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CHARACTERIZATION OF IMMORTALIZED AND MALIGNANT ADULT HUMAN PROSTATE EPITHELIAL CELL LINES: PWR-1E, RWPE-1 AND RWPE-2

By

Diana Bello

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ABSTRACT

CHARACTERIZATION OF IMMORTALIZED ADULT HUMAN PROSTATE EPITHELIAL CELL LINES: PWR-1E, RWPE-1 AND RWPE-2

By

Diana Bello

Prostate cancer is the most common cancer in adult men in the United States. With an estimated 244,000 new cases for 1995, prostate cancer has become a major health concern, but little is known about its etiology. Research has been slow due to the lack of suitable human cell models. To establish suitable cell models, non-neoplastic adult human prostatic epithelial cells were immortalized with an AD12/SV-40 hybrid virus (PWR-1E) or a HPV-18 virus (RWPE-1). RWPE-1 cells were subsequently transformed by Ki-*ras* to establish the RWPE-2 tumorigenic cell line. PWR-1E, RWPE-1 and RWPE-2 cell lines express cytokeratins 8 and 18, prostate specific antigen and androgen receptor as determined by immunoperoxidase staining. All three cell lines show growth stimulation in response to androgens, EGF and bFGF and growth inhibition in response to TGF-β, as determined by a microplate growth response assay. PWR-1E and RWPE-1 cells are not, but RWPE-2 cells are tumorigenic. These cell lines provide useful cell models for studies on prostate carcinogenesis and chemoprevention.

To my family and my mentor

Acknowledgments

I take this opportunity to recognize all the people who directly and indirectly contributed to the work in this thesis. I convey my gratitude to my mentor, Dr. Mukta M. Webber for her encouragement, advice, support and overall tenacity without which I would have not been able to complete my work. I extend to her my thanks for allowing and encouraging me to explore new avenues whenever possible. I thank my committee members, Dr. David D. Wartinger, Dr. Chia-cheng Chang and Dr. Emmanuel Hackle for their assistance, advice and support. I thank W. Scott Metcalfe and Melissa Carey for their assistance in familiarizing me with cell culture work and Anurada Waghray and Salmaan Quader for their invaluable assistance, support and friendship.

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Abbreviations

ABC	Avidin-Biotin Complex
AD12-SV40	Adenovirus-12/Simian Virus-40 Hybrid Virus
AR	Androgen Receptor
bFGF	basic Fibroblast Growth Factor
BPE	Bovine Pituitary Extract
BSA	Bovine serum albumin
cK-18	Cytokeratin 18
cK-8	Cytokeratin 8
DCS	Donor Calf Serum
D-PBS	Dulbecco's Phosphate Buffered Saline
EGF	Epidermal Growth Factor
FBS	Fetal Bovine Serum
F-VIII	Factor VIII (von Willebrand Factor)
IF	Immunofluorescence
KGM	Keratinocyte Growth Medium
K-SFM	Keratinocyte Serum Free Medium
MoAb	Monoclonal Antibody
PSA	Prostate Specific Antigen
PSF	Penicillin-Streptomycin-Fungizone
PWR	Prostate Worthington
RWPE	Rockville Worthington Prostate Epithelial
TGF-β	Transforming Growth Factor-β

Abbreviations

5 α-DHT	5-alpha-dihydrotestosterone
ABC	Avidin-Biotin Complex
AD12-SV40	Adenovirus-12/Simian Virus-40 Hybrid Virus
AR	Androgen Receptor
bFGF	basic Fibroblast Growth Factor
BPE	Bovine Pituitary Extract
BSA	Bovine serum albumin
cK-18	Cytokeratin 18
cK-8	Cytokeratin 8
DCS	Donor Calf Serum
D-PBS	Dulbecco's Phosphate Buffered Saline
EGF	Epidermal Growth Factor
FBS	Fetal Bovine Serum
F-VIII	Factor VIII (von Willebrand Factor)
IF	Intermediate Filaments
KGM	Keratinocyte Growth Medium
K-SFM	Keratinocyte-Serum Free Medium
MoAb	Monoclonal Antibody
PSA	Prostate Specific Antigen
PSF	Penicillin, Streptomycin and Fungizone
PWR	Prostate Webber Rhim
RWPE	Rhim Webber Prostate Epithelial
TGF-ß	Transforming Growth Factor beta

Introduction

Cancer is the second leading cause of death in the United States. With an estimated 244,000 new cases for 1995, adenocarcinoma of the prostate is the most common cancer diagnosed in adult men in the United States. It is estimated that over 40,000 men will die from prostate cancer in 1995 (1), making prostate cancer the second leading cause of cancer death in American men. Incidence of prostate cancer increases with age and about 80% of prostatic carcinomas are diagnosed in men over the age of 60 (2). The increase in incidence is partly due to improved methods for detection and a large aging male population. For these reasons, prostate cancer has become a major health concern in the United States. Autopsy studies in the United States show that approximately 11 million men 45-50 years old have latent carcinoma of the prostate. It is predicted that one in ten men will develop prostate cancer in his lifetime (2,3). Although precancerous lesions, referred to as prostatic intraepithelial neoplasia (PIN), appear in men in their 20's and 30's, 60% of prostate cancer cases are diagnosed in men in their 60's and 70's, when invasion and metastasis have already occurred (4). Presently, not much is known about the

causes of prostate cancer. Therefore, a great deal of importance and attention has been placed on the need for information regarding the mechanisms involved in the progression of precancerous latent lesions to clinical cancer as well as effective strategies for the treatment of advanced disease. Research in these areas, however, has been slow.

A major obstacle faced by researchers studying prostate cancer is the lack of suitable human cell models. Although tumors of rat prostate are available for study (5), the use of human prostate epithelial cells for the study of prostate cancer is the best model system aside from human experimentation which may not be feasible or ethical. Rodent model systems are derived from animals whose prostate is morphologically different from humans and results produced would have to be extrapolated to humans. This can be overcome by the use of human prostate cell culture models. It would be ideal to use epithelial cells isolated from fresh human prostate tissues, but unfortunately, these cultures can not be used for long term studies, because normal cells generally can not be maintained as replicative cultures beyond 4 to 5 passages over a period of a few weeks. Therefore, it would be extremely beneficial to develop human prostatic cell lines that demonstrate the characteristics of normal prostate epithelial cells but do not undergo senescence. This is accomplished by transfecting primary and secondary prostate epithelial cells with viral immortalizing genes. The immortalized cells can be further made tumorigenic by the introduction of a second oncogene. These immortalized as well as malignant cell lines would then provide uniform, standardized and reproducible models to study the multistep process of carcinogenesis and the genetic alterations in malignant transformation.

Three such cells lines have been developed in Dr. Mukta M. Webber's laboratory. Prostatic epithelial cells from two human donors have been immortalized with an adenovirus 12/simian 40 hybrid virus and the human papillomavirus-18, respectively. These cell lines are respectively termed PWR-1E and RWPE-1. Cells from the RWPE-1 immortalized cell line were further transformed by the addition of a Ki-*ras* oncogene to produce the tumorigenic RWPE-2 cell line. The primary objective of the work presented in this thesis is to characterize PWR-1E, RWPE-1 and RWPE-2 cell lines as human prostatic epithelial cell lines. Therefore, the following hypotheses are proposed:

- **PWR-1E and RWPE-1 cell lines exhibit charateristics of non-neoplastic epithelial cells.**

- **PWR-1, RWPE-1 and RWPE-2 cell lines retain and express differentiated functions of prostatic epithelial cells.**

- **Ki-*ras* transfected RWPE-2 cells show malignant characteristics.**

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Chapter One

Literature Review

In order to characterize any cell line, one must have a basic understanding of the organ of origin with regard to its anatomy, histology and embryology. Such an understanding lays the foundation necessary for the proposed investigations on the three cell lines relating to the expression of intermediate filament proteins, Factor VIII, androgen receptor and prostate specific antigen (PSA) expression and growth responses to androgens and growth factors that regulate the growth and development of the prostate. These subjects are reviewed in this chapter.

Part One:

Anatomy, Histology and Embryology of the Human Prostate

Anatomy and Histology of the Human Prostate

It is necessary to have a basic anatomical and histological, as well as, a functional understanding of the organ under investigation. This is especially true in the case of a small and usually overlooked organ such as the prostate, becuase different diseases that affect the prostate show association with a particaular anatomical region of the prostate.

Anatomy

The prostate is an exocrine accessory sex gland. It weighs about 20 grams and is roughly the size of a large walnut. The prostate lies caudal to the urinary bladder and cranial to the external urethral sphincter (figure 1) (1). It is composed of glands, muscle and fibrous tissue and is encapsuled by connective tissue (1,2). Using the urethra as the point of reference, the prostate is divided into the ventral fibromuscular portion and a dorsal glandular portion. It is also divided into proximal and distal segments by the 35 degree angulation the urethra takes as it runs through the prostate (2). The secretion of the prostate glands constitutes a major component of semen. The prostatic glands empty their secretion into prostatic ducts, that in turn, empty into the prostatic urethra and the secretion exits the body during ejaculation. This prostatic secretion functions as a vehicle for spermatozoa.

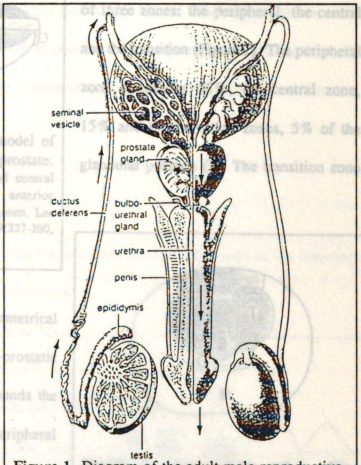

Figure 1. Diagram of the adult male reproductive system. Starr, C. and McMillian, B. Human Biology, New York, Wadworth Publishing Co. p. 297, 1995.

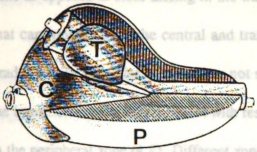

Figure 2. Three-dimensional model of the three glandular zones of the prostate. Peripheral (P), transitional (T) and central (C) zones of the prostate. The anterior fibromuscular stroma is shown in green. Lee et al. Ca-A Cancer J. Clinicians, 39:337-360, 1989.

The glandular prostate is composed of three zones: the peripheral, the central and the transition (Figure 2). The peripheral zone constitutes 75%, the central zone, 15% and the transition zones, 5% of the glandular prostate (2). The transition zone is formed by two bilaterally symmetrical lobules lateral to the pre-prostatic sphincter. The central zone surrounds the ejaculatory ducts.(figure 3) The peripheral zone, located posteriorly, runs from the apex to the base. (figure 2) (2,4) Although carcinoma has been found in all three zones, the majority (72%) of the carcinomas occurs in the peripheral zone. The transition zone only develops 20% of carcinomas but it is the primary location where benign prostatic hyperplasia arises. Only 8% of carcinomas arise in the central zone (2,4,5). There is some controversy as to whether or not there are distinct differences between carcinomas that arise in the peripheral

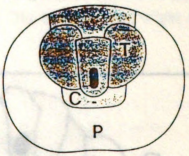

Figure 3. Cross-section of a three-dimensional model of the glandular prostatic zones. Cross-section at mid gland. Peripheral (P), Transitional (T) and central (C) zones of the prostate. Lee et al. Radiology. 170: 609-615, 1989.

zone as opposed to those arising in the transition and central zones. It has been suggested that carcinomas from the central and transition zones are less malignant and of a lower grade (4,6,7). This idea is, however, not supported by reports which show that tumors of the transition zone are no different with respect to metastatic potential from those that arise in the peripheral zone (4,8). Different zones of the prostate show histological differences, which are described below.

Histology

Adenocarcinoma of the prostate arises from the epithelial cells of the secretory glands or acini. The prostatic acini are known to contain two types of epithelial cells: basal and luminal (3). While, the basal cells are considered to be the stem cells from which luminal cells arise, luminal cells are terminally differentiated secretory cells (3) (figure 4). According to the stem cell theory, a third population of cells, termed amplifying cells, are thought to exist. These amplifying cells are considered to be cells in transition from the basal to the luminal type (3). Some evidence supporting the existence of the amplifying cell type has been demonstrated by work on cytokeratin expression. This will

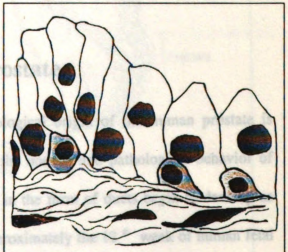

Figure 4. Pseudo-stratified prostatic epithelial cell layer. Basal cells (gray) and luminal cells (white) are illustrated attached to the extracellular matrix (ECM). Black cells in the ECM represent fibroblast.

be addressed in the cytokeratin section.

Although all prostatic acini contain both basal and luminal epithelial cells, distinct morphological differences are found between the three zones. The peripheral zone is composed of small, simple acini lined by columnar secretory epithelial cells, while the acini in the central zone are large, irregularly contoured and are lined by low columnar to cuboidal epithelium. The transition zone, which is histologically similar to the peripheral zone, differs in that its stroma is more dense and compact (2,4).

The functioning prostatic acinar and ductal epithelial cells of all three zones produce prostate specific antigen. The epithelial cells of the central zone differ in that they contain a gastric proenzyme called pepsinogen II (4). The embryological origin of the prostate will be discussed in the next section.

Embryology of the Human Prostate

A basic understanding of the embryological origin of the human prostate is necessary, because it gives one insights into the normal and pathological behavior of prostate cells in the adult man. Prostate cells in the form of ducts begin to develop as outgrowths from the urethral epithelium at approximately the 12 th week of human fetal development (2). These prostatic ducts grow out into the adjacent splanchnic mesenchyme both above and below the entrance of the mesonephric ducts (1, 2, 9) (Figure 5). The budding of prostatic ducts from the urethral epithelium is stimulated by testosterone from the embryonic testis, but the target of the androgenic influence is the adjacent mesenchyme not the urethral epithelium, thus making the mesenchyme the primary inductive influence.

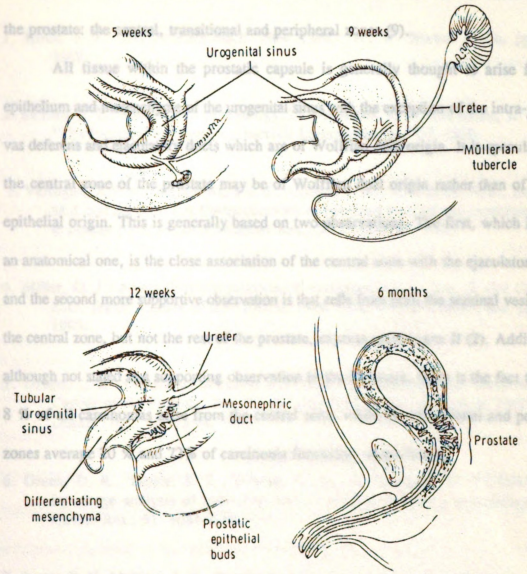

Figure 5. Differentiation of the urogenital sinus in the human male.
Smith, D.R. General urology. Toronto, Lange Medical Publications, p. 20, 1972.

The inductive ability of the mesenchyme ceases with adulthood (2). Prostatic ducts first form as tubes which then branch and rebranch to form the three distinct ductal zones of the prostate: the central, transitional and peripheral zones (9).

All tissue within the prostatic capsule is generally thought to arise from the epithelium and mesenchyme of the urogenital sinus with the exception of the intra-prostatic vas deferens and ejaculatory ducts which are of Wolffian duct origin. It is speculated that the central zone of the prostate may be of Wolffian duct origin rather than of urethral epithelial origin. This is generally based on two observations. The first, which is purely an anatomical one, is the close association of the central zone with the ejaculatory ducts, and the second more supportive observation is that cells from both the seminal vesicles and the central zone, but not the rest of the prostate, express pepsinogen II (2). Additionally, although not stated as a supporting observation in the literature, there is the fact that only 8 % of all carcinomas arise from the central zone, while the transitional and peripheral zones average 20 % and 72% of carcinoma formation, respectively.

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Part Two

Characterization

Characterization of the newly established cell lines is one of the most important steps that must be undertaken. Cell lines should be characterized to the point that no doubt concerning their origin could arise. This is because the validity of results obtained from the cell lines rests on it. For this reason PWR-1E, RWPE-1 and RWPE-2 cell lines were characterized on the basis of their expression of intermediate filament proteins, Factor VIII, androgen receptor and prostate specific antigen and growth response to growth factors which are known to regulate growth of prostate epithelial cells *in vivo*.

The expression of intermediate filament proteins and Factor VIII was investigated to establish epithelial origin. The expression of cytokeratin intermediate filaments would establish epithelial cell origin, while the absence of the intermeditate filament proteins, desmin and vimentin, and of Factor VIII, a hemostatic factor, would exclude origin from non-epithelial cells. Androgen receptor and prostate specific antigen expression would establish their luminal prostatic epithelial origin, while the appropriate growth response to androgens and growth factors would illustrate their ability to mimic the response of normal prostatic epithelial cells. Characterization by these criteria requires that one have a basic understanding of intermediate filament proteins, effects of androgens on the human prostate, the nature of prostate specific antigen and androgen receptor expression and of the growth factors such as epidermal growth factor, basic fibroblast growth factor and transforming growth factor-β.

Intermediate Filaments and Factor VIII

Intermediate filament proteins and Factor VIII were investigated to establish the epithelial origin of PWR-1E, RWPE-1 and RWPE-2 cell lines. Factor VIII is a hemostatic factor produced by endothelial cells. Intermediate filaments are a major cytoskeletal component and are organized as a network that surrounds the nucleus and spreads out into the cytoplasm terminating at the plasma membrane (1,2). They are involved in the formation and maintenance of cell shape, intracellular support and cell locomotion, as well as receptor mediated endocytosis and positioning of cellular organelles. There are five classes of intermediate filament proteins: cytokeratins (tonofilaments), desmin, vimentin, neurofilaments and glial filaments (1,2). Intermediate filament proteins, unlike the other two cytoskeletal components, microtubule and microfilament, are expressed in a cell type specific manner. Cytokeratins are a characteristic of epithelial cells, vimentin of cells of mesenchymal origin such as fibroblasts, and desmin of muscle cells. Neurofilaments and glial filaments, found in neurons and astroglia cells, respectively, will not be discussed (1,2).

Intermediate Filaments

Cytokeratins

Cytokeratins are cytoskeletal components of epithelial cells, therefore, to establish epithelial cells origin, expression of cytokeratins is necessary. Cytokeratins are the first intermediate filament protein to appear during embryonic development. The synthesis of the cytokeratin polypeptides occurs at about day four, in late morula, as the trophectoderm

begins to forms in the blastocyst. The blastocyst is composed of two layers. An outer layer of differentiated cells, the trophectoderm, and an inner mass of cells that are multipotential showing no expression of intermediate filament proteins. The appearance of cytokeratin is concurrent with the first formation of the desmosome-like junctions between neighboring cells. Desmosomes complex with bundles of cytokeratin filaments on the inner plasma membrane, while the outer membrane surface interacts with the cell surface of other cells to form regions of cell to cell contact that are involved in the adhesion of epithelial cells. The number of these desmosome-cytokeratin complexes increase as the blastocyst develops. The appearance of cytokeratins in the trophectoderm represents the initial step in epithelial cell differentiation (2).

Cytokeratins are a multigene family of about 20 biochemically and immunologically distinct yet closely related polypeptides. These polypeptides range in their molecular weight from 40,000 to 68,000 Da and in their isoelectric points from 5 to 8. They are usually expressed in pairs, which are composed of a basic and an acidic cytokeratin protein (1). These acidic and basic combinations are then expressed in a specific set that are characteristic of specific cell types. This specificity of acid and basic pair expression is seen in the human prostate. For example, cytokeratin 8 (basic) and cytokeratin 18 (acidic) are characteristic of prostatic luminal, secretory cells, while cytokeratin 5 and cytokeratin 14 or 15 are characteristic of prostatic basal cells (1-3). To date, prostatic basal cell are known to express cytokeratins 1, 5, 10, 14, 15 and 16 (4-6). Luminal cells are known to express cytokeratin 8, 18 and 19 (4,7,8). Some cells have been found to express cytokeratins 5, 8, 15, 16 and 18 which supports the existence of an amplifying cell population in the prostatic epithelium. This observation is consistent with the proposed role for amplifying cells as cells

in transition from basal to luminal cells. There are no differences in cytokeratin expression between epithelial cells in the central and peripheral zones (7,9).

The differences in cytokeratin expression between basal and luminal cells of the prostate has been an extremely useful tool in the diagnosis of prostate cancer. It is generally accepted that prostate cancer arises from the luminal cells of the prostate. This is supported *in vitro* and *in vivo* by the fact that prostatic carcinoma cells do not express cytokeratin 1, 5, 10, 14, 15 and 16, which are associated with basal cells, but do express cytokeratins 8 and 18, which are associated with luminal cells (3-7,9).

A decrease in cytokeratin 8 and 18 expression in some prostate carcinomas, as compared to normal and hyperplastic prostate, has been observed (10). This decrease in cytokeratin expression may be one of the consequence of the de-differentiation process which cell may undergo during transformation from a normal to a malignant phenotype (10). It is interesting to note that treatment of prostate and breast cancer cells with differentiation inducing agents, such as retinoids, can increase cytokeratin expression concomitantly with the re-acquisition of epithelial morphology (11,12).

In cancers, such as breast and prostate, the decrease in cytokeratin expression is sometimes associated with the expression of a second type of intermediate filament protein, vimentin (10, 13). Vimentin and its possible role in prostate cancer is the topic of discussion in the next section.

Vimentin

Vimentin is a normal cytoskeletal component of cells of mesenchymal origin such as fibroblasts (2). Vimentin is not normally expressed in epithelial cells. However, carcinomas arising from epithelial cells may co-express cytokeratins and vimentin. This co-expression may sometimes be seen when epithelial cells are placed in cell culture. So, the question is "Do immortalized epithelial cells express vimentin and, if so, why?" In order to answer this question, one must have a clear understanding of vimentin itself, why mesenchymal cell express it, when do other non-mesenchymal cell express vimentin and why do they express it?

In the course of embryogenesis, vimentin is first expressed shortly after implantation at about day nine with the formation of the mesoderm (2). Mesoderm formation is initiated when cells, mostly from the ectoderm and some from the endoderm layers, start to express vimentin in response to new yet unknown differentiation signals. Concurrently, within hours of the start of vimentin expression, these cells cease synthesis of cytokeratins and desmosome proteins (2). The ability of newly formed mesoderm cells to co-express cytokeratins and vimentin between days 9 and 14 is due to reduced cell:cell contact (2). With this switch from cytokeratin to vimentin, the newly formed mesoderm cells have acquired the additional characteristic of motility, a characteristic also exhibited by invasive carcinoma cells (2).

The association of vimentin with cellular differentiation is illustrated by the central nervous system of the newborn human infant and in an *in vitro* myoblast cell model which mimics the developing muscular system. Immature cells of the central nervous system in the newborn express vimentin but with postnatal development the amount of vimentin

expression decreases until it is not measurable in the mature spinal cord and brain of the adult (2). *In vitro* studies of myogenesis show that while desmin is expressed at very low levels in dividing myoblast type cells, vimentin expression is high. It is only in post- mitotic myoblasts when cells begin to fuse that desmin is synthesized at a higher rate than vimentin (2).

Vimentin, unlike cytokeratins, is encoded by a single chromosomal gene. Vimentin has a molecular weight of 53,500 Da. Although vimentin is characteristic of cells of mesenchymal origin such as fibroblasts, when more than one type of intermediate filament protein is expressed in the same cell, it is vimentin that is the other intermediate filament protein (2). I suggest that the reason why vimentin is always the other intermediate filament co-expressed by non-mesenchymal cancer cells may be because the expression of vimentin facilitates the process of cancer progression. These cells seem to need the co-expression of vimentin to be motile in order to invade and metastasize (13).

Normal differentiated epithelial cells are characterized by the expression only of cytokeratins and an absence of vimentin expression (14). There is some controversy as to whether or not prostate epithelial cells are part of a minority of epithelial cell types that co-express cytokeratins and vimentin. Nagle et al. (15) have reported co-expression of vimentin and cytokeratins in normal acinar luminal cells, but it is unclear from the literature whether their staining was focal or general. In sections of normal prostate, other investigators (16, 17), including myself, have found only focal staining of vimentin in prostate tissue sections. Ramaekers et al. (10) have found that an increase in positive staining for vimentin was consistently associated with a more advanced tumor stage. In the well characterized Dunning rat prostate cell lines, the protien pBUS51, which shows 96% homology to human vimentin,

is expressed at low levels in normal and benign prostatic epithelium and in well differentiated prostatic tumors but is highly expressed in hormone independent, highly metastatic tumors (18). They conclude that pBUS51, i.e. vimentin expression, correlates with tumor progression and more specifically with the degree of loss of differentiation (18). Additionally, Sommers et al. (19) observed vimentin expression in drug resistant, aggressive human breast carcinoma cell lines where vimentin expression in epithelial cells was associated with an aggressive, invasive malignant phenotype (19). This is in agreement with the observation that during transformation from a normal to malignant phenotype, cells undergo pronounced changes in their basic cytoskeletal components as part of a de-differentiation process frequently observed in advanced malignant disease (10). Desmin and its role in cell characterization is discussed in the next section.

Desmin

Desmin intermediate filament protein is expressed by muscle cells. Epithelial cells do not express desmin, therefore, an absence of desmin expression would verify that the our prostate cells were not of muscle origin. Desmin has a molecular weight of 53,000 Da (2). It was first isolated from chicken gizzard smooth muscle but its expression has been found in skeletal muscle, cardiac muscle, visceral smooth muscle and smooth muscle of many blood vessels. Desmin is absent in normal epithelial cells, myoepithelial cells or normal mesenchymal tissue other than muscle (2, 20). Its expression remains tissue specific during neoplastic transformation. Desmin expression is present in leiomyomas, rhabdomyomas, rhabdomyosarcomas and leiomyscarcomas, but absent in epithelial tumors (2, 20).

Factor VIII

Factor VIII, an important hemostatic factor, is synthesized and secreted by endothelial cells but epithelial cells do not (21). Factor VIII is a 220,000 Da plasma glycoprotein (22). Following vascular injury, Factor VIII functions to promote the adhesion of platelets to the blood vessel wall (21). Expression of Factor VIII is commonly used as a marker for endothelial cells (22). Therefore an absence of Factor VIII expression in our prostate cell lines would eliminate endothelial cell origin.

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Prostate Specific Markers

In order to fully characterize the PWR-1E, RWPE-1 and RWPE-2 cell lines, prostatic as well as epithelial origin must be established. Epithelial origin is established by analysis of the presence of cytokeratins and the absence of desmin and factor VIII. Prostatic epithelial origin is established by analysis of three prostate specific characteristics: 1) analysis of androgen receptor expression;2) induction of prostate specific antigen expression and 3) growth response to androgens, which demonstrates the ability of cells to mimic the response of normal prostatic epithelial cells.

Androgens and Androgen Receptor

Androgens are necessary for normal differentiation, development and maintenance of the male reproductive system including the accessory sex glands, the prostate and the seminal vesicles (1-3). Androgen effects occur in a cell and tissue specific manner (2). Androgen dependance of the prostate is demonstrated by the observation that after removal of the testes (castration) the prostate undergoes involution (4,5). However, adminstration of androgen to the castrated animal induces the proliferation of these androgen deprived, involuting prostatic cells (6). Androgen affects cell growth both directly and indirectly (6). Androgens stimulate the synthesis of receptors for growth factors, such as epidermal growth factor, thus, making the cells more sensitive to the growth factor (6). Metabolism of testosterone in the prostate results in the production of 5α-dihydrotestosterone (5α-DHT) and androstanediol. 5α-DHT is an active metabolite while androstanediol is an

inactive metabolite of testosterone (2,7). The action of 5α-DHT is mediated through a ligand activated androgen receptor (1,2). The androgen receptor has been detected in the luminal secretory cells but not in the in basal cells of the prostatic epithelium (8). The androgen receptor is present at lower levels in stroma of the prostate (8). Although it is generally accepted that the androgen receptor is a nuclear receptor (7, 9), it should be noted that some investigators state that there is both a nuclear receptor and a cytosolic receptor (10). Ekman et al. (11) found that metastatic prostatic epithelial cells had a higher cytosolic androgen receptor content than primary tumor cell which in turn had higher content than normal cells. The reverse was true as far as the nuclear androgen receptor is concerned (11).

The androgen receptor belongs to a large family of hormone-induced transcription factors called the nuclear receptor superfamily (2). The androgen receptor gene is located on the human X chromosome (12, 13). The androgen receptor is estimated to be 110,000 Da (14). The androgen receptor is extremely labile and susceptible to proteolytic degradation (6). Although androgen receptor expression is up-regulated in response to androgen, upon prolonged exposure, androgen receptor mRNA may be down regulated (15). Growth factors modulate androgen receptor mRNA expression, for example, transforming growth factor β up-regulates androgen receptor mRNA, while epidermal growth factor down regulates it (16).

The androgen receptor is thought to have at least three distinct functional regions: the transactivation domain, the DNA-binding region and the ligand-binding sequence (2). The transactivation domain contains the sequence information needed to specify gene recognition in the transcriptional regulation of responsive genes. It also contains information needed to optimize the transactivation capabilities of the receptor. The transactivation domain also

contains a long homopolymeric amino acid sequence containing CAG repeats. An enlargement of this CAG polyglutamine region in this domain seems to diminish the function of the androgen receptor and has been associated with Kennedy's disease which is characterized by the degeneration of motor neurons (2). Point mutations are rare in this region (2). The DNA-binding domain interacts with cis-acting elements of the regulated genes known as hormone response elements (HREs) which are 15 nucleotide long palindromic sequences usually located in the 5'flanking region of the gene. This domain consists of two cystine rich Zinc fingers. The first finger is responsible for the receptor's DNA recognition and the second finger is responsible for the dimerization of two receptor molecules during their association with DNA. If the second finger is absent then dimerization of two receptors does not occur and there is no elicitation of transcriptional activation. Mutations are common in this region of the androgen receptor (2). The ligand-binding sequence is involved in binding with the hormone. A complete deletion of this area causes the androgen receptor to be active all the time, even in the absence of the androgen. However a single amino acid substitution result in total or partial androgen insensitivity (2).

Although testosterone binds with the androgen receptor, its more active metabolite 5α-DHT has a 3-fold stronger affinity, forms a more stable complex, and may sometimes be necessary for an effect to occur (2,6). Testosterone diffuses freely into the cells and moves toward the nucleus either free or bound to a transport protein. At the nuclear envelope it is metabolized by 5 α-reductase enzyme into active 5α-DHT and inactive androstenedione. 5α-DHT is then translocated into the nucleus where it complexes with the androgen receptor (7). The receptor then undergoes a conformational change which allow the receptor to recognize and interact with specific regulatory DNA sequences, located in the flanking regions of target

genes. This interaction results in an up- or down-regulation of the expression of several genes or gene networks (6, 17). This occurs in a simultaneously or a temporally defined sequence to eventually result in the expression of an androgen induced phenotype. One such phenotype in the human prostate is the synthesis and secretion of prostate specific antigen (6).

Prostate Specific Antigen

Prostatic secretory epithelial cells synthesize and secrete prostatic specific antigen *in vivo* (4). Prostate specific antigen is a major component of the semen. Its function is to liquefy the semen after ejaculation (18,19). It is a serine protease with molecular weight of about 33,000 Da. It is composed of a single 240 amino acid glycoprotein chain. The prostate specific antigen gene is located on chromosome 19 (18,20).

The effects of androgen on prostate specific antigen production are clearly demonstrated by the prostatic cancer cell line LNCaP (21). In LNCaP cells, prostate specific antigen is constitutively secreted, even in the absence of an androgen stimulus, due to a mutation in its androgen receptor. Addition of androgens into the medium causes an increase of prostate specific antigen expression (21, 22). Additionally, growth factors, such as basic fibroblast growth factor and epidermal growth factor are known to slightly decrease the secretion of prostate specific antigen. Epidermal growth factor down regulates prostate specific antigen mRNA and interferes with androgen regulation of the gene (16). Clinically, serum prostate specific antigen levels, which are elevated in association with prostate cancer, are used for the diagnosis of prostate cancer as well as an early detection marker (18, 23, 24). Recently, prostate specific antigen has been shown to be involved in invasion and metastasis

of prostate cancer due to its ability to degrade components of the extracellular matrix (18).

As stated previously, it is the luminal secretory cells of the mature prostatic epithelium that synthesize and secrete prostate specific antigen in response to androgens (4, 18). Since the expression of prostate specific antigen is a differentiated function of normal prostate epithelial cells, it is used as a marker for prostatic differentiation. Therefore, cells that express prostate specific antigen in response to androgen treatment can be said to be of prostatic epithelial origin.

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Peptide Growth Factors

During the process of immortalization and development of PWR-1E and RWPE-1 cells and subsequent transfection of RWPE-1 by Ki-*ras* to derive RWPE-2 cells, response to growth factors may be affected. Therefore, it would be beneficial to characterize the new cell lines with regard to their response to growth factors to determine if their responses mimic those of normal prostatic epithelial cells or they differ. I examined response to epidermal growth factor, basic fibroblast growth factor and transforming growth factor-β because they are the three major growth factors involved in the regulation of the normal prostatic epithelium (1).

Growth factors function as peptide signaling molecules that enable cells to maintain local homeostasis and adjust to their biological environment. In this manner, cells are able to control cellular functions such as cellular differentiation, promotion or inhibition of cellular proliferation and many others functions, such as up or down regulation of prostate specific antigen and androgen receptor (2). Growth factors may be secreted by cells which may affect their own behavior (autocrine) or that of a neighboring target cells (paracrine). Growth factors exert their biological function by binding to cellular receptors which initiate a cascade of reactions that reflects the particular functions of the growth factor in question (3). During malignant transformation, initiated cells may accomplish selective growth by autocrine over-expression of growth factors (4,5). This has been observed in numerous types of cancers such as gastric, esophageal, breast and prostate cancers (5).

Epidermal growth factor, basic fibroblast growth factor and transforming growth factor-β are the three major growth factors involved in the regulation of the normal prostatic

epithelium (1). Changes in the levels of all three growth factors have been observed in malignant prostate cells (4,5). Prostatic epithelial cells are normally under androgen regulation. The loss of hormone sensitivity is thought to be due to the ability of malignant cells to produce growth factors in an autocrine manner (6). One characteristic of progression from a normal to a malignant phenotype is reflected in the increased rate of cell proliferation caused by the autocrine secretion of growth factors (5, 7). The subsequent step to a metastatic phenotype maybe be facilitated, for example, by the ability of epidermal growth factor to increase the production of proteases such as urokinase-type plasminogen activator (8).

Epidermal Growth Factor (EGF)

Effects of epidermal growth factor were examined to determine if the immortalized prostatic epithelial cell lines mimic the proliferative response observed in normal prostatic epithelial cells. In the course of purifying nerve growth factor from mouse submaxillary glands during the 1960's, Chen and Levi-Montalcini observed that an isolated fraction, not containing nerve growth factor, had distinct biological activity when injected into newborn mice (9). This factor was subsequently isolated and termed tooth-lid factor because it caused the eruption of incisors and precocious opening of the eyes. When it was found that the opening of the eyes was due to an increase in epidermal thickening and keratinization, the factor was renamed epidermal growth factor (9).

The epidermal growth factor gene is located on human chromosome 4. Epidermal growth factor mRNA levels are age dependant. They are activated at birth and steadily increase throughout life. Epidermal growth factor is a mitogen which exerts its biological

activity by way of the epidermal growth factor receptor. The epidermal growth factor receptor was originally isolated from A-431, an epidermoid human carcinoma cell lines. It is an integral membrane glycoprotein of 170,000 Da. Its gene is located on human chromosome 7 (9). The intracellular domain of the epidermal growth factor receptor has tyrosine kinase activity (8). Upon binding of EGF to its receptor, which is located in the plasma membrane, the epidermal growth factor receptor autophosphorylates and clusters, leading to dimerization of the receptor. This in turn activates second messenger pathways such as, diacylglycerol (DAG) and inositol 1, 4, 5-triphosphate (IP_3), and modulates the activity of several intracellular kinases by tyrosine phosphorylation. Thus, epidermal growth factor modulates the expression of a number of early response genes such as c-*fos* and c-*jun*. In prostatic epithelial cells, epidermal growth factor modulates prostate specific antigen and androgen receptor genes (8,9). The epidermal growth factor-receptor bound complex is, itself, endocytosed and sorted through various intracellular compartments to eventually reach the lysosome, where the receptor either is degraded or recycled (9).

The autocrine over expression of epidermal growth factor has been implicated in neoplastic transformation (5). In primary cultures of normal prostate cells and in the prostate cancer cell line LNCaP, exogenous epidermal growth factor causes an increase in cell proliferation (11, 12). In other prostate cancer cell lines, such as PC-3 and DU-145, exogenous epidermal growth factor causes little to no effect on cell proliferation. The lack of growth response to epidermal growth factor in DU-145 may be because they themselves produce epidermal growth factor. Although little growth stimulation is seen in PC-3 cells, exogenous epidermal growth factor causes an increase in their *in vitro* invasive ability (5). This apparently occurs as a result of an increase in urokinase-type plasminogen activator, a

serine protease involved in the regulation of pericellular proteolysis and extracellular matrix degradation (5). Androgens have been implicated in the regulation of EGF in the prostate (9). The next section will address basic fibroblast growth factor which is another inducer of prostatic epithelial cell proliferation.

Basic Fibroblast Growth Factor (bFGF)

Effects of basic fibroblast growth factor, which is involved in growth regulation of normal prostatic epithelium (1), were investigated to determine if the immortalized prostatic epithelial cell lines mimic the proliferative response observed in normal prostatic epithelial cells. Although the existence of substances from the brain and pituitary extracts that promoted the growth of cultured fibroblast has been known since the 1940's, the term fibroblast growth factor was not coined until its re-discovery during the 1970's (13, 14). Since then, seven member of the fibroblast growth factor family have been identified (acidic FGF, basic FGF, INT-2, HST/K- FGF, FGF-5,FGF-6 and FGF-7) of which basic fibroblast growth factor and acidic fibroblast growth factor are the best characterized (14, 15). Growth factors in the fibroblast growth factor family are embryonic inducers of cellular proliferation and differentiation, they have a short effective range and are non-humoral. Very low levels of fibroblast growth factor are required for biological activity (13). Members of the fibroblast growth factor family show potent mitogenic activity as well as non-mitogenic activities such as induction of motility, differentiation, protease production and angiogenesis (15,16). They induce a mitogenic response in prostatic epithelial cells and in myoblasts and show non-mitogenic activity in central nervous system neurons, cells of the adrenal medulla and in

various types of tumor cells (14). Interestingly, members of the fibroblast growth factor family have also been reported to cause both mitogenic and non-mitogenic responses in fibroblast and endothelial cells. Basic fibroblast growth factor is found in many different tissues including the prostate, while acidic fibroblast growth factor is found primarily in the central nervous system (17).

Basic fibroblast growth factor is a single chain protein composed of 154 amino acid with a molecular weight of 16, 500 Da. The basic fibroblast growth factor gene is located on human chromosome 5 (14). Basic fibroblast growth factor exerts its biological activity through a specific cell membrane receptor, a fibroblast growth factor-receptor. The fibroblast growth factor-receptors are single chain polypeptides with a molecular weight ranging from 110, 000 to 150, 000 Da depending on the cell type. The molecular weight differences may explain why, in some cases, cells will only bind either acidic fibroblast growth factor or basic fibroblast growth factor. Generally, fibroblast growth factor-receptors have a higher affinity for basic fibroblast growth factor than acidic fibroblast growth factor, resulting in basic fibroblast growth factor being 10 to 100 times more potent than acidic fibroblast growth factor. Although a substantial amount of information is available, investigators have not been able to produce a general signal transduction pathway for the members of the fibroblast growth factor family, because different cell types seem to use different signaling pathways. Like epidermal growth factor, the members of the fibroblast growth factor family have been found to induce the expression of a number of early response genes such as *fos* and *myc* (14). Additionally, basic fibroblast growth factor as well as epidermal growth factor, have been shown to slightly decrease prostate specific antigen secretion (7). Very little basic fibroblast growth factor is secreted into the conditioned medium of cultured cells. This is due to the

absence of a typical amino-terminal signal peptide in basic fibroblast growth factor needed to mediate classic protein secretion (14). It seem that most of the basic fibroblast growth factor secreted by cells is accumulated in the extracellular matrix they produce. In accordance with this observation, basic fibroblast growth factor has been detected in the basement membrane of various tissues, where it is thought to bind to the heparin sulfate proteoglycans (14,15, 18). Members of the fibroblast growth factor family show strong binding to heparin (14).

The growth of prostatic epithelial cells is stimulated by basic fibroblast growth factor (1, 14, 15, 18). The established prostate cancer cell lines LNCaP, which is androgen responsive, and DU145 cell line which is androgen non-responsive, also show growth stimulation in response to exogenous basic fibroblast growth factor (3). In another prostate cancer cell line, PC-3 cells, no effect on cell growth is seen. The lack of growth response in PC-3, in spite of the presence of fibroblast growth factor-receptor, is thought to be due to some alteration in the post-receptor signal transduction pathway (3). This idea is consistent with the observation that c-*myc* mRNA is not elevated in response to exogenous basic fibroblast growth factor in PC-3 cells, as it is in DU145 treated cells (3). Next I will disscuss a growth factor that inhibits prostatic epithelial cell growth, the transforming growth factor-β.

Transforming Growth Factor-β (TGF-β)

Effects of transforming growth factor-β, one of the three major growth factors involved in the growth regulation of normal prostatic epithelium (1), were examined to ascertain if the immortalized prostatic epithelial cell lines mimic the inhibitory response observed in normal prostatic epithelial cells. Transforming growth factor-β was first purified from human platelets and placenta and bovine kidney during the early 1980's (13). The members of the transforming growth factor-β family are negative regulators of growth in many cell types, especially epithelial cells (19, 20). They are also known to regulate differentiation, chemotaxis and metabolism of many different types of cells. To date, three different mammalian forms of TGF-β have been identified, transforming growth factor-β 1, 2 and 3. All three forms share similar biological functions but the effects of TGF-β_1 are 100 fold more potent than TGF-β_2 on certain types of cells (19,20). According to Sporns and Roberts, "Transforming growth factor-β is the prototypical multifunctional growth factor" (19). Its biological effect on a given target cell is dependant on the cell type, the growth conditions and the presence of other growth factors. Although a bifunctional response to TGF-β by both fibroblast and epithelial cells has been reported, TGF-β generally stimulates the growth of fibroblasts but inhibits the growth of epithelial and endothelial cells (19-21). In addition to inhibiting epithelial cell growth,TGF-β stimulates the production of extracellular matrix components and protease inhibitors and causes a decreases in the net activity of extracellular matrix degrading proteolytic enzymes (19, 20).

The genes for transforming growth factor-β 1, 2 and 3 are located on human

chromosome 19, 1 and 14, respectively (19). It is believed that all of the members of the TGF-β family are derived from duplication of a common ancestral gene (22). Like most growth factors,TGF-β exerts its biological activity through a specific cell membrane receptor, a transforming growth factor β-receptor. Thus far, three classes of binding sites, that coexist on cells, have been recognized (22). The most common is type I receptor which has a molecular weight that ranges from 60,000 to 70,000 Da and has a high affinity for TGF-β_1 but low affinity for TGF-β_2 (20). Type II receptors are less common and have a molecular weight ranging from 85,000 to 110,000 Da (22). Both type I and II receptors are glycoproteins and binding of growth factor to both sites correlates with biological activity. Type III binding sites are membrane bound proteoglycans of 100,000 - 140,000 Da. They are believed to bind to the extracellular matrix and thus mediate organization of the cellular cytoskeleton. All forms of TGF-β bind to type III receptor with equal affinity (19, 22). In addition to the already cited activities, TGF-β causes an increase in androgen receptor expression (8).

The growth of prostatic epithelial cells is regulated by TGF-β (1). Growth inhibition by TGF-β *in vitro* has been seen in normal and malignant prostatic cells (8, 20). Likewise, established prostate cancer cell lines DU-145 and PC-3, both of which are considered to be androgen non-responsive, show growth inhibition in response to exogenous TGF-β (23). On the other hand, the LNCaP cell line, which is androgen responsive, dose not show a direct growth inhibition but may be considered responsive, because TGF-β is able to counter the proliferative effects of EGF in these cells (8).

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Chapter Two

Characterization of the Immortalized Adult Human Prostate Epithelial Cell Line: PWR-1E

Data presented in this chapter have been submitted for publication:

Webber, M.M., Bello, D., Kleinman, H.K., Wartinger, D.D., Williams, D.E., and Rhim, J.S.: Prostate specific antigen and androgen receptor induction and characterization of an immortalized adult human prostatic epithelial cell line. Carcinogenesis (submitted), December 1995.

Abstract

Prostate cancer is the most common type of cancer diagnosed and the second leading cause of cancer death in adult men in the United States. Research in prostate cancer has been slow due to the lack of suitable human cell models. The use of human prostate epithelial cells *in vitro* for the study of prostate cancer is the best model system aside from human experimentation. In order to establish suitable human cells models, normal adult human prostatic epithelial cells from a white male donor were immortalized with an AD12-SV40 hybrid virus and resulted in the selection and establishment of PWR-1E cell line. The PWR-1E cell line was characterized on the basis of intermediate filament proteins, Factors VIII, prostate specific antigen and androgen receptor expression by immunoperoxidase staining, and growth response to androgen and growth factors. The cells show growth stimulation and prostate specific antigen and androgen receptor expression in response to androgen treatment. PWR-1E cells shows strong expression of cytokeratins 8 and 18 and weak expression of basal cell cytokeratins. They do not express desmin or FactorVIII. These characteristics undoubtably establish their prostatic epithelial origin. PWR-1E cell line shows a dose dependant growth stimulation by epidermal growth factor and basic fibroblast growth factor and growth inhibition when exposed to transforming growth factor-β. The PWR-1E cell line provides a useful cell culture model for studies on prostate carcinogenesis and chemoprevention.

Background

Simian Virus 40 Virus and Adenovirus 12

The simian virus 40 has been used to immortalize human keratinocytes (1), thyroid follicular cells (2), mammary (3) and human neonatal prostate epithelial cells (4), and bone marrow stromal cells (5). In most cases the epithelial cells retain many of their differentiated functions (2,4). It is believed that simian virus-40 confers immortality through the expression of SV-40 large T antigen. SV-40 large T antigen binds and inactivates the retinoblastoma gene product, the Rb protein, thereby preventing Rb protein from performing its normal cell regulatory activities (6). Additionally, SV-40 large T antigen complexes with and sequesters p53 tumor suppressor protein (6). When cells are infected with the SV-40 virus the following may occur. If the cells are permissive, they will allow the virus to multiply, however, if the cells are non-permissive for virus replication, viral integration into the host genome may occur. The permissive cells die, while the non-permissive cells are conferred an extended life span or immortality (7). A fraction of the SV-40 infected cells which have acquired an extended life span, undergo a crisis stage during which a majority of the cells die (6, 8). This crisis stage may last for many months. Those cells that do not die off during the crisis period will become immortal. The surviving immortalized cells generally do not produce the infectious SV-40 virus, but express high levels of large-T antigen. Large-T antigen expression is necessary for extended life span and immortality (6). To date only two human adult prostate cell lines have been successfully immortalized by SV-40 infection (9, 10).

In an attempt to overcome the crisis stage experienced by infection with SV-40 alone, the adenovirus 12/ simian virus 40 (AD12/SV-40) hybrid virus was used in our

studies. This AD12/SV40 virus is composed of recombinant DNA containing all or part of the SV-40 genome and part or all of the adenovirus genome enclosed in the adenovirus capsid. The AD12/SV40 hybrid virus immortalizes the cells without the culture going through a crisis phase. This is illustrated by the establishment of our PWR-1E adult human prostate cell line.

Isolation of Primary Prostatic Epithelial Cells and Their Immortalization with AD12/SV40 Hybrid Virus

The PWR-1 cell line was derived from a tissue specimen of "normal" prostate from a 67 year old white male patient undergoing radical cystoprostatectomy for transitional cell carcinoma of the bladder. Pathologic evaluation of the surgical specimen did not reveal any prostatic malignancy; but, mild nodular hyperplasia was seen. No neoplasms were detected. Epithelial cells were isolated by Dr. Mukta M. Webber following a collagenase technique she developed to isolate prostatic epithelial cells (11). The tissue was cut into 2-3 mm cubes and placed in one 100 mm Petri dish with 30 ml RPMI-1640 containing 5% FBS and 400 Units/ml of collagenase. After 48 h digestion in collagenase, the tissue was triturated until all lumps were broken up, and centrifuged for 5 min to remove collagenase. The pellet was suspended in saline G and allowed to settle in the refrigerator for 1 h. The supernatant was removed and the sediment was suspended again in saline G and allowed to settle at 4°C. This was repeated 4 times. The sediment after the last wash containing prostatic epithelial acini was suspended in storage medium (11) containing spermine (2 μg/ml) and left at 4°C overnight to destroy any fibroblasts that may be present in the preparation (11). The acini were centrifuged, suspended in growth medium and plated in culture flasks/plates coated with a mixture of 10 μg/ml each of fibronectin and type IV collagen. Primary cultures of prostatic epithelium were established.

Primary and secondary cultures were infected by an AD12/SV40 preparation (1:10 dilution). After 10 days the infected cultures were subcultured and plated in coated plates and the medium was changed every 48 h. One month after infection, colonies began to appear. One isolated clone did not show any cytopathic effects and its cells had an epithelial morphology. This clone was further subcloned by dilution plating of single cells in 96-well plates. PWR-1E cell line was derived from one of these clones. PWR-1E cells have undergone over 250 cell generations (population doubling).

Characterization

The PWR-1E cell line was characterized on the basis of the expression of intermediate filament proteins, Factor VIII, androgen receptor and prostate specific antigen and growth response to an androgen and growth factors (epidermal growth factor, basic fibroblast growth factor and transforming growth factor-β).

Intermediate filament proteins (cytokeratins, vimentin and desmin) and Factor VIII were investigated using monoclonal antibodies to cytokeratins, vimentin, desmin and Factor VIII, by an immnunoperoxidase staining method. This was done to establish their epithelial origin by the presence of cytokeratins but the absence of desmin, vimentin and Factor VIII. Androgen receptor and prostate specific antigen expression was also demonstrated by immunoperoxidase staining after the cells were treated with mibolerone, a synthetic, non-metabolizable androgen, to induce their synthesis. This was done to establish luminal prostatic epithelial origin of PWR-1E cells. The expression of large-T antigen was examined using monoclonal antibodies by immunoperoxidase to verify SV-40 associated protein expression. Growth response to androgens and epidermal growth factor, basic fibroblast growth factor and transforming growth factor-β was determined using a

microplate assay to illustrate their ability to mimic the response of normal prostatic epithelial cells to these agents. Additionally, the invasive ability was examined to establish the absence or presence of malignant characteristics.

Materials

The materials used were: keratinocyte growth medium (KGM) serum-free medium, CC-3001 from Clonetics; keratinocyte-serum free media (K-SFM), 17005-042 and antibiotic/antimycotic mixture (PSF), 600-5240AG from Gibco-BRL; charcoal treated fetal bovine serum (FBS) 0.2μ filtered, 52-0115 from Cocalico Biologicals, Inc.; Dulbecco's phosphate buffered saline without Ca^{++}/Mg^{++}(D-PBS), 28374 from Pierce; mibolerone, W-300 from BIOMOL Biomolecular Research Laboratories; epidermal growth factors (EGF), 40001 and transforming growth factor beta (TGFβ), 40039 from Collaborative Research; recombinant basic fibroblast growth factor (rbFGF), 234-FS from R & D Systems; monoclonal antibody to prostate specific antigen (PSA), M-0750 from DAKO; polyclonal antibody to androgen receptor (AR), PA1-110 from Affinity BioReagents; monoclonal antibody to cytokeratin 8, C-5301, monoclonal antibody to cytokeratin 18, C-8541, monoclonal antibody to desmin, D-1033, monoclonal antibody to vimentin, V-6630, monoclonal antibody to Factor-VIII, F-3520, 5-a-dihydrotestosterone (5α-DHT), D-5027 and bovine serum albumin, A-2153 from Sigma; monoclonal antibody to cytokeratin 1, 5, 10 and 14 (Keratin-903), C34903 from Enzo Diagnostics; monoclonal antibody to SV-40 Large T antigen, OP-09 from Oncogene Science; Vectastain Elite ABC Peroxidase Kit, PK-6102 and 3,3-diamino-benzidine (DAB) Substrate Kit, SK-4100 from Vector Laboratories; Hema 3 Stain Set, 67-56-1 from Curtin-Matheson Scientific; 12 mm circle microscope cover glass (coverslips), 12-545-80 from Fisher Chemicals; Nuclepore

membrane, 8 μ pore size, 1550446 and 96 well plates, 3596 from Costar; 24 well plates, 3047 from Falcon, Beckon-Dickinson; Matrigel, gift from Dr. H.K. Kleinman, National Institues of Health.

Methods

Cell Culture

PWR-1E cells were maintained in the complete K-SFM medium which contains 50 mg/ml of bovine pituitary extract (BPE) and 5 ng/ml EGF, and to this is added 1% PSF (penicillin G sodium, 10,000 units/ml, streptomyosin sulfate 10,000 mg/ml and amphotericin B as Fungizone, 25 mg/ml in 0.85% saline). Cells were passaged upon confluence. For passaging, cell were washed with D-PBS, then incubated with 3 ml of trypsin/EDTA mixture (0.05% trypsin, 0.02 mM EDTA) diluted 1:1 with D-PBS for 8-10 min. Cells were dislodged by tapping, neutralized by the addition an equal volume of 2% donors calf serum (DCS) in D-PBS and recovered by centrifugation (2000 rpm) for 5 min. Cell counts were performed with a Coulter Counter and cell were seeded at 2 to 5 x 10^6 cells/ T-75 flask. All experiments were conducted on PWR-1E cells between passage 36 and 46.

Cell Proliferation Studies

Analysis of Growth Characteristics and Cell Doubling Time by Growth Curve Assay. In order to examine the growth characteristic and the doubling time of PWR-1E cells, growth curves was generated. A microplate assay using 96-well plates was used. This assay is based on the direct relationship between absorbance and cell number (12).

A wide range of cell densities were used. Cells were plated at low (625, 1,250, 2,500 and 5,000 cells) and high (10, 000, 20,000, and 40,000 cells) densities in complete K-SFM medium. Three replicate wells were plated per cell density. A total of 8 plates were prepared. Medium was changed every 48 h. Plates were fixed and stained at days 1, 2, 4, 6, 8, 10, and 14 after plating. Plates were washed twice with normal saline to remove the dead cells and media, fixed in 95% ethanol for 1 h and stained with 0.5 % methylene blue, which is a protein binding dye, for 45 min. During this time the dye is taken up by the fixed cells. The cells were then incubated at 37^{0}C for 24 h in 1% SDS to extract the dye. Absorbance was read at 620 nM using a Titertek microplate reader (13). Becuase of the direct relationship between cell number and absorbance (12), the absorbance data were plotted against time, analyzed and cell doubling times were determined for low and high cell densities.

Androgen Dose Response Assay. Since androgens regulate prostatic epithelial growth *in vivo* and *in vitro,* the effects of mibolerone on cell growth were investigated using the dose response assays as described by Waghray and Webber (13). Mibolerone, a synthetic non-metabolizable androgen, was used instead of the naturally occurring androgen, 5-α dihydrotestosterone, to insure the continued presences of the androgen in the media. 5-α DHT has a half life of only about 2 hours (14). Mibolerone was dissolved in ethanol, protected from light and stored at - 20 ^{0}C. The final concentrations of ethanol in the medium was 0.1% and it was not inhibitory to the growth of the cells. Concentrations of mibolerone tested were 0.01, 0.05, 0.1, 0.5, 1, 5, and 10 nM.

PWR-1E cells were plated at a density of 10,000 cells per well in a 96 well plate in complete K-SFM medium. Forty-eight hours after plating, medium was changed to K-SFM (no EGF or BPE added) containing 0.1% bovine serum albumin and supplemented

with mibolerone. Plates were fixed at 5 and 7 days after treatment and prepared for absorbance reading as described previously.

Growth Factor Dose Response Assay. Since epidermal growth factor, basic fibroblast growth factor and transforming growth factor-b are the three major growth factors involved in the regulation of the normal prostatic epithelium (14), their effects on cell growth were investigated using the same dose response assay described above. Epidermal growth factor was dissolved in sterile distilled water, basic fibroblast growth factor in 0.1% BSA in D-PBS and transforming growth factor-ß in 4 mM HCl in D-PBS containing 1mg/ml of bovine serum albumin. All growth factor stocks were stored at -20°C. The final concentrations of the vehicle in the medium was 0.1% for EGF and bFGF and 1% for TGF-ß. The vehicle was not inhibitory to the growth of cells. Concentrations of all growth factors tested were 0.625, 1.25, 2.5, 5, and 10 ng/ml.

PWR-1E cells were plated at a density of 5,000 cells per well in a 96 well plate in complete K-SFM medium. Forty-eight hours after plating, medium was changed to K-SFM medium supplemented with BPE (no EGF) and the test growth factor. Plates were fixed at 5 and 7 days after treatment and prepared for absorbance reading as described previously.

Analysis of Intermediate Filament Proteins, Factor VIII and Large-T Antigen by Immunocytochemistry

Intermediate filament proteins cytokeratins, vimentin and desmin, large-T antigen and Factor VIII were investigated by monoclonal antibodies to cytokeratins, vimentin, desmin, Factor VIII and large-T antigen, using an indirect immuno-peroxidase method, to establish the epithelial cell origin of PWR-1E cells. PWR-1E cells were grown on sterile,

alcohol washed coverslips in 24 well plates at a density of 20,000 cells in complete K-SFM medium/well. Coverslips were soaked in 95% ethanol overnight, individually wiped with low lint paper wipes and autoclaved for 30 min. Medium was changed every 48 h. Upon 70% confluence, 5 to 7 days after plating, cells were rinsed twice with D-PBS and fixed in a 1:1 solution of methanol and acetone for 2 min with gentle agitation. The coverslips were then either processed the same day or stored in a -80^{0}C freezer. Immunocytochemistry was performed using an indirect immuno-peroxidase method following a modified Vector protocol as follows: Cells were blocked in normal horse serum for 1 h at room temperature and incubated with the appropriate monoclonal antibody (Ab) diluted in normal horse serum. Table 1 shows specific antibody dilution, incubation time and temperature and source of each antibody used. Cells were subsequently processed using the Vectorstain Elite ABC Peroxidase kit. Cells were treated with 200 μl/coverslip of the biotinylated anti-mouse or anti-rabbit IgG secondary Ab, as appropriate (see Table 1), for 30 min. Prior to Avidin-biotin-peroxidase complex (ABC) application, cells were treated with 200 µl/coverslip of a 3% H_2O_2 solution for 3 min to quench all endogenous peroxidase activity. Cells were then incubated in 200 µl/coverslip of the ABC solution for 30 min and developed with diaminobenzidine-nickel chloride substrate kit for peroxidase for 5-7 min, (200 µl/coverslip). Cells were washed twice in 500 µl D-PBS over 10 min between all successive steps after primary Ab application. Cells were then dehydrated in two washes of 95% ethanol and two of 100% ethanol, for 3 min each, through xylol (a 1:1 solution of xylene and 100% ethanol), for 5 min and twice through xylene, for 3 and 5 min, respectively. Coverslips were mounted on alcohol washed slides using Permount. All of the preceding steps were carried out at room

Table 1. Antibody source and conditions for immunoperoxidase staining

Antibody	Immunogen	Dilution	Source
Anti-Cytokeratin 8 [1, 2, 3]	Cytokeratin 8	1:200	Sigma
Anti-Cytokeratin 18 [1, 2, 3]	Cytokeratin 18	1:800	Sigma
Keratin-903[1, 2, 3]	Cytokeratin 1, 5, 10 and 14	1:50	Enzo Diagnostics
Anti-Desmin[1, 2, 3]	Desmin	1:50	Sigma
Anti-Vimentin[1, 2, 3]	Vimentin	1:40	Sigma
Anti-Factor VIII[1, 2, 4]	F-VIII	1:1000	Sigma
Anti-SV40 Large-T Antigen[1, 2, 3]	SV40 Large- T Antigen	1:500	Oncogene Science

[1] Incubation time of 1 hour at room temperature.

[2] Monoclonal

[3] Mouse

[4] Rabbit

temperature unless otherwise indicated. Using a high power light microscope, cells were determined to be either positive or negative for a given antigen as compared to controls (see section on Controls).

Analysis of Androgen Receptor and Prostate Specific Antigen by Immunocytochemistry

PWR-1E cells were grown on alcohol washed, sterile, 12 mm circular coverslips in 24 well plates at a density of 20,000 cells/ well and treated with 5 nM mibolerone for 6 days to induce prostate specific and androgen receptor expression. Preliminary experiments showed that treatment beyond 6 days resulted in no expression of prostate specific antigen. This absence is most likely due to a down regulation of the prostate specific antigen gene. The experimental design for inducing prostate specific antigen expression was as follows: twenty-four hours after plating, the 24 well plates were divided into 3 groups. One group was treated with 5 nM mibolerone in complete K-SFM medium. The remaining two groups consisted of ethanol vehicle treated and untreated controls. Media were changed every 48 h. After 6 days of mibolerone treatment, cells were washed twice with D-PBS, fixed in a 1:1 solution of methanol/acetone and stored in a -80^{0}C freezer or processed the same day. Prostate specific antigen and androgen receptor expression was demonstrated by indirect immuno-peroxidase staining. Table 2 shows specific Ab dilution, incubation time and temperature and source of prostate specific antigen and androgen receptor antibodies. Two separate experiments, using mibolerone, were preformed to induce PSA and androgen receptor expression. To confirm results, immunostaining was repeated twice for each experiment.

Table 2. PSA and androgen receptor antibody source and conditions for immunoperoxidase staining

Antibody	Immunogen	Dilution	Time/Temp.
Anti-Prostate Specific Antigen (PSA)[1, 3]	Human PSA	1:20	24 h / 4 °C
Anti-Androgen Recptor [2, 4]	Androgen Receptor	1:100	1 h / 21 °C

[1] Mouse, Monoclonal

[2] Rabbit, Polyclonal,

[3] Dako

[4] Affinity BioReagent

Invasion Assay Using Boyden Chambers

The *in vitro* invasion assay using Boyden chambers was developed by Drs. Albini and Kleinman (15). This assay examines the ability of cells to invade through a reconstituted basement membrane, "Matrigel" layered on 8 μ pore size Nuclepore filter (figure 6). Matrigel, secreted by Engelbreth-Holm-Swarm (EHS) mouse tumor cells, is used as an *in vitro* basement membrane. Conditioned medium from NIH/3T3 fibroblast cultures is used in the bottom chamber as a chemoattractant. NIH/3T3 cells were plated in 10% fetal bovine serum (FBS) in Dulbeccos's minimum essential medium (DMEM). At sub-confluence, cultures were rinsed with D-PBS and placed on serum-free DMEM containing 50 μg/ml of ascorbic acid which increases matrix protein synthesis (15). Conditioned medium was collected 24 h later.

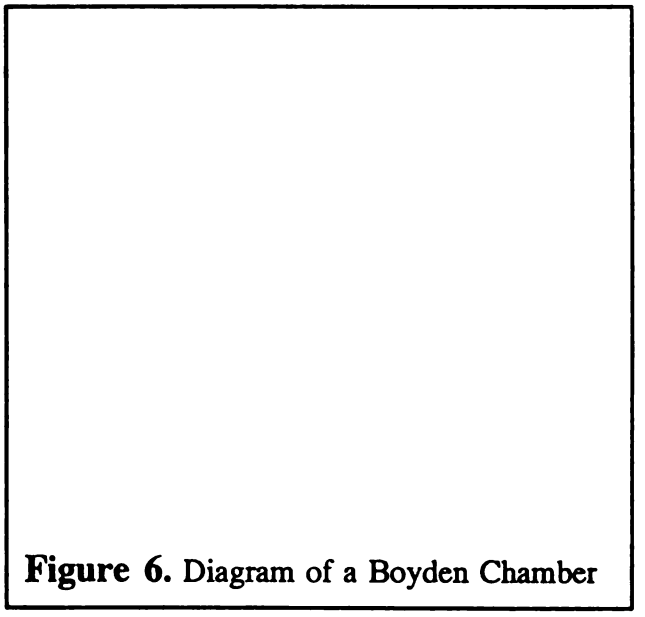


Figure 6. Diagram of a Boyden Chamber

We have made three modifications to the original assay. First, the cells are allowed to invade for 24 h instead of 5 h. We have found that this time frame allows the cells ample time to invade. Second, instead of determining the number of invaded cells on the basis of counts of only a few random representative microscopic fields, as has been done for most studies described in the literature, we stain the nuclei, extract the dye and read the absorbance. Since there is a direct relationship between cell number and absorbance (12), we believe that in this manner we are able to account for all of the cells that are on

the invaded side of the filter. Third, in addition to analyzing only those cells that are attached to the invaded side of the filter, we also count the cell that detach and fall into the bottom chamber. In this manner all of the invading cell are taken into account.

Two human prostatic carcinoma cell lines, DU-145 showing high and LNCaP showing low invasive ability, were used as controls to ensure the success of the assay. The modified assay was conducted as follows: On the day prior to running the invasion assay, PWR-1E, DU-145 and LNCaP cells were plated in 10 ml of medium into 100 mm plates under the following conditioned. PWR-1 cells were plated in complete K-SFM medium at a density of 3 million cells per plate. DU-145 were plated in RPMI medium containing 5 % DCS at a density of 2 million cells per plate. LNCaP cells were plated in RPMI medium containing 12% FBS at a density of 4 million per plate. "Matrigel" was diluted 1:20 with cold (4^0C), sterile distilled water on ice to avoid coagulation of the "Matrigel", for a final concentration of 500 μg/ml. Each Nuclepore filter was coated with 25 μg of "Matrigel" in a 50 μl solution and left to dry overnight at room temperature under sterile conditions.

Twenty-four hours after plating, cells were washed with D-PBS, incubated with 3 ml of 1 mM EDTA for 8-10 min, dislodged by tapping, neutralized by the addition of 3 ml of RPMI containing 0.1% BSA and recovered by centrifugation (2000 rpm) for 7 min. Pellets were resuspended to obtain 2 million cells per ml in RPMI medium containing 0.1% BSA. 220 μl of NIH/3T3 cell conditioned medium, which served as the chemoattractant, was added to the bottom chamber. The "Matrigel" coated filters were placed, coated side up, in the chambers and the top chamber piece was tightly screwed. 200 μl of the 2 million/ml suspension containing 40,000 cells was added to the top chamber and allowed to remain undisturbed for 5 min before overlaying with 650 μl of

RPMI medium containing 0.1% BSA. Cells were allowed to invade for 24 h. Triplicate chambers were prepared for each cell line. The control chambers contained only RPMI medium containing 0.1% BSA. Twenty-four hours later, the medium from the top chambers was carefully aspirated. The filters were carefully removed with forceps and placed, "Matrigel" coated side down, invaded cells up, in a 100 mm petri dish that had been previously layered with ½ inch of paraffin wax. Cell on the filters and in the bottom chambers were processed as follows:

Filters: Filters were kept immobilized with insect pins. They were stained using the HEMA 3 staining kit by fixing for 5 min in the fixative solution (100% methanol), 1 min in solution I (0.1% eosin Y), 1 min in solution II (0.1% methylene Blue) and rinsed with distilled water. Filters were placed on an alcohol washed glass slide invaded side down and, using a cotton tip applicator, all of the "Matrigel" and non-invaded cell were wiped off. Each filter was then placed in a well containing 300 µl of 0.1 N HCl in 24 well plates and incubated for 15 min at room temperature to extract the dye. 200 µl from each well was then placed in a 96 well plate and the absorbance read using a Titertek microplate reader.

Bottom Chamber: Medium in the bottom chambers was triturated five times and 100 µl was taken from each of the three chambers, pooled into cuvettes containing 10 ml of isotone and triturated ten time. Cell counts were preformed using a Coulter counter. The DU-145 cells that invaded and were attached to the underside of the filter and those counted in the bottom well were each taken as 100%. The two percentages were added to obtain total invasion. Percent absorbance and cell counts for PWR-1 and LNCaP cells were calculated using DU-145 as 100%.

Controls

For dose response assays to examine the effects of mibolerone, two different controls consisting of cells treated with K-SFM (no BPE or EGF) supplemented with 0.1% bovine serum albumin with and without ethanol were used. For dose response assays to examine the effects of EGF, bFGF and TGF-β, two different controls consisting of cells treated with K-SFM supplemented with BPE (no EGF) with and without vehicle were used. Vehicle treated controls were used to calculate growth response to test agents.

For all immunoperoxidase experiments, coverslips receiving no primary antibody but incubated with the secondary Ab followed by the ABC complex and the DAB- nickel solution in the same manner as the experimental coverslips, were used as controls, to insure that the resulting staining was not due to non-specific binding of the reagents. Additionally, in experiments which examined prostate specific antigen and androgen receptor expression induced by mibolerone, cells treated with only K-SFM and K-SFM containing ethanol were also stained. For the invasion assay experiments two chambers containing RPMI medium with 0.1% BSA severed as blank controls.

Results

Growth Requirements

Growth curves for PWR-1E cells were generated for cell densities ranging from 625 cells to 40,000 cells. Cells were fixed, stained and absorbance read for days 1, 2, 4, 6, 8, 10, and 14 using the microplate assay. Absorbance data were plotted against time (days) for each cell density. Figure 7 is a growth curve graph representing one of the four growth experiments conducted. All four experiment showed the same growth trends.

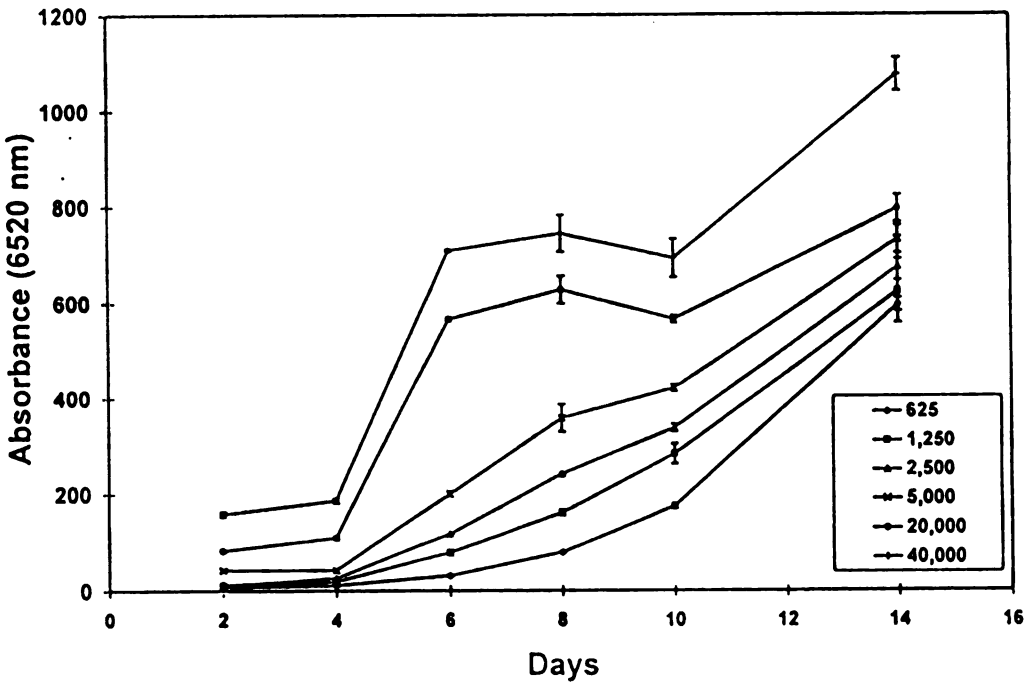


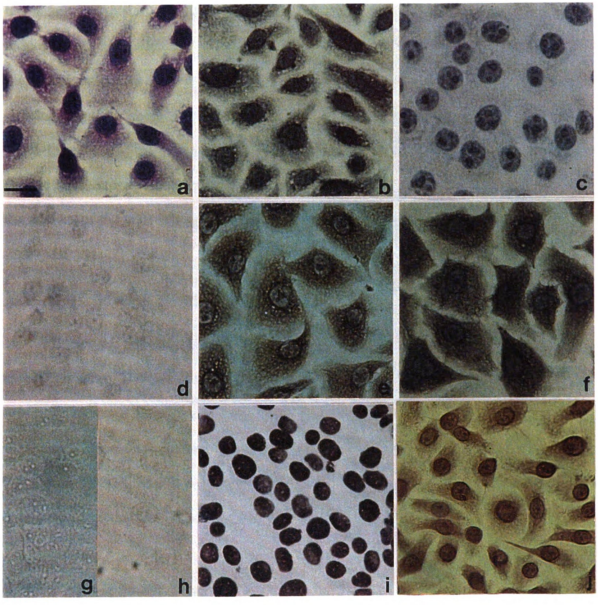
Figure 7. **Growth Curves for PWR-1E.** Cells were plated at densities ranging from 625-40,000 cells in complete K-SFM in 96 well plates. Plates were stained at 1, 2, 4, 6, 8, 10 and 14 days and absorbance was plotted against time.

Because of the direct relationship between absorbance and cell number (12), the plotted data were used to determine cell doubling times. Cell doubling time in log phase was estimated as 30 h when cells are plated at high densities of 10, 000, 20,000, and 40,000 cells, and 53 h at low densities of 625, 1,250, 2,500 and 5,000 cells/ well. The average doubling time was estimated to be 42 h.

Expression of Intermediate Filament Proteins, Factor VIII and Large-T Antigen

PWR-1E cells have an epithelial cell morphology as demonstrated by hematoxylin & eosin staining in Figure 8a. The expression of cytokeratin 8, cytokeratin 18, basal cell cytokeratins 1, 5, 10 and 14, desmin and Factor VIII was determined by immunoperoxidase staining using monoclonal antibodies. PWR-1E cells showed strong cytoplasmic staining for cytokeratin 8 and cytokeratin 18 (Figure 8 e, and f) but moderate to weak staining for vimentin (Figure 8 j). Expression of basal cell cytokeratins was weak and heterogeneous in distribution, i.e. some cells did not show expression (data not shown). Desmin and Factor VIII expression was absent. (Figure 8 g and h). PWR-1E cell show strong nuclear expression of large-T antigen (Figure 8 i).

Figure 8. **Characterization of PWR-1E Cells on the Basis of Cellular Proteins.** Proteins were detected by immunoperoxidase staining. a. haematoxylin and eosin staining; b. positive staining for PSA; c. positive staining for nuclear androgen receptor. Cells for b. and c. were pretreated for 6 days with 5 nM mibolerone; d. a control lacking primary antibody; e. and f. positive staining for cytokeratin 8 and 18, respectively; g. and h. absence of staining for desmin and Factor VIII, respectively; i. positive staining for SV-40 large T antigen; j. positive staining for vimentin. Bar, 20mM, (625X).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Expression of Prostate Specific Antigen and Androgen Receptor

The prostate specific antigen and androgen receptor expression are considered to be markers for prostatic epithelial cells. The expression of PSA and androgen receptor was induced by 5 nM mibolerone treatment for 6 days and detected using monoclonal and polyclonal antibodies to PSA and androgen receptor, respectively, by immunoperoxidase staining. Prostate specific antigen expression was strong in mibolerone treated cells PWR-1E cells (Figure 8 b) as compared to the control which lacked primary antibody (Figure 8 d). Expression of androgen receptor in mibolerone treated cells was present in the nucleus of the cells (Figure 8 c). Controls did not show nuclear staining (Figure 8 d).

Effect of Mibolerone on Growth

Effect of mibolerone on the growth of PWR-1 cells was examined by the microplate assay. The dose response curve represented in Figure 9 shows the average of two experiments. Absorbance is plotted against mibolerone concentration. PWR-1E cell show a dose dependant increase in cell growth at mibolerone concentrations ranging from 0.5 nM to 10 nM. Maximal growth stimulation of 130% of control for five days of mibolerone treatment is seen at 10 nM concentration (Figure 9).

Effect of Growth Factors

Epidermal Growth Factor. The effect of EGF on the growth of PWR-1E cells was examined by the microplate assay. Results are shown in Figure 10 where absorbance is plotted against growth factor concentration. The EGF dose response curve represented in Figure 10 is the average of two experiments after five days of treatment. PWR-1E cell

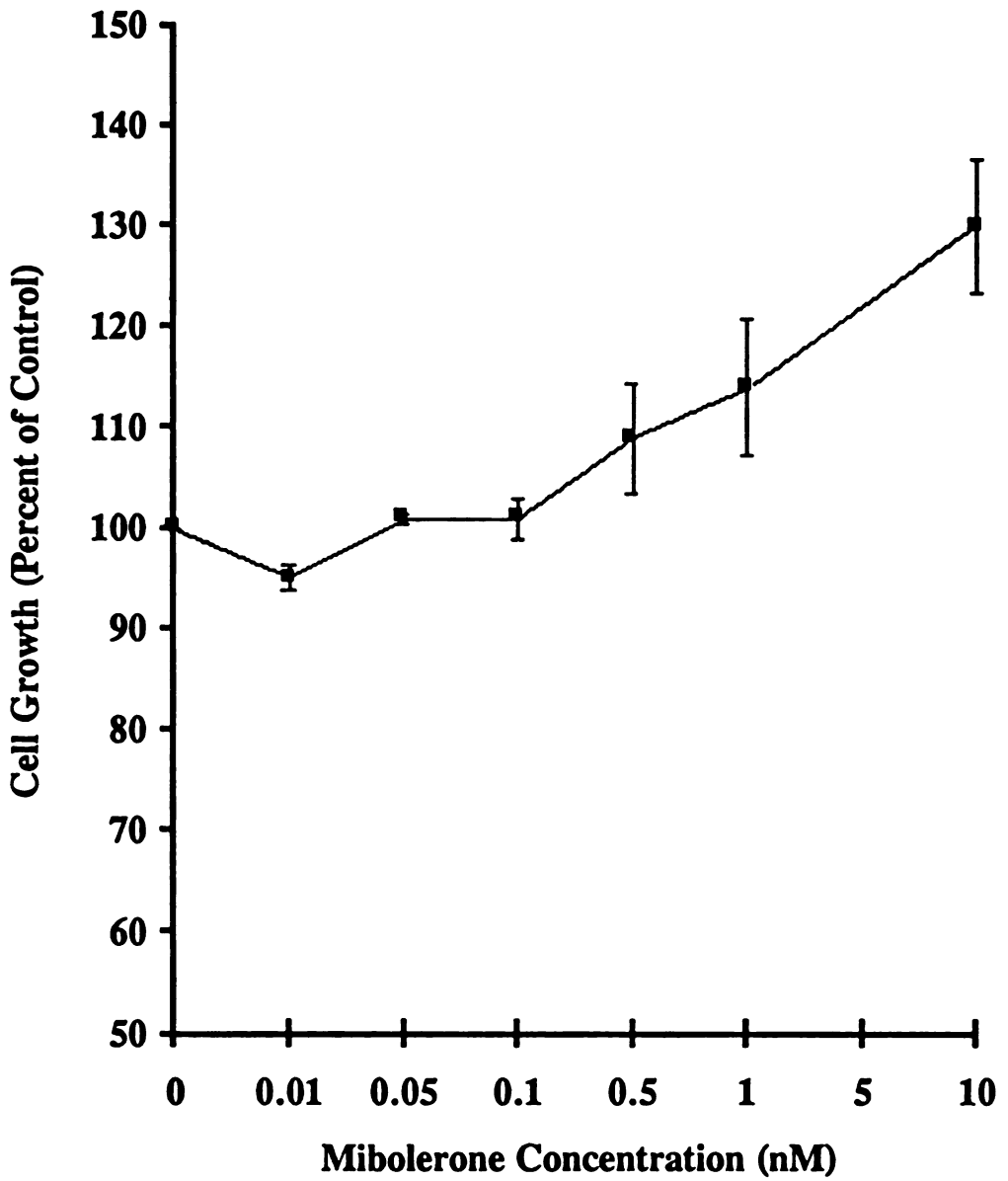

Figure 9. **Effects of Mibolerone on the Growth of PWR-1E cell. 10,000 cells were plated in K-SFM medium containing 0.1% BSA in 96 well plates and treated with mibolerone for 5 days. Absorbance was plotted against mibolerone concentrations.**

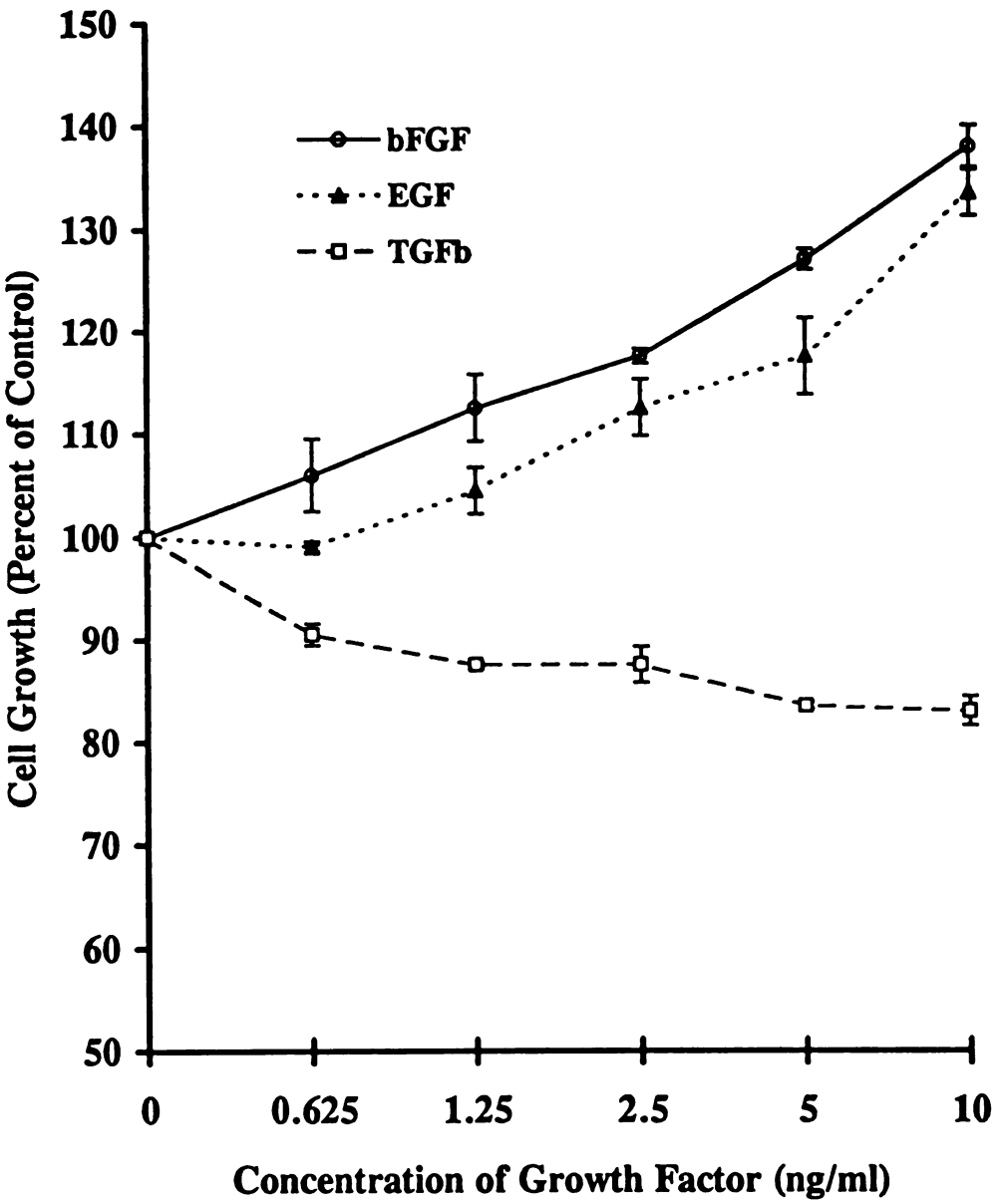

Figure 10. **Growth Factors.** Effects of bFGF (0), EGF (◊) and TGF-β (□) on the growth of PWR-1E cell. 5,000 cells were plated in BPE supplemented K-SFM in 96 well plates. Plates were stained at five days and absorbance was plotted against growth factor concentrations.

show a dose dependant increase in cell growth in response to EGF concentrations with a maximal growth increase to 134% of control at 10 ng/ml of EGF (Figure 10).

Basic Fibroblast Growth Factor. The effect of bFGF on the growth of PWR-1E cells was, also, examined by the microplate assay. Effect of bFGF on PWR-1 cells after five days of treatment is shown in Figure 10 with absorbance plotted against growth factor concentration. The bFGF dose response curve represented in Figure 10 is the average of two experiments. After five days of treatment, a dose dependent stimulation of cell growth was observed for bFGF concentrations ranging from 0.625 ng/ml to 10 ng/ml with a maximal growth increase to 138% of control at 10 ng/ml of bFGF.

***Transforming Growth Factor*-β.** The effect of TGF-β on the growth of PWR-1E cells after five days of TGF-β treatment is shown in Figure 10 with the absorbance plotted against growth factor concentration. The TGF-β dose response curve represented in Figure 10 is the average of two experiments. A dose dependant inhibition of cell growth is observed for concentrations of TGF-β from 0.625 to 10 ng/ml. Maximal inhibition to 83% of control was observed at 10 ng/ml of TGF-β.

In vitro Invasion

Using the *in vitro* Boyden chamber invasion assay, the invasive ability PWR-1E cells was determined and compared with the invasive ability of DU-145 and LNCaP cells, where DU-145 invasion was set at 100%. Figure 11 shows that in comparison to the highly invasive DU-145 cell line, PWR-1E cells show a 12% invasion, while the low invasive LNCaP cell line shows a 19% invasion.

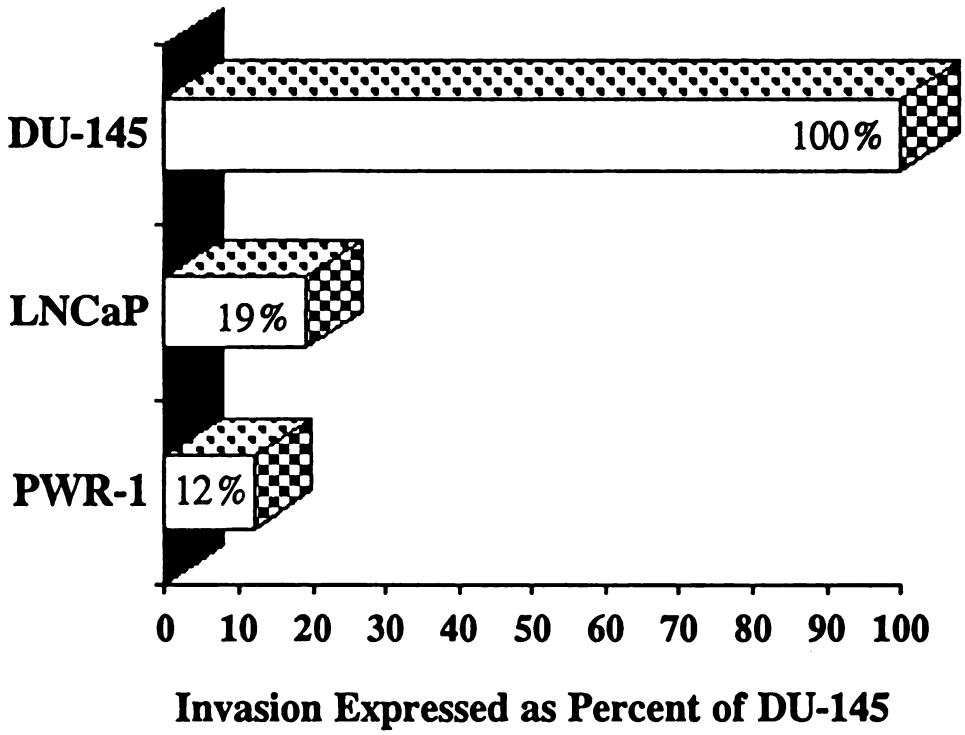

Figure 11. ***In Vitro*** **Invasion.** Invasive ability of DU-145, LNCaP and PWR-1E cell lines was examined by the Boyden chamber *in vitro* invasion assay. 400,000 cells were plated on each "Matrigel"-coated filter and allowed to invade for 24 h. The invasive ability of the highly invasive DU-145 cell line was set at 100 % invasion.

Discussion

Epithelial cells are characterized by the expression of cytokeratin intermediate filaments (16,17). Luminal prostatic epithelial cells, which are terminally differentiated secretory cells, express cytokeratin 8 and 18 (18, 19, 20). Using immunoperoxidase staining, I have established the expression of cytokeratin 8 and 18 (Figure 8) in PWR-1E cells. This indicates that PWR-1E cells are derived from luminal cells of prostatic acini. Possible derivation from muscle or endothelial cells was excluded by their lack of desmin and Factor VIII expression (Figure 8) (21, 22). The fact that PWR-1E cells showed weak staining for vimentin, a mesenchymal cell marker, is not surprising since epithelial cells that have been placed in culture are known to sometimes show vimentin expression (16). However, when cells of mesenchymal origin are placed in culture, they do not express cytokeratins. Therefore, I feel confident in excluding a mesenchymal origin due to the expression of cytokeratins. PWR-1E cells also heterogeneously express basal cell cytokeratins. This may be due to the presence of a small population of cells that express characteristics similar to those of amplifying cells. Amplifying cell are thought to represent cells in transition from stem or basal to luminal cells and they may express cytokeratins that are characteristic of both basal and luminal cells such as cytokeratins 5, 8, 15, 16 and 18 (18, 20, 23-25).

Prostatic epithelial origin was established by analysis of three prostate specific characteristics: 1) response to androgens which demonstrates the ability of cells to mimic the response of normal prostatic epithelial cells; 2) expression of androgen receptor and 3) induction of prostate specific antigen expression. The reported lack of androgen responsiveness of most established prostatic epithelial cells lines may be because of the experimental design used or the inclusion of growth factors in the medium (14). No

systematic attempts have been made by other investigators to explore the basis of this apparent absence of growth response. We have found that the use of 5 α-DHT as the test androgen may not provide enough androgenic stimulus to induce cell proliferation. 5 α-DHT has a half life of about 2 hours. Therefore, unless the medium is changed frequently, continuous androgenic stimulus will not be present. This is reflected by the small growth stimulation observed when PWR-1E cells were treated with 5 α-DHT (data not shown). For this reason, I used mibolerone, a synthetic non-metabolizable androgen, to insure the presence of androgen in the medium during the experimental period. I was, therefore able to demonstrate the androgen responsiveness of PWR-1E cell.

The addition of growth factors to the medium may also interfere with possible androgenic response. This interference by growth factors was observed in preliminary experiment in which PWR-1E cells were treated with mibolerone in K-SFM medium that was supplemented with EGF and BPE. In these experiments, no growth stimulation was observed. I suggest that this lack of response to mibolerone may be due to the fact that EGF down regulates androgen receptor. The decrease or absence of stimulation by the added androgen could be explained on the basis of the down regulation of androgen receptor and the masking of androgen response due to EGF-induced cell proliferation (26). When PWR-1E cells were treated with 10nM mibolerone in K-SFM medium containing 0.1% bovine serum albumin but lacking any growth factors, a 30% increase in cell proliferation was observed (Figure 9). This 30% growth increase of PWR-1E cells by mibolerone clearly establishes that the PWR-1E cell line is androgen responsive.

Because androgens exert their influence on prostatic epithelial cells via the androgen receptor, the expression of androgen receptor in PWR-1E cells was examined by immunoperoxidase using polyclonal antibody to androgen receptor. PWR-1E cells showed nuclear expression of androgen receptor after 6 days of treatment with

5 nM mibolerone (Figure 8c). Pre-treatment with mibolerone was done for two reasons; first to up regulate the receptor and thereby facilitate the detection of the low abundance of nuclear androgen receptor and second, to attempt to mimic the conditions present during the androgen response growth assay.

Expression of prostate specific antigen is a differentiated function of normal prostate epithelial cells and it is used as a marker for prostatic epithelial cell differentiation. Prostatic secretory epithelial cells synthesize and secrete PSA *in vivo* in response to androgens (14). With the exception of the three cell lines presented in this thesis and the two malignant cell lines, LNCaP and ALVA101, none of the established immortalized or malignant cell lines have been shown to express PSA (14). I believe that this inability to demonstrate PSA expression, in at least some cases, may be due to the experimental conditions. Because PSA expression is induced by androgen stimulation, it would follow that in order to detect PSA expression, cells should be treated with androgens. Therefore, I was able to induce PSA expression by treating cells with 5 nM mibolerone for 6 days and demonstrate its presence by immunoperoxidase staining using monoclonal antibodies to human PSA. PWR-1E cells clearly show strong expression of prostate specific antigen which provides strong evidence for their prostatic epithelial origin. (Figure 8 b).

During the process of immortalization, the response of PWR-1E cells to growth factors may be affected. Therefore, the PWR-1E cell line was characterized with regards to its response to growth factors. This was done to ascertain that their responses either mimic normal prostatic epithelial cells or to document how they differ. The effects of EGF, bFGF and TGF-β, the three major growth factors involved in the regulation of the normal prostatic epithelium, were examined (14).

In response to exogenous epidermal growth factor, PWR-1E cells showed a dose dependant increase in cell proliferation (Figure 10). A maximal growth stimulation to 134% of control was seen at 10 ng/ml of EGF. A dose dependant increase in cell growth was also seen when PWR-1E cells were treated with basic fibroblast growth factor (Figure 10), with a maximum increase in growth to 138 % of control at 10 ng/ml of bFGF. The finding of EGF stimulatory response for PWR-1E is consistent with other SV-40 immortalized epithelial cell lines (27, 10). It is believed that one of the many mechanism by which SV-40 suppresses senescence involves an increase in the levels of EGF receptor which makes the cells more sensitive to EGF (10). It is interesting to note that PWR-1E cell are more sensitive to bFGF than to EGF.

Normal prostatic epithelial cells respond to exogenous TGF-β by showing a marked growth inhibition. However, PWR-1E cells showed only a slight inhibition with a decrease in growth to 83% of control at 10 ng/ml TGF-β (Figure 10). Other investigators have also observed a lack of a strong inhibition in SV-40 immortalized cells. For example, SV-40 immortalized normal thyroid follicle cells show a loss of TGF-β response along with a partial loss of its receptor (2). But in the SV-40 immortalized prostate epithelial cell line developed by Lee et al., TGF-β inhibition was more evident (10).

SV-40 immortalization does not interfere with normal differentiated functions as demonstrated by the ability of PWR-1E cells to: 1) express prostate specific antigen in response to androgens; 2) express androgen receptor; 3) demonstrate a growth response to androgens and 4) express luminal prostatic epithelial cytokeratins 8 and 18. However immortalization resulted in some changes in the ability of PWR-1E cells to respond to growth factors. The observed differences in response to growth factors may be caused by

the induction of chromosome instability after SV-40 immortalization (28). The immortalized cells incorporate the SV-40 virus in a random manner. Because the effects of the virus immortalization are random, immortalized cells may develop along different epigenetic pathways (28). This is clearly seen in the T series of prostate epithelial cells transformed by SV-40 by Ohnuki et al. (28). When the response of the SV-40 immortalized prostatic cells to growth factor were examined, it was found that in comparison to each other, all of the clones of the T series showed different growth response. I propose that the low inhibitory response seen in PWR-1E cells by TGF-β may be due to immortalization by SV-40.

The invasive potential of PWR-1E cells was determined by their ability to invade a reconstituted basement membrane *in vitro* using the Boyden chamber assay. Invasion by cancer cells through the basement membrane *in vivo* is one of the first steps in the progression to metastasis (29). Since normal prostatic epithelial cells *in vivo* do not invade but simply rest on the basement membrane, it would follow that PWR-1E cells should not be invasive or at least show markedly lower invasive ability than the highly invasive prostate cancer cell line DU-145 and the low invasive prostate cancer cell line, LNCaP. The invasive ability of PWR-1E cells was, therefore, compared to that of DU-145 and LNCaP. The invasive ability of each cell line was expressed as a percentage of the highly invasive DU-145 cell line, which was set at 100 % invasion. In comparison to DU-145 cells, LNCaP cells showed a 19% invasion and PWR-1E cells showed a 12 % invasion (Figure 11). We were surprised to see that their was not much difference in the invasive ability between low invasive LNCaP cell line and the immortalized PWR-1E cell line. The low invasion shown by PWR-1E cells again may be due to immortalization by SV-40. Perhaps the yet unknown genetic changes common to SV-40 immortalized cells, are responsible for the low invasive characteristic shown by PWR-1E cells. Nevertheless,

results obtained from work which is not a part of this thesis showed that when PWR-1E cells are injected into nude mice they did not form tumors even after a period of six months, hence they are non-tumorigenic.

The epithelial origin of PWR-1E cells was established by their expression of epithelial cell specific cytokeratins 8 and 18, and the lack of non-epithelial cell markers, desmin and Factor VIII. The prostatic epithelial origin of the PWR-1E cell line was established by the expression of three prostate specific characteristic: 1) the expression of prostate specific antigen in response to androgen stimulation, a marker of differentiated prostatic epithelial cell function; 2) the presence of androgen receptor and 3) the ability of PWR-1E cells to respond to androgens. Additionally, I have demonstrated, the ability of PWR-1E immortalized cells to respond to prostatic regulatory growth factors EGF, bFGF and TGF-β. PWR-1E cells have also been shown to be non-tumorigenic.

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Chapter Three

Characterization of Immortalized Adult Human Prostate Epithelial Cell Lines: RWPE-1 and RWPE-2

Data presented in this chapter have been submitted for publication:

Bello, D., Webber, M.M., Kleinman, H.K., Wartinger, D.D. and Rhim, J.S.: Androgen responsive and prostate specific antigen positive adult human prostatic epithelial cells immortalized by human papilloma virus and transformed by v-Ki-*ras*. (Submitted), 1996 .

Abstract

Prostate cancer is the most common type of cancer diagnosed and the second leading cause of cancer death in adult males in the United States. Research on prostate cancer has been slow due to the lack of suitable human cell models. The use of human prostate epithelial cells *in vitro* for the study of prostate cancer is the best model system aside from human experimentation. In order to establish suitable human cells models, normal adult human prostatic epithelial cells from a white male donor were immortalized with the human papilloma virus 18 and resulted in the selection and establishment of the RWPE-1 cell line. RWPE-1 cells were further transformed by Ki-*ras* infection to establish a tumorigenic cell line termed RWPE-2. RWPE-1 and RWPE-2 cell lines were characterized on the basis of intermediate filament proteins, Factors-VIII, prostate specific antigen and androgen receptor expression by immunoperoxidase staining and growth response to growth factors. RWPE-1 and RWPE-2 cell lines show growth stimulation and prostate specific antigen and androgen receptor expression in response to androgen treatment. Both cell lines show strong expression of cytokeratins 8 and 18 and weak expression of basal cell cytokeratins. They do not express desmin or Factor VIII. These characteristics undoubtably establish their prostatic epithelial origin. RWPE-1 and RWPE-2 cell lines show a dose dependant growth stimulation by epidermal growth factor and basic fibroblast growth factor and growth inhibition when exposed to transforming growth factor-β. Other research, not reported in this thesis has shown that RWPE-1 cells are not tumorigenic while RWPE-2 cells form tumors when injected into nude mice. The RWPE-1 and RWPE-2 cell lines provide useful cell culture models for studies on prostate carcinogenesis and chemoprevention.

Background

Human Papillomavirus

Several human papillomaviruses are sexually transmitted and have been associated with a number of urogenital cancers such as cervical and penile cancers (1,2). Human Papillomaviruses have been successfully used for immortalization of cells *in vitro*, for example human cervical epithelial cells (3,4), foreskin epithelial cells (4), and mammary epithelial cells (5). It is believed that human papillomavirus imparts immortality through the expression of E6 and E7 proteins. E7 inactivates the retinoblastoma gene product, the Rb protein, thereby preventing Rb protein from preforming its normal cell regulatory activities (4,7). The E6 protein complexes with and inactivates p53, also a tumor suppressor protein (1). Retinoblastoma and p53 protein inactivation or mutations in Rb and p53 genes are known to contribute to the development of cancer (8). Because human papillomavirus infections are common in the precursor lesions of anogenital cancers, (6) immortalization of primary cell by human papillomavirus has been used to develop a model system, to investigate neoplastic transformation from a normal to a tumorigenic phenotype in the study of cervical cancer (4). In the human prostate, human papillomavirus infections have been associated with cancer (4,9). This association is best illustrated in Japanese men, where 41% of prostate carcinomas contain human papillomavirus DNA along with the co-expression of *ras* mutations (10). To date only one other human prostate epithelial cell line (1), in addition to our RWPE-1 cell line (11), has been successfully immortalized with human papillomavirus-18.

Infections of non-tumorigenic human papillomavirus immortalized cells with Ki-*ras* has been shown to cause transformation to tumorigenic cells (11). Genes of the *ras* family

are proto-oncogenes, which when activated, are believed to play a role in carcinogenesis (7). The c-*ras* gene encoded proteins play an important role in the normal growth and differentiation of cells (7). The combination of human papillomavirus infection and *ras* mutation are associated with advanced stages of prostate cancer in Japanese men (10). Despite the low overall frequency of *ras* mutations in non-Japanese men, when *ras* expression occurs in prostate carcinomas, it is associated with tumor progression (12). Additionally, *ras* activation is found in almost all the established prostate cancer cell lines (12). To date, the only human papillomavirus-18 immortalized, *ras* transformed, malignant adult human prostatic epithelial cell line is RWPE-2, which was established in our laboratory (11).

Isolation and Development of RWPE-1

A tissue specimen of normal prostate from a 54 year old white male patient undergoing radical cystoprostatectomy for transitional cell carcinoma of the bladder was used. Pathology report found the prostate to be normal with mild prostatic nodular hyperplasia. Epithelial cells were isolated by Dr. Mukta M. Webber following a modified collagenase technique (13). The tissue was cut into 2-3 mm cubes and placed in one 100 mm Petri dish with 30 ml RPMI-1640 containing 5% FBS and 400 Units/ml of collagenase After 48 h digestion in collagenase, the tissue was triturated until all lumps were broken up, and centrifuged for 5 min to remove collagenase. The pellet was suspended in Saline G and allowed to settle in the refrigerator for 1 h. The supernatant was removed and the sediment was suspended again in saline G and allowed to settle at 4°C. This was repeated 4 times. The sediment, after the last wash, containing the acini was suspended in storage medium containing spermine (2 μg/ml) and left at 4°C overnight

to destroy any fibroblasts that may be present in the preparation. The acini were centrifuged, suspended in growth medium and plated in culture flasks/plates for growing these cells. Culture flasks/plates were coated with a mixture of 10 μg/ml each of fibronectin and collagen IV (13). Primary cultures of prostatic epithelium were established. Primary cultures were subcultured and cells were plated in coated plates. Medium was changed every 48 h. These cells were further subcultured to determine the number of passages until senescence. These cells could not be propagated beyond five to six passages. Secondary cultures were transfected with a plasmid pSHV-18m containing the entire human papilloma virus-18 genome (HPV-18) inserted into the EcoRI site of pSV2 neo by polybrene-induced DNA transfection method as described by Rhim et. al. (11) Cells were transfected with 10 μg of pSHPV-18m using polybrene at a concentration of 10 μg/ml and incubated overnight. These cells were then shocked with 30% dimethyl sulfoxide for 4 min. After 5 days, cells were subcultured at a 1:2 ratio weekly. Medium was changed twice weekly. The resulting HPV-18 immortalized cell line was designated RWPE-1.

Development of RWPE-2

Clones derived from RWPE-1 cells were infected at a multiplicity of infection of ~100 with the Ki-MuSV [BeSV] (baboon endogenous virus psuedotype) that contains an activated Ki-*ras* oncogene (11). The resulting HPV-18 immortalized cell line was designated RWPE-2.

Characterization

The RWPE-1 and RWPE-2 cell lines were characterized on the basis of the expression of intermediate filament proteins, Factor VIII, androgen receptor, prostate specific antigen, growth response to an androgen and three growth factors, epidermal growth factor, basic fibroblast growth factor and transforming growth factor-β.

Intermediate filament proteins cytokeratins, vimentin and desmin and Factor VIII were investigated using monoclonal antibodies to cytokeratins, vimentin, desmin and Factor VIII, by an immunoperoxidase staining method. This was done to establish their epithelial origin by the presence of cytokeratins but the absence of desmin, vimentin and Factor VIII. Androgen receptor and prostate specific antigen expression was also demonstrated by immunoperoxidase staining after the cells were treated with mibolerone, a synthetic, non-metabolizable androgen, to induce their synthesis. This was done to establish luminal prostatic epithelial origin of RWPE-1 and RWPE-2 cells. Growth response to androgens and epidermal growth factor, basic fibroblast growth factor and transforming growth factor-β was determined using a microplate assay to illustrate their ability to mimic the response of normal prostatic epithelial cells to these agents. In addition, the invasive ability was examined to establish the absence or presences of malignant characteristics.

Materials

The materials used were: keratinocyte growth medium (KGM) serum-free medium, CC-3001 from Clonetics; keratinocyte-serum free media (K-SFM), 17005-042 and antibiotic/antimycotic mixture (PSF), 600-5240AG from Gibco-BRL; charcoal treated fetal

bovine serum (FBS) 0.2μ filtered, 52-0115 from Cocalico Biologicals, Inc.; Dulbecco's phosphate buffered saline without Ca^{++}/Mg^{++}(D-PBS), 28374 from Pierce; mibolerone, W-300 from BIOMOL Biomolecular Research Laboratories; epidermal growth factors, 40001 and transforming growth factor beta, 40039 from Collaborative Research; recombinant basic fibroblast growth factor, 234-FS from R & D Systems; monoclonal antibody to prostate specific antigen (PSA), M-0750 from DAKO; polyclonal antibody to androgen receptor, PA1-110 from Affinity BioReagents; monoclonal antibody to cytokeratin 8, C-5301, monoclonal antibody to cytokeratin 18, C-8541, monoclonal antibody to desmin, D-1033, monoclonal antibody to vimentin, V-6630, monoclonal antibody to Factor-VIII, F-3520, 5-a-dihydrotestosterone (5α-DHT), D-5027 and bovine serum albumin, A-2153 from Sigma; monoclonal antibody to cytokeratin 1, 5, 10 and 14 (Keratin-903), C34903 from Enzo Diagnostics; Vectastain Elite ABC Peroxidase Kit, PK-6102 and 3,3'-diamino-benzidine (DAB) Substrate Kit, SK-4100 from Vector Laboratories; Hema 3 Stain Set, 67-56-1 from Curtin-Matheson Scientific; 12 mm circle microscope cover glass (coverslips), 12-545-80 from Fisher Chemicals; Nuclepore membrane, 8 μ pore size, 1550446 and 96 well plates, 3596 from Costar; 24 well plates, 3047 from Falcon, Beckon-Dickinson; "Matrigel", gift from Dr. H.K. Kleinman, National Institutes of Health.

Methods

Cell Culture

RWPE-1 and RWPE-2 cells were maintained in the complete K-SFM medium which contains 50 μg/ml of bovine pituitary extract (BPE) and 5 ng/ml EGF, and to this is added 1% PSF (penicillin G sodium, 10,000 units/ml, streptomyosin sulfate

10,000 mg/ml and amphotericin B as Fungizone, 25 µg/ml in 0.85% saline). Cells were passaged upon confluence. For passaging, cell were washed with D-PBS, then incubated with 3 ml of trypsin/EDTA mixture (0.05% trypsin, 0.02 mM EDTA) diluted 1:1 with D-PBS for 8-10 min. Cells were dislodged by tapping, neutralized by the addition an equal volume of 2% donors calf serum (DCS) in D-PBS and recovered by centrifugation (2000 rpm) for 5 min. Cell counts were performed with a Coulter Counter and cell were seeded at 2 to 5 x 10^6 cells/ T-75 flask. All experiments were conducted on RWPE-1 cells between passage 36 and 46. All experiments except growth factors dose response assay, conducted on RWPE-2 cells were done on passages between 35-45, dose response assay experiments were conducted on cell between passages 15-25.

Cell Proliferation Studies

Effects of Serum-containing and Serum-free Media on the Growth of RWPE-1 Cells by Microplate Assay. In order to identify the best medium for the growth and maintenance of RWPE-1 cells, effects of the following media on growth were examined: complete K-SFM medium which is supplemented with EGF and BPE and K-SFM medium with the addition of 10% FBS. This assay is based on the direct relationship that exists between absorbance and cell number (14). RWPE-1 or RWPE-2 cells were plated at cell densities of 625, 1,250, 2,500, 5,000, 10,000 and 20,000 cells. Cells were plated in 96-well plates using six replicate wells/cells density. Medium was changed every 48 h and the plates were fixed and stained at day 5 after plating. Plates were washed twice with normal saline to remove the dead cells and media, fixed in 95% ethanol for 1 h and stained with 0.5 % methylene blue, which is a protein binding dye, for 45 min. During this time the dye is taken up by the fixed cells. The cells were then incubated at 37^{0}C for

24 h in 1% SDS to extract the dye. Absorbance was read at 620 nM using a Titertek microplate reader (13). Becuase of the direct relationship between cell number and absorbance (14), the absorbance data were plotted against time and analyzed.

Analysis of Growth Characteristics and Cell Doubling Time by Growth Curve Assay- In order to examine the growth characteristic and the doubling time of RWPE-1 and RWPE-2, growth curves were generated. A microplate assay using 96-well plates was used. This assay is based on the direct relationship between absorbance and cell number (14, 15). A wide range of cell densities was used. Cells were plated at low (625, 1,250, 2,500 and 5,000 cells) and high (10, 000, and 20,000 cells) densities in complete K-SFM medium. Three replicate wells were plated per cell density. A total of 7 plates were prepared. Medium was changed every 48 h. Plates were fixed and stained at days 2, 4, 6, 8, 10, and 14 after plating. Plates were prepared for absorbance reading as described previously. Absorbance was read at 620 nM using a Titertek microplate reader (15). Becuase of the direct relationship between cell number and absorbance (14), the absorbance data were plotted against time, analyzed and cell doubling times were determined for low and high cell densities.

Growth Factor Dose Response Assay- Since epidermal growth factor, basic fibroblast growth factor and transforming growth factor-β are the three major growth factors involved in the regulation of the normal prostatic epithelium (2), their effects on cell growth were investigated using the same dose response assay described above. Epidermal growth factor was dissolved in sterile distilled water, basic fibroblast growth factor in 0.1% BSA in D-PBS and transforming growth factor-ß in 4 mM HCl in D-PBS containing 1 mg/ml of bovine serum albumin. All growth factor stocks were stored at -20^{0}C. The final concentrations of the vehicle in the medium was 0.1% for EGF and bFGF

and 1% for TGF-ß. The vehicle was not inhibitory to the growth of cells. Concentrations of all growth factors tested were 0.625, 1.25, 2.5, 5, and 10 ng/ml.

For the examination of EGF and bFGF effects, RWPE-1 and RWPE-2 cells were plated at a density of 5,000 cells per well in a 96 well plate in complete K-SFM medium. Because TGF-β inhibits cells growth, cells were plated at a density of 10,000 cells per well in a 96 well plate in complete K-SFM medium. Forty-eight hours after plating, medium was changed to medium containing test agent. Medium for EGF and bFGF experiments consisted of K-SFM medium supplemented with BPE (no EGF). Medium for TGF-β experiments consisted of complete K-SFM medium. Plates were fixed at 5 and 7 days after treatment and prepared for absorbance reading as described previously.

Analysis of Intermediate Filament Proteins and Factor VIII by Immunocytochemistry

Intermediate filament proteins cytokeratins, vimentin and desmin and Factor VIII were investigated by monoclonal antibodies to cytokeratins, vimentin, desmin and Factor VIII, using an indirect immuno-peroxidase method, to establish the epithelial cell origin of RWPE-1 and RWPE-2 cells. RWPE-1 and RWPE-2 cells were grown on sterile, alcohol washed coverslips in 24 well plates at a density of 20,000 cells in complete K-SFM medium/well. Coverslips were soaked in 95% ethanol overnight, individually wiped with low lint paper wipes and autoclaved for 30 min. Medium was changed every 48 h. Upon 70% confluence, 5 to 7 days after plating, cells were rinsed twice with D-PBS and fixed in a 1:1 solution of methanol and acetone for 2 min with gentle agitation. The coverslips were then either processed the same day or stored in a -80^{0}C freezer.

Immunocytochemistry was performed using an indirect immuno-peroxidase method following a modified Vector protocol as follows: Cells were blocked in normal horse serum for 1 h at room temperature and incubated with the appropriate monoclonal Ab diluted in normal horse serum. Table 3 shows specific antibody dilution, incubation time and temperature and source of each antibody used. Cells were subsequently processed using the Vectorstain Elite ABC Peroxidase kit. Cells were treated with 200μl/ coverslip of the biotinylated anti-mouse or anti-rabbit IgG secondary Ab, as appropriate (see Table 3), for 30 min. Prior to Avidin-biotin-peroxidase complex (ABC) application, cells were treated with 200µl/coverslip of a 3% H_2O_2 solution for 3 min to quench all endogenous peroxidase activity. Cells were then incubated in 200 µl/coverslip of the ABC solution for 30 min and developed with diaminobenzidine-nickel chloride substrate kit for peroxidase for 5-7 min, (200 µl/coverslip). Cells were washed twice in 500 µl D-PBS over 10 min between all successive steps after primary Ab application. Cells were then dehydrated in two washes of 95% ethanol and two of 100% ethanol, for 3 min each, through xylol (a 1:1 solution of xylene and 100% ethanol), for 5 min and twice through xylene, for 3 and 5 min, respectively. Coverslips were mounted on alcohol washed slides using Permount. All of the preceding steps were carried out at room temperature unless otherwise indicated. Using a high power light microscope, cells were determined to be either positive or negative for a given antigen as compared to controls (see section on Controls).

Table 3. Antibody source and conditions for immunoperoxidae staining

Antibody	Immunogen	Dilution	Source
Anti-Cytokeratin 8 [1, 2, 3]	Cytokeratin 8	1:200	Sigma
Anti-Cytokeratin 18 [1, 2, 3]	Cytokeratin 18	1:800	Sigma
Keratin-903[1, 2, 3]	Cytokeratin 1, 5, 10 and 14	1:50	Enzo Diagnostics
Anti-Desmin[1, 2, 3]	Desmin	1:50	Sigma
Anti-Vimentin[1, 2, 3]	Vimentin	1:40	Sigma
Anti-Factor VIII[1, 2, 4]	F-VIII	1:1000	Sigma

[1] Incubation time of 1 hour at room temperature.

[2] Monoclonal

[3] Mouse

[4] Rabbit

Analysis of Androgen Receptor and Prostate Specific Antigen by Immunocytochemistry

RWPE-1 and RWPE-2 cells were grown on alcohol washed, sterile, 12 mm circular coverslips in 24 well plates at a density of 20,000 cells/well and treated with 5 nM mibolerone for 6 days to induce prostate specific and androgen receptor expression. Preliminary experiments showed that treatment beyond 6 days resulted in no expression of prostate specific antigen. This absence is most likely due to a down regulation of the prostate specific antigen gene. The experimental design for inducing prostate specific antigen expression was as follows: twenty-four hours after plating, the 24 well plates were divided into 3 groups. One group was treated with 5 nM mibolerone in complete K-SFM medium. The remaining two groups consisted of ethanol vehicle treated and untreated controls. Media were changed every 48 h. After 6 days of mibolerone treatment, cells were washed twice with D-PBS, fixed in a 1:1 solution of methanol/acetone and stored in a -80^{0}C freezer or processed the same day. Prostate specific antigen and androgen receptor expression was demonstrated by indirect immuno-peroxidase staining. Table 4 shows specific Ab dilution, incubation time and temperature and source of prostate specific antigen and androgen receptor antibodies. Two separate experiments, using mibolerone, were preformed to induce PSA and androgen receptor expression. To confirm results, immunostaining was repeated twice for each experiment.

Table 4. PSA and androgen receptor antibody source and conditions for immunoperoxidase staining

Antibody	Immunogen	Dilution	Time/Temp.
Anti-Prostate Specific Antigen (PSA)[1, 3]	Human PSA	1:20	24 h / 4 °C
Anti-Androgen Recptor [2, 4]	Androgen Receptor	1:100	1 h / 21 °C

[1] Mouse, Monoclonal

[2] Rabbit, Polyclonal,

[3] Dako

[4] Affinity BioReagent

Invasion Assay Using Boyden Chambers

The *in vitro* invasion assay using Boyden chambers was developed by Drs. Albini and Kleinman (16). This assay examines the ability of cells to invade through a reconstituted basement membrane, "Matrigel" layered on 8 μ pore size Nuclepore filter (figure 6). "Matrigel", secreted by Engelbreth-Holm-Swarm (EHS) mouse tumor cells, is used as an *in vitro* basement membrane. Conditioned medium from NIH/3T3 fibroblast cultures is used in the bottom chamber as a chemoattractant. NIH/3T3 cells were plated in 10 % fetal bovine serum in Dulbeccos's minimum essential medium (DMEM). At sub-confluence, cultures were rinsed with D-PBS and placed on serum-free DMEM containing 50 μg/ml of ascorbic acid which increases matrix protein synthesis (15, 16). Conditioned medium was collected 24 h later.

We have made three modifications to the original assay. First, the cells are allowed to invade for 24 h instead of 5 h. We have found that this time frame allows the cells ample time to invade. Second, instead of determining the number of invaded cells on the basis of counts of only a few random representative microscopic fields, as has been done for most studies described in the literature, we stain the nuclei, extract the dye and read the absorbance. Since there is a direct relationship between cell number and absorbance (14, 15), we believe that in this manner we are able to account for all of the cells that are on the invaded side of the filter. Third, in addition to analyzing only those cells that are attached to the invaded side of the filter, we also count the cell that detach and fall into the bottom chamber. In this manner all of the invading cell are taken into account.

Two human prostatic carcinoma cell lines, DU-145 showing high and LNCaP showing low invasive ability, were used as controls to ensure the success of the assay. The

modified assay was conducted as follows: On the day prior to running the invasion assay, RWPE-1, RWPE-2, DU-145 and LNCaP cells were plated in 10 ml of medium into 100 mm plates under the following conditioned. RWPE-1 and RWPE-2 cells were plated in complete K-SFM medium at a density of 3 million cells per plate. DU-145 were plated in RPMI medium containing 5 % DCS at a density of 2 million cells per plate. LNCaP cells were plated in RPMI medium containing 12% FBS at a density of 4 million per plate. "Matrigel" was diluted 1:20 with cold (4^0C), sterile distilled water on ice to avoid coagulation of the "Matrigel", for a final concentration of 500 μg/ml. Each Nuclepore filter was coated with 25 µg of "Matrigel" in a 50μl solution and left to dry overnight at room temperature under sterile conditions.

Twenty-four hours after plating, cells were washed with D-PBS, incubated with 3 ml of 1 mM EDTA for 8-10 min, dislodged by tapping, neutralized by the addition of 3 ml of RPMI containing 0.1% BSA and recovered by centrifugation (2000 rpm) for 7 min. Pellets were resuspended to obtain 2 million cells per ml in RPMI medium containing 0.1% BSA. 220 μl of NIH/3T3 cell conditioned medium, which served as the chemoattractant, was added to the bottom chamber. The "Matrigel" coated filters were placed, coated side up, in the chambers and the top chamber piece was tightly screwed. 200 μl of the 2 million/ml suspension containing 40,000 cells was added to the top chamber and allowed to remain undisturbed for 5 min before overlaying with 650 μl of RPMI medium containing 0.1% BSA. Cells were allowed to invade for 24 h. Triplicate chambers were prepared for each cell line. The control chambers contained only RPMI medium containing 0.1% BSA. Twenty-four hours later, the medium from the top chambers was carefully aspirated. The filters were carefully removed with forceps and placed, "Matrigel" coated side down, invaded cells up, in a 100 mm petri dish that had

been previously layered with ½ inch of paraffin wax. Cell on the filters and in the bottom chambers were processed as follows:

Filters: Filters were kept immobilized with insect pins. They were stained using the HEMA 3 staining kit by fixing for 5 min in the fixative solution (100% methanol), 1 min in solution I (0.1% eosin Y), 1 min in solution II (0.1% methylene Blue) and rinsed with distilled water. Filters were placed on an alcohol washed glass slide invaded side down and, using a cotton tip applicator, all of the "Matrigel" and non-invaded cell were wiped off. Each filter was then placed in a well containing 300 μl of 0.1 N HCl in 24 well plates and incubated for 15 min at room temperature to extract the dye. 200 μl from each well was then placed in a 96 well plate and the absorbance read using a Titertek microplate reader.

Bottom Chamber: Medium in the bottom chambers was triturated five times and 100 μl was taken from each of the three chambers, pooled into cuvettes containing 10 ml of isotone and triturated ten time. Cell counts were preformed using a Coulter counter. The DU-145 cells that invaded and were attached to the underside of the filter and those counted in the bottom well were each taken as 100%. The two percentages were added to obtain total invasion. Percent absorbance and cell counts for RWPE-1, RWPE-2 and LNCaP cells were calculated using DU-145 as 100%.

Controls

For dose response assays to examine the effects of EGF and bFGF, two different controls consisting of cells treated with K-SFM supplemented with BPE (no EGF) with and without vehicle were used. For dose response assays to examine the effects of TGF–β,

two different controls consisting of cells treated with complete K-SFM with and without the 4 mM HCl in D-PBS containing 1mg/ml of bovine serum albumin vehicle were used. Vehicle treated controls were used to calculate growth response to test agents

For all immunoperoxidase experiments, coverslips receiving no primary antibody but incubated with the secondary Ab followed by the ABC complex and the DAB- nickel solution in the same manner as the experimental coverslips, were used as controls, to insure that the resulting staining was not due to non-specific binding of the reagents. Additionally, in experiments which examined prostate specific antigen and androgen receptor expression induced by mibolerone, cells treated with only K-SFM and K-SFM containing ethanol were also stained. For the invasion assay experiments two chambers containing RPMI medium with 0.1% BSA severed as blank controls.

Results

Growth Requirements

Effects of serum-free and serum containing media on growth of RWPE-1 cells were examined by microplate assay. RWPE-1 cells were plated at densities ranging from 625 cells to 20,000 cells per well in 96 well plates in the two test media, respectively. Results show (Figure 12), that cell growth was almost two times greater when cells were grown in 10% FBS, than when grown in complete K-SFM medium for 5 days. Cells grown in media containing serum could not be maintained beyond 2 passages. Therefore, the best medium for the maintenance of RWPE-1 was complete K-SFM medium.

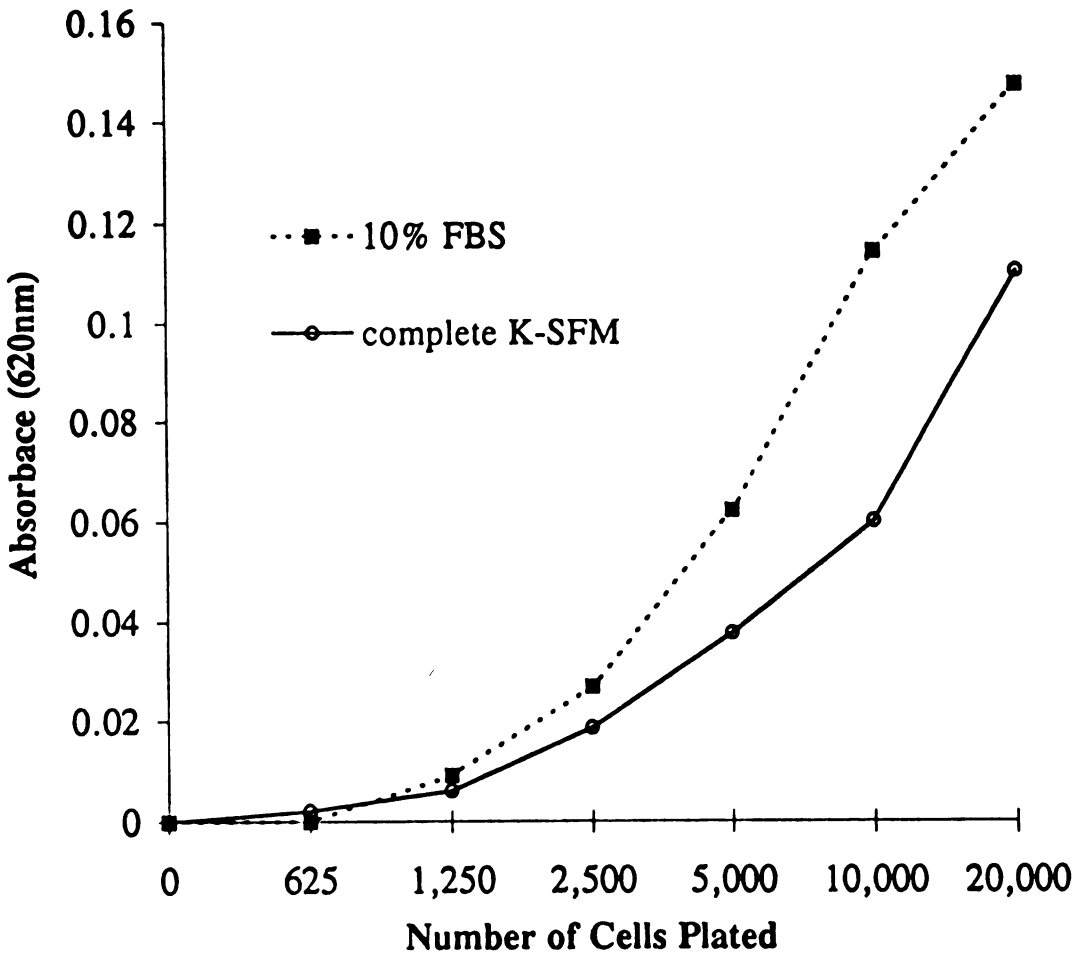

Figure 12. Effects of Serum -containing and Serum-free Media on the Growth of RWPE-1 Cells. Effects of K-SFM containing 10% FBS (■) and complete K-SFM (○) on the growth of RWPE-1 cells were examined. Cells were plated at densities ranging from 625-20,000 cells. Plates were fixed 5 days after plating and absorbance was plotted against cells density.

To establish cell doubling times, growth curves for RWPE-1 and RWPE-2 cells were generated for cell densities ranging from 625 cells to 20,000 cells using the microplate assay. Absorbance data were plotted against time (days) for each cell density. Figure 13 and 14 are growth curve graphs representing one of the four growth experiments conducted for each cell line. Because of the direct relationship between absorbance and cell number (12), the plotted data were used to determine cell doubling times. Cell doubling time in log phase for RWPE-1 was estimated to be 48 h when cells were plated at high densities of 10, 000 and 20,000 and 72 h at low densities of 625, 1,250, 2,500 and 5,000 cells (Figure 13). For the RWPE-2 cells, the doubling time in log phase was estimated to be 32 h at high density and 50 h at low density (Figure 14). The average doubling times for RWPE-1 and RWPE-2 cells were estimated to be 60 h and 41 h, respectively.

Expression of Intermediate Filament Proteins and Factor VIII

RWPE-1 and RWPE-2 cells have an epithelial cell morphology as demonstrated by hematoxylin & eosin staining in Figure 15 a and 16 a, respectively. Expression of cytokeratin 8, cytokeratin 18, basal cell cytokeratins 1, 5, 10 and 14, desmin and Factor-VIII was determined by immunoperoxidase staining using monoclonal. RWPE-1 and RWPE-2 cells showed strong cytoplasmic staining for cytokeratin 8 and cytokeratin 18 (Figure 15 e and 16 e, 15 f and 16 f, respectively) but moderate to weak staining for vimentin (Figure 15 i and 16 i, respectively). Expression of basal cell cytokeratins was weak and heterogeneous in distribution, i.e. some cells did not show expression (data not shown). Desmin and Factor-VIII expression was absent. (Figure 15g and 16 g, 15 h and 16 h, respectively).

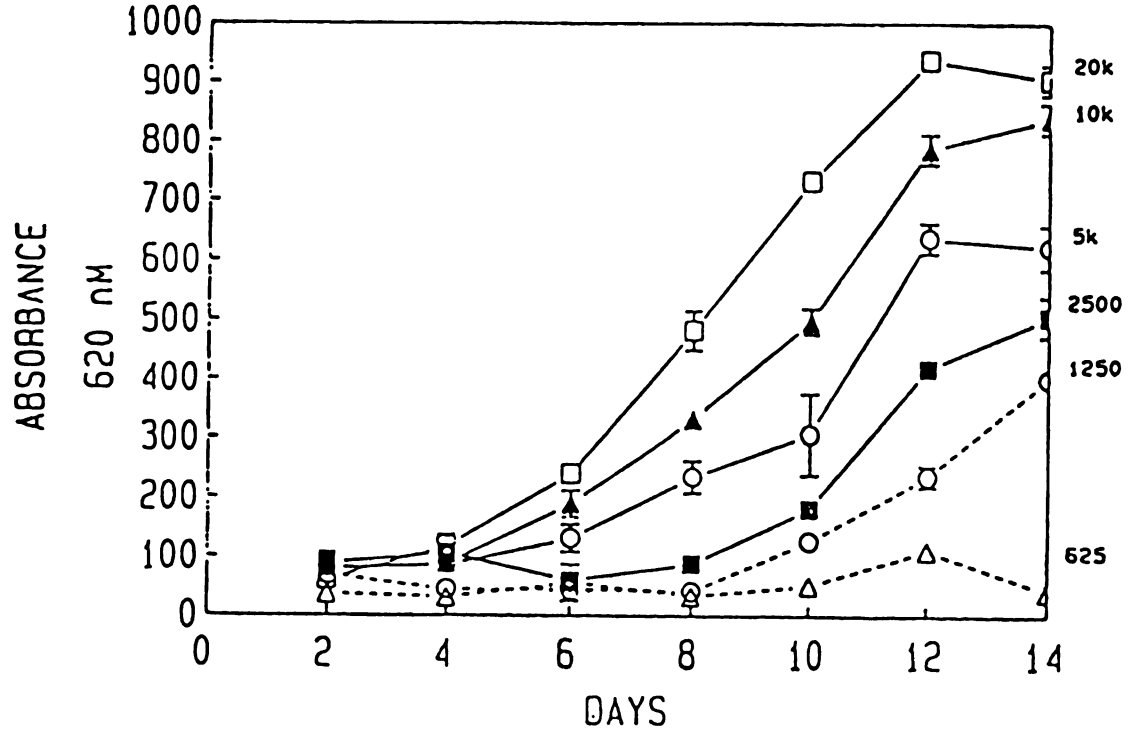

Figure 13. **Growth Curves for RWPE-1.** Cells were plated at densities ranging from 625-20,000 cells in complete K-SFM in 96 well plates. Plates were fixed at 2, 4, 6, 8, 10 and 14 days and absorbance was plotted against time.

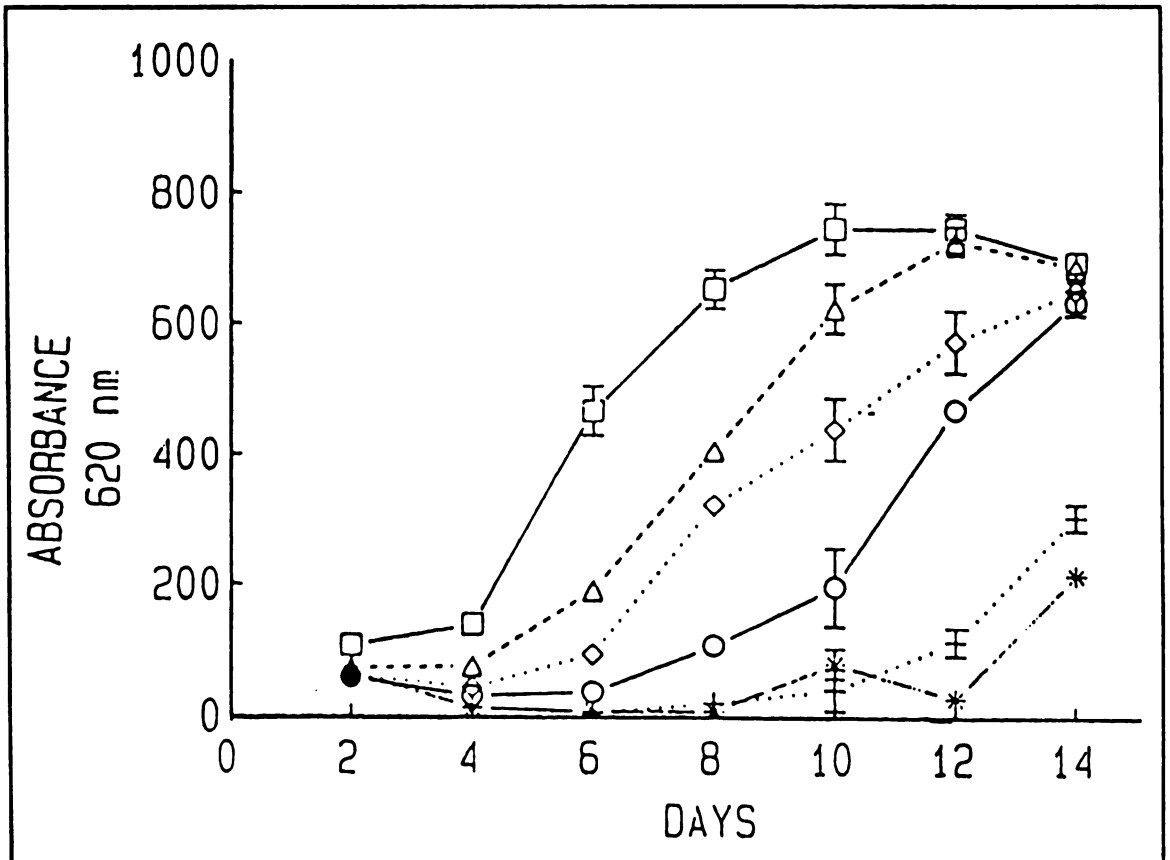

Figure 14. **Growth Curves for RWPE-2.** Cells were plated at densities ranging from 625-20,000 cells in complete K-SFM in 96 well plates. Plates were fixed at 2, 4, 6, 8, 10 and 14 days and absorbance was plotted against time.

Figure 15. Characterization of RWPE-1 Cells on the Basis of Cellular Proteins. Proteins were detected by immunoperoxidase staining. a. hematoxylin and eosin staining; b. positive staining for PSA; c. positive nuclear staining for androgen receptor. Cells for b. and c. were pretreated for 6 days with 5 nM mibolerone; d. a control lacking primary antibody; e. and f. positive staining for cytokeratin 8 and 18, respectively; g. and h. absence of staining for desmin and Factor VIII, respectively; i. positive staining for vimentin. Bar, 20mM, (625X).

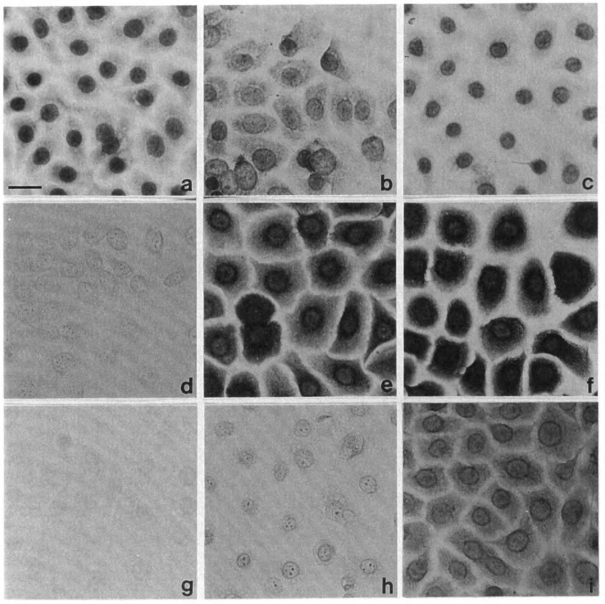
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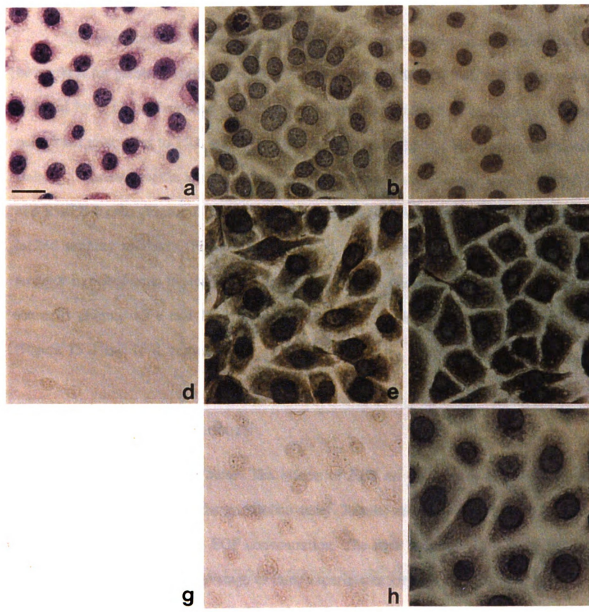
Figure 16. Characterization of RWPE-2 Cells on the Basis of Cellular Proteins. Proteins were detected by immunoperoxidase staining. a. hematoxylin and eosin staining; b. positive staining for PSA; c. positive nuclear staining for androgen receptor. Cells for b. and c. were pretreated for 6 days with 5 nM mibolerone; d. a control lacking primary antibody; e. and f. positive staining for cytokeratin 8 and 18, respectively; g. and h. absence of staining for desmin and Factor VIII, respectively; i. positive staining for vimentin. Bar, 20mM, (625X).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Expression of Prostate Specific Antigen and Androgen Receptor

The prostate specific antigen and androgen receptor expression are considered to be markers for prostatic epithelial cells. The expression of PSA and androgen receptor was induced by 5 nM mibolerone treatment for 6 days and detected using monoclonal and polyclonal antibodies to PSA and androgen receptor, respectively, by immunoperoxidase staining. Prostate specific antigen expression was detected in mibolerone treated RWPE-1 and RWPE-2 cells (Figure 15 b and 16 d, respectively) as compared to the control which lacked primary antibody (Figure 15 d and 16 d, respectively). Expression of androgen receptor in mibolerone treated RWPE-1 and RWPE-2 cells was present in the nucleus of the cells (Figure 15 c and 16 c, respectively). Controls did not show nuclear staining (Figure 15 d and 16 d, respectively).

Effect of Growth Factors

Epidermal Growth Factor. The effects of EGF on the growth of RWPE-1 and RWPE-2 were examined by the microplate assay. Results are shown in Figure 17 where absorbance is plotted against EGF concentration. The dose response curves represented in Figure 17 for EGF are the average of three experiments for each cell line after five days of treatment. A dose dependant stimulation of cell growth was observed for EGF in RWPE-1 cells with a maximal growth increase to 150% of control 10 ng/ml. In contrast, RWPE-2 cells showed an initial increase in growth to 113% of control at 0.625 ng/ml, but did not show further increase with increasing concentration up to 5 ng/ml. Exposure to 10 ng/ml, however, caused a further increase to 133% of control.

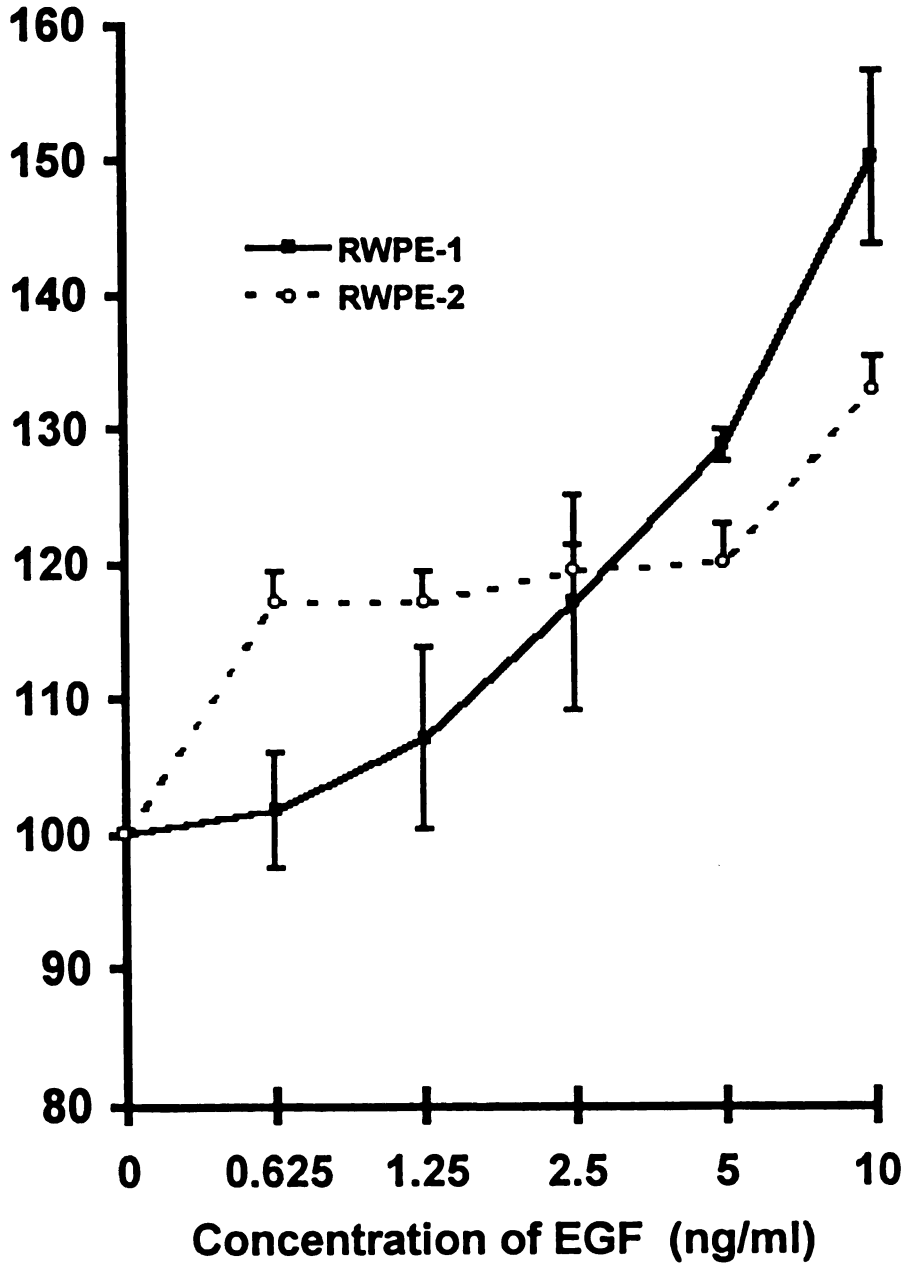

Figure 17. **Epidermal Growth Factor.** Effects of EGF on the growth of RWPE-1 (■) and RWPE-2 (◯) cells. 5,000 cells were plated in K-SFM medium supplemented with BPE in 96 well plates. Plates were fixed at seven days and absorbance was plotted against EGF concentrations.

Basic Fibroblast Growth Factor. The effects of bFGF on the growth of RWPE-1 and RWPE-2 cells were, also, examined by the microplate assay. Effects of bFGF on RWPE-1 and RWPE-2 cells after seven days of treatment are shown in Figure 18 with the absorbance plotted against bFGF concentration. The dose response curves represented in Figure 18 are the average of two experiments for each cell line. After seven days of treatment, stimulation of cell growth for RWPE-1 was observed at 2.5 ng/ml and higher concentrations with maximal growth increase to 152% of control at 10 ng/ml. On the other hand RWPE-2 cells did not show growth stimulation at concentrations lower than 10 ng/ml, with a maximal increase to 115% of control at this concentration.

Transforming Growth Factor-β. Effects of TGF-β on the growth of RWPE-1 and RWPE-2 cells were examined by the microplate assay. The effects of TGF-β on RWPE-1 and RWPE-2 cells after seven days of treatment are shown in Figure 19 with the absorbance plotted against TGF-β concentration. The dose response curves represented in Figure 10 are the average of three experiments for each cell line. After seven days of treatment, a marked inhibition of cell growth for RWPE-1 was observed at 0.625 ng/ml with maximal growth inhibition to 63% of control at 10 ng/ml. Similar inhibition of cell growth for RWPE-2 was also observed at 0.625 ng/ml, with a maximal growth inhibition to 68% of control at 10 ng/ml. However, the overall decrease in RWPE-2 cells was slightly lower than that of RWPE-1 cells.

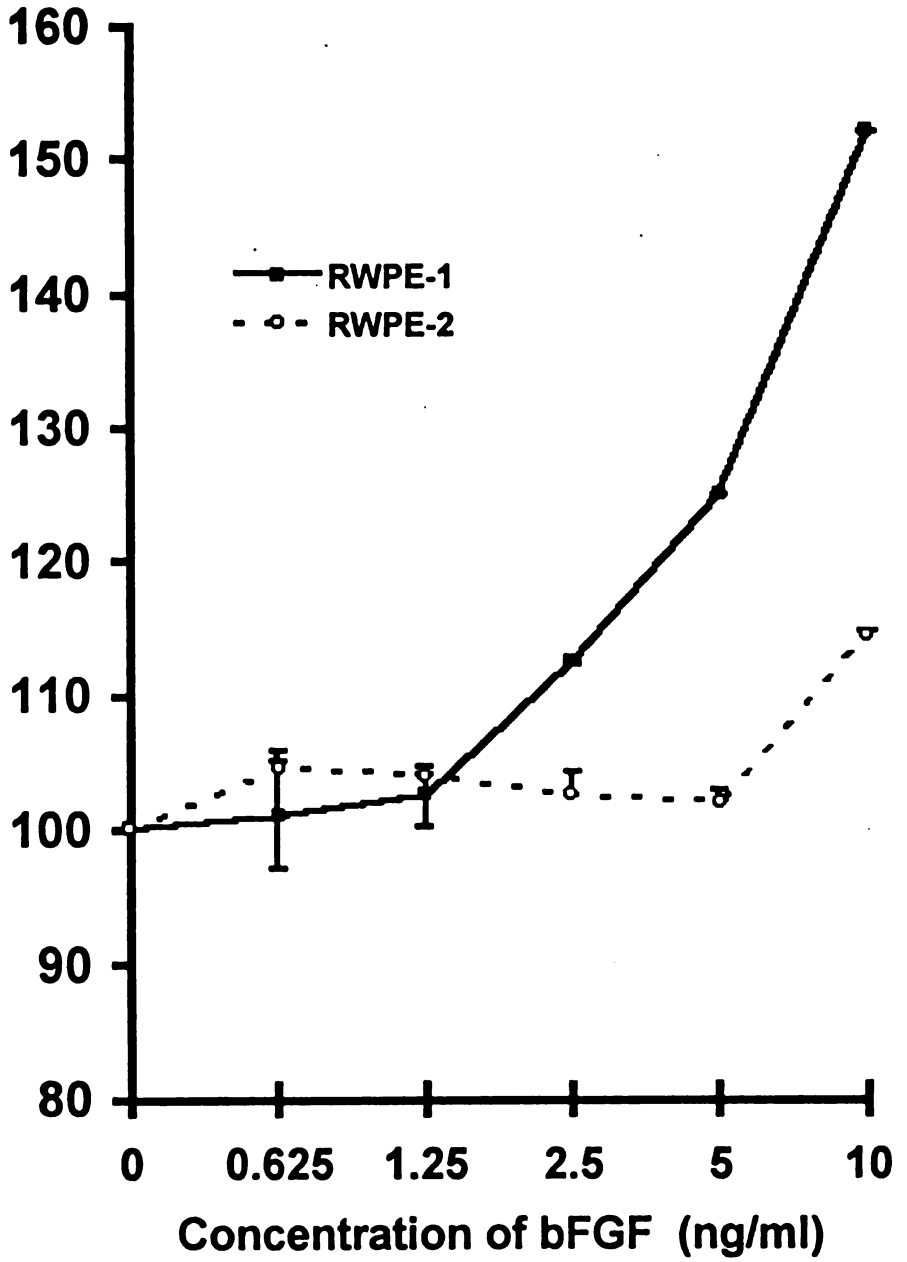

Figure 18. **basic Fibroblast Growth Factor.** Effects of bFGF on the growth of RWPE-1 (■) and RWPE-2 (○) cells.5,000 cells were plated in K-SFM medium supplemented with BPE in 96 well plates. Plates were fixed at seven days and absorbance was plotted against bFGF concentrations.

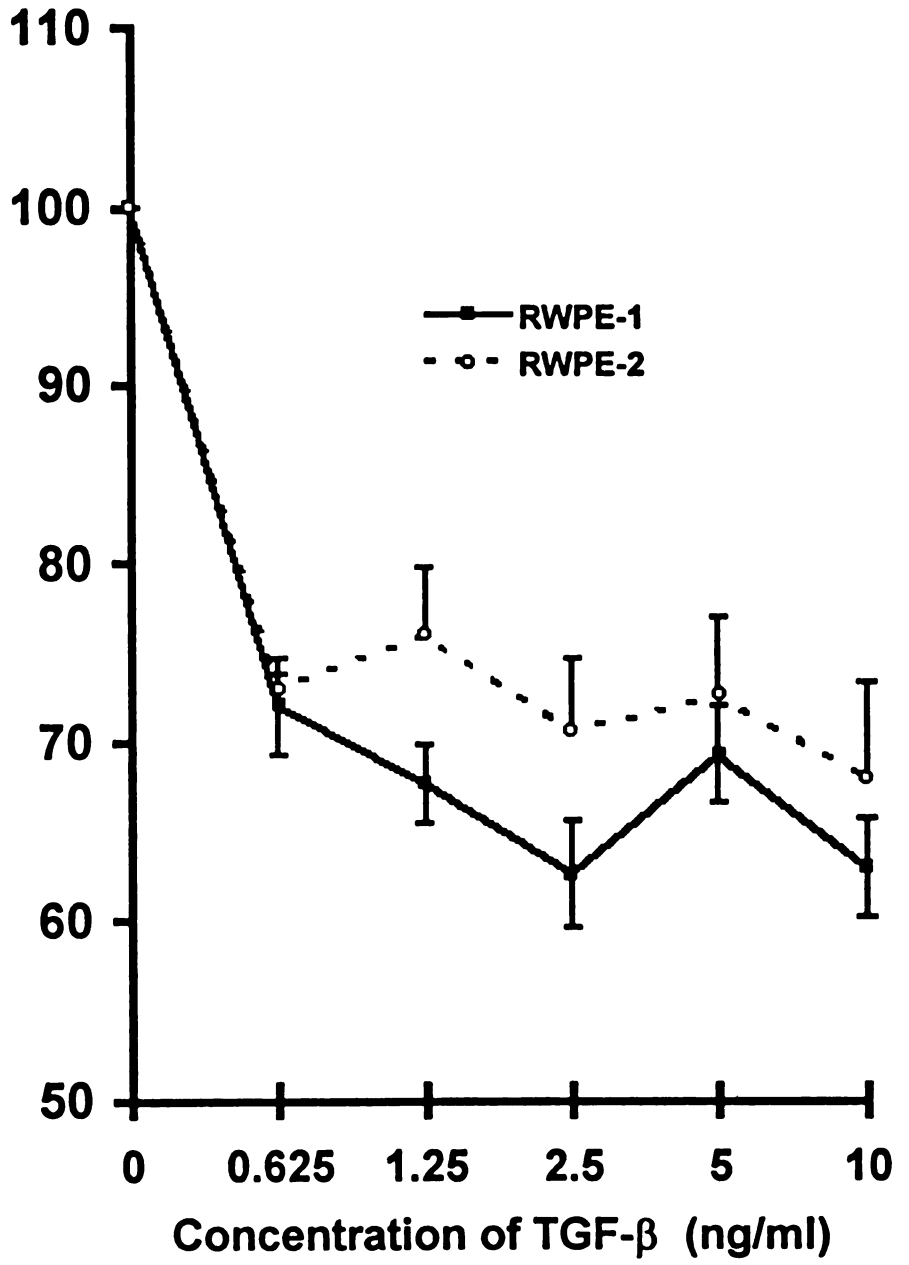

Figure 19. Transforming Growth Factor-β. Effects of TGF-β on the growth of RWPE-1 (■) and RWPE-2 (◯) cells. 10,000 cells were plated in complete K-SFM medium in 96 well plates. Plates were fixed at seven days and absorbance was plotted against TGF-β concentrations.

In Vitro Invasion

The invasive ability RWPE-1 and RWPE-2 cells was determined and compared with the invasive ability of DU-145 and LNCaP cells, where DU-145 invasion was set at 100%. Results show (Figure 20) that in comparison to the highly invasive DU-145 cell line, the low invasive LNCaP cell line showed 19% invasion. RWPE-1 cells were found to be non-invasive showing only a negligible 1% invasion, while RWPE-2 cells were invasive, showing a 49% invasion.

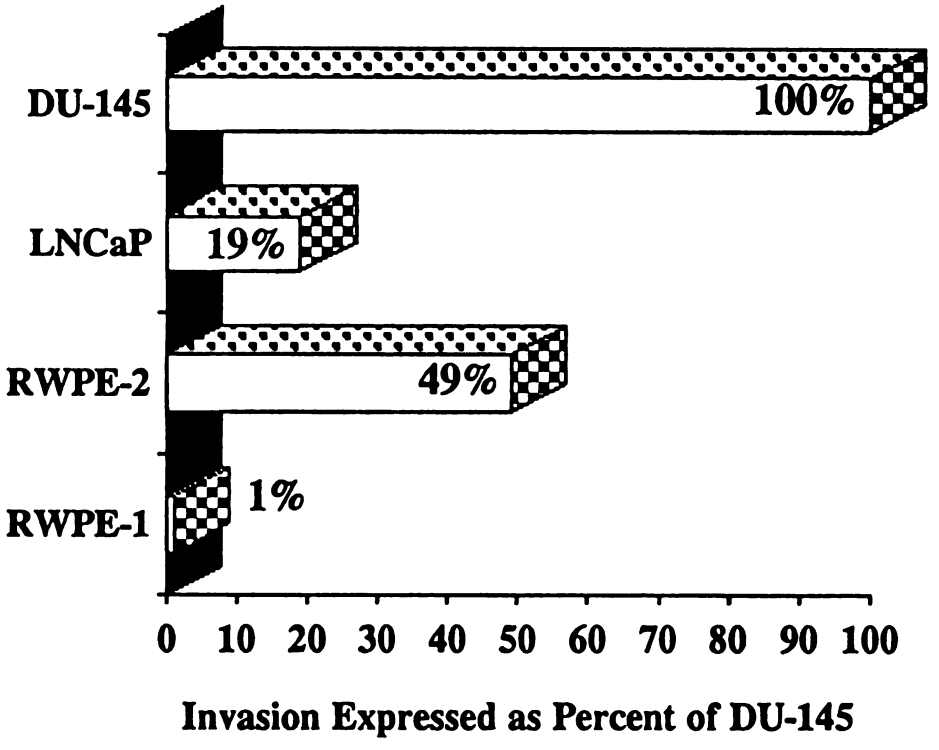

Figure 20. ***In Vitro* Invasion.** Invasive ability of DU-145, LNCaP, RWPE-1 and RWPE-2 cell lines was examined by the Boyden chamber assay. 400,000 cells were plated on each "Matrigel"-coated filter and allowed to invade for 24 hours. Invasion by the highly invasive DU-145 cell line was set at 100%.

Discussion

Expression of cytokeratin intermediate filament is a characteristic of epithelial cells (17,18). Secretory, luminal prostatic epithelial cells express cytokeratin 8 and 18 (19, 20, 21). Using immunoperoxidase staining, I have established the expression of cytokeratin 8 and 18 in RWPE-1 and RWPE-2 cells (Figure 14 and 15). This indicates that RWPE-1 and RWPE-2 cells are derived from the luminal, secretory cells of the prostatic epithelium. Possible derivation from muscle or endothelial cells was excluded by their lack of desmin and Factor VIII expression (Figure 14 and 15). The fact that RWPE-1 and RWPE-2 cells showed for weak expression of vimentin, a mesenchymal cell marker, is not surprising since epithelial cells that have been placed in culture are known to sometimes show vimentin expression (18). Both RWPE-1 and RWPE-2 cells also heterogeneously express basal cell cytokeratins. This may be due to the presence of a small population of cells that express characteristics similar to those of amplifying cells. Amplifying cells are thought to represent cells in transition from stem or basal to luminal cells and they may express cytokeratins that are characteristic of both basal and luminal cells such as cytokeratins 5, 8, 15, 16 and 18 (19, 21, 24-26).

Prostatic epithelial origin was established by analysis of two prostate specific characteristics; 1) induction of prostate specific antigen expression and 2) espression of androgen receptor. Expression of prostate specific antigen is a differentiated function of normal prostate epithelial cells (2,27). With the exception of the three cell lines presented in this thesis and the two malignant prostatic cell lines, LNCaP and ALVA 101, none of the established immortalized or malignant cell lines have been shown to express PSA. I believe that this inability to demonstrate PSA expression, in at least some cases, may be

due to the experimental conditions. Initial experiments to demonstrate PSA expression in RWPE-1 and RWPE-2 cells by immunoperoxidase, using monoclonal antibodies to human prostate specific antigen, showed no PSA staining. Based on the fact that PSA expression is induced by androgen stimulation, I reasoned that the lack of PSA staining was due to the absence of androgens in the medium. Thus, in order to detect PSA expression, RWPE-1 and RWPE-2 cells should be pre-treated with androgens. Therefore, I supplemented the complete K-SFM medium with the naturally occurring androgen, 5α-DHT at 5nM concentration. After growing RWPE-1 cells in the presence of 5α-DHT for several days, I was able to demonstrate weak PSA staining. 5 α-DHT has a half life of approximately 2 hours in culture medium (2). Therefore, unless the medium was changed frequently, continuous androgenic stimulus will not have been present. To insure the presence of androgen in the medium, mibolerone, a synthetic non-metabolizing androgen, was used for subsequent experiments. I was able to successfully induce higher levels of prostate specific antigen expression in RWPE-1 and RWPE-2 cells by treating the cells with 5 nM of mibolerone. Both RWPE-1 and RWPE-2 cells clearly show expression of prostate specific antigen (Figure 14 and 15).

Because androgens exert their influence on prostatic epithelial cells via the androgen receptor, the expression of androgen receptor in RWPE-1 and RWPE-2 cells was also examined by immunoperoxidase staining using polyclonal antibodies. RWPE-1 and RWPE-2 cells showed nuclear androgen receptor expression after 6 days of treatment with 5 nM of mibolerone (Figure 14 and 15) Pre-treatment with mibolerone was done for two reasons; first to up-regulate the receptor and thereby facilitate the detection of the low abundance of nuclear androgen receptor and second, to attempt to mimic the conditions present *in vivo*.

During the process of immortalization of RWPE-1 cells and as a result of their further transformation with Ki-*ras* to produce RWPE-2 cells, response to growth factors may be affected. Therefore, the RWPE-1 and RWPE-2 cell lines were characterized with regards to their response to growth factors. This was done to ascertain if their responses either mimic normal prostatic epithelial cells or to document how they differ. The effects of epidermal growth factor, basic fibroblast growth factor and transforming growth factor- b, the three major growth factors involved in the regulation of the normal prostatic epithelium, were examined.(2)

In response to exogenous EGF, RWPE-1 showed a dose dependant increase in cell proliferation (Figure 17). RWPE-1 cells were more sensitive to growth stimulation with EGF than RWPE-2 cells as indicated by a maximal growth stimulation to 150% of control for RWPE-1 and 132% for RWPE-2 at 10 ng/ml of EGF. Also RWPE-2 did not show a dose response at EGF concentrations between 0.625 to 5 ng/ml. This difference in sensitivity between RWPE-1 and RWPE-2 cells is even more evident in their response to bFGF (Figure 18). When exogenous bFGF is added, RWPE-1 cells show a dose-dependent increase with a maximal growth stimulation to 152% of control at 10 ng/ml of bFGF. However, RWPE-2 cells did not show any response in the dose-range between 0.625 to 5 ng/ml and only an increase to 115% of control was seen at 10 ng/ml. Comparison of growth factor response with the other human papillomavirus immortalized prostatic cell line, PZ-HPV-7, was not possible, due to the lack of published data for this cell line (2). The differences in response to EGF and bFGF between the two cells lines reflect the transformation of RWPE-2 cells with Ki-*ras*. Since autocrine production of growth factors by cancer cells is a recognized phenomenon, it is possible that RWPE-2 cells have lost their sensitivity to growth factors due to autocrine growth factor production.

RWPE-1 and RWPE-2 cells showed growth inhibition when exposed to exogenous TGF-β. Both cell lines show a dose dependant decrease in growth in response to TGF-β (Figure 19). Although there was little difference in the response between RWPE-1 and RWPE-2 cells, RWPE-1 cells appeared to be slightly more sensitive to TGF-β than RWPE-2 cells. It should be noted that for examining the effects of TGF-β, experiments were conducted in medium which contained EGF. Therefore, the observed inhibitory response consists of the following: 1) that TGF-β inhibited EGF-induced growth and 2) that TGF-β caused further inhibition in both cell lines.

My results show that human papillomavirus immortalization did not interfere with normal prostatic differentiated functions as demonstrated by the ability of RWPE-1 and RWPE-2 cells to: 1) express prostate specific antigen in response to androgens, 2) express androgen receptor and 3) express luminal prostatic epithelial cytokeratins 8 and 18. However transformation with Ki-*ras* altered the response of RWPE-2 cell to growth factors as compared to RWPE-1 cells.

The invasive potential of RWPE-1 and RWPE-2 cells was determined by their ability to invade a reconstituted basement membrane *in vitro* using the Boyden chamber assay. Invasion by cancer cells through the basement membrane *in vivo* is one of the first steps in the progression to metastasis (27). Since normal prostatic epithelial cells do not invade but simply rest on the basement membrane, it would be anticipated that RWPE-1 cells would not be invasive, while RWPE-2 cells, because of the Ki-*ras* infection, would be invasive. The invasive ability of RWPE-1 and RWPE-2 was compared with that of the highly invasive prostate cancer cell line DU-145 and low invasive LNCaP. Invasion by DU-145 cell line was set at 100%, where LNCaP cells showed a 19% invasion. Results

show that the HPV-18 immortalized RWPE-1 cells were not invasive. However, RWPE-2 cells showed 49% invasion compared to DU-145 cells (Figure 20). Other research on RWPE-1 and RWPE-2, not reported in this thesis, investigated their tumorigenicity in nude mice and anchorage independence by growth in soft agar. Results obtained from these studies were found to correlate with the invasive ability. The non-invasive RWPE-1 cell line was found to be anchorage dependant and non-tumorigenic. In contrast, the invasive RWPE-2 cell line was tumorigenic and anchorage independent. The differences between RWPE-1 and RWPE-2 with regard to invasive ability, the ability to grow in soft agar and tumorigenicity in nude mice, are apparently due to the presence of Ki-*ras* oncogene in RWPE-2 cells. This further demonstrates the association between *ras* expression and tumorigenicity. Therefore, it can be concluded that RWPE-1 cells do not posses malignant characteristics, while RWPE-2 cells do express malignant characteristics.

The epithelial origin of RWPE-1 and RWPE-2 cells has been established by the expression of epithelial cell specific cytokeratins 8 and 18, and absence of desmin and Factor VIII expression. The prostatic epithelial origin of the RWPE-1 and RWPE-2 cell lines has been established by the expression of two prostate specific characteristics: 1) the presence of androgen receptor and 2) the ability of RWPE-1 and RWPE-2 cells to induce prostate specific antigen expression in response to androgens. Additionally, different responses of RWPE-1 and RWPE-2 cells to prostatic regulatory growth factors EGF, bFGF and TGF-β have been demonstrated. RWPE-1 cells were found to be non-invasive, anchorage dependant and non-tumorigenic, while RWPE-2 cells were found to be invasive, anchorage independent and tumorigenic.

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Conclusion

Characterization of newly established human prostatic cell lines is an essential step for establishing their origin and their ability to express differentiated functions, before determining their utility for studies on prostate carcinogenesis and prevention. PWR-1E, RWPE-1 and RWPE-2 cell lines were characterized on the basis of their expression of intermediate filament proteins, hemostatic Factor VIII, androgen receptor and prostate specific antigen and growth response to growth factors which are known to regulate prostate cells *in vivo*.

Intermediate filament proteins cytokeratins, vimentin and desmin and hemostatic Factor VIII were investigated using monoclonal antibodies by immunoperoxidase staining. This was done to establish their epithelial origin by expression of cytokeratins and absences of desmin, vimentin and Factor VIII. Androgen receptor and prostate specific antigen expression was also demonstrated by immunoperoxidase staining after cells were treated with mibolerone, a synthetic androgen, to induce their expression. This was done to establish luminal prostatic epithelial origin. Growth response to androgens, in the case of PWR-1E, and to epidermal growth factor, basic fibroblast growth factor and transforming growth factor-β for all three cell lines, was determined by microplate assay to determine their ability to mimic the response of normal prostatic epithelial cells.

absence or presences of malignant characteristics.

PWR-1E, RWPE-1 and RWPE-2 cells express epithelial specific cytokeratins 8 and 18, and do not express desmin and Factor VIII. These characteristics establish the epithelial origin of the three cell lines. All three cell lines express androgen receptor and prostate specific antigen in response to treatment with mibolerone. PWR-1E, RWPE-1 and RWPE-2 cells all showed stimulation of cell growth in response to EGF and bFGF, and inhibition in response to TGF-β. Additionally, PWR-1E immortalized cells showed low invasive ability but did not form tumors in nude mice. RWPE-1 immortalized cells were non-invasive and non-tumorigenic, while RWPE-2 cells were both tumorigenic and invasive. From these results, it can be concluded that the three cell lines are of prostatic epithelial origin and they retain the ability to respond to androgens and expression of differentiated function as shown by PSA expression. The PWR-1E, RWPE-1 and RWPE-2 cell lines provide fully characterized and highly valuable *in vitro* cell culture models for studies on prostate carcinogenesis and chemoprevention.