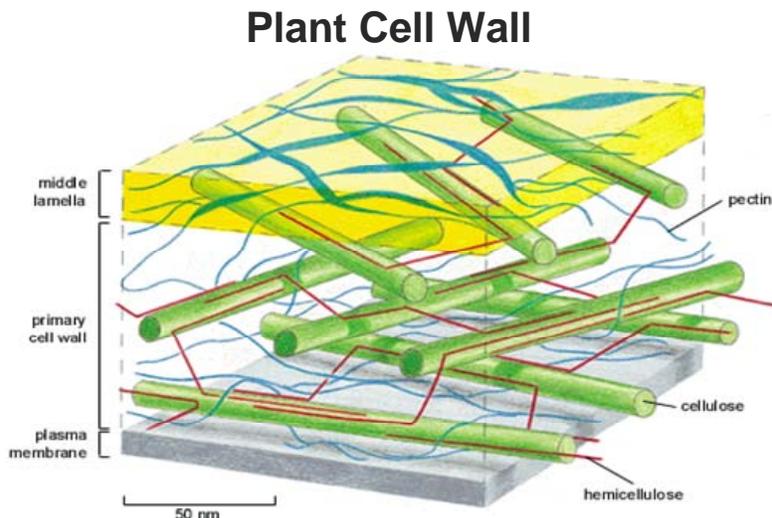
(Molecular Biology of the Cell, Alberts et al. 1994)

Figure 1.10. The polysaccharides of the plant primary cell wall. 1) Cellulose microfibrils (green tubes) (1,4-linked β-D-glucose residues), 2) hemicelluloses (red strings) (polysaccharides that H-bond to cellulose), and 3) the structurally complex pectins (blue strands) (also known as matrix polysaccharides).

Plants: Pectin and pectic polysaccharides

Pectins are the only major class of plant polysaccharides that appear to be restricted to primary cell walls (Willats *et al.,* 2001). Three types of polysaccharides comprise the pectin present in plant primary walls: a linear homopolymer known as homogalacturonan (HGA), the branched polymer rhamnogalacturonan I (RG-I), and the substituted galacturonans of which the ubiquitous member is rhamnogalacturonan II (RG-II) (Ridley *et al.*, 2001; Albersheim *et al.*, 1996) (Fig 1.11).
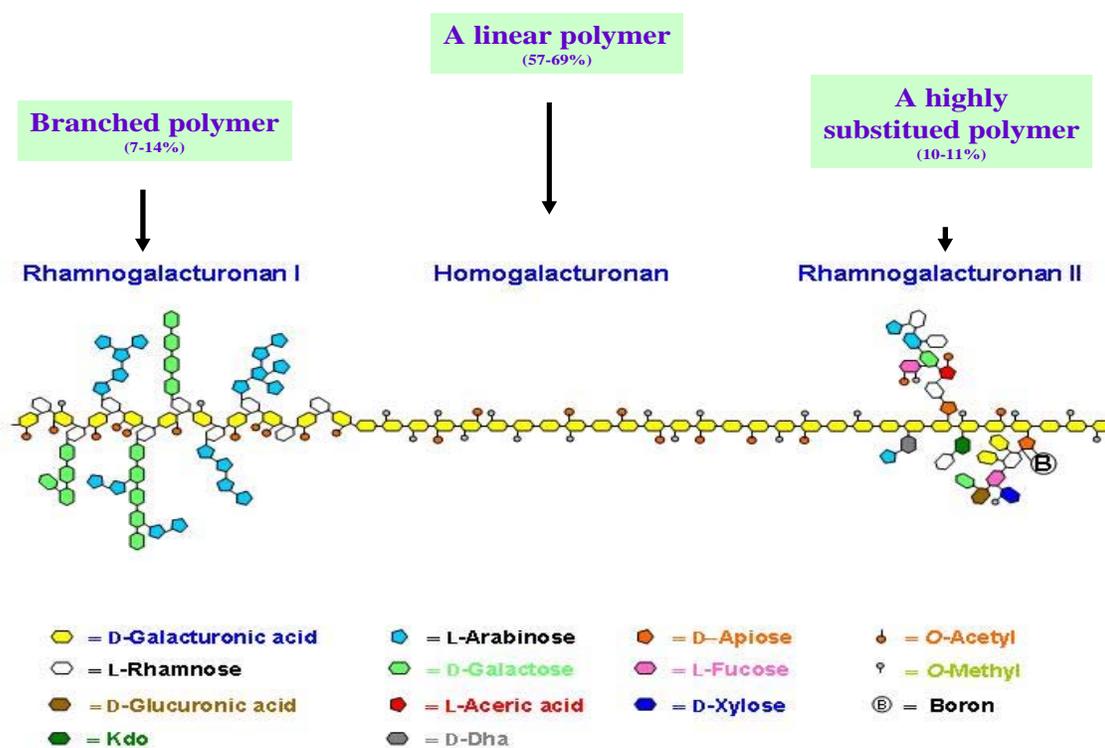
Figure 1.11. The pectic polysaccharides. 1) Homogalacturonan (HGA) is a linear polymer, 2) Rhamnogalacturonan I (RG-I) is a branched polymer and 3) the substituted galacturonan Rhamnogalacturonan II (RG-II)). www.plbio.kvl.dk/ plbio/cellwall.htm

Homogalacturonan accounts for 57%-69% of total pectin (Mohnen, 2002) and is thought to range from 30-200 galacturonic acid (Gal A) residues in length (reviewed in Mohnen, 1999). It is a linear chain of 1,4-linked α-D-galactopyranosyluronic acid (GalA) in which 70-80% of the

Gal A residues may be methyl esterified at the C-6 carboxyl (Voragen *et al.*, 1986). HGA may also be partially O-acetylated at C-3 or C-2 (Ishii, 1995, 1997a) (Fig 1.12). The length of HGA affects its tertiary structure and removal of methyl esters within the wall matrix permits calcium cross-linking of HGA allowing the formation of supramolecular assemblies and gels, a characteristic of great importance to the food and cosmetic industries.
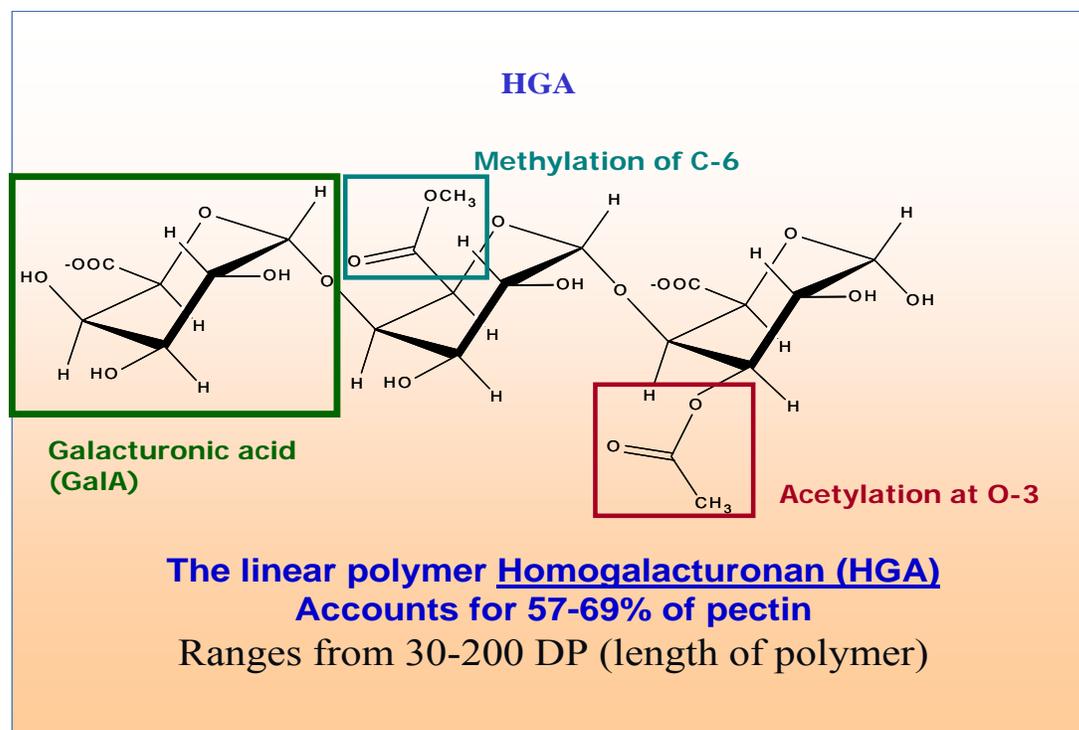
Figure 1.12. The pectic polysaccharide Homogalacturonan (Ridley *et al.* 2001). Homogalacturonan is a linear chain of 1,4-linked α-D-galactopyranosyluronic acid (GalA) which can have 70-80% of GalA residues methyl esterified at the C-6 carboxyl (Voragen *et al.*, 1986). HGA may also be partially O-acetylated at C-3 or C-2 (Ishii, 1995, 1997a).

Rhamnogalacturonan-I (RG-I) is a family of pectic polysaccharides with a backbone of the repeating dissacharide [→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→] that accounts for 7-14% of pectin (Fig 1.13). Roughly 20-80% of the rhamnose residues of RG-I are substituted by L-arabinose, D-galactose, L-arabinans, galactans, or arabinogalactans (Ridley *et al.*, 2001; Mohnen, 1999; O'Neill *et al.*, 1990). The side branches include α1,5- and α1,3-linked arabinans, β-1,4

linked or β-1,3 and β-1,6 linked galactans, and arabinogalactans of diverse linkages (reviewed in Mohnen, 1991). The average MW of sycamore RG-I is estimated to be 10^5-10^6 Da (O'Neill *et al.*, 1990). Rhamnogalacturonan II (RG-II) is a substituted galacturonan that accounts for 10-11% of pectin and its structure is highly conserved across plant species (Fig 1.14). RG-II consists of a homogalacturonan backbone to which is attached four side branches of complex structure. It is arguably the most complicated polysaccharide in nature consisting of 11 different types of sugars joined in over 20 different linkages and contains unusual sugars such as 2-O-methyl-xylose, 2-O-methyl fucose, KDO, DHA and apiose (Ridley *et al.*, 2001).

Representative structure of RG-I
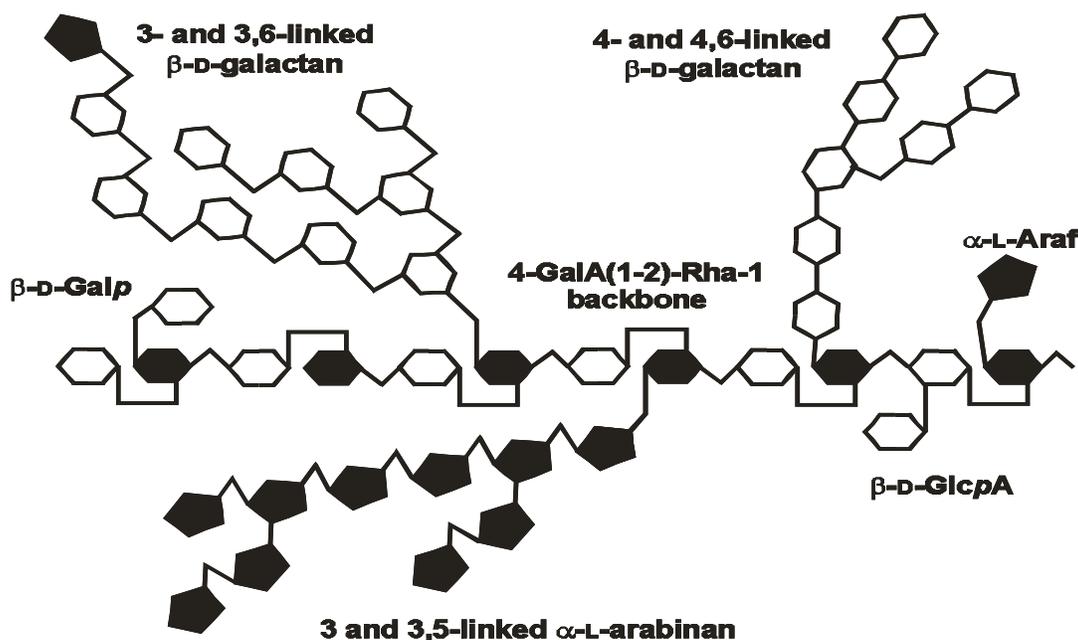
Figure 1.13. The pectic polysaccharide Rhamnogalacturonan I (Ridley *et al.* 2001). Rhamnogalacturonan-I (RG-I) is a family of pectic polysaccharides with a backbone of the repeating dissacharide [→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→] that accounts for 7-14% of pectin.

Representative structure of RG-II
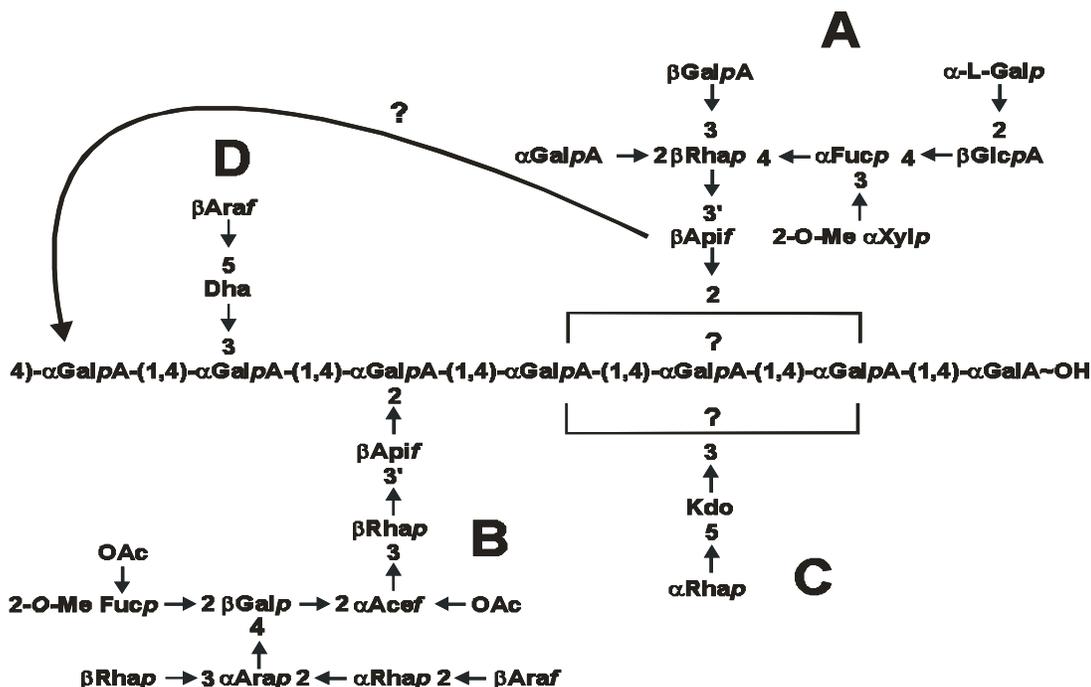
Figure 1.14. The pectic polysaccharide Rhamnogalacturonan II (Ridley *et al.* 2001). Rhamnogalacturonan II (RG-II) consists of a homogalacturonan backbone to which is attached four side branches of complex structure. It is believed to be the most complicated polysaccharide in nature consisting of 11 different types of sugars joined in over 20 different linkages.

Whereas the structure of RG-II is conserved, the structures of HGA and RG-I are more variable in different plants and in different cell types, due to differences in the polymer size, in the pattern of acetyl, methyl and other modifications of the GalA residues in the backbone of HGA, and due to variations in the length and type of side branches of the RG-I backbone.

HGA, RG-I and RG-II are generally purified from intact purified cell walls by treatment with endopolygalacturonase, which cleaves a stretch of four or more contiguous non-methylesterified α-1,4-linked galacturonic acid residues in HGA (Fig 1.15). The ability to cleave the pectic polysaccharides from the intact wall has been used as evidence to support the model that the backbones of the three pectic polysaccharides are covalently linked together in the wall.

Nakamura *et al.* (2002) have provided structural evidence to support this linkage. Additional mechanisms for the association of the pectic polysaccharides include the formation of borate ester cross-linked RG-II dimers (Kobayashi *et al.*, 1996; O'Neill *et al.*., 1996), HGA-Ca^{2+} interchain salt bridges (Morris *et al.*, 1982), and possible feruloyl polysaccharide ester crosslinks (Fry, 1982; Ishii, 1997). However, a complete understanding of the larger 3D structure of pectin in the wall, and how the polymers are associated in the wall, is still lacking. For example, the role of proposed ester cross links between the carboxyl moiety of galacturonic acid in HGA and other pectic or wall polymers remains unclear (Kim and Carpita, 1992; Brown and Fry, 1993; Iiyama *et al.*, 1994; Hou and Chang, 1996; Djelineo 2001). Although the structural identity of the proposed ester(s) linkage at the carboxyl group of galacturonic acid in pectin is not known, the data presented in this thesis support a role for this linkage in the ability of pectin to induce apoptosis in prostate cancer cells.
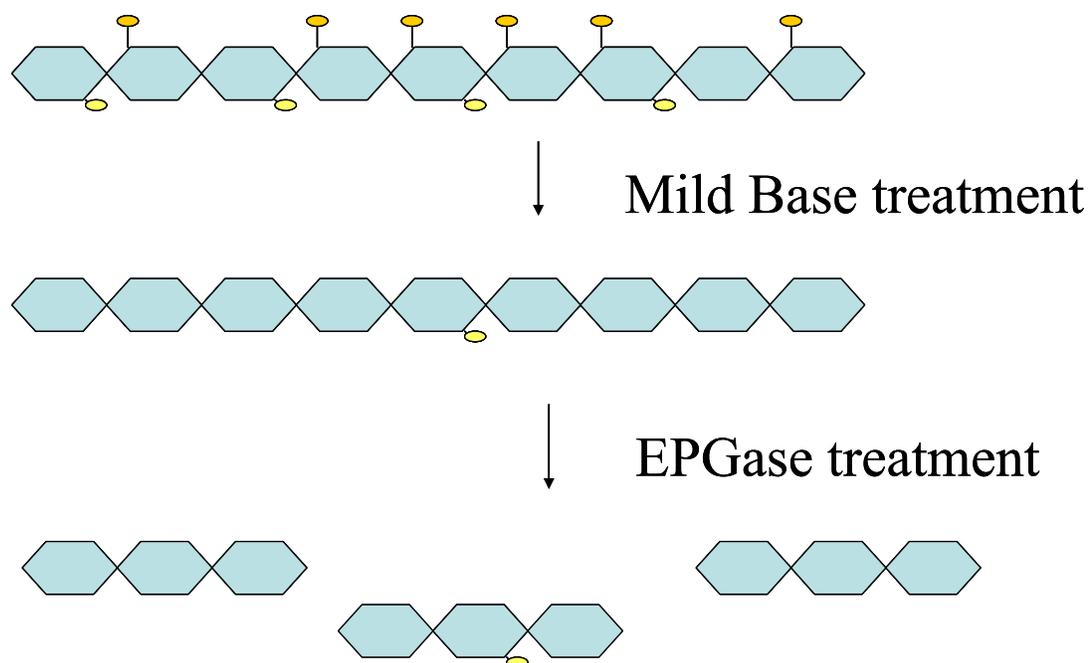
Figure 1.15. Isolation of pectic polysaccharides from the plant cell wall. When HGA, RG-I, and RG-II are isolated from plant cell walls, they are treated with mild base, resulting in deesterification (e.g. removal of methyl ester), and with the enzyme endopolygalacturonase (EPG) which cleaves regions of nonmethylesterified HGA.

The production of commercial pectin, which is used by the food and cosmetic industries, usually involves an acid extraction of pectin from dried citrus peels or apple pomace (Thakur *et al.*, 1997), a process that results in the destruction and loss of RG-II and of some RG-I. In some cases, commercial citrus pectin may be treated with base or heat to yield fragmented and structurally modified pectin. Since many biomedical and nutraceutical pectins are generated by such treatments, the interpretation of biomedical effects of pectin must be accompanied by an understanding of the structure of the pectin that exerts a particular effect.

Literature Review: pectin and cancer metastasis

Published studies show that pectin, a natural component of all fruits and vegetables, inhibits breast and colon cancer primary tumor growth and metastasis in mice. In a study performed by Nangia-Makker *et al*. (2002), athymic mice were injected in the mammary fat pad region with 750,000 MDA-MB-435 human breast cancer cells or in the cecum with 5 million metastatic LSLiM6 human colon adenocarcinoma cells. Since these mice lacked a thymus they were immunologically deficient, and their ability to maintain human tissues made them good models for studying human cancers. When the mice were fed 10 mg/ml pH-modified citrus pectin (MCP) in their drinking water starting 1 week prior to injection of the cancer cells, the number of breast or colon cancer infected mice with lung and lymph node/liver metastases, respectively, were drastically decreased compared to control mice given no pectin (Fig. 1.16 and 1.17). Furthermore, the primary tumor volume at 7 weeks was considerably smaller in the pectin-fed breast cancer mice and the primary tumor weight at 6 weeks was lower in the pectin-fed colon cancer mice compared to controls (mice given water without pectin) (Fig. 1.16 and 1.17).
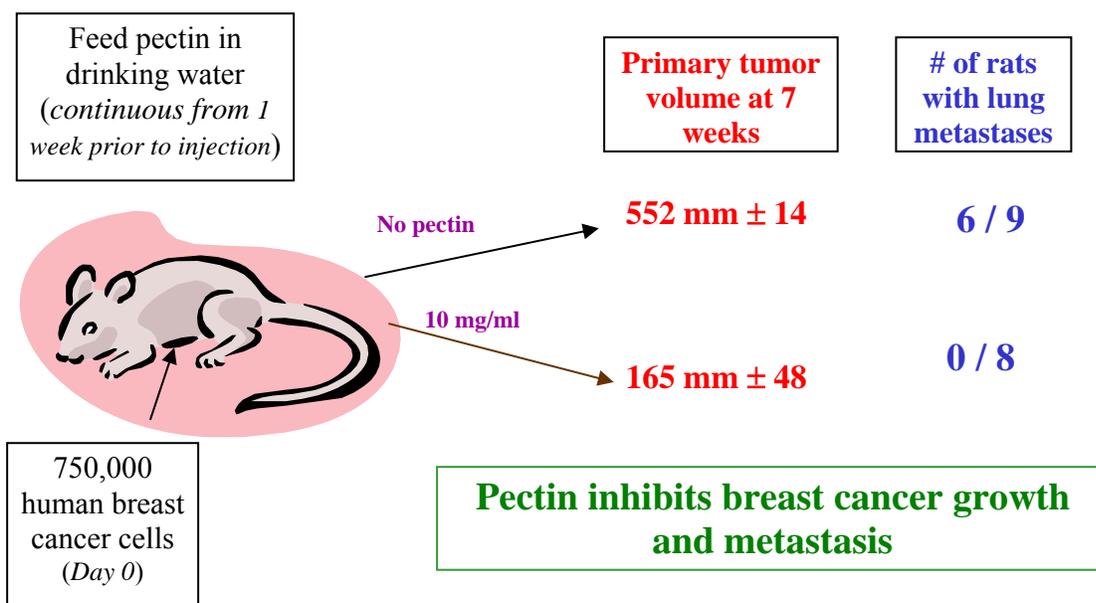
Figure 1.16. Inhibition of human breast cancer cell growth and metastasis in nude mice by oral intake of modified citrus pectin (Nangia-Makker *et al.*, 2002)**.** Athymic mice were injected in the mammary fat pad region with 750,000 MDA-MB-435 human breast cancer cells. Some mice were fed 10 mg/ml pH-modified citrus pectin (MCP) in their drinking water starting 1 week prior to injection of the cancer cells. The number of breast cancer-infected mice with lung metastases was drastically decreased compared to control mice given no pectin. The primary tumor volume at 7 weeks was also considerably smaller in the pectin-fed breast cancer mice compared to controls (mice given water without pectin). (Figure gift of Debra Mohnen)

Feed pectin in drinking water (*continuous from 1 week prior to injection*)		Primary tumor weight at 6 weeks	Intra-abdominal tumor weight	# of rats with lymph node/liver metastases
No pectin		1.16 g	2.0 g	9/9, 6/10
10 mg/ml pectin		0.65 g	0.88 g	2/8, 0/9

5,000,000 human colon carcinoma cells (*Day 0*)

Pectin inhibits colon cancer growth and metastasis
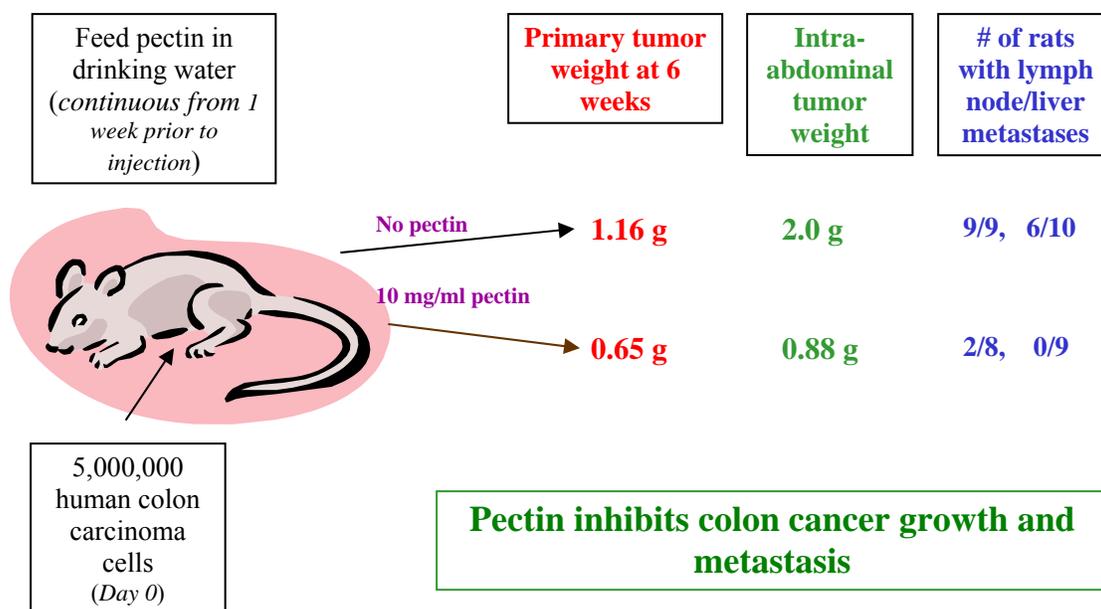
Figure 1.17. Inhibition of human colon cancer cell growth and metastasis in nude mice by oral intake of modified citrus pectin (Nangia-Makker *et al*., 2002)**.** Athymic mice were injected in the cecum with 5 million metastatic LSLiM6 human colon adenocarcinoma cells. Some mice were fed 10 mg/ml pH-modified citrus pectin (MCP) in their drinking water starting 1 week prior to injection of the cancer cells. The number of colon cancer-infected mice with lymph node/liver metastases was drastically decreased compared to control mice given no pectin. The primary tumor weight at 6 weeks and intra-abdominal tumor weight was also lower in the pectin-fed colon cancer mice compared to controls (mice given water without pectin). (Figure of Debra Mohnen)

The breast cancer-infected, pectin-fed mice also showed a decrease in the average number of blood vessels per primary tumor compared to the control. Growing and metastasizing tumors are dependent on tumor vasculature which provides the aberrant cells with needed oxygen and nutrients. Human umbilical vein endothelial cells (HUVECs) are known to bind to galectin-3, a carbohydrate (galactose)-binding protein (Barondes *et al.,* 1994)) found in the cytoplasm and/or secreted from the cell that binds to the surface proteins and extracellular matrix components of cells (Sato and Hughes 1992). Galectins aid in cell-cell and cell matrix adhesion (Hughes, 2001) and galectin-3 is known to be present on MDA-MB-435 human breast cancer cells. Galectin-3 mediate angiogenesis by binding to HUVECs *in vitro* (Nangia-Makker *et al.,* 2000). Studies performed by the same group, Nangia-Makker *et al.,* 2002, show that capillary tubule formation

by HUVECs, and HUVEC chemotaxis and binding to galectin-3 was inhibited by modified citrus pectin in a dose-dependent manner. These results demonstrate that MCP inhibits cancer growth and metastasis and galectin-3-mediated angiogenesis *in vivo* and *in vitro.*

Pectins have also been shown to bind to melanoma cells *in vitro* (Platt and Raz, 1992). It was found that large, unmodified citrus pectin (CP) increased lung colonization of murine B16-F1 melanoma cells while relatively small, pH-modified citrus pectin (MCP) significantly decreased this colonization. For *in vivo* studies, 1 million B16-F1 cells, incubated with CMF-PBS (Ca^{2+}- and Mg^{2+}- free phosphate buffered saline) (control), CP or MCP, were injected intravenously into the tail vein of 8-week-old female C57BL/6 syngeneic, genetically identical mice (with respect to antigens or immunological reactions). After 17 days, the mice were autopsied and the number of lung colonies per mouse was counted. When injected with B16-F1 melanoma cells previously incubated with 5 mg/ml CP, the number of lung tumor colonies per mouse increased threefold (mean 139) compared to controls (mean 43). Contrastingly, B16-F1 cells, incubated with 5.0 mg/ml MCP, intravenously injected into mice led to a significant reduction in the number of lung tumor colonies formed (mean 0) compared to controls (mean 33) (Platt and Raz, 1992). In the same study, one hour agitation of B16-F1 melanoma cells with 0.5 mg/ mL CP increased spontaneous homotypic aggregation of the cells while 1 hour incubation with MCP did not. Furthermore, in a subsequent study the relatively small, pH-modified pectin (MCP) inhibited melanoma cell adhesion to laminin, a basal lamina protein, in a dose-dependent manner (Inohara and Raz, 1994), while both CP and MCP inhibited anchorage-independent growth (dose-dependently) of B16-F1 cells in semisolid medium, an *in vitro* method thought to mimic metastasis *in vivo* (Inohara and Raz, 1994). It is interesting that these anti-metastatic effects of pectins occurred in the absence of cell toxicity (Inohara and Raz, 1994). In some

cases, disruption of cell-cell or cell-matrix adhesion through MCP interference with galectin-3-mediated carbohydrate recognition is thought to play a key role in the reduction of cancer metastasis. Taken together, these data support the role of pectin as an anti-metastatic agent and show that there is a differential response of cells to pectin, depending upon the type of pectin used.

Modified citrus pectin has also been shown by Pienta *et al.* (1995) to reduce prostate cancer metastasis. One million fast-growing, poorly differentiated rat prostate adenocarcinoma cells, Dunning (R3327) MAT-LyLu subline (Dunning, 1963; Isaacs *et al.*, 1986), were injected into the hind limb of male Copenhagen rats. When continuously given 0.1% modified citrus pectin in their drinking water from day four to necropsy, there was at least a 50% reduction in the number of rats with lung metastases and a reduction in the number of metastases per lung (Fig 1.18). MCP did not affect primary tumor growth. MCP also was found to strongly inhibit MAT-LyLu cell adhesion to rat aortic endothelial cells in a dose dependent manner when incubated with the cells for 90 minutes. This is thought to occur by disrupting the binding of galectin-3, which is expressed on the surface of MAT-LyLu cells, to its normal ligand (Pienta *et al.*, 1995). The above described data validates the anti-metastatic effects of MCP by preventing cell-cell and/or cell-matrix adhesion in prostate cancer.
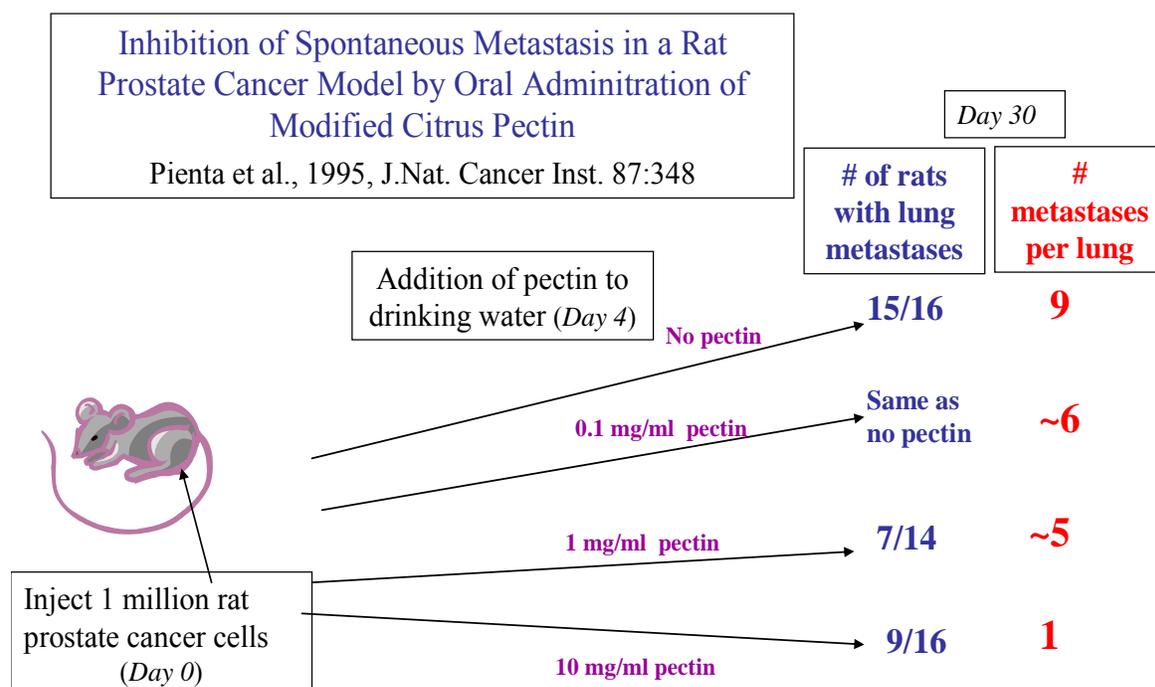
Figure 1.18 Inhibition of prostate cancer metastasis by MCP. One million rat prostate adenocarcinoma cells were injected into male Copenhagen rats. When continuously given 0.1% modified citrus pectin in their drinking water from day four to necropsy, there was at least a 50% reduction in the number of rats with lung metastases and in the number of metastases per lung. **(Pienta *et al.*, 1995)** (Figure from Debra Mohnen 2004)

Literature Review: pectin and apoptosis

Several studies have shown that pectins not only inhibit metastatic lesions, but also that some commercially available citrus pectin can induce apoptosis. Azoxymethane-injected rats fed a citrus pectin or a fish oil/pectin diet, had a greater number of apoptotic cancerous cells per colon crypt column, compared to control rats fed with corn oil and/or cellulose (Chang *et al.*, 1997a) (Fig 1.19). Furthermore, pectin-fed rats had a lower incidence of adenocarcinoma (51.5%) than control animals (75.6%) (Chang *et al.*, 1997a, b). The apoptotic index in the distal colon of rats fed pectin was higher than in rats fed a standard diet; this was accompanied by reduced

expression of anti-apoptotic Bcl$_2$ and activation of caspase-1 and poly(ADP)ribose, a substrate of caspases (Aviv-Green *et al.*, 2000a,b,c). Similarly, supplementation of pectin-rich orange-pulp to the diet of dimethylhydrazine-injected Sprague Dawley rats resulted in activation of caspase-3 in rat tumors and an increased activity of killer T cells in the tumors (Kossoy *et al.*, 2001). In colon adenocarcinoma HT29 cells, caspase-3 activity increased significantly when these cells were treated with 10 mg/ml low methylated apple pectin (Olano-Martin *et al.*, 2003). Taken together these results suggest that exposure of colon cancer cells to pectin results in increased apoptosis and reduced tumor growth.

Oral administration of commercially available citrus pectin causes increased apoptosis
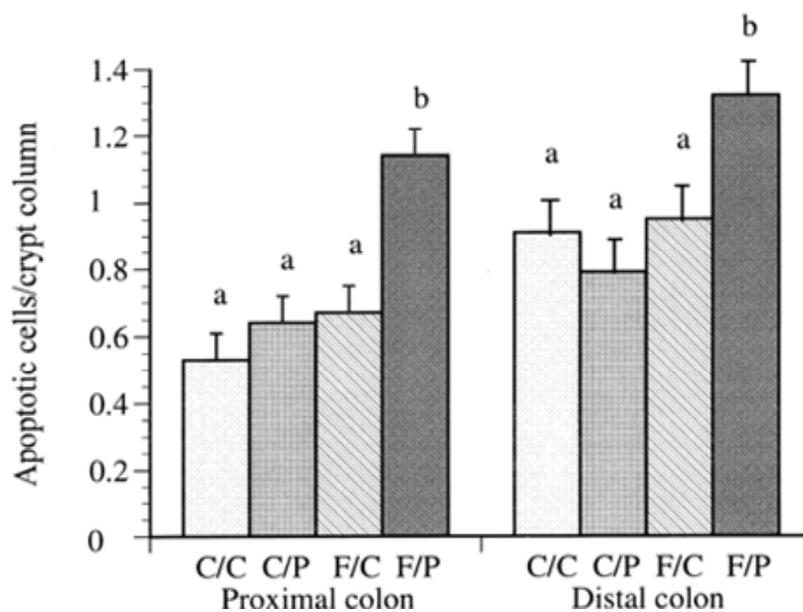
Figure 1.19 The interactive effects of fat and fiber on number of apoptotic cells per crypt column in proximal and distal colon of rats. Values are means ± pooled SEM, $n = 40$. Bars with different letters are significantly different, $P < 0.05$. Rats fed fish oil/pectin, F/P, had more apoptotic cells per crypt column than rats fed corn oil/cellulose, C/C, corn oil/pectin, C/P, or fish oil/cellulose, F/C. (Chang et al. 1997)

Chapter 2: SPECIFIC AIMS OF PROJECT

The goal of the research project was to address the following question:

Does pectin induce apoptosis in human prostate cancer cells?

The specific aims for this project were:

1) to test different pectins on two different prostate cancer cell lines to determine if the pectins induced apoptosis in the cells

2) to determine the structural characteristics of any apoptotic pectins identified

Cell types used in study

There are eight immortalized prostate cancer cell lines available, only two of which were derived from primary tumors (Muraki *et al.,* 1990; Narayan and Dahiya 1992). LNCaPs, or lymph node-derived prostate cancer cells were established from a metastatic lesion of human prostatic adenocarcinoma. More specifically, the patient was a 50 year old Caucasian male with moderately differentiated carcinoma (Horoszewicz *et al.,* 1980; Kozlowski and Sensibar 1999). The LNCaP cells respond to androgens and do not contain the cell surface protein galectin-3 (Califice *et al.,* 2004). LNCaP C4-2s are a sister cell line which are androgen insensitive and undergo apoptosis through a different pathway of initiators than LNCaP. Because of these properties, LNCaPs and LNCaP-C4-2 cells were chosen for this study.

Pectins used in study

We chose three pectins for our initial studies based on the positive results from previous publications that showed that modified citrus pectin inhibits cancer progression in multiple types of cancer cells. Specifically, we used citrus pectin (Sigma P-91356, 8% methyl esterified), which was the type of pectin from which the other two pectins were derived. We also tested two different pectins that were derived by either heat treatment of CP (fractionated pectin powder, (FPP) from Thorne Research 2020582), or by treatment with acid and base (pectasol from EcoNugenics). We chose the latter type of pectin because it is most closely related to the MCP used in the pectin anti-metastatsis studies described earlier.

Commercial pectins are often generated by treating dried citrus or apple with acid, followed by precipitation and drying. The resulting commercial pectin is mainly HGA with some RG-I. Most or all of RG-II is lost. Such citrus pectin (CP) will be referred to as the mother pectin in this thesis since it is the starting pectin used to generate the two fragmented pectins (FPP and PeS) used in the studies reported here. Fragmentation of CP by heat or pH treatment yields FPP and PeS, respectively (Fig. 2.1).
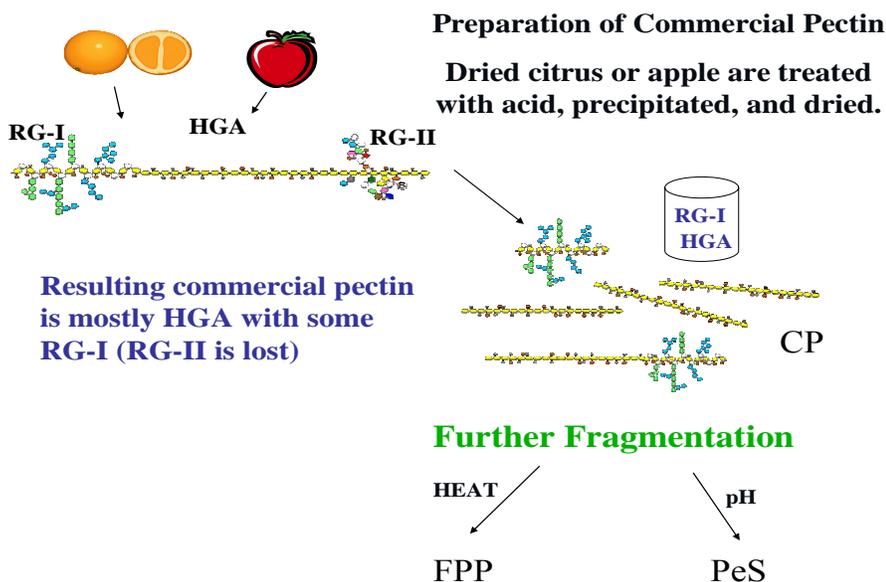
Figure 2.1 Preparation of commercial pectin. Commercial pectins are often generated by treating dried citrus or apple with acid, followed by precipitation and drying. The resulting commercial pectin is mostly HGA with some RG-I. Most or all of RG-II is lost. Further fragmentation by heat or pH treatment yields FPP and PeS, respectively, two of the pectins used in studies reported in this thesis.

Apoptosis

Apoptosis was first introduced as a form of cell death in mature cells in 1972 (Kerr *et al.*). The mechanisms of how apoptosis is induced and executed have only become clear in the past 15 years. Unlike necrosis, apoptosis is a programmed cell suicide. It is a critical event in early development and in the maintenance of homeostasis throughout life. The pathogenesis of many diseases can be attributed to either too much or too little apoptosis. In cancer, the level of apoptosis is greatly diminished and cells divide quicker than they die.

Apoptosis is often characterized by chromatin condensation (due to cell shrinkage), membrane inversion leading to the exposure of phosphatidyl serine residues, blebbing, and DNA fragmentation. Apoptosis occurs through two distinct cellular pathways. The extrinsic pathway is initiated when death activators (e.g. tumor necrosis factor (TNF), tumor necrosis factor-related

apoptosis inducing ligand (TRAIL)) bind to the cell surface and cause the aggregation of death receptors. In the second pathway, the stress-induced intrinsic pathway, signals inside of the cell, including the withdrawal of growth factors, radiation damage, and disturbances in the cell cycle, cause loss of mitochondrion membrane permeability, leading to apoptosis. In both pathways, the endogenous family of caspase proteases is activated to degrade cellular components. The components are then digested by neighboring cells. Caspases are cysteine proteases that exist within the cell as zymogens or inactive proenzymes. Proenzymes must be cleaved at aspartate residues and assembled into heterotetramers before they can be converted into the active enzymes that are capable of cleaving cellular proteins and of causing morphological changes in the cell (Tapia-Vieyra and Mas-Oliva, 2000). Caspases act in a heirarchial fashion, as initiator caspases (caspase-2, caspase-8, caspase-9) activate effector caspases (caspase-3, caspase-6, caspase-7). More specifically, in type I cells, those cells that are stimulated extrinsically, an apoptotic stimulus activates an initiator caspase, caspase-8, which propagates death signals directly through the activation of effector caspases, procaspase-3 and procaspase-7 (Fig 2.3). Alternatively in type II cells, wherein intrinsic apoptotic stimuli trigger the mitochondrial-dependent pathway of apoptosis, Bid, a proapoptotic cell death regulator, is truncated following binding by caspase-8. The truncated protein, tBid, translocates to the mitochondria to induce the oligomerization of proapoptotic agents Bax and Bak, consequently triggering the release of an apoptogenic factor, Cytochrome C (Cyt C) (Wang *et al.*, 1996). Cyt C, also essential for mitochondrial respiration and energy production, binds to cytosolic apoptosis protease activating factor (Apaf-1). A conformational change is induced and a wheel-like structure consisting of seven molecules of Apaf-1, cyt c and adenosine triphosphate (ATP), known as the apoptosome,

is formed**. Caspase recruit domain (CARD) binds to Apaf-1 to sequester caspase-9. Activated caspase-9 activates caspase-3 and leads to rapid cell death (Fig. 2.3).
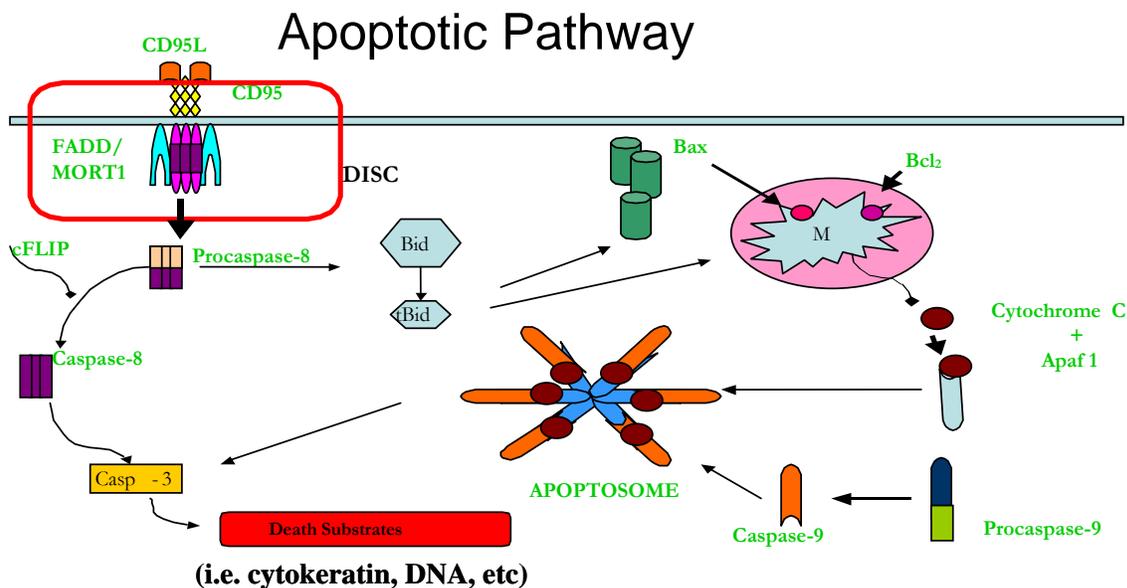
Figure 2.2 The apoptotic pathway. In type I cells, an apoptotic stimulus activates caspase-8, which propagates death signals directly through the activation of procaspase-3 and subsequently the effector caspase, caspase-3. In type II cells, caspase-8 induces apoptosis by direct activation of caspase-3 as well as by the involvement of the mitochondria.

In prostate cancer cells, apoptosis is known to be induced by the activation of several pathways including Bcl_2, TRAIL and $TNF\alpha$ family members. TRAIL induces apoptosis through its interaction with death receptors DR4 and DR5, activators of caspase-8. The success of this apoptotic pathway requires the involvement of several members of the Bcl_2 family. Thus, the apoptotic response of prostate cancer cells is the culmination of interactions between several apoptotic pathways.

Because the cells of the prostate require androgens to grow, usually one of first steps in prostate cancer treatment is androgen depletion therapy (ADT). Androgen deprivation therapy in prostate cancer is rarely curative because the metastatic cancer is heterogenous, consisting of

both androgen-dependent and androgen-independent prostate cancer cells. In fact, in prostate cells subjected to androgen withdrawal, only the secretory epithelial cells are forced to undergo apoptosis (Kyprianou *et al., 1991*). Furthermore, antiproliferative chemotherapeutic agents for prostate cancer only lead to cell death when cells are proliferating (in the synthesis phases of the growth cycle). Greater than 90% of prostatic cancer cells are not actively proliferating and are, therefore, resistant to standard cytotoxic chemotherapy (Tapia-Vieyra and Mas-Oliva 2001). Thus, the identification of methods other than androgen ablation and chemotherapy are of great importance for inducing apoptosis in metastatic prostate cancer. In this thesis it is demonstrated that a product of plant cell walls, pectin, is capable of inducing apoptosis in both androgen-responsive (LNCaP) and androgen-independent (LNCaP C4-2) human prostate cancer cells.

Chapter 3

PECTIN INDUCES APOPTOSIS IN HUMAN PROSTATE CANCER CELLS:

CORRELATION OF APOPTOTIC FUNCTION WITH PECTIN STRUCTURE[1]

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Summary

Fractionated pectin powder (FPP) induced significant apoptosis (approximately 40-fold above non-treated cells) in both androgen-responsive (LNCaP) and androgen-independent (LNCaP C4-2) human prostate cancer cells as determined by the Apoptosense assay and activation of caspase-3 and its substrate, PARP. Citrus pectin and pH-modified pectin, PectaSol, had little or no apoptotic activity, suggesting a structure-dependence to pectin's apoptotic activity. Glycosyl residue composition and linkage analyses revealed no significant differences between the pectins. Mild base treatment to remove ester linkages destroyed FPP's apoptotic activity and yielded HGA oligosaccharides. Treatment of FPP with pectinmethylesterase to remove galacturonosyl carboxymethylesters and/or with endopolygalacturonase to cleave non-methylesterified homogalacturonan caused no major reduction in apoptotic activity, implicating the requirement for a base-sensitive linkage other than the carboxymethylester.

SIGNIFICANCE

Treatment options for androgen-independent prostate cancer cells are limited. Therefore, it is critical to identify agents that induce the death of both androgen-responsive and androgen-insensitive cells. Here it is demonstrated that a product of plant cell walls, pectin, is capable of inducing apoptosis in both androgen-responsive (LNCaP) and androgen-independent (LNCaP C4-2) human prostate cancer cells. For the first time, specific structural features of pectin responsible for inducing apoptosis are identified. These findings provide the foundation for future research on the mechanism by which specific pectic structures induce apoptosis in cancer

cells and provide a basis for the rational development of pectin-based pharmaceuticals, nutraceuticals, or recommended diet changes aimed at reducing/inhibiting the occurrence and progression of cancer.

Introduction

Prostate cancer is the most common malignancy and the second leading cause of death from cancer in American men. The goal of many cancer therapies, such as antihormone therapy and chemotherapy, is to induce apoptosis in tumor cells. Androgen deprivation therapies induce cell death in androgen-sensitive cells (Colombel and Buttyan, 1995; Buttyan *et al.*, 1997; Perlman *et al.*, 1999; Bruckheimer *et al.*, 1999) while androgen-insensitive cells remain unaffected (Kozlowski and Grayhack, 1991; Santen, 1992; Kreis W, 1995). However, androgen-insensitive cells are capable of undergoing apoptosis. Thus, the identification of novel methods to induce apoptosis in prostate cancer cells irrespective of their androgen response has great therapeutic value. In this paper we demonstrate that pectin, a plant polysaccharide fiber, induces apoptosis in both androgen-responsive and androgen-independent prostate cancer cells. This is the first extensive analysis correlating the structural features in pectin with apoptosis-inducing activity in cancer cells.

The role of dietary components in cancer prevention and progression is an area of increasing clinical and scientific interest. Both the American Institute of Cancer Research (AICR) and the World Cancer Research Fund (WCRF) estimated that 30-40% of cancer cases worldwide are preventable by dietary means (Liang *et al.*, 2001). Pectin is a natural complex plant polysaccharide present in all higher plant primary cell walls and consequently, is a dietary

component of all fruits and vegetables. Pectin accounts for approximately 30% of the primary walls of all higher plants except the grass family, where it makes up about 10% of the primary wall. Pectin has multiple roles in plant growth, development and disease resistance (Ridley *et al.*, 2001) and is used as a gelling and stabilizing agent in the food industry (Thakur *et al.*, 1997).

Previous research has shown that pectin can suppress colonic tumor incidence in rats (Heitman *et al.*, 1992) and inhibit cancer cell metastasis in mice and rats (Platt and Raz, 1992; Pienta *et al.*, 1995; Nangia-Makker *et al.*, 2002). Pectin has been shown to bind to B16-F1 melanoma cells *in vitro* (Platt and Raz, 1992). Furthermore, when injected intravenously in mice, relatively large commercial pectin increased homotypic cell-cell aggregation and metastasis to the lung while pH-modified, relatively small pectin inhibited lung metastasis (Platt and Raz, 1992), demonstrating a differential response depending upon the type of pectin used. Oral administration of a pH-modified citrus pectin significantly reduced metastasis of rat prostate adenocarcinoma MAT-LyLu to the lung (Pienta *et al.*, 1995). It is interesting that anti-metastatic effects of pectins occurred in the absence of cell toxicity (Inohara and Raz, 1994). From such data, it has been hypothesized that pectins can bind to cancer cell surface galectins (galactose binding lectins) and interfere with cell-cell or cell-matrix adhesion, inhibiting metastatic lesions (Inohara and Raz, 1994).

Several studies have shown that pectins not only inhibit metastatic lesions, but that some commercially available citrus pectins induce apoptosis. Azoxymethane-injected rats fed a citrus pectin or fish oil/pectin diet, had a greater number of apoptotic cells per colon crypt column, compared to rats fed corn oil and/or cellulose (Chang *et al.*, 1997a). Furthermore, pectin/fish oil-fed rats had a lower incidence of adenocarcinoma (51.5%) than animals fed cellulose/corn oil (75.6%) (Chang *et al.*, 1997a,b). The apoptotic index in the distal colon of pectin-fed rats was

higher than in rats fed a standard diet. This was accompanied by reduced expression of the anti-apoptotic protein Bcl-2 and activation of caspase-1 and poly(ADP)ribose polymerase, substrates of caspases (Aviv-Green *et al.*, 2000a,b,c). Similarly, administration of a pectin-rich 15% orange-pulp diet to dimethylhydrazine-injected Sprague Dawley rats resulted in a decreased number of endophytic tumors, an activation of caspase-3, and an increased activity of T cell killers in the tumors; all characteristic anti-tumor effects (Kossoy *et al.*, 2001). In human colon adenocarcinoma HT29 cells, caspase-3 activity increased significantly when treated with 10 mg/ml low methylated apple pectin (Olano-Martin *et al.*, 2003). Taken together these results suggest that exposure of malignant colon cells to pectin results in increased apoptosis and reduced tumor growth.

The interpretation of studies with pectin are complicated by i) the structural complexity of this plant-derived cell wall polysaccharide, ii) the modifications in pectin structure that result from the commercial extraction from plants, and iii) the additional modifications of pectin structure that result from the diverse fragmentation techniques used to produce specialized pectins (e.g. high pH (base) treatment; Platt and Raz, 1992; Pienta *et al.*, 1995; Eliaz, 2001; Nangia-Makker *et al.* 2002). Pectin is a complex family of polysaccharides that contain 4-linked α−D-galacturonic acid residues (O'Neill *et al.*, 1990). It is generally accepted that three types of polysaccharides comprise pectin: a linear homopolymer known as homogalacturonan (HGA), the branched polymer rhamnogalacturonan I (RG-I), and the substituted galacturonans of which the ubiquitous member is rhamnogalacturonan II (RG-II) (Ridley *et al.*, 2001; Albersheim *et al.*, 1996).

Homogalacturonan (HGA) accounts for 57-69% of pectin (Mohnen, 2002). HGA is a linear polymer of 1,4-linked α-D-galactopyranosyluronic acid (GalA) in which some (8-74%;

Voragen *et al.*, 1986) of the carboxyl groups may be methyl esterified. HGA may also be partially O-acetylated at C-3 or C-2. The length of HGA remains unclear, but degrees of polymerization of 30 to 200 have been reported (reviewed in Mohnen, 1999). Rhamnogalacturonan-I (RG-I) is a family of pectic polysaccharides that accounts for 7-14% of pectin and consists of a backbone of the repeating disaccharide [→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→]. Roughly 20-80% of the rhamnoses of RG-I are substituted by L-arabinose, D-galactose, L-arabinans, galactans, or arabinogalactans (Ridley *et al.*, 2001; Mohnen, 1999; O'Neill *et al.*, 1990). The side branches include α-1,5- and α-1,3-linked arabinans, β-1,4 linked or β-1,3 and β-1,6 linked galactans, and arabinogalactans of diverse linkages (reviewed in Mohnen, 1991). The average MW of sycamore RG-I is estimated to be 10^5-10^6 Da (O'Neill *et al.*, 1990). Rhamnogalacturonan II (RG-II) is a substituted galacturonan that accounts for 10-11% of pectin and whose structure is highly conserved across plant species. RG-II consists of a homogalacturonan backbone to which are attached four side branches of complex structure. It is arguably the most complicated polysaccharide in nature consisting of 12 different types of sugars joined in over 20 different linkages and contains unusual sugars such as 2-O-methyl xylose, 2-O-methyl fucose, KDO, DHA and apiose (Ridley *et al.*, 2001).

HGA, RG-I and RG-II are generally purified from intact purified cell walls by treatment with the enzyme endopolygalacturonase, which cleaves a stretch of four or more contiguous non-methylesterified α-1,4-linked galacturonic acid residues in HGA (Chen and Mort, 1996). The ability to cleave the pectic polysaccharides from the intact wall has been used as evidence to support the model that the backbones of the three pectic polysaccharides are covalently linked together in the wall. Nakamura *et al.* (2002) have provided structural evidence to support this linkage. Additional mechanisms for the association of the pectic polysaccharides include the

formation of borate ester cross-linked RG-II dimers (Kobayashi *et al.*, 1996; O'Neill *et al.*, 1996), HGA-Ca^{2+} interchain salt bridges (Morris *et al.*, 1982), and possible feruloyl polysaccharide ester crosslinks (Fry 1982; Ishii, 1997). However, a complete understanding of the larger 3D structure of pectin in the wall, and how the polymers are associated in the wall, is still lacking. For example, the role of proposed ester crosslinks between the carboxyl moiety of galacturonic acid in HGA and other pectic or wall polymers remains unclear (Kim and Carpita, 1992; Brown and Fry, 1993; Iiyama *et al.*, 1994; Hou and Chang, 1996; Djelineo 2001). Although the structural identity of the proposed ester(s) linkage at the carboxyl group of galacturonic acid in pectin is not known, the data presented in this paper support a role for this linkage in the ability of pectin to induce apoptosis in prostate cancer cells.

Whereas the structure of RG-II is conserved, the structures of HGA and RG-I are more variable in different plants and cell types due to differences in the polymer size, in the patterns of acetyl, methyl and other modifications of the GalA in the backbone of HGA, and due to variations in the length and type of side branches of the RG-I backbone. Furthermore, the production of commercial pectin usually involves an acid extraction of pectin from dried citrus peels or apple pomace (Thakur *et al.*, 1997), a process which results in the destruction and loss of RG-II and of some RG-I. In some cases, commercial citrus pectin may be further treated with base or heat to yield a more fragmented and structurally modified pectin. Thus, an appreciation of the biomedical benefits of pectin must be accompanied by an understanding of the structure of the pectin.

The goal of the present research is to examine the potential use of pectin in cancer therapy. The rationale is that pectin has been shown to induce apoptosis in colonic cancer cells (see above); pectins have multiple health promoting effects (Yamada *et al.*, 2003; Yamada,

1996); and the extraordinary structural complexity of pectin (Ridley et al, 2001) makes it a potential multi-functional therapeutic agent. Here we report that Fractionated Pectin Powder (FPP) induces apoptosis in both androgen-sensitive and androgen insensitive cells. Furthermore, modifications of the structure of pectin demonstrate a structure-function relationship in the apoptogenic activity of FPP.

Results

Effect of three commercial pectins on apoptosis in human prostate cancer cell lines LNCaP and LNCaP C4-2

The androgen-response of prostate cancer cells is an important criterion for devising appropriate therapy. Therefore, we utilized two prostate cancer cell lines, androgen-responsive LNCaP and androgen insensitive LNCaP C4-2, to determine the effects of pectin. Since the structure of commercially available pectin differs depending on the source and method of preparation, we first examined the effects of several commercially available pectin preparations: Citrus Pectin (CP), Pectasol (PeS) and Fractionated Pectin Powder (FPP). CP represents the starting pectin (i.e. "mother pectin") used to make the pH modified pectins, while PeS is a pH-modified pectin generated by base treatment. FPP represents another type of commercial pectin generated by heat treatment of pectin at 100-132ºC for 20 min to 5.5 hr. Cells incubated in media devoid of pectin served as negative controls, while cells treated with thapsigargin, a compound that induces apoptosis in prostate cancer cells, were the positive controls. Induction of apoptosis was assessed using an M30 Apoptosense assay that measures the amount of neoepitope generated by cleavage of cytokeratin-18 by caspases that are activated during apoptosis.

Figure 1 shows that FPP induced significant apoptosis in both androgen-responsive LNCaP (Figure 1A) and androgen-insensitive LNCaP C4-2 (Figure 1B) cells, while PeS and CP induced little or no apoptosis. As expected thapsigargin, utilized as positive control, induced significant apoptosis. To confirm the induction of apoptosis, cell extracts were analyzed by immuno-blots, which showed activation of caspase-3, an important inducer of apoptosis, in both LNCaP and LNCaP C4-2 cells treated with FPP (Figure 1C). The 35 KDa procaspase-3 was cleaved into 19 KDa, 17 KDa and 12 KDa products when treated with FPP, confirming significant apoptotic response. None of the other pectins induced a similar response, supporting the M30 assay data. As expected, thapsigargin-treated cells showed activation of caspase-3.
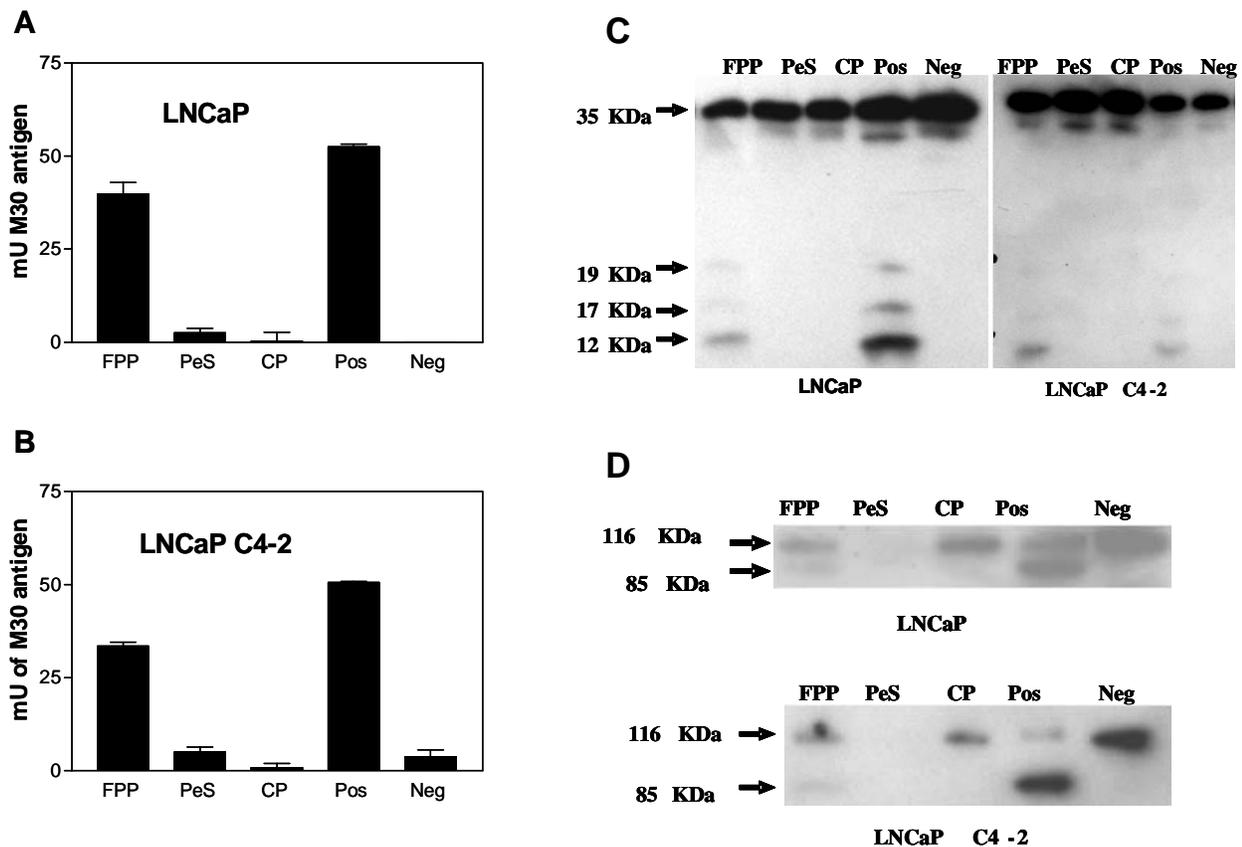

Figure 3.1. Induction of apoptosis by FPP **A:** Apoptosis induced in LNCaP cells was measured by ELISA using an M30-Apoptosense assay. The ELISA measures antibody binding to a neoepitope generated following cleavage of cytokeratin-18 by activated caspases. Cells were

treated with 1mg/ml Fractionated Pectin Powder (FPP), Pectasol (PeS), Citrus Pectin (CP), or with 0.01 mM thapsigargin (Pos) for 48 hr. Incubation with media alone served as the negative control (Neg). Equal amounts of cell extracts (12 μg protein) were used in ELISA. Data are the average of duplicate apoptosis assays of duplicate cell extracts ± SEM. Comparable results were obtained in at least two experiments. **B:** Induction of apoptosis in LNCaP C4-2 cells by FPP. For detailed description, please see A above. **C:** Activated Caspase-3 in LNCaP and LNCaP C4-2 cells treated with FPP. Western blot analysis of 30 μg protein from LNCaP and LNCaP C4-2 cells treated as described in Figure 1A using anti-caspase-3 antibody (see experimental procedures). Procaspase-3 is shown at 35 KDa. The 19 KDa, 17 KDa and 12 KDa cleaved products have been indicated. **D:** Activated PARP in LNCaP and LNCaP C4-2 cells treated with FPP. Western analysis was done using anti-PARP antibody. For detailed description see C above and experimental procedures. Poly ADPribose-polymerase is shown at 116 KDa and its cleavage product is shown at 85 KDa.

Analysis of the cell extracts for the presence of PARP (poly(ADP-ribose polymerase), a substrate of activated caspases, showed the presence of an 85 KDa cleaved product in the positive control (thapsigargin-treated cells) and in cells treated with FPP (Figure 1D), but not with PeS or CP. These results confirm that in both androgen-responsive and androgen-insensitive prostate cancer cells, FPP induced apoptosis, while PeS and CP did not have any effect.

As the above results showed that among the pectins tested, only FPP induced apoptosis, experiments were conducted to identify the effective dose of FPP required for apoptosis. Treatment of LNCaP cells with increasing concentrations of FPP showed that 3 mg/ml FPP induced maximum apoptosis (Figure 2). Lower concentrations of 0.01, 0.10 and 0.50 mg/ml FPP did not affect LNCaP cells significantly, while 1.0 mg/ml induced significant apoptosis. As no significant differences in apoptosis were noted between 1.0 mg/ml and 3.0 mg/ml, all subsequent experiments were conducted using 1 mg/ml FPP.
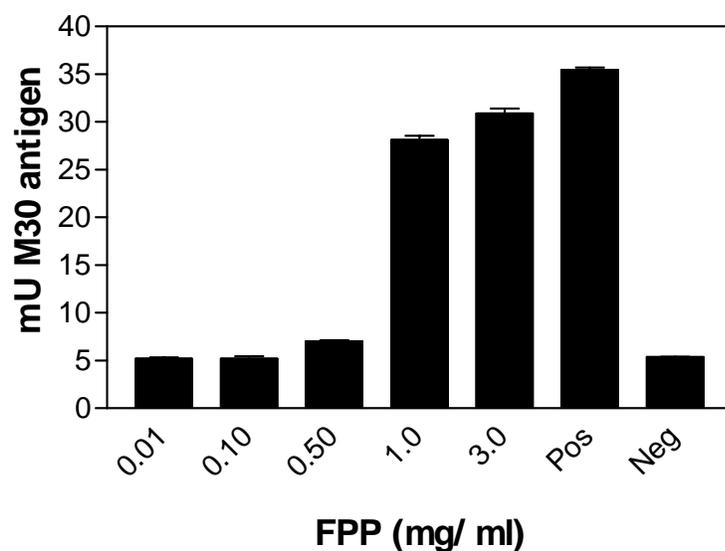
Figure 3.2. Concentration curve for apoptosis-inducing effect of FPP. LNCaP cells were treated for 48 hr with 0.01 mg/ml, 0.1 mg/ml, 0.5 mg/ml, 1 mg/ml, 3 mg/ml FPP, with 1 μM Thapsigargin (Pos) or with media alone (Neg). Apoptosis was measured using the M30 Apoptosense assay. Data are the average of duplicate apoptosis assays of duplicate cell extracts (29 μg protein) ± SEM. Comparable results were obtained in two independent experiments.

Defining the pectic structure(s) in FPP that induce apoptosis

Pectin is a complex polymer consisting of the polysaccharides homogalacturonan (HGA), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) that are held together in the plant wall by incompletely defined covalent and/or noncovalent interactions. We hypothesized that the apoptosis-inducing activity resided in one or more of the pectic polysaccharides HGA, RG-I and RG-II. To test this hypothesis, and to determine which structural features of pectin were required to induce apoptosis, LNCaP and LNCaP C4-2 cells were treated for 48 hr with 1 mg/ml of pectin fractions enriched for HGA, RG-I or RG-II. The HGA fraction consisted primarily of HGA oligosaccharides (oligogalacturonides) of degrees of polymerization of 7-23. Figure 3 shows that none of the purified pectic polysaccharides induced significant apoptosis in either the LNCaP (Figure 3A) or the LNCaP C4-2 cells (Figure 3B), suggesting that the apoptosis-inducing activity did not reside in the individual HGA, RG-I or RG-II polysaccharides. Thus, we hypothesized that some aspect of pectin structure lost during the preparation of the

purified RG-I, RG-II and HGA was responsible for the apoptosis-inducing activity observed with FPP.

To determine whether major structural differences among FPP, CP and PeS accounted for the apoptosis-inducing activity, the relative sizes of FPP, PeS, and CP were established by separation over a Superose 12 HR10 size exclusion chromatography (SEC) column in 50 mM sodium acetate and 5 mM EDTA. The eluted pectins were detected using a uronic acid colorimetric assay. Figure 3C shows that FPP was intermediate in size between the polydisperse and large CP (Figure 3E), which has an estimated molecular mass range of 23-71 kDa (www.sigmaaldrich.com) and the relatively uniformly sized and low molecular weight PeS (Figure 3D), which has a molecular mass range of 10-20 kD (www.econugenics.com). The polydisperse and intermediate size of FPP may indicate that the apoptosis-inducing activity requires an intermediate size polymeric structure, although proof of this requires elucidation of the specific apoptosis-inducing moiety.
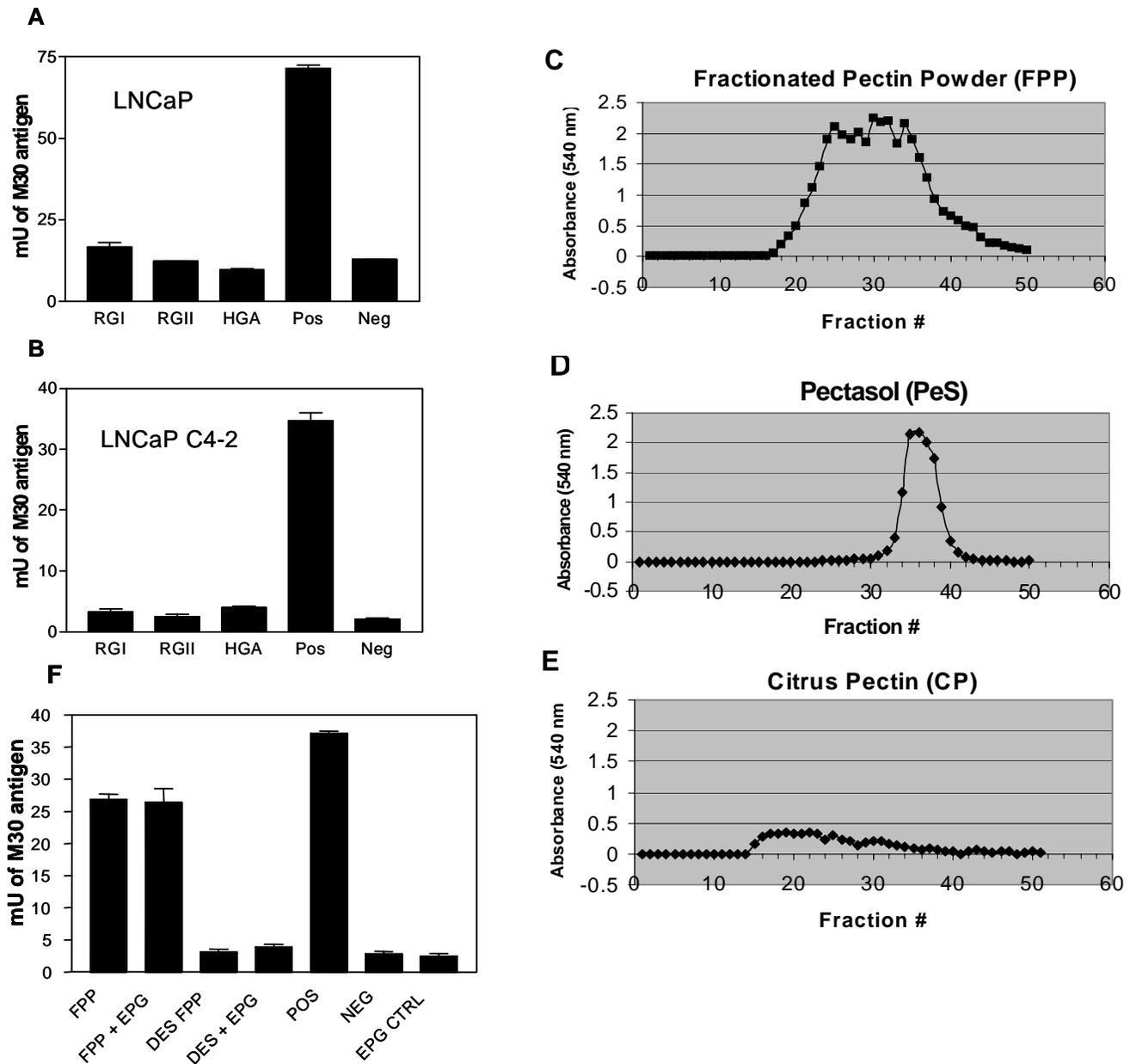
Figure 3.3. Determination of the active structural components in FPP that contribute to its apoptosis-inducing activity. **A:** Effects of treatment of LNCaP cells with the purified pectins. LNCaP cells were treated with 1 mg/ml homogalacturonan (HGA), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) for 48 hr and equal amounts of protein (12 μg) were assayed for apoptosis using the M30 Apoptosense assay. Data are the average of duplicate apoptosis assays of duplicate cell extracts ± SEM. Comparable results were obtained in three independent experiments. **B:** Same as A above except that LNCaP C4-2 cells were used. **C, D, E:** The size distribution of FPP as determined by size exclusion chromatography over a Superose 12 HR10 column in 50 mM sodium acetate and 5 mM EDTA and detection by a uronic acid colorimetric assay. **C:** data for FPP; **D:** data for PeS; **E** data for CP. **F:** Effect of mild base deesterification and/or endopolygalacturonase treatment of FPP on its ability to induce apoptosis. LNCaP cells were treated with 1 mg/ml FPP, FPP treated with endopolygalacturonase (EPG+FPP), FPP

deesterified by mild base treatment (DES+FPP), deesterified FPP that was subsequently treated with endopolygalacturonase (DES+EPG), 0.01 mM thapsigargin (Pos), or media only (Neg). Data are the average of duplicate apoptosis assays of duplicate cell extracts (30 μg protein) ± SEM. Similar results were obtained in two independent experiments.

The individual pectic polysaccharides HGA, RG-I and RG-II are purified from wall-derived pectin by a combination of chemical and enzyme treatments. A common initial purification scheme to isolate HGA, RG-I and RG-II is to subject plant walls to a mild base treatment to remove ester linkages within pectin. Such a treatment removes, for example, methyl esters on the galacturonic acid (GalA) residues in HGA rendering the pectin more accessible to the action of enzymes, such as endopolygalacturonanses (EPGs). EPGs cleave HGA in regions with contiguous nonesterified GalA residues (Chen and Mort, 1996), thereby fragmenting pectin and releasing RG-I and RG-II. FPP was therefore deesterified by mild base treatment to determine if ester linkages are required for its apoptosis-inducing activity. FPP was brought to pH 12 for 4 hr at 0°C on ice, neutralized, and repeatedly lyophilized against water, as described in the Experimental Procedures. Alternatively, the FPP was either treated with EPG to fragment the pectin, or the deesterified FPP was treated with EPG. LNCaP cells were treated with the enzyme-treated FPP to determine whether cleavage of an HGA-containing region of FPP affected its apoptosis-inducing activity. Apoptosis assays showed that EPG treatment of FPP had little or no effect on its apoptosis-inducing activity (Figure 3F). On the contrary, mild base treatment to remove ester linkages almost completely abolished the pectin-induced apoptotic response, indicating the importance of one or more base-sensitive linkages (e.g. ester linkages) in the apoptotic function of FPP.

To determine whether specific carbohydrates were uniquely enriched in FPP and contributed to its apoptosis-inducing activity, the glycosyl residue composition of FPP, PeS and

CP were compared. Figure 4 shows the average mole % glycosyl residue composition of unmodified and base-treated FPP, CP and PeS. As expected, galacturonic acid (GalA) was the major component in all three pectins tested. No consistent correlation was found between the apoptosis-inducing activity and the glycosyl residue composition of FPP compared to unmodified CP and PeS. For example, FPP was arabinose-rich compared to PeS, but had comparable amounts of Ara to CP. Likewise, both FPP and PeS had less Gal, slightly less Rha, and more GalA than CP.
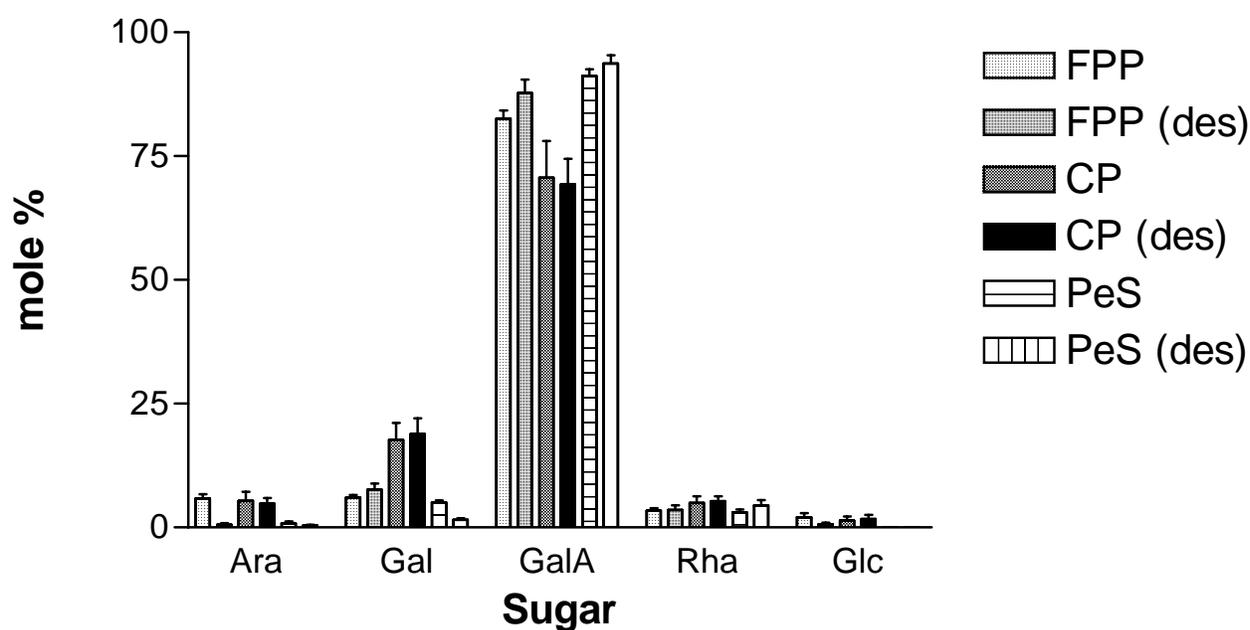
Figure 3.4. Glycosyl residue composition analyses of unmodified and base-treated FPP, PeS and CP. The glycosyl residue composition of untreated and base-treated FPP, CP and PeS. Composition analyses were done by GC/MS of TMS derivatives of methyl glycosides produced by acid methanolysis (York W.S. *et al.* 1985). Data are the average ± SEM mole % specific sugar from duplicate analyses from two separate experiments (N = 4).

As noted in Figure 3F, mild base treatment to remove ester linkages in FPP destroyed its apoptosis-inducing activity. To determine whether this treatment altered the glycosyl residue composition, FPP, CP and PeS were brought to pH 12 for 4 hr, neutralized, and lyophilized (as described in the Experimental Procedures) and the glycosyl residue composition was determined.

Surprisingly, the deesterification step led to a large reduction (90%) in the amount of Ara recovered in FPP (Figure 4), but did not alter Ara significantly in CP and PeS. At this time, the reason for the loss of Ara in deesterified FPP is unclear.

The fine structural differences between FPP, CP and PeS were probed further by determining their glycosyl residue linkages. The glycosyl residue linkages of FPP, CP and PeS obtained using both a single (Table 1) and double (Table 2) methylation procedure were compared. The disadvantage of the single methylation method is incomplete methylation leading to incomplete linkage results, but the advantage is avoidance or reduction of fragmentation of the pectin due to ß-elimination of the glycosyluronic acid linkages in the HGA. On the other hand, the double methylation method leads to more complete methylation but can lead to ß-elimination of the glycosyluronic acid linkages (York *et al.*, 1985), and thus, to an apparent increase in the amount of terminal GalA and a loss in the apparent 4-linked GalA (Table 2). Comparison of the linkage data for the unmodified pectins showed that all three pectins contain primarily HGA (the presence of 4-linked GalA) and RG-I (2-linked Rha and 2,4-linked Rha). FPP and CP contained 5-linked arabinan and 4-linked galactan, which are known to occur as side chains of RG-I, whereas these linkages were absent or greatly reduced in PeS. As expected, in the double methylation procedure (Table 2) there was an unrealistically high apparent terminal-GalA content, likely due to fragmentation of the HGA because of ß-elimination. The amount of terminal GalA was significantly less in the single methylation method. Likewise, as expected there was more apparent undermethylation of the pectin in the single methylation method, identified as apparent 2,3,4,-linked GalA. Undermethylation likely also explains the greater amounts of 2,3-linked and 3,4-linked GalA obtained in the single methylation method compared to the double methylation method. The only linkage data that correlated with the apoptosis-

inducing activity was the higher amounts of terminal and 5-linked Ara in FPP. These linkages were lost in the base-treated FPP. Whether these glycosyl residues are involved in the apoptosis response remains to be confirmed.

Table 3.1. Glycosyl linkage analysis of untreated and base-treated FPP, CP and PeS by the single methylation method.

Glycosyl Residue	Relative amount					
	FPP	FPP (des)	CP	CP (des)	PeS	PeS (des)
T-Ara (f)	4 ± 0	0	2.5 ± 0.5	1 ± 0	0	0
5-Ara	3 ± 0	0	2 ± 0	1 ± 0	0	0
T-Gal	4 ± 0	2 ± 0	2.5 ± 0.5	1.5 ± 0.5	5 ± 0	2 ± 0
3-Gal	0.5 ± 0.5	0	1.5 ± 0.5	0.5 ± 0.5	0	0
4-Gal	12 ± 0	7.5 ± 2.5	11.5 ± 1.5	8 ± 1	3 ± 0	0
2,4-Gal	3 ± 0	1.5 ± 1.5	2.5 ± 0.5	1.5 ± 0.5	0	0
3,4-Gal	1.5 ± 0.5	1 ± 1	2 ± 0	1 ± 1	0	0
T-GalA	9 ± 2	4.5 ± 2.5	2.5 ± 0.5	2 ± 0	23.5 ± 0.5	7 ± 0
4-GalA	36.5 ± 5.5	37.5 ± 6.5	32 ± 1	40.5 ± 4.5	28 ± 0	45 ± 4
2,4-GalA	7.5 ± 0.5	10.5 ± 0.5	10 ± 0	10.5 ± 1.5	7 ± 0	10 ± 1
3,4-GalA	8 ± 1	13.5 ± 1.5	10.5 ± 1.5	14 ± 1	7.5 ± 0.5	14.5 ± 1.5
GalA (U)[2]	5 ± 1	8 ± 1	6 ± 1	10 ± 1	7.5 ± 0.5	11.5 ± 1.5
T-Rha	T[3]	0	T	T	0	0
2-Rha	7 ± 1	4 ± 0	5 ± 0	3 ± 0	10± 0	5 ± 0
2,3-Rha	2 ± 0	1.5 ± 0.5	2 ± 0	1.5 ± 0.5	3.5 ± 0.5	2 ± 0
2,4-Rha	3 ± 0	1 ± 1	3 ± 0	2 ± 0	3.5 ± 0.5	2 ± 0
2,3,4-Rha	1.5 ± 1.5	1 ± 0	1.5 ± 0.5	1 ± 0	1.5 ± 0.5	1 ± 0
4-Glc	2 ± 0	2.5 ± 1.5	3 ± 1	1 ± 0	0	0

[1]Linkage analysis was carried out by GC/MS of partially methylated alditol acetates (PMAAs) produced by permethylation, depolymerization, reduction, and acetylation as described by York *et al.* 1985. Data are average % of glycosyl residues with specified linkages ± SEM from duplicate analyses (N = 2).

[2]U: undermethylated; apparent 2,3,4,-linked GalA

[3]T: trace; <1

Table 3.2. Glycosyl linkage analysis of untreated and mild base-treated FPP, CP and PeS by the double methylation method.

Glycosyl Residue	Relative amount					
	FPP	FPP (des)	CP	CP (des)	PeS	PeS (des)
T-Ara (f)	1.5 ± 1.5	0	0	0	0	0
5-Ara	0.5 ± 0.5	0	0	0	0	0
T-Gal (f)	0	0	0	0	5.5 ± 0.5	0
T-Gal	2 ± 0	3.5 ± 1.5	2.5 ± 0.5	1 ± 0	1.5 ± 0.5	1 ± 0
4-Gal	5 ± 0	11.5 ± 6.5	4.5 ± 1.5	3.5 ± 0.5	0	0
2,4-Gal	1 ± 0	T[3]	1 ± 0	0.5 ± 0.5	0	T
T-GalA	20.5 ± 4.5	12 ± 1	20.5 ± 1.5	12.5 ± 1.5	17.5 ± 3.5	17.5 ± 1.5
4-GalA	43 ± 1	48 ± 9	47.5 ± 2.5	58.5 ± 1.5	55 ± 7	58.5 ± 1.5
2,4-GalA	1.5 ± 0.5	1.5 ± 1.5	2.5 ± 0.5	4 ± 1	2.5 ± 0.5	3.5 ± 1.5
3,4-GalA	4.5 ± 1.5	2.5 ± 2.5	3.5 ± 0.5	6 ± 0	3 ± 0	3.5 ± 0.5
GalA (U)[2]	3.5 ± 1.5	1.5 ± 1.5	4.5 ± 0.5	5.5 ± 0.5	2.5 ± 0.5	2.5 ± 1.5
T-Rha	1 ± 1	0	0	0	0	0
2-Rha	2.5 ± 0.5	5.5 ± 3.5	4 ± 2	2 ± 0	3.5 ± 0.5	2 ± 0
2,4-Rha	3 ± 1	3.5 ± 0.5	2.5 ± 0.5	4 ± 0	2 ± 0	2 ± 1
T-Glc	3.5 ± 0.5	0	0	0	2 ± 0	2 ± 2
4-Glc	5 ± 0	9.5 ± 5.5	4.5 ± 0.5	4 ± 4	2.5 ± 0.5	4 ± 0

[1] Linkage analysis was carried out as in Table 1, except that following the first methylation the permethylated material was reduced by super-deuteride and the reduced sample was re-methylated using the NaOH/MeI method (see Experimental Procedures). Data are the average % of glycosyl residues with the specified linkages ± SEM from duplicate analyses (N = 2).
[2]U: undermethylated; apparent 2,3,4,-linked GalA

[3]T: trace; <1

Evidence for a base-sensitive cross link in FPP required for apoptotic activity

As described earlier, mild base treatment of FPP to remove ester-linked moieties in pectin, destroyed its apoptosis-inducing activity, while treatment with EPG had little on no effect on its apoptosis-inducing activity (Figure 3F). To determine how the base treatment affected the function of FPP, intact, EPG-treated, base-treated (deesterified), and deesterified plus EPG-treated FPP were separated over high percentage polyacrylamide gels. These gels are particularly useful to separate HGA oligosaccharides (oligogalacturonides, OGAs) (Djelineo, 2001). The PAGE gels were stained with alcian blue and silver stain to detect the pectins. The alcian blue is a positively-charged dye that binds the negatively charged GalA in the pectin.

PAGE showed that FPP separated as a smear of dark staining polymeric pectin near the top of the gel (Figure 5A, lane 1). These lanes also contained discrete bands in the bottom third of the gel that represent oligogalacturonides (OGA) of degrees of polymerization of ~ 6-14. EPG-treated FPP looked similar to unmodified FPP except for the loss of OGAs at the bottom of the gel. The loss of the OGAs was expected due to cleavage by EPG into mono-, di- and tri-galacturonic acid (Doong *et al.*, 1985) which can not be visualized in the alcian stained gels. The similarity of the larger polymeric portion of untreated and EPG-treated FPP suggests that the bulk of the polymeric portion of FPP was not accessible to cleavage by EPG, possibly due to esterification. Unexpectedly, treatment of FPP with mild base to deesterify the pectin led to a major change in the appearance of the FPP as determined by PAGE. Mild base treatment of FPP resulted in the loss of the large polymeric alcian-blue stained material and the appearance of dark-staining bands near the bottom of the gel (compare lanes 3 and 1 in Figure 5A). To test whether these bands represented HGA oligosaccharide (i.e. OGAs), the deesterified FPP was subsequently treated with EPG, which reduced dark-staining bands at the bottom of the gel (compare lanes 4 and 3 of Figure 5A). These results confirm that the dark-staining bands were indeed OGAs. Since the base treatment also led to the loss of the apoptosis-inducing activity (Figure 3F), we conclude that the linkage of the OGAs into a polymeric structure and/or the specific base-sensitive linkage itself, is required for the apoptosis-inducing activity of FPP.

C

D
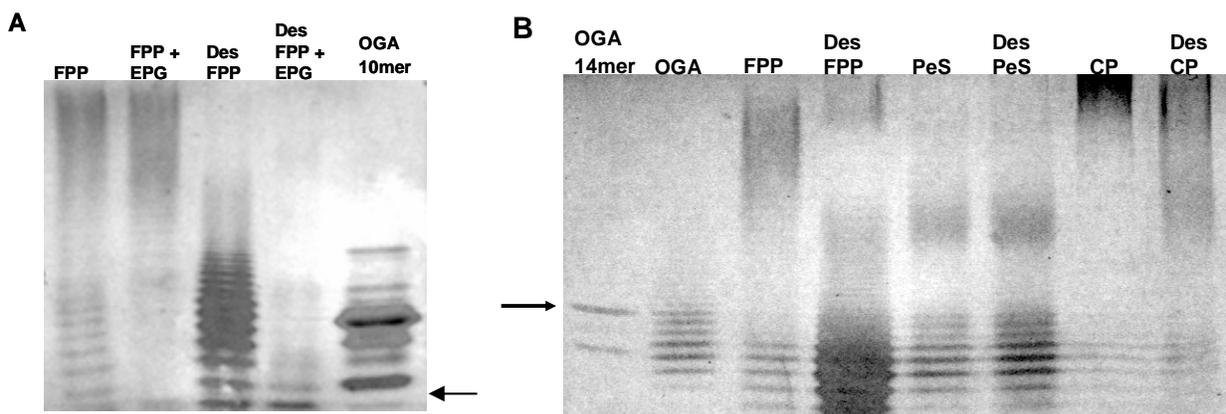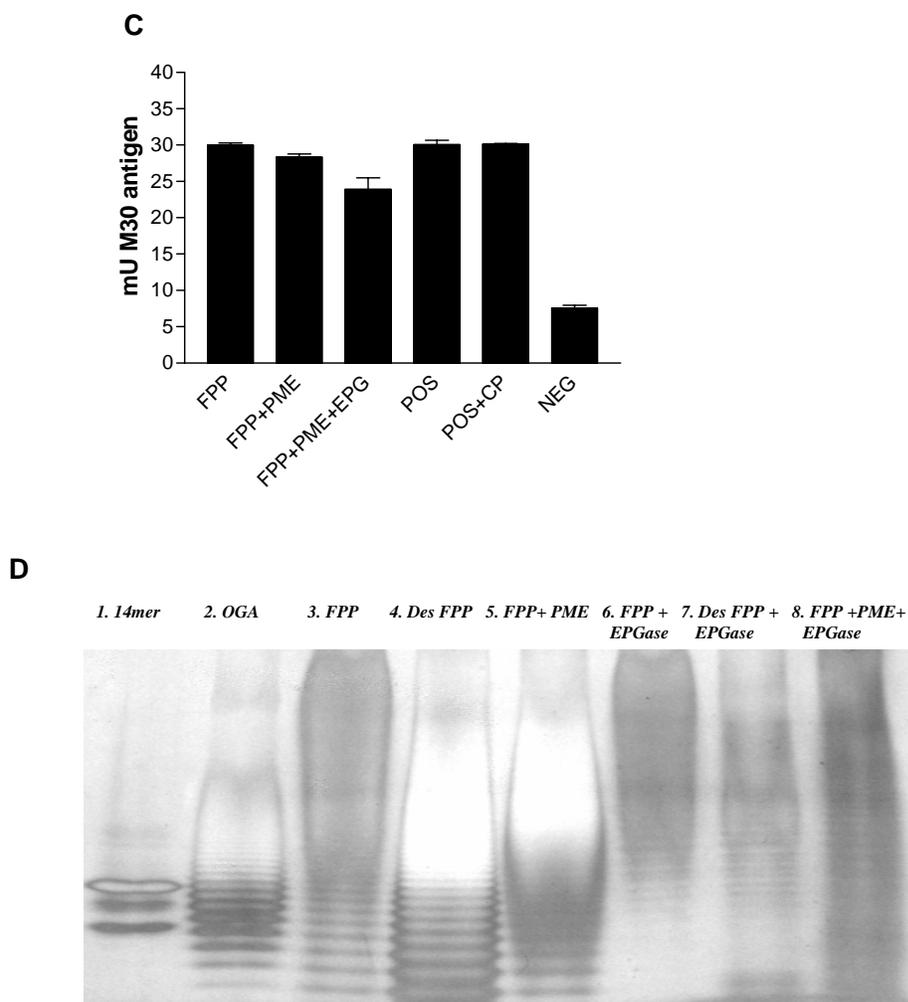
Figure 3.5. Use of PAGE and M30-Apoptosense ELISA to probe the FPP structure/function relationships. **A:** Intact FPP, FPP treated with endopolygalacturonase (FPP+EPG), base-deesterified FPP (Des FPP) and deesterified FPP that was subsequently treated with EPG (Des FPP + EPG) separated by PAGE (see Experimental Procedures). Lanes 1-4, 10 μg of treated or untreated FPP; Lane 5: 0.1 μg of oligogalacturonide (OGA) of degree of polymerization (DP) 10 (see arrow). **B:** Intact FPP, PeS and CP and the respective base-treated pectins (Des) were separated by PAGE. Lane 1, 0.1 μg OGA of DP 14 (see arrow); Lane 2, 1 μg mixture of OGAs of DP ~7-23; lanes 3-8, 10 μg of the treated or untreated pectins. **C:** Effect of pectinmethylesterase treatment of FPP on its on its ability to induce apoptosis in LNCaP cells. Equal amounts of protein (30 μg) from cells treated with 1 mg/ml FPP, pectin methylesterase-treated FPP (FPP+PME), methylesterase and endopolygalacturonase-treated FPP (FPP+PME+EPG), 0.001 mM thapsigargin (Pos), 0.001 mM thapsigargin + 1 mg/ml CP (Pos+CP), or media only (Neg) were tested for their ability to induce apoptosis in LNCaP cells as measured cells by M30 antibody binding. Data are the average of duplicate apoptosis assays of duplicate cell extracts ± SEM. Similar results were obtained in at least two separate experiments. **D:** PAGE analysis of intact FPP, base-deesterified FPP (Des FPP), pectin methylesterase-treated FPP (FPP+PME), endopolygalacturonase-treated FPP (FPP+EPG),

deesterified FPP that was subsequently treated with EPG (Des FPP + EPG) and pectin methylesterase and endopolygalacturonase-treated FPP (FPP+PME+EPG). Lane 1, 0.1 µg OGA of DP 14 (see arrow); Lane 2, mixture of OGAs of DP ~7-23; Lanes 3-8, 6 µg of the treated and untreated FPP samples.

Since FPP, but not PeS or CP, had apoptosis activity, intact and mild-base treated FPP were compared to treated and untreated PeS and CP to identify any structural differences that correlated with FPP's apoptotic activity. PAGE analysis of PeS revealed a narrow-range of dark-staining smear near the center of the gel and a series of OGAs, consistent with its relatively low mass of 10-20 kD. Mild base treatment of PeS had no effect on PeS (see lanes 5 and 6, Figure 5B). This was expected since PeS is generated from CP by a base treatment, and thus, further base treatment had no affect. A similar analysis of CP revealed that the bulk of untreated CP barely entered the PAGE gel, as indicated by the dark staining material at the top of the gel, although a relatively small amount of OGAs were also detected (Lane 7, Figure 5B). Deesterification of CP lead to the appearance of a broad smear of stained material in the upper half of the gel, and to a slight increase in the amount of OGAs (compare lanes 7 and 8, Figure 5B). The change in the appearance of CP upon base treatment suggests that i) the generated material was more negatively charged due to an increased number of HGA carboxyl groups following removal of methyl esters, and/or ii) that base treatment led to a reduction in the size of the polymer.

Mild base treatment is non-specific and may remove several types of esters including carboxymethyl, acetyl, and other esters. Therefore, to achieve specific cleavage of HGA carboxymethyl esters, FPP was treated with pectinmethylesterase (PME). LNCaP cells were treated with intact FPP, with FPP treated with PME, or with FPP treated with both PME and EPG and the apoptotic activity was assayed. Removal of methyl esters present in FPP resulted in only a very small reduction (4%) in the apoptotic activity of FPP (Figure 5C). Apoptotic activity

was reduced 20% when FPP was treated with both PME and EPG, although the loss of apoptotic activity was considerably less than the effects of mild base treatment (compare Figures 5C and 3F). These results suggest that base-sensitive linkages other than the HGA carboxymethylesters play a dominant role in the apoptosis-inducing activity of FPP.

The effects of the above manipulations on FPP were further analyzed by PAGE, which showed that PME treatment shifted the bulk of the dark staining material from the top of the gel (Figure 5D, lane 3) to the bottom half of the gel (lane 5). The mild base treatment led to the generation of fast moving FPP fragments (lane 4) suggesting that this treatment not only cleaved methyl esters, but also cleaved additional linkages (possibly esters) leading to extensive fragmentation of the FPP and the loss of apoptotic activity. Interestingly, although PME treatment of the FPP also generated fragments that moved faster into the gel, a large proportion of that material migrated more slowly than the base-treated material (Figure 5D). Since the PME-treated FPP retained apoptotic activity (Figure 5C), it is likely that the moderately-sized material near the center of the gel contains fragmented FPP that is responsible for apoptotic activity.

To determine the effect EPG on the structure of the PME-treated FPP, intact, PME-treated, and base-treated FPP were treated with EPG and separated by PAGE (Figure 5D, lanes 6-8). As expected, the OGAs present in intact FPP (lane 3, Figure 5D) were lost upon treatment with EPG (lane 6, Figure 5D). Likewise, treatment of deesterified FPP with EPG resulted in the loss of OGAs (compare lanes 4 and 7, Figure 5D) and the movement of the polymeric fragments further into the gel. Finally, removal of the HGA methyl esters by PME treatment produced a broad band of slow migrating material (lane 5, Figure 5D). Treatment of this material with EPG led to the loss of OGAs and the appearance of a broad band of stained material suggesting that a

significant amount of the pectin remained cross-linked as a diverse size range of oligosaccharides and polysaccharides (lane 8, Figure 5D). An important observation was that less pectic material was cleaved by EPG following PME treatment of FPP (lane 8, Figure 5D) compared to base treatment (lane 7, Figure 5D), suggesting that base treatment causes the loss of linkages in addition to methyl esters. The presence of an ester-like cross-linking in the HGA backbone is further supported by the observation that EPG treatment of FPP led to the loss of only OGA bands (lane 2, Figure 5A), indicating that EPG does not possess the ability to degrade the larger pectin components in FPP, while EPG treatment of base-treated FPP leads to the loss of the vast majority of the FPP (lane 4, Figure 5A).

Discussion

Cancer therapy is aimed at either the primary tumor or the metastatic state. Due to the differences in the characteristics of the cancer cell in the primary and the metastatic cancers, most therapies do not address both these cancer types. Pectin, a natural plant polysaccharide present in all higher plant cell walls, and thus in all fruits and vegetables and in most plant derived foods, is a compound that appears to be able to inhibit cancer metastasis and primary tumor growth in multiple types of cancer in animals. Although, pectins were initially recognized as compounds capable of inhibiting metastatic lesions (Heitman *et al.*, 1992; Platt and Raz, 1992; Pienta *et al.*, 1995; Nangia-Makker *et al.* 2002), recently, pectins have been shown to reduce primary tumor growth (Nangia-Makker *et al.* 2002). It has been suggested that the inhibitory effects of pectin on metastatic lesions in the lung are mediated through their binding to galectin-3 (Pienta *et al.* 1995). Galectins are galactoside-binding lectins. Galectin-3 is a specific

carbohydrate-binding lectin present on the surface of cancer cells, particularly metastatic cells. Galectin-3 aids in cell-cell interactions by binding to galactose-containing molecules on neighboring cancer cells. In human colon, stomach and thyroid cancers, the amount of galectin-3 increased as the cancer progressed. Blocking galectin-3 expression in highly malignant human breast, papillary and tongue carcinoma cells led to reversion of the transformed phenotype and suppression of tumor growth in nude mice (Honjo *et al.*, 2000; 2001). It has been proposed that pH-modified citrus pectin blocks binding of galectin-3, and thus, inhibits tumor cell-cell interactions (Pienta *et al.* 1995). The potential impact of blocking galectin-3 action includes inhibition of the aggregation of cancer cells to each other and to normal cells, thus inhibiting metastatic lesions. However, LNCaP cells do not express galectin-3 suggesting that the apoptotic effects of pectins observed in the present work, are due to a mechanism not mediated through galectin-3.

The interpretation of the effects of pectin on cancer cells are complicated by the structural complexity of pectins. Most of the published reports on the anticancer effects of pectin utilized pectins that were modified by alterations in pH in an effort to fragment pectin structure to facilitate biological effects. Therefore, as a first step to determine whether the size of pectin and/or its method of preparation affected its biological activity, we examined the effects of several commercial pectins, fractionated pectin powder (FPP), citrus pectin (CP) and PectaSol (PeS), on prostate cancer cells. The results showed that only FPP had significant apoptosis-inducing activity in prostate cancer cells. CP and PeS did not affect the cells, agreeing with published data showing no apoptotic effects of pH-modified pectin on prostate cancer cells and xenografts (Pienta *et al.*, 1995). As pectins consist of homogalacturonan and rhamnogalacturonans, we hypothesized that these polysaccharides in FPP may be responsible for

its apoptotic effects. However, experiments to determine the apoptotic effects of HGA, RG-I and RG-II, indicated that these structural components were not responsible for the apoptosis-inducing activity of FPP. To further examine the structural differences among the tested pectins, the effect of pectin size, and glycosyl residue composition and linkage, on the apoptotic activity was tested. Analysis showed no correlation of these parameters among the pectins. We therefore conclude that the apoptosis-inducing activity of FPP is related to some fine structural constituent not detected by the analyses used.

To further understand the structure-function relationship of apoptotic pectin, FPP was specifically fragmented using endopolygalacturonase (EPGase), which cleaves at contiguous non-esterified GalA residues in HGA. Cleavage of FPP with EPGase did not have a major effect on its apoptotic activity. Thus, two methods were use to remove ester linkages from FPP prior to EPG treatment and thereby, make FPP more susceptible to EPG cleavage: chemical deesterification by mild base treatment and specific enzymatic hydrolysis of methyl esters by treatment with pectin methylesterase (PME). Chemical deesterification of FPP resulted in significant loss of apoptosis suggesting that a base sensitive structure, such as an ester, is necessary for the apoptotic activity of FPP. However, specific cleavage of methyl esters by pectin methylesterase did not destroy FPP's apoptosis inducing activity, suggesting that linkages other than methyl esters are required for apoptotic function.

PAGE analysis of intact and treated FPP showed that a base-sensitive linkage in a polymeric/oligomeric FPP structure and/or a specific base-sensitive linkage itself is required for the apoptotic activity of FPP. Taken together the results suggest that an ester-based (or related) cross-link in pectin is important for the apoptosis-inducing activity of FPP.

In conclusion, we have demonstrated that among the pectins tested, FPP is able to induce apoptosis in both androgen-responsive and androgen-insensitive prostate cancer cells. Furthermore, for the first time, we have analyzed structural characteristics of pectin that are responsible for its apoptotic activity. Further experiments are being conducted to identify the specific apoptotic structure in FPP and to explore structure-function aspects of the anti-cancer activities of pectins.

Experimental Procedures

Materials

Androgen-responsive prostate cancer cells, LNCaP were obtained from American Type Culture Collection (Rockville, MD) and androgen-refractory LNCaP C4-2 cells were purchased from Grocer Inc., Oklahoma City, OK. Fetal Bovine Serum (FBS), penicillin/streptomycin, unmodified citrus pectin, sodium hydroxide and alcian blue were purchased from Sigma-Aldrich. Fungizone was obtained from Invitrogen. Bio-Rad Protein Assay Dye Reagent concentrate was purchased from Bio-Rad. M-30 Apoptosense ELISA is from Peviva AB (Sweden). Sodium carbonate and sodium acetate were purchased from J. T. Baker and acetic acid from EM Science. Pectasol was purchased from EcoNugenics and Fractionated Pectin Powder purchased from Thorne Research. Pectinmethylesterase (PME; from *Aspergillus niger* 2.2 μg/μl, 1.0 U/μg, 1U=1 μmol/min) and endopolygalacturonase (EPG; from *Aspergillus niger* 0.5 μg/ μl, 1.2 U/μl, 1U=1μmol/min) were obtained from Carl Bergmann, Complex Carbohydrate Research Center, University of Georgia. All other chemicals, unless otherwise stated, were from Fisher Scientific.

Purified pectins

Purified HGA, RG-I, and RG-II were a gift of Stefan Eberhard, Complex Carbohydrate Research Center, University of Georgia. The homogalacturonan was a mixture of oligogalacturonides of degrees of polymerization of ~7-23 that were produced by partial endopolygalacturonase treatment of commercial polygalacturonic acid as described by Spiro *et al.* (1993). RG-I was isolated from sycamore (*Acer pseudoplatanus*) suspension culture cells as described (Marfà *et al.*, 1991). RG-II was isolated from red wine basically as described by Pellerin *et al.* (1996).

Endopolygalacturonase treatment of pectins

Ammonium formate, pH 4.5, was added to 500 ul of 20 mg/ml deesterified Fractionated Pectin Powder (desFPP) and Fractionated Pectin Powder (FPP) to give a final ammonium formate concentration of 10 mM. Two μl of 1.2 U/ μl, 0.5 mg/ml *Asperillus niger* endopolygalacturonase (EPG) was added. As a negative control, 500 ul of 20 mg/ml FPP in 10 mM ammonium formate was also prepared. The FPP samples were incubated overnight at RT, frozen at -80 $^{\circ}$C, and lyophilized to dryness. The dry samples were analyzed by high % acrylamide PAGE and tested for apoptotic activity in an M-30 apoptosense ELISA.

Pectinmethylesterase treatment of pectins

One μl of 1U/ μg, 2.2 μg/ μl *Aspergillus niger* pectinmethylesterase (PME) was added to 1 ml of 20 mg/ml Fractionated Pectin Powder (FPP) in 10 mM ammonium formate. One ml of 20 mg/ml FPP in 10 mM ammonium formate served as a negative control. A combined PME+EPG treatment involved mixing 2 μl of 1.2 U/ μl, 0.5 mg/ml *Asperillus niger* EPG and 1 μl of 1U/ μg, 2.2 μg/ μl *Aspergillus niger* pectinmethylesterase PME in 10 mM ammonium formate. The FPP

samples were incubated overnight at RT, frozen at -80 $^{\circ}$C, and lyophilized to dryness. The dry samples were analyzed by high % acrylamide PAGE and tested for apoptotic activity in an M-30 apoptosense ELISA.

Pectin de-esterification

Fifty milligrams pectin was dissolved in 50 ml of de-ionized water and placed on ice. The starting pH of the solution (3.83) was measured and recorded using a pH meter (Orion Research, Inc. Beverly, MA, model SA520). The pH of the pectin solution was brought to pH of 12 by adding cold 1.0 M NaOH, and a pH of 12 was maintained for four hours by the addition of 0.1 M NaOH. The solution was kept on ice to retain a temperature of 0 $^{\circ}$C. After four hours, the pH was adjusted to 5.2 by the addition of glacial acetic acid. The deesterified pectin solution was frozen at -80°C, and lyophilized. The dry material was dissolved in water, frozen, and re-lyophilized to remove any remaining residual material.

Cell culture, pectin treatments and quantification of apoptosis

LNCaP and LNCaP C4-2 cells were grown in RPMI-1640 medium supplemented with 25 mM HEPES, 2.0 mM L-Glutamine, 10% fetal bovine serum (Hyclone, Logan, Utah), 50 U/ml Penicillin, 0.05 mg/ml Streptomycin, and 0.25 μg/ml Fungizone and grown in the presence of 5% CO_2 at 37 $^{\circ}$C. Cells were maintained in logarithmic growth phase by routine passage every 10-12 days (LNCaP) or 6-7 days (LNCaP C4-2). Cells were plated at a density of 1.6 x 10^5 cells per well in 6-well culture plates and allowed to adhere for 24 hr. The medium was removed and medium containing filter-sterilized pectin (0.20 um nylon filters; Fisher Scientific) was added. Treated cells were incubated in media containing the following compounds at the indicated

concentrations for the times indicated (see Figure legends): Fractionated Pectin Powder (FPP), Pectasol (PeS), Citrus Pectin (CP), deesterified fractionated pectin powder (desFPP), pectin methylesterase-treated fractionated pectin powder (PME+FPP), endopolygalacturonase (EPG)-treated fractionated pectin powder (EPG+FPP), PME-treated desFPP, EPG-treated desFPP, combined enzyme treatments, rhamnogalacturonan-I, rhamnogalacturonan-II, purified homogalacturonan, and Thapsigargin (positive control, Sigma, St. Louis, MO). The negative controls were untreated cells cultured in media alone. Cells were harvested after incubation for 48 hr by centrifugation, the cell pellet was resuspended in ice cold lysis buffer (10 mM Tris-HCL, pH 7.4, 10 mM $MgCl_2$, 150 mM NaCl), incubated on ice for 5 min, and soluble protein collected by centrifugation at 4°C. Protein concentration was determined using a Bradford/Bio-Rad Protein Assay (see below). Apoptotic activity was assayed using the M-30 Apoptosense ELISA (see below).

Bradford/Bio-Rad Protein assay

Bovine serum albumin (BSA) (1-10 μg) or cell lysate samples were added in triplicate to wells in a 96-well plate and brought to 160 ul/well with autoclaved deionized filtered water. Forty ul Bio-Rad Protein Assay Dye Reagent Concentrate (cat # 500-0006) was added and the plate was gently agitated on a plate shaker for 15 min at RT. OD 595 nm was determined using a Finstruments Model 347 (Vienna, Virginia, USA) microplate reader. Protein concentration was determined based on the BSA standard curve.

Apoptosense assay

Cells were plated in culture plates and treated as described above. Upon completion of the experiments, cells were harvested and total protein extracted as described above. Protein was assayed for the presence of the apoptosis-specific cytokeratin-18 neoepitope (generated by cleavage of cytokeratin-18 by apoptotic caspases) using the M30-Apoptosense ELISA (Peviva AB, Sweden). The assay and reagents were as provided by the manufacturer. In brief, protein extract was added to 96-well plates coated with mouse monoclonal M30 antibody, horseradish peroxidase tracer solution was added to the wells in a dark room illuminated with a green safety light, and the plate was incubated with agitation for 4 hr at room temperature. Color was developed by adding tetramethyl benzidine solution and incubating in darkness for 20 min. Optical density was determined at 450 nm using Spectra MAX 340 (Molecular Devices, Menlo Park, CA) or Finstruments Model 347 (Vienna, Virginia, USA) microplate readers. The amount of cytokeratin-18 neoepitope was determined based on standard curves generated using standards provided by the manufacturer.

Preparation of cell lysates for western blotting

Cells were harvested by trypsinization and washed cell pellets were resuspended in lysis buffer (1X PBS, 1% Triton X 100, 0.5% sodium deoxycholate, 0.1% SDS, 1mM EDTA, 0.5 μg/μl leupeptin, 1 μg/μl pepstatin, 1 μg/μl phenylmethyl sulfonyl fluoride (PMSF), 1 μg/ml aprotinin) and incubated on ice for 30 min. The lysed cells were centrifuged at 10,000g for 10 min at 4°C and the supernatant was collected. Protein concentration was determined as described above.

Oligogalacturonide-polyacrylamide gel electrophoresis

Pectin samples were separated by PAGE and analyzed by alcian blue staining using a modification of the procedures of Corzo *et al.* (1991) and Reuhs *et al.* (1993, 1998) as described by Djelineo (2001). Pectin samples were mixed in a 5:1 ratio with 6X sample buffer (0.63 M Tris-Cl/ pH 6.8, 0.05% phenol red, 50% glycerol), loaded onto a resolving gel (0.38 M Tris pH 8.8, 30% (wt/vol) acrylamide [37.5:1 acrylamide: bis-acrylamide, wt/wt]) overlaid with a stacking gel (5% acrylamide, 0.64 M Tris pH 6.8 M) and separated at 17.5 mA for 60 minutes or until the phenol dye was within 1 cm of the end of the gel. The gel was stained 20 min with 0.2% alcian blue in 40% ethanol, washed thrice for 20 seconds and then 20 minutes in water. The gel was incubated with shaking in 0.2% silver nitrate containing 0.075% formaldehyde, rinsed thrice for 20 seconds with water, and then incubated in 4% sodium carbonate containing 0.05% formaldehyde until bands appeared. The carbonate solution was immediately removed and the gel was stored overnight in 5% acetic acid and then stored in water or dried.

Size exclusion chromatography

Five mg of fractionated pectin powder (FPP), pectasol (PeS), and citrus pectin (CP), were separated at 0.5 ml/min in 50 mM sodium acetate and 5 mM EDTA over a Superose 12 HR10 (10-300 mm) size exclusion chromatography (SEC) column using a Dionex DX500 system. The eluted pectin was detected using an uronic acid colorimetric assay (Blumenkranz and Asboe-Hansen, 1973).

Glycosyl residue composition analysis

Pectin samples were analyzed for glycosyl residue composition at the Complex Carbohydrate Service Center at the University of Georgia, Athens by combined gas chromatography/mass

spectrometry (GC/MS) of the per-O-trimethylsilyl (TMS) derivatives of the monosaccharide methyl glycosides produced from the sample by acid methanolysis basically as described in York *et al.* (1985). Methyl glycosides were prepared by methanolysis in 1M HCl in methanol at 80ºC for 18-22 hr, followed by re-*N*-acetylation with pyridine and acetic anhydride in methanol for detection of amino sugars. The samples were per-*O*-trimethylsilylated by treatment with Tri-Sil (Pierce) at 80ºC for 0.5 hr. GC/MS analysis of the TMS methyl glycosides was performed on an HP 5890 GC interfaced to a 5070 MSD using a Supelco DB1 fused silica capillary column.

Glycosyl residue linkage analysis

Pectin samples were analyzed for glycosyl residue linkage at the Complex Carbohydrate Service Center at the University of Georgia, Athens basically as described by York *et al.* (1985). Glycosyl residue linkage analyses were conducted using both single and double methylation procedures. For the "single methylation" linkage analysis, the samples were permethylated, depolymerized, reduced, acetylated and the resultant partially methylated alditol acetate residues analyzed by GC-MS. Specifically, the samples were permethylated by the method of Hakomori (1964) by treatment with dimethylsulfinyl anion and methyl iodide in DMSO, the permethylated material reduced by super-deuteride, hydrolyzed in 2M trifluoroacetic acid (TFA) for 2 hr at 121ºC, reduced with $NaBD_4$, and acetylated using acetic anhydride/TFA. The resulting partially methylated alditol acetates were separated on a 30 m Supelco 2330 bonded phase fused silica capillary column and analyzed on a Hewlett Packard 5890 GC interfaced to a 5970 mass detector in selective electron impact ionization mode. For the "double methylation" linkage analysis, the methods were as described above except that following the first methylation the permethylated material was reduced by super-deuteride and the reduced sample was re-methylated using the

NaOH/MeI method of Ciucanu and Kerek (1984). The remethylated samples were hydrolyzed using 2M TFA and processed as described above.

Western blot analysis

Proteins (50 μg, unless stated otherwise) were separated on NuPAGE 10% Bis-Tris gels (Novex pre-cast mini gels, Invitrogen, Carlsbad, CA) at 100 volts for 1 hour in the presence of 1x MES-SDS running buffer (InVitrogen, Carlsbad, CA). Separated proteins were transferred to (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA) at 42 volts for 2.5 hr using a Novex XCell II blotting apparatus in MES transfer buffer in the presence of NuPAGE antioxidant. Transfer of the proteins to the PVDF membrane was confirmed by staining with Ponceau S (Sigma). The blots were blocked in 5% non-fat dry milk in TBS, washed twice for 10 min each with TBS containing 0.01% Tween-20 and incubated for 2 hr at RT with primary antibody diluted in TBS containing 0.5% milk. The following antibodies were used in the immunoblots: rabbit polyclonal anti-caspase-3 antibody (BD Pharmingen, San Diego, CA), rabbit polyclonal anti-PARP antibody (Cell Signaling Technology, Beverly, MA) and anti-actin antibody (Sigma). Immunoreactive bands were visualized using the ECL detection system (Amersham, Pharmacia Biotech, Arlington Heights, IL) and signals were developed after exposure to X-ray film (X-Omat films, Eastman Kodak Company, Rochester, N.Y).

Acknowledgements

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CONCLUSIONS

Prostate cancer is the most common malignancy in American men (*Surveillance, Epidemiology, and End Results Program (SEER),* 1975-2000, Division of Cancer Control and Population Sciences, National Cancer Institute, 2003) and the second leading cause of death from cancer in American men (American Cancer Society, 2004). The goal of many cancer therapies is to induce apoptosis in tumor cells. Usually one of first steps in prostate cancer treatment is androgen depletion therapy (ADT). In hormonally responsive prostate cancer, androgen deprivation therapies induce cell death in androgen-sensitive cells (Colombel and Buttyan, 1995; Buttyan *et al..*, 1997; Perlman *et al..*, 1999; Bruckheimer *et al..*, 1999), while androgen-insensitive cells remain unaffected (Kozlowski and Grayhack, 1991; Santen, 1992; Kreis W, 1995). Androgen deprivation therapy in prostate cancer is rarely curative because the metastatic cancer is heterogenous, consisting of both androgen-dependent and androgen-independent prostate cancer cells. Although, ADT is ineffective against androgen-insensitive cells, the cells are still capable of undergoing apoptosis. Thus, the identification of novel methods to induce apoptosis in prostate cancer cells, irrespective of their androgen response, is of great importance.

Although the etiology of prostate cancer is poorly understood, according to the American Institute of Cancer Research, 30-40% of all cancer cases are preventable by dietary means. A product of plant cell walls and a dietary component of all fruits and vegetables, pectin, has been found to suppress colonic tumor incidence in rats (Heitman *et al.*, 1992) and inhibit cancer cell metastasis in mice and rats (Platt and Raz, 1992; Pienta *et al.*, 1995; Nangia-Makker *et al.* 2002).

It has been suggested that the inhibitory effects of pectin on metastatic lesions in the lung are mediated through their binding to galectins. Cancer cells, particularly metastatic cells, have specific carbohydrate-binding protein molecules on their cell surfaces, called galectins (or galactoside-binding lectins), galectin-3 being particularly well known. Galectins aid in cell-cell interactions by binding to galactose molecules on neighboring cancer cells. In human colon, stomach and thyroid cancers, the amount of galectin-3 increases as the cancer progresses. It has been proposed that pH-modified citrus pectin blocks binding of galectin-3 to its ligands and thus, inhibits tumor cell-cell interactions. The potential impact of blocking galectin action includes inhibition of the aggregation of cancer cells to each other and to normal cells, thus inhibiting metastatic lesions. However, LNCaP cells, used in our studies, do not express galectin-3, suggesting that the apoptotic effects of pectins observed in this thesis, are due to a mechanism not mediated through galectin-3.

The interpretation of studies with pectin are complicated by i) the structural complexity of this plant-derived cell wall polysaccharide, ii) the modifications in pectin structure that result from the commercial extraction of pectin from plants, and iii) the further modifications of pectin structure that result from the diverse fragmentation techniques used to produce specialized pectins (e.g. high pH (base) treatment; Platt and Raz, 1992; Pienta *et al.*, 1995; Eliaz, 2001; Nangia-Makker *et al.* 2002). Most of the published reports utilized pectins that are either modified by alterations in pH or by treatment with heat. The goal of the treatments is to fragment pectin structure to facilitate biological effects. Therefore, as a first step to determine whether size and method of preparation affected the biological activity of the pectins, we examined the effects of several commercial pectins, FPP, citrus pectin and PectaSol, on prostate cancer cells. The results showed that only FPP had high apoptosis-inducing activity in prostate cancer cells. CP

and PeS did not affect the cells. We hypothesized that, as pectins consist of homogalacturonan and rhamnogalacturonans, these polysaccharides in FPP may be responsible for its apoptotic effects. Experiments to determine the apoptotic effects of HGA, RGI, and RGII, indicated that the enzymatically separated and purified structural components of pectin were not responsible for the apoptosis-inducing activity of FPP. To further examine the structural differences among the tested pectins, the size and glycosyl residue composition of different pectins were compared. These analyses showed no correlation of these parameters among the pectins, even though FPP induced apoptosis while the other pectins did not. Therefore, we concluded that the apoptosis-inducing activity of FPP was probably related to specific fine structural features of FPP that are not present in PeS and CP.

To further understand the structure-function relationship of apoptotic pectin, FPP was specifically fragmented using endopolygalacturonase (EPGase), which cleaves at contiguous non-esterified GalA residues in HGA. Cleavage of FPP with EPGase did not have a major effect on its apoptotic activity. Thus, two methods were use to remove ester linkages from FPP prior to EPG treatment and thereby, make FPP more susceptible to EPG cleavage: chemical deesterification by mild base treatment and specific enzymatic hydrolysis of methyl esters by treatment with pectin methylesterase (PME). Chemical deesterification of FPP resulted in significant loss of apoptosis activity suggesting that a base sensitive structure, such as an ester, is necessary for the apoptotic activity of FPP. However, specific cleavage of methyl esters by pectin methylesterase did not destroy FPP's apoptosis inducing activity, suggesting that linkages other than carboxymethylesters are required for apoptotic function.

PAGE analysis of intact and treated FPP showed that a base-sensitive linkage in a polymeric/oligomeric FPP structure and/or a specific base-sensitive linkage itself is required for

the apoptotic activity of FPP. Taken together the results suggest that an ester-based (or related) cross-link in pectin is important for the apoptosis-inducing activity of FPP. In conclusion, we have demonstrated that among the pectins tested, FPP is able to induce apoptosis in both androgen-responsive and androgen-insensitive prostate cancer cells. Further experiments are being conducted to identify the specific apoptotic structure in FPP and to explore structure-function aspects of the anti-cancer activities of pectins.

The fractionation of fractionated pectin powder treated with pectinmethylesterase and endopolygalacturonase over anion exchange and size exclusion columns should allow us to identify the smallest active pectin that induces apoptosis. The availability of the smallest active structure would also allow studies aimed at identifying the molecular mechanism of action of the apoptotic pectin. One possible mechanism of action is that pectin binds a growth factor and inhibits binding to its receptor(s) on cancer cells. For example, fibroblast growth factors (FGFs), which normally mediate developmental processes in the embryo and homeostasis in the adult, are expressed at increased levels in prostate cancer cells (Kwabi-Addo, 2004). Furthermore, a protease-dependent degradation of the extracellular matrix causes increased mobilization of endogenous growth factors (Saksela and Rifkin, 1990; Whitelock *et al.*, 1996) in cancerous tissues, which increases the availability of these growth factors to cancer cells (Kwabi-Addo 2004). FGF signaling promotes tumor progression by enhancing multiple biological processes including the motility and invasiveness of cancer cells, tumor angiogenesis, resistance to cell death, metastasis, and androgen independence (Kwabi-Addo 2004). Heparin, a sulfated polysaccharide with structural similarity to pectin (Rillo *et al.*, 1992), and heparin sulfate proteoglycans play a critical role in FGFs binding to their receptors, though the manner by which this happens is not fully understood. Pectin is thought to competitively inhibit heparin binding in

the FGF/FGFR complex, and therefore prevent signal transduction, by ionically interacting with FGF-FGFR through carboxyl groups and/or hydrogen binding through hydroxyl groups (Liu *et al.*, 2001). Another heparin-dependent growth factor-growth factor receptor interaction that could potentially be inhibited by the competitive binding of pectin is that of VEGF (vascular endothelial growth factor) to its receptor (VEGFR). VEGF is an endothelial cell-specific growth factor and the principal regulator of angiogenesis under normal and pathological conditions (Yu *et al.*, 2002). Like FGF, VEGF is also overexpressed in a large majority of human cancers (Ferrara and Davis-Smith 1997). Competitive inhibition of heparin-mediated binding of VEGF to VEGFR by pectin could prevent tumor angiogenesis and therefore, tumor progression.

In this thesis we demonstrate that pectin, a plant polysaccharide fiber, induces apoptosis in both androgen-responsive and androgen-independent human prostate cancer cells. Furthermore, for the first time, we have identified specific structural features of pectin responsible for inducing apoptosis. These findings provide the foundation for future research targeted at identifying the mechanism by which specific pectic structures induce apoptosis in cancer cells and provide a basis for the rational development of pectin-based pharmaceuticals, neutraceuticals, or recommended diet changes aimed at reducing/inhibiting the occurrence and progression of cancer.

LITERATURE CITED[1]

([1]Literature cited in the Introduction, Specific Aims (Chapter 2) and Conclusions only. The references for Chapter 3 are found at the end of that chapter.)

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