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ACINAR MORPHOGENESIS IN NON-MALIGNANT
HUMAN PROSTATIC EPITHELIAL CELLS
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ACINAR MORPHOGENESIS IN NON-MALIGNANT HUMAN PROSTATIC EPITHELIAL CELLS AND ITS LOSS IN MALIGNANT CELLS

By

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ABSTRACT

ACINAR MORPHOGENESIS IN NON-MALIGNANT HUMAN PROSTATIC EPITHELIAL CELLS AND ITS LOSS IN MALIGNANT CELLS

By

Diana Bello-DeOcampo

During the multi-step process of carcinogenesis in epithelial cells, multiple, cumulative, genetic and epigenetic changes occur as cells progress from a normal to a malignant, invasive and metastatic phenotype. During tumor progression, additional changes lead to the development of autonomous, growth factor and hormone independent, highly invasive metastatic cells. Little is known about the multiple steps in prostate carcinogenesis and tumor progression. For the present study, it was hypothesized that during prostate carcinogenesis and progression, the following changes occur: 1) that there is a progressive loss of the ability of epithelial cells to undergo acinar morphogenesis, by polarization, to form well defined acini with lumens; 2) that adhesion of cells to laminin is altered and this occurs as a result of changes in the expression of laminin integrin receptors which bind cells to laminin; 3) that the loss of abnormal expression of integrins imparts to the cells increased invasive ability; and 4) that cells show altered response to both stimulatory and inhibitory growth factors, which occurs as a result of changes in the level of expression of growth factors and their receptors.

Using a family of seven human prostate epithelial cell lines, which include a human papillomavirus 18 immortalized, non-tumorigenic cell line RWPE-1, and several progressively more tumorigenic cell lines, derived from RWPE-1 cells, a novel, 3-dimensional human prostate cell culture model was developed to test the hypotheses. In this cell model, RWPE-1 cells mimic normal epithelial acinar morphogenesis as it occurs *in vivo*. Immunocytochemical and confocal microscopy methods were employed. Results show that RWPE-1 cells form acini only when plated in a matrix of Matrigel or laminin, but not in collagen or fibronectin. When binding of cells to laminin is blocked by antibody to laminin, cells fail to form acini. RWPE-1cells show strong expression of the laminin-specific $\alpha 6\beta 1$ integrin receptor at their

basal end. When the α6 or β1 integrin subunits were blocked by their anti-functional antibody, the ability of cells to form acini was reduced. This loss was more dramatic with the β1 integrin antibody. The six tumorigenic cells lines showed a progressive loss of acini forming ability with increasing malignancy. While the least malignant WPE1-NA22 cells showed α6β1 integrin expression similar to that seen in RWPE-1 cells, a loss of α6 and diffuse expression of β1 intergrin was observed in the most malignant, WPE1-NB26 cells. This abnormal integrin expression showed a direct relationship to the loss of ability of cells to undergo acinar morphogenesis and their increased invasive ability.

Exposure of RWPE-1 cells to growth stimulatory exogenous EGF caused a dose-dependent decrease in acinar formation. These results suggest that, because of increased cell proliferation, malignant cells lose the ability to form acini and the degree of loss is related to abnormal integrin expression and increased invasive ability. Further, the growth inhibitory, exogenous TGF-β1 and β2 were more effective inhibitors of growth of RWPE-1 and WPE1-NA22 cells than that of WPE11-NB26 cells. Both TGF-β1 and β2 enhanced the ability of WPE1-NA22 cells to form acini and exposure of cells to anti-functional TGF-β antibody resulted in a loss of polarization and acini forming ability. While RWPE-1 cells expressed endogenous TGF-β (TFG-β1>TGF-β2>TGF-β3), and TGF-β RII and III, there was a progressive loss of expression of both the TGF-β and its receptors with increasing malignancy.

Taken together, these results demonstrate that during prostate carcinogenesis and tumor progression, there is a progressive loss of the normal expression of laminin integrin receptors. Increased autocrine stimulation by growth factors, such as EGF, and the decrease or loss of TGF-β and its receptors, the key negative regulators, leads to a loss of growth homeostasis. Thus, changes in the expression of laminin integrins, growth factors and their receptors, contribute to the inability of cancer cells to undergo acinar morphogenesis. This is associated with increased invasive ability. These results facilitate the development of agents which may be used to restore normal acinar morphogenesis and, thus, block carcinogenesis, invasion and metastasis.

To my family and my mentor

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TABLE OF CONTENTS

List of Tables..... *xi*

List of Figures..... *xii*

List of Abbreviations..... *xv*

Introduction..... 1

Hypotheses..... 3

Chapter One. The Human Prostate: background..... 5

Anatomy..... 6

Development of the Human Prostate..... 7

Prostate Cancer 18

Chapter Two. Materials & Methods..... 29

Materials..... 30

Media and Cell Culture Reagents..... 30

Antibodies..... 30

Growth Factors and Hormones 31

Extracellular Matrix (ECM) 31

Other Materials 31

Methods..... 32

Cell Lines 32

Cell Culture 33

Cell Proliferation in Monolayer Cultures..... 34

EGF and mibolerone combined dose response assay..... 34

Transforming growth factor (TGF)-β1, β2 & β3 dose response assay 35

Immunocytochemical analysis of using monolayer cultures 35

Laser scanning microscopy 37

In Vitro 3-Dimensional Culture Assays 38

Acinar morphogenesis assay 38

Immunocytochemical analysis of Matrigel cultures 39

Induction of functional differentiation by androgen in Matrigel cultures 41

Analysis of individual basement membrane components..... 42

Induction of acinar formation assay..... 43

Inhibition of acinar formation assay..... 44

EGF effects on acinar morphogenesis..... 44

Invasion assay 46

Chapter Three. Extracellular Matrix and Integrins: background 50

Epithelial cell polarity 51

Extracellular matrix (ECM)..... 52

Integrins 59

Prostate epithelial markers..... 69

Chapter Four. Acinar Differentiation by Non-malignant Immortalized Human Prostatic Epithelial Cells and Its Loss by Malignant Cells 76

Abstract 77

Introduction 78

Materials and methods 81

Materials 81

Methods 81

Results 84

Morphogenesis in 3-Dimensional cultures of RWPE-1, non-tumorigenic cells and immunostaining for laminin, α6 and β1 integrins, and PSA 84

Comparison of the ability for structural morphogenesis by the non-tumorigenic RWPE-1 cells with that of the tumorigenic RWPE-2 and DU-145 cells in 3-D cultures 88

The invasive ability shows an inverse relationship with the ability to undergo acinar morphogenesis 88

Discussion 90

Chapter Five: Extracellular Matrix and Integrins Modulate Acinar Morphogenesis by RWPE-1 Cells 101

Abstract 102

Introduction 103

Results 106

Laminin is required for acinar morphogenesis 106

β1 integrin is essential for acinar formation 111

Discussion 114

Chapter Six. Malignant Human Prostatic Epithelial Cells Lose the Ability to Undergo Acinar Morphogenesis 125

Abstract 126

Introduction 127

Results 129

Loss of acinar morphogenesis in 3-D cultures by malignant cell lines 129

Invasive ability shows an inverse relationship with the ability to undergo acinar morphogenesis..... 133

Alterations in integrin α 6β1 expression in malignant cell lines.....134

Deregulation of growth by EGF inhibits acinar morphogenesis.....137

Discussion 141

Chapter Seven. Regulation of Acinar Morphogenesis by TGF-β in Non-malignant and Malignant Human Prostatic Epithelial Cells.....152

Abstract 153

Introduction 155

Results 157

Effects of TGF-β isoforms on the growth of RWPE-1 cells ..157

RWPE-1 cells express TGF-β1, 2 and 3 and the TGF-β RII and RIII receptors 159

TGF-β1 and TGF-β2 are necessary for acinar morphogenesis.159

Effects of TGF-β isoforms on the growth of WPE1-NA22 and WPE1-NB26 cells.....162

Alteration in TGF-β1, 2 and 3 and TGF-β RII and
TGF-β RIII receptor expression in malignant WPE1-NA22
and WPE1-NB26 cells 164

Restoration of acinar-forming ability by
TGF-β1 and TGF-β2.....168

Discussion 169

List of Tables

Table 2-1. Antibodies and conditions for immunocytochemistry on monolayer cultures 37

Table 2-2. Antibodies and conditions for immunocytochemistry of Matrigel cultures 41

Table 2-3. Neutralizing antibodies and conditions for inhibition of acinar formation assay 46

Table 3-1. Properties of basement membrane components 55

Table 3-2. Integrins and their ECM binding sites 63

List of Figures

Figure 1-1. Three-dimensional model of the three glandular zones of the prostate..... 6

Figure 1-2. Differentiation of the urogenital sinus in the human male..... 8

Figure 1-3. High grade PIN demonstrates 4 architectural patterns 21

Figure 1-4. Proposed model for prostatic carcinogenesis..... 22

Figure 3-1. Pseudo-stratified prostatic epithelial cell layer..... 54

Figure 3-2. A current model of basement membrane structure..... 55

Figure 3-3. Schematic model of Laminin-1..... 57

Figure 3-4. A model of laminin-entactin/nidogen complex..... 58

Figure 3-5. A schematic structure of integrins..... 61

Figure 3-6. The integrin family..... 61

Figure 3-7. Integrin receptor binding to basement membrane 62

Figure 3-8. Active sites for integrins..... 65

Figure 4-1. Appearance of RWPE-1 cells on Matrigel..... 85

Figure 4-2. 3-D cultures..... 87

Figure 4-3. Cell organization on Matrigel and relationship with invasion..... 89

Figure 5-1. Acinar morphogenesis by RWPE-1 cells in Matrigel..... 107

Figure 5-2. Optical serial sections of RWPE-1 acinus grown in Matrigel..... 107

Figure 5-3. Optical serial sections of RWPE-1 acinus grown in laminin-1..... 109

Figure 5-4. RWPE-1 cells on ECM matrices. 109

Figure 5-5. Acinar formation on matrix components 110

Figure 5-6. Inhibition of acinar formation by anti-laminin neutralizing antibody 110

Figure 5-7. α6 Integrin expression 112

Figure 5-8. IgG control 112

Figure 5-9. β1 Integrin expression 112

Figure 5-10. α2 Integrin expression..... 113

Figure 5-11. β4 Integrin expression 113

Figure 5-12. Inhibition of acinar formation by anti-integrin neutralizing antibodies 115

Figure 6-1. Acinar formation of immortalized RWPE-1 cell line and malignant prostate epithelial cell lines..... 131

Figure 6-2 Acinar formation of immortalized non-tumorigenic and tumorigenic prostate epithelial cell lines grown in Matrigel 132

Figure 6-3. Invasive ability of non-tumorigenic immortalized and malignant human prostate epithelial cells..... 135

Figure 6-4. Relationship between acini-forming ability and invasive ability..... 135

Figure 6-5. α6β1 laminin integrin expression 136

Figure 6-6. Effects of EGF on the growth of RWPE-1 cells and its modulation by androgen mibolerone.....138

Figure 6-7. Reduction of acinar formation by EGF.....140

Figure 6-8. Enhancement of acinar formation by EGF neutralizing antibody.....140

Figure 7-1. Effects of TGF-β1, TGF-β2 and TGF-β3 on growth of RWPE-1 cells in monolayer cultures.....158

Figure 7-2. TGF-β1, TGF-β2 and TGF-β3 expression in non-malignant RWPE-1 and malignant human prostatic epithelial cells.....160

Figure 7-3.TGF-β RII and TGF-β RIII expression in non-malignant RWPE-1 and malignant human prostatic epithelial cells.....161

Figure 7-4.Reduction of acinar formation by anti-TGF-β neutralizing antibodies 163

Figure 7-5. Reduction of acinar formation by anti-TGF-β neutralizing receptor antibodies.....163

Figure 7-6. Effects of TGF-β1, TGF-β2 and TGF-β3 on growth of WPE1-NA22 cells in monolayer cultures.....165

Figure 7-7. Effects of TGF-β1, TGF-β2 and TGF-β3 on growth of WPE1-NB26 cells in monolayer cultures.....166

Figure 7-8. Effects of TGF-β1 on the growth of RWPE-1, WPE1-NA22 and WPE1-NB26 cells in monolayer cultures.....167

Figure 7-9. Enhancement of acinar formation by TGF-β.....170

Abbreviations

5 α-DHT	5-α-dihydrotestosterone
ABC	Avidin-Biotin Complex
AR	Androgen Receptor
BPE	Bovine Pituitary Extract
BSA	Bovine serum albumin
CK-14	Cytokeratin 14
CK-18	Cytokeratin 18
D-PBS	Dulbecco's Phosphate Buffered Saline
DCS	Donor Calf Serum
EGF	Epidermal Growth Factor
HPV-18	Human Papilloma Virus 18
IF	Intermediate Filaments
INT	P-Iodo-nitrotetrazolium violet
K-SFM	Keratinocyte-Serum Free Medium
LSM	Laser Scanning Microscope
MoAb	Monoclonal Antibody
NMU	*N*-nitroso-*N*-methlyurea
PIN	Prostatic intraepithelial neoplasia
PSA	Prostate Specific Antigen
PSF	Penicillin, Streptomycin & Fungizone
TGF-ß	Transforming Growth Factor β

Introduction

Adenocarcinoma of the prostate is the most common type of cancer, excluding skin cancer, in adult men in the United States. It is estimated that over 184,500 men will be diagnosed and 39,200 men will die of prostate cancer in 1998, thus, making prostate cancer the second leading cause of cancer death in the U.S. (Landis et al., 1998). Incidence of prostate cancer increases with age and about 80% of prostate cancers are diagnosed in men over the age of 60 (Carter and Coffey, 1990). Autopsy studies show that approximately 11 million men older than 45-50 years in the U.S. have latent carcinoma of the prostate and it is predicted that one in ten men will develop prostate cancer in his lifetime (Carter and Coffey, 1990, Foster, 1990). Although cases of precancerous lesions, referred to as prostatic intraepithelial neoplasia (PIN), appear in men in their 20's and 30's, the majority of prostate cancer cases are diagnosed in men their 60's and 70's at an advanced stage of the disease when invasion and metastasis have already occurred (Bostwick, 1992). Presently, little is known about the causes of prostate cancer. Therefore, a great deal of importance and attention is being placed on the need for information on the mechanisms involved in carcinogenesis and on treatment strategies for the prevention of the progression of precancerous, latent lesions to clinical cancer, as well as, effective strategies for the treatment of the advanced disease. However, research in these areas has been slow.

One of the major obstacles faced by researchers is the lack of suitable human cell models and *in vitro* culture systems which would mimic the *in vivo* micro-environment. Currently, the use of human prostate epithelial cells for the study of prostate cancer is the best available model system aside from human experimentation which is basically unfeasible and unethical. Immortalized human prostatic cell lines provide uniform, standardized and reproducible models for the study of the multi-step process of carcinogenesis and the alterations that occur in malignant transformation. Such cells lines have been developed and fully characterized in Dr. Webber's laboratory at Michigan State University. Prostatic epithelial cells from a donor were immortalized with the human papilloma virus-18 (HPV-18), thus establishing the RWPE-1 cell line (Rhim et al., 1994 and Bello et al., 1997). Additional cell lines, which represent different stages of carcinogenesis, were developed by transfection with the *ras*-oncogene or exposure of RWPE-1 cells to a carcinogen. RWPE-1 cells were transformed by the addition of a Ki-*ras* oncogene to produce the tumorigenic RWPE-2 cell line (Rhim et al., 1994 and Bello et al., 1997). Other RWPE-1 cells were treated with the chemical carcinogen, *N*-nitroso-*N*-methlyurea (NMU), to establish cell lines of varying tumorigenicity: WPE1-NA22, WPE1-NB11, WPE1-NB14, WPE1-NB26 and WPE1-NB27 (Webber, personal communication). Collectively, these cells lines represent different stages of neoplastic transformation from initiated non-invasive, non-tumorigenic to highly invasive, tumorigenic cells.

Hypotheses:

The main focus of this study is the development of a 3-dimensional (3-D) culture system, which mimics *in vivo* prostatic acinar morphogenesis, and its application to study the factors involved in the progression of carcinogenesis utilizing the unique characteristics of the newly developed cell lines. Specifically, the role of the individual extracellular matrix components and their integrin receptors, as well as growth factors and their receptors, in epithelial polarization and acinar formation and how they are altered in malignant cells, was examined. The proposed hypotheses are that:

- **RWPE-1 cells undergo structural and functional differentiation when grown on Matrigel, a reconstituted basement membrane.**
- **Laminin is the key extracellular matrix component necessary for acinar morphogenesis.**
- **Cell attachment and epithelial polarization require the basal expression of $\alpha 6\beta 1$ laminin integrin receptor.**
- **TGF-β and EGF play an essential role in the epithelial organization and formation of acini.**
- **Neoplastic cells lose the ability to undergo acinar morphogenesis.**
- **Loss of normal $\alpha 6\beta 1$ laminin integrin expression results in the loss of ability to undergo acinar morphogenesis.**
- **Progression through the multi-step processes of carcinogenesis involves at least two sequentially unique steps:**
    1. **Deregulation of growth involving alterations in the expression and responsiveness to growth factors.**
    2. **Alterations in the $\alpha 6\beta 1$ laminin integrin receptor expression.**

References

Bello D, Webber MM, Kleinman HK, Wartinger DD and Rhim JS: Androgen responsive adult human prostatic epithelial cell lines immortalized by human papillomavirus 18. Carcinogenesis 18: 1212-1223, 1997.

Bostwick, D.G.: Prostatic intraepithelial neoplasia (PIN): current concepts. J. Cell. Biochem. 164:10-19, 1992.

Carter, H.B. and Coffey, D.S.: The prostate: increasing medical problem. Prostate 16:39-48, 1990.

Foster, C.S.: Predictive factors in prostatic hyperplasia and neoplasia. Hum. Pathol. 21:575-577, 1990.

Landis SH, Murray T, Bolden S and Wingo PA: Cancer Stats, 1998. CA Cancer J. Clin. 48:1, 1998.

Rhim JS, Webber MM, Bello D, Lee MS, Arnstein P, Chen L and Jay G: Stepwise immortalization and transformation of adult human prostate epithelial cells by a combination of HPV-18 and v-Ki-ras. Proc. Nat. Acad. Sci. USA, 91:11874-11878, 1994.

Chapter One

The Human Prostate: Background

The Human Prostate

The prostate is an exocrine gland with an average weight of 20 grams. The adult prostate gland lies caudal to the urinary bladder and cranial to the external urethral sphincter (Rous et al., 1988). It is composed of glands, muscle and fibrous tissue encapsulated by connective tissue (McNeal, 1989, Rous et al., 1988). Although the prostate gland is not a primary sex gland, it is considered an accessory sex gland because of its indirect involvement in procreation. The secretion of the prostate gland constitutes a major component of semen. The prostatic glands empty their secretion into prostatic ducts, that in turn, empty into the prostatic urethra, where this secretion mixes with sperm from the testes that arrive via the ejaculatory ducts. The prostatic secretion functions as a buffered vehicle for spermatozoa and provides them nourishment (Rous, et al., 1988). This review will focus on the normal and the malignant prostate.

Anatomy

The prostate is divided into the ventral fibromuscular portion and a dorsal glandular portion. The glandular prostate is composed of three zones: the peripheral (75%), the central (15%) and the transition (5%) (Figure 1-1). The peripheral zone surrounds the posterolateral peripheral aspect and extends from the apex to the base. It is

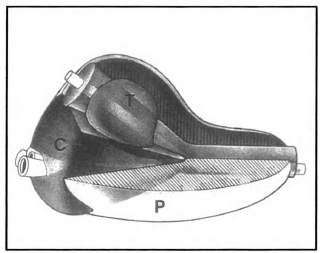


Figure 1-1. Three-Dimensional model of the three glandular zones of the prostate. Peripheral (P), transitional (T) and central (C) zones of the prostate (Lee et al., 1989).

aspect and extends from the apex to the base. It is composed of small, simple acini lined by columnar secretory epithelial cells. The central zone surrounds the ejaculatory ducts. The acini in the central zone are large, irregularly contoured and are lined by low columnar to cuboidal epithelium. The transition zone is formed by two bilaterally symmetrical lobules lateral to the pre-prostatic sphincter. The transition zone is histologically similar to the peripheral zone but differs in that its stroma is more dense and compact (McNeal, 1989, Miller, 1993). The prostatic stroma is primarily composed of smooth muscles cells and fibroblasts which reside in the extracellular matrix (ECM). Due to the lack of suitable *in vitro* human models and the obvious moral and ethical constraints on the use of live human subjects for experimentation, the fetal and post-natal development of the prostate has been studied extensively in the rodent model and the information has been extrapolated to the human prostate.

Development of the Human Prostate

Embryonic development: The first indication of prostatic development in humans is seen at the twelfth week (3rd month) of fetal life as outpouchings from the urethral epithelium in the distal portion of the urogenital sinus (Figure 1-2). These urethral epithelial cells bud out as prostatic buds into the adjacent urogenital mesenchyme both above and below the entrance of the mesonephric ducts and the openings of the ejaculatory duct (McNeal, 1989, Rous, 1988, Smith, 1972). By the fourth month of fetal life the prostate buds reach their final number. Five paired buds are formed in all. The top pairs of buds are thought to be of mesodermal origin and form the inner zones of the prostate.

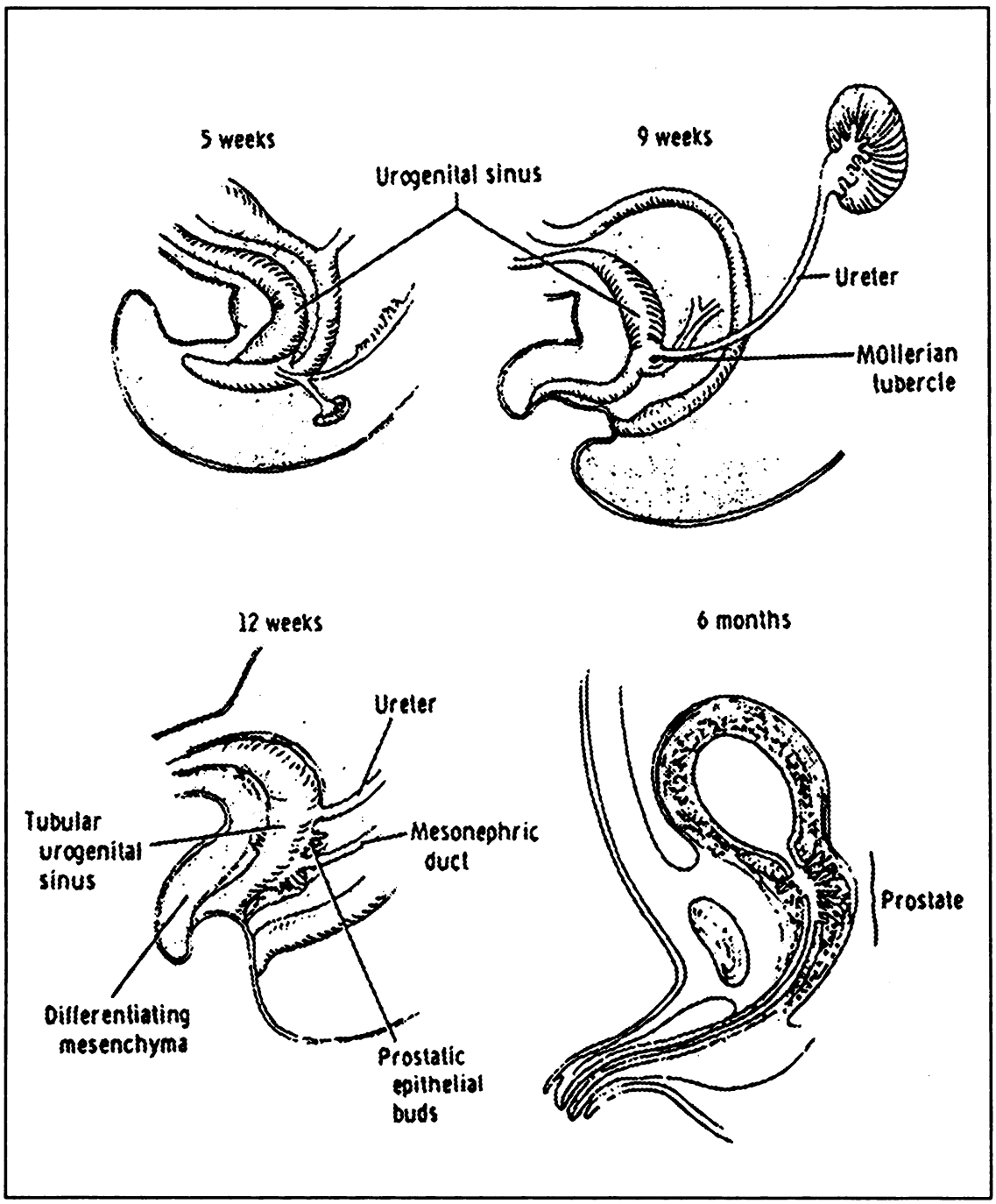


Figure 1-2. Differentiation of the urogenital sinus in the human male (Smith, 1972).

The outer zone of the prostate is formed by the lower buds and is thought to be of endodermal origin (Aumuller, 1979, Coffey, 1992). These buds branch and re-branch, to form determinate glands that are directed toward the bladder, where they wind spirally around the urethra. They will eventually comprise three distinct zones: the central, transitional and peripheral zones. The fibromuscular tissue that was originally continuous with the bladder, becomes entangled with the branching buds and becomes the prostatic stroma (Aumuller, 1979, Coffey, 1992, Smith, 1972).

The initial "budding" of prostatic ducts from the urethral epithelium is stimulated by 5α-dihydrotestosterone (5α-DHT). Testosterone from the fetal testis is metabolically converted to 5α-DHT by 5α-reductase. Although both the embryonic prostatic epithelial cells and the surrounding mesenchymal cells are capable of converting testosterone to 5α-DHT, the epithelium produces significantly more 5α-DHT than the mesenchyme. Interestingly enough, the final target of the androgenic influence is the adjacent mesenchyme not the epithelium. The epithelium converts testosterone to 5α-DHT, which then diffuses out to the mesenchyme. Although both the epithelial and mesenchymal cells express androgen receptor, the androgen receptor in the epithelial cells in the developing prostate does not bind 5α-DHT. It has been shown that the androgen receptor mRNA found in the epithelial cells of the prostatic buds is, at least, several hundred bases shorter than that of the androgen receptor mRNA in the mesenchymal cells. Exactly why this so is not yet clear (Timme et al., 1994). The fact that the mesenchyme, and not the epithelium, is the target of androgen action has been demonstrated quite elegantly by Cuhna and associates (1987 and 1996) using epithelial-mesenchymal recombinant experiments. In such experiments, androgen-sensitive urogenital sinus mesenchyme and androgen-insensitive urogenital epithelium, with the addition of

androgens, were able to form prostatic acini, while the converse, androgen-insensitive urogenital mesenchyme and androgen-sensitive urogenital epithelium failed to form acini.

In other experiments using pregnant females, anti-androgens were used at different stages of pregnancy to determine the importance of androgens in eliciting prostatic budding. It was found that when anti-androgens were administered on day 16.5 day postcoitum, prostatic budding was completely abolished. But if the anti-androgens were administered on day 18.5, prostatic budding occurred. The action of androgen in this case, is thought of to be a *turn on* switch. Once you turn on the switch for morphogenesis you do not need to keep your hand on it, i.e. further stimulation *in vivo* by androgen is not needed beyond the initial induction (Donjacour and Cuhna, 1988).

To date, the generally accepted mechanism for prostatic budding from the urogenital sinus may be visualized as follows: testosterone from the embryonic testis is converted to 5α-DHT by the "prostatic" epithelial cells, 5α-DHT then diffuses to the mesenchymal cells where it binds to the androgen receptor and induces (turns on) the production of an unknown growth factor(s), which, in turn, stimulates the urogenital epithelial cells to form prostatic buds. Two questions arise from this explanation: what are the growth factors secreted by the mesenchyme that induce the budding and what role do adhesion molecules, ECM bound proteases and growth factors play in modifying the extracellular matrix (ECM) which the prostatic buds are invading? Unfortunately, very little information about human fetal and postnatal budding and branching is available due to the lack of functional model systems, but work in postnatal rat branching morphogenesis, has been done and is generally extrapolated to the human condition to fill in the gaps. The 3-D human prostate epithelial cell model developed and described in the present work should serve as an *in vitro* model for human

prostate morphogenesis.

Postnatal Development of the Prostate: In general, postnatal development of the prostate may be divided into three phases: 1) postnatal involution (regression) phase (1 month after birth to 2 months); 2) infantile resting phase (2 months to 10-13 years); 3) maturation phase (14 to 18-21 years). During postnatal development no new lobe formation is observed (Aumuller, 1979 and 1991).

At birth, acini are predominantly lined with epithelium showing squamous epithelial metaplasia which is thought to be caused by maternal estrogen. There is some scattered secretory activity but these secretions are strictly mucosal. The postnatal involution phase following birth occurs in response to the cessation of maternal steroids. During this time the metaplastic epithelium is replaced by cuboidal psuedostratified epithelium (Aumuller, 1979 &1991, Coffey, 1992).

During the first three months after birth there are large hormone surges that result in hormone imprinting of the prostate. These hormonal surges involve transient increases in the serum levels of estrogen, androgen and progesterone in the early postnatal life in both humans and rats. The estrogen surge is represented by the high serum estradiol levels seen at birth. Estrogen levels dramatically fall in the first few days after birth which results in involution (Coffey, 1992). In human males, a surge in testosterone is observed between the second and third months. Testosterone levels increase up to 60 times higher than normal pre-pubertal levels. In fact, at the time of the surge, testosterone levels almost match adult testosterone levels. Progesterone levels are high at birth in both animals and are thought to be of placental origin. In the human neonate, there is another surge of progesterone at two months (Coffey, 1992). From rat studies, we know that theses surges are critical in hormonally imprinting the

prostate. Such imprinting subsequently determines the gland's future growth regulation by steroid hormone. This growth regulation by imprinting is illustrated by experiments where exogenous estrogen administration to the neonatal rat reduced the sensitivity of the adult animal to androgens (Naslund and Coffey, 1986). On the other hand, exogenous administration of androgen or progesterone to the neonatal rat resulted in an increase in sensitivity to androgens in adulthood. It is proposed that the neonatal hormone surges are altering the properties of the prostatic stem cells or that they are perhaps altering stem cell numbers. Although the mechanism remains unclear, it does appear that the hormone surges act directly on the prostate, and not through the hypothalamic-pituitary axis (Naslund and Coffey, 1986). Since this phenomena of neonatal and pre-pubertal steroids imprinting of the prostate has been established as a critical factor in rats, it is thought that the same process occurs in the human male.

Following the post-involution/regression phase, only minimal growth, development and histological differentiation occur in the infantile prostate. When boys reach 10 years of age the prostate begins to develop its typical external shape from the spherical shape it had at birth. With the onset of puberty at 11 to 13 years of age, the maturation of the glandular epithelium begins. This maturation phase lasts from 11-13 to 18-22 years of age. During this time, the glandular ducts begin to grow in diameter, and they branch (branching morphogenesis) and develop new glands. The usually thin infantile epithelium starts to transform into a multilayered columnar epithelium. In the mouse this phase, which occurs within the first 15 days of postnatal life, is very well studied. It had always been assumed that branching morphogenesis was an androgen-dependant process, but that notion is being challenged. When mice were castrated 24 hours after birth, a decrease in the number of tips

and branch points in both dorsal and ventral prostates was observed but both lobes still underwent significant branching (25-45%). In order to counter any residual androgen activity or androgen contribution from the adrenal gland, anti-androgen was administered to the castrated animals. This treatment did not cause any additional suppression of branching. Therefore, it can be concluded that branching morphogenesis is sensitive to, but does not require, chronic androgen stimulation. The idea is that unlike the fetal condition, where androgen levels are necessary at critical periods for initial prostatic budding, postnatal branching morphogenesis does not require androgen stimulation. Even if the preceding is true, androgen is essential for functional differentiation of the prostate in the adult male (Aumuller, 1979 &1991, Coffey, 1992, Naslund and Coffey, 1986).

Branching morphogenesis stops at 15 days after birth in rats. What gives the stop command? Cessation of branching is not likely due to the increase in androgen levels, because branching stopped at 15 days in both the non-castrated and castrated animals alike. One attractive proposed mechanism is that the decrease in the ratio of stroma to epithelium causes branching to stop, but in the castrated neonates branching stops at 15 days even though there is an abundance of stroma available. But it must be noted that the stroma of castrated animals is abnormal, in that it has larger amounts of collagen and other ECM materials (Donjacour and Cuhna, 1988). This is possibly due to improper ECM remodeling, a process which may indirectly require androgens for proper functioning. Furthermore, although it is tempting to extrapolate this lack of androgenic response to the human condition, it must be noted that, unlike humans, rats have a very low sensitivity to testosterone, especially during this branching period.

Because postnatal branching morphogenesis increases the number of glands, the amount and degree of maturation of its secretory products also increases. At birth the few secretory cells present secrete mucous, which is continued throughout infancy. Functional secretory maturation usually occurs between ages 18-22, as acini with distinct lumens are developed after postnatal branching morphogenesis. Secretions go from mucosal to prostate-specific. As early as 14 years of age, most glands have a more or less duct-like appearance and show some immunoreactivity for prostatic acid phosphate (PAP) and prostate specific antigen (PSA) . There are no mucous producing cells in the adult prostate as there are in infancy and in the early post-pubertal prostate (Aumuller, 1979 &1991).

At the completion of maturation, the adult prostatic acini are said to generally contain three types of cells: basal epithelial cell, luminal epithelial cells and neuroendocrine cells (Verhagen et al., 1992). The basal cells are considered to be the stem cells from which luminal cells arise. Luminal cells are the terminally differentiated secretory cells of the prostate gland. A fourth type of cell population, called amplifying cells, is thought to exist in the prostate gland. These amplifying cells are considered to be cells in transition from the basal to the luminal type (Verhagen et al., 1992).

Growth factors involved in prostatic development: Recently, keratinocyte growth factor (KGF) and transforming growth factor-β (TGF-β) have been proposed to mediated the mesenchymal-epithelial interaction in the developing prostate (Cuhna, 1996). KGF is a member of the fibroblast growth factor family and is primarily produced by fibroblasts and mesenchymal cells but has a high degree of specificity for epithelial cells (Cuhna, 1996). While, KGF is primarily produced by the mesenchymal cells, the KGF receptor is principally expressed by epithelial cells, where it induces cell proliferation. *In vitro* organ

culture experiments, using the ventral prostate of new born rats, have been used to investigate the role of KGF during postnatal branching. KGF transcripts were found in the new born ventral prostate; additionally, transcripts for its receptor were found specifically in the epithelium. When organ cultures were grown in the presence of testosterone, the ventral prostate ducts showed extensive branching but when grown in the absence of testosterone branching was modest and eventually stopped all together. The addition of antibodies against KGF into the culture medium containing testosterone inhibited ductal branching. The branching patterns of organ cultures grown in the presence of testosterone and anti-KGF antibodies resembled the pattern seen when cultures were grown in the absence of testosterone. Thus, the anti-KGF antibodies inhibited the trophic effects of testosterone. Most striking was the observation that cultures grown in the absence of testosterone, but in the presence of KGF, demonstrated ductal growth and branching morphogenesis patterns similar to those patterns seen in cultures treated with testosterone alone. That is to say, KGF almost completely mimicked the growth and branching morphogenesis seen in response to androgens (Cuhna, 1996). Based on the above evidence, it is proposed that one of the unknown growth factors produced by the mesenchymal cells of the urogenital sinus in response to 5α-DHT induced prostatic budding is KGF.

Transforming growth factor-β (TGF-β): Another growth factor that has been associated with prostatic development is TGF-β. Because of its apparent important role in branching and acinar morphogenesis *in vivo*, I have investigated its effects on acinar morphogenesis in the 3-D cell culture model. These results are reported in chapter seven.

We have previously reported the effects of TGF-β_1 on prostatic epithelium in monolayer cultures (Webber et al., 1996, Bello et al., 1997).

TGF-βs are members of a superfamily of polypeptides that regulate cell cycle progression, differentiation, chemotaxis and metabolism of many different types of cells (Habib and Chem, 1994). Their 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 (Franzen et al., 1993, Roberts and Sporn, 1990, Webber et al., 1996). 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 (Roberts and Sporn, 1990; Franzen et al., 1993). Presently three form of TGF β (TGF-β_1, TGF-β_2, and TGF-β_3) have been identified in the prostate.

Although TGF-β is expressed by both the urogenital mesenchyme and the urogenital epithelium of the fetal mouse, the highest levels are detected in the mesenchyme and not the epithelium. Specifically, the highest levels are in the areas of active epithelial duct formation. TGF-β expression actually follows the path the epithelial cells take as they penetrate into the surrounding mesenchyme. For proteolytic remodeling of the mesenchyme, serine proteases, such as tissue and urokinase plasminogen activator (tPA & uPA), plasmin (from plasminogen by uPA) and some metalloproteinases are the best candidates, since they have also been found to be increased in the urogenital mesenchyme

(Timme et al., 1994, Wilson et al. 1992). Cathepsin D expression has been found in the basal portion of the prostatic epithelial cells as they penetrate, but this expression is lost with formation of lumens (Wilson, 1995). The reciprocal relationship between TGF-β and proteases has been somewhat elucidated. TGF-β isoforms must be cleaved from a latent form into active molecules. Therefore, one of the functions of these proteases is to activate matrix bound growth factors, such as TGF-β (Aumuller, 1991 and Timme et al., 1994). The other function is to degrade the ECM. TGF-β and proteases work together in prostatic morphogenesis in the following manner: TGF-β induces prostatic epithelial cells to produces matrix proteins and causes an up-regulation of their integrin receptor expression, thus, providing invading epithelial cells attachment sites and the needed ECM receptors which enable them to move. Meanwhile, proteases degrade the newly laid down ECM, keep the epithelial cells from differentiating by removing the matrix and release growth factors, such as bFGF, thus, maintaining their proliferative state.

In summary, there is little change in the actual size of the prostate gland from the time between birth till puberty. At puberty, in response to the increased levels of testosterone, the prostate again begins to grow. As discussed above, this growth is the result of significant hyperplasia of the ductal epithelium and the formation of bud sites. This branching morphogenesis, which leads to the elaboration of the duct system, the development of new acini and a condensation of the stroma, is accompanied by a slow and continuous increase in the size and mass of the prostate. By age 30, when growth stops, the prostate has grown to twice its pre-pubertal size primarily due to the development of new acini and related ducts. The prostate remains largely unchanged in size until 45-55

years of age. By age 60, most men develop benign prostatic hyperplasia (BPH) and/or prostate cancer (Aumuller, 1979, Rous, 1988, McNeal, 1989). One objective of the present study is to examine changes in integrin expression and possible associated changes in the ability of prostate cells to polarize and undergo acinar morphogenesis. Such changes may also affect the invasive ability of prostate cancer cells. Some characteristics of prostate cancer, invasion and the precancerous changes, characterized by prostatic intraepithelial neoplasia (PIN) will now be described. It should be noted that the set of cell lines used in the present study may represent a continuum of neoplastic changes from normal epithelium to invasive cancer (see chapter 6).

Prostate Cancer

With the ever increasing life span in the United States, adenocarcinoma of the prostate has become a major health concern. If prostate cancer is detected when it is pathologically confined to the prostate, that is, it has not yet metastasized, it can be surgically removed or successfully treated. Therefore, it is the goal of modern medicine to not only be able to accurately diagnose prostate cancer but to do so early, while it is still confined to the prostate. That is why the American Cancer Society continues to recommend yearly prostate check-ups to men over 40 as a regular annual assessment for prostate cancer (Cupp et al., 1993). Unfortunately, even with the advances in early detection, 60% of prostate cancer cases are diagnosed in men in their 60's and 70's, when invasion and metastasis have already occurred (Miller, 1993; Cupp et al., 1993).

Invasion: Invasion by cancer cells involves invasion of the stroma, of the bladder, and/or of the seminal vesicles. Stromal invasion is noted by the lack of basal cells and the basement membrane which separates the acini from the stroma. The acini may also appear to be haphazardly dispersed in the stroma and the acini themselves frequently have irregular shapes. Additionally, there might be signs of individual or groups of cells growing out of the acini and invading into the stroma. Bladder and periurethral invasion is characterized by the presence of malignant cells in the lamina propria that have no relationship to the surface epithelium (Mostofi et al., 1992). Seminal vesicle invasion is usually associated with cancers that also have lymph node metastasis and are larger than 12 mm, however this association does not negate the probability of small cancers being over-looked (Villers et al. 1991).

Pathology: Prostatic carcinoma has a great variety of architectural patterns that have been arranged into a "linear scale of progressive dedifferentiation" (McNeal et al., 1986). These patterns have been used to establish an architectural grading system. Some of these unique patterns may also serve as criteria for the diagnosis of prostate cancer (McNeal et al., 1986). The pathologist looks for the following architectural disturbances (Mostofi et al., 1992). In the normal prostate, glands are uniformly large, convoluted, and are comprised of basal and luminal cells arranged in a pseudostratified pattern (Miller, 1993). Each gland is surrounded by a basement membrane and is distributed in a nodular pattern (Mostofi et al., 1992). In prostate cancer, the acini are small and the cells are arranged in a single layer devoid of basal cells. These small acini are packed closely together or they may form large acini of a single layer of cells with no convolutions. A pattern of large and small acini arranged side by side is also seen (Mostofi et al., 1992).

Another distinct pattern is that of small or large single layered acini fused to each other without any stroma between them. Other arrangements such as cribriform or gland in gland patterns also denote prostate cancer (Mostofi et al., 1992).

It is interesting to note that a number of benign lesions may be confused with or perhaps even mis-diagnosed as prostate cancer. These benign lesions are basal cell hyperplasia, atrophy, unusual variants of hyperplasia and cribriform hyperplasia.

Precursor changes- prostatic intraepithelial neoplasia (PIN): Progress has been exceedingly slow in identifying the precursor to prostate cancer due to the inability to accurately re-biopsy the prostate (Miller, 1993). Candidates for precursor lesions have been proposed such as Roman bridging, adenosis, clear cell cribriform hyperplasia, and intraductal dysplasia. All but interductal dysplasia have been discredited as possible prostatic precancerous lesions (Miller, 1993).

Intraductal dysplasia was first described by McNeal and Botswick in 1986 (McNeal et al., 1986). They felt it was a precursor lesion to prostate cancer because of its similarities in nuclear pleomorphorphism and nuclear prominence to cancer and its association with concurrent prostate cancer in specimens (Miller, 1993). In 1989, the term prostatic intraepithelial neoplasia (PIN) was proposed and replaced intraductal dysplasia which had also been referred to as large acinar atypical hyperplasia, hyperplasia with malignant change, marked atypia and duct-acinar dysplasia (Miller, 1993, Bostwick, 1995).

Prostatic intraepithelial neoplasia is generally divided into two grades: high and low (Bostwick, 1995). Low grade PIN is characterized by glands that show epithelial cell crowding with enlarged nuclei that vary in size. An undisrupted basal epithelial cell layer

is present. High grade PIN displays four architectural patterns: micro papillary, tufting, cribriform and flat (Figure 1-3). PIN spreads much like prostate carcinoma. Three patterns of spread have been described for PIN. In the first pattern, neoplastic PIN cells appear to replace the luminal epithelial cells and the basal cells appear unaltered. In the second pattern, the neoplastic luminal-like PIN cells appear to invade the acinar wall, disrupting some basal cells (Bostwick, 1995). In the third pattern, which is rarely found, the PIN cells have invaginated between the basal cell layer and columnar secretory cell layer. Some high grade PIN show severe fragmention of the basal layer and invasion. In fact, it can not be distinguish from carcinoma *in situ* (Bostwick, 1995).

The incidence of PIN, similar to prostate cancer, increases with age. However, unlike prostate cancer, which shows a higher prevalence in blacks than in whites, the prevalence of PIN is equal in both (Botswick, 1995). PIN has been found in men in their

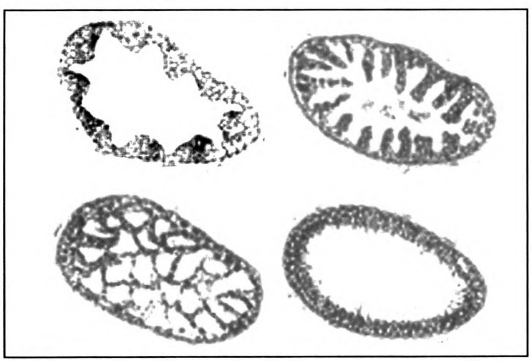


Figure 1-3. High grade PIN demonstrates 4 architectural patterns : micropapillary, tufting, cribriform and flat (Miller, 1993).

20's (9%) and 30's (22%) and is thought to predate the onset of carcinoma by 5 to 10 years (Bostwick, 1995). As in prostate cancer, the peripheral zone of the prostate is the most common site for PIN. Other similarities between PIN and prostate cancer are: elevated levels of collagenase type IV, EGF receptor, and growth factors, such as EGF and TGF-α. Oncogene products such as erbB-2 and erbB-3 and bcl-2 have been shown to be elevated in PIN. In contrast, differentiated markers such as cytokeratins and neuroendocrine cells are lost with increasing grades of PIN. Although serum PSA levels are increased in PIN (Kim et al., 1995), PSA staining of PIN cells is decreased or lost (Bostwick, 1995). An elegant model for prostate carcinogenesis has been proposed, based on the multistep theory of transformation, using the morphologic continuum of PIN (Figure 1-4). In this model, low grade PIN corresponds to very mild and mild dysplasia while high grade PIN encompasses the progression from mild to severe dysplasia including *in situ* carcinoma. PIN ends and prostate cancer begins when the neoplastic cells invade the stroma after the basal cell layer is completely disrupted (Botswick et al., 1987).

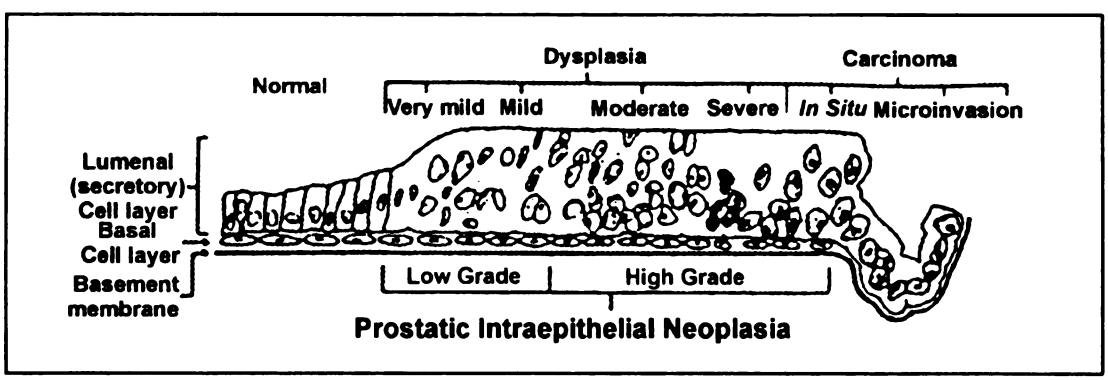


Figure 1-4. Proposed model for prostatic carcinogenesis (Bostwick, 1996).

Clinically, PIN has become of very important because it has a strong predictive value as a marker for prostate cancer (Botwick, 1995). In a study where 37 patients were biopsied, 14 (38%) showed presence of PIN. When they were biopsied again within eight years, they had all developed prostate cancer (Bostwick, 1995). Another study showed similar results, where 39% of the 104 men that had been diagnosed with PIN, had prostate cancer upon follow up a few years later (Park et al., 1989 see Bostwick, 1995). In fact one study found that the probability of developing cancer was also depended on the type of PIN diagnosed in the initial biopsy. Forty percent of men diagnosed with high grade PIN had cancer when re-biopsied 18 months later, while only 13% of those with low grade PIN showed cancer (Bostwick, 1995). One could argue that the cancer was present at the first biopsy but was just not sampled until the second, but that is highly unlikely if an adequate amount of locations are initially biopsied. Although it is very much implied, no studies to date have definitively been able to determine whether or not PIN progresses to cancer as in the carcinogenesis model. It is felt that this is just a matter of time (Bostwick, 1995).

It should be noted that the set of cell lines used in the present study may represent a continuum of neoplastic changes from normal epithelium to invasive cancer (see chapter six).

References

Aumuller G: Prostate gland and seminal vesicles. Berlin: Springer-Verlag. pp. 12-88. 1979.

Aumuller G: Postnatal development of the prostate. Bullletin de l'association des anatomistes, 74:229, 39-42, 1991.

Bello D, Webber MM, Kleinman HK, Wartinger DD and Rhim JS: Androgen responsive adult human prostatic epithelial cell lines immortalized by human papillomavirus 18. Carcinogenesis 18: 1212-1223, 1997.

Bostwick DG: Prostatic intrapeithelial neoplasia (PIN): Current concepts. J Cell. Molec. Biol. suppl. 16H10-19, 1992.

Bostwick DG: Prospective origins of prostate carcinoma. Prostatic intraepithelial neoplasia and atypical adenomatous hyperplasia. Cancer. 78:330-6, 1995.

Bostwick DG: Progression of prostatic intraepithelial neoplasia to early invasive adenocarcinoma. Eur. Urol. 30:145-52, 1996.

Coffey DS: Physiology of the male reproduction. In Walsh, , P.C., Retik, A.B., Stamey, T.A., Vaughan, E.D.(eds): "Campbell's Urology"sixth edition. Pennsylvania: W.B. Saunders Company, pp. 224-246, 1992.

Cunha G: Growth factors as mediators of androgen action during male urogenital development. Prostate supplement. 6: 22-25, 1996.

Cunha G and Donjacour A: Stromal-epithelial interactions inn normal and abnormal prostatic development. Current concepts and approaches to the study of prostate cancer. 239: 251-272, 1987.

Cupp MR and Oesterling JE: Prostate-specific antigen, digital rectal examination, and transrectal ultrasonography: their roles in diagnosing early prostate cancer. Mayo Clin. Proc.68:297-306, 1993.

Donjacour A and Cunha G:The effects of androgen depravation on branching morphogenesis in the mouse prostate. Dev. Biol., 128: 1-14, 1988.

Franzen P, Ichijo H and Miyazono D: Different signals mediate transforming growth factor-β 1-induced growth inhibition and extracellular matrix production in prostate carcinoma cells. Exp. Cell Res., 207: 1-7, 1993.

Habib FK and Chem C: Pathogenesis of benign prostatic hyperplasia. In: Handbook on benign prostate hyperplasia. Chisholm, G.D. (ed.), New York, Raven Press, pp 19-31, 1994.

Henttu P and Vinkop P: Growth factor regulation of gene expression in the human prostatic carcinoma cell line LNCaP. Cancer Res., 53: 1051-1058, 1993.

Kim ED, Smith ND and Grayhack JT: Prostate specific antigen in the expressed prostatic fluid of men with benign prostatic hyperplasia and prostate carcinoma. J. Urol. 154:1802-5, 1995.

Lee F, Torp-Pedersen ST and Siders DB: The role of transrectal ultrasound in the early detection of prostate cancer. CA-Cancer-J-Clin. 39:337-60. 1989.

McNeal J E: Anatomy an embryology. In Fitzpatrick, J. M. and Krane, R. J. (eds): "The Prostate" Edinburgh: Churchill Livingstone, pp 3-7, 1989.

McNeal JE, Bostwick DG, Kindrachuk RA, Redwine EA, Freiha FS and Stamey TA: Patterns of progression in prostate cancer. Lancet. 1:60-3, 1986.

Miller GJ: Anatomical and pathological considerations. In Das, S. and Crawford, E. D. (eds): "Cancer of the Prostate" New York: Marcel Dekker, Inc, pp 13-17, 1993.

Mostofi FK, Davis CJ Jr, and Sesterhenn IA: Pathology of carcinoma of the prostate. Cancer. 70(1 Suppl):235-53, 1992.

Naslund MJ and Coffey DS: The differential effects of neonatal androgens, estrogen and progesterone on adult rat prostate growth. J. Urol., 136: 1136-1140, 1986.

Roberts AB and Sporn MB: The transforming growth factor-βs. In: Peptide growth factors and their receptors I. Sporn, M.B. and Roberts, A.B. (eds.), New York, Springer-Verlag, pp. 419-472, 1990.

Rous SN: The Prostate Book. New York : W. W. Norton & Co, pp 19-25, 1988.

Smith DR: General Urology. Canada: Lange Medical Publications, p. 22, 1972.

Timme TL, Trouong LD, Merz VW, Krebs T, Kadmon D, Flanders KC, Park SH and Thompson T: Mesenchymal-epithelial interaction and transforming growth factor-β expression during mouse prostate morphogenesis. Endocrinology, 134: 1039-1045, 1994.

Verhagen APM, Ramaekers FCS, Aalders TW, Schaafsma HE, Debruyne MJ and Schlken JA: Co-localizationn of basal and luminal cell-type cytokeratins in human prostate cancer. Cancer Res., 52: 6182-6187, 1992.

Villers A, McNeal JE, Freiha FS and Stamey TA: Multiple cancers in the prostate. Morphologic features of clinically recognized versus incidental tumors. Cancer. 70: 2313-8, 1992.

Webber MM, Bello D and Quader S: Immortalized and tumorigenic adult human prostatic epithelial cell lines: characteristics and applications. Part I. Cell markers and immortilized nontumorigenic cell lines. Prostate 26:386-394, 1996.

Wilson JD: Recent studies of the mechanism of action of testosterone. N. England J. Med., 287: 1284, 1972.

Chapter Two

Materials & Methods

Materials

Media & cell culture reagents

Keratinocyte-serum free medium (K-SFM) 17005-042, Antibiotic/antimycotic mixture (PSF) 15240-013, Dulbecco's modified Eagle's medium (DMEM) 21063-029 and RPMI-1640 11875-119 (Gibco-BRL, Grand Island, NY); Dulbecco's phosphate buffered saline Ca2+/Mg2+-free (PBS) 28374 (Pierce, Rockford, IL); donor calf serum 1110-90 (Intergin, Purchase, NY).

Antibodies

Monoclonal antibody (mAb) to α6 integrin, MAB1972, mAb to α2 integrin, AB1936 and mAb to β4 integrin, AB1922 (Chemcon, Temecula, CA); integrin mAb to α6 integrin, clone GoH3 (AMAC Inc., Westbrook, ME); mAb to β1 integrin (gift of Dr. S.K.Akiyama, NIDR, NIH); mAb to laminin, C-827, mouse IgG, M8273, rat IgG, I4131, goat IgG, I5256 and cytokeratin 18 , C-8541 (Sigma, St. Louis, MO); mAb to cytokeratin 14, 10003 (Cappel, Durham, NC); mAb to prostate specific antigen (PSA), M-0750 (Dako, Carpinteria, CA); polyclonal Ab to androgen receptor (AR), PA1-110, (Affinity BioReagents, Neshanic Station, NJ); Vectastain Elite ABC Peroxidase Kit, PK-6102, 3,3'-diamino-bezidine (DAB) Substrate Kit, SK-4100, Vectashield mounting medium, H1000, Vectashield mounting medium with PI, H1300, Anti-mouse Fluorescent Kit, FI-2100, Anti-rat Fluorescein, FI-4000, Anti-goat Fluorescein, FI-5000, Mouse IgG, I-2000, Fluorescein Avidin D, A-2001, biotinylated anti-goat IgG, BA5000 and biotinylated anti-rabbit IgG, BA1000 (Vector laboratories, Burlingame, CA); anti-TGF–β1 mAb, MAB240,

anti-TGF-β2, AB-112-NA, TGF-β3, AB-244-NA, anti-EGF, MAB236, anti-TGF-β1 mAb, MAB246, anti-TGF-β2, MAB 612, TGF-β3, M643, anti-TGF-RII, AF-241-NA and TGF-RIII, AF-242-PB (R&D Systems, Minneapolis, MN); anti FAK, F15020 (Transduction Laboratories, Lexington, KY).

Growth factors and hormones

Epidermal growth factors (EGF), 40001, transforming growth factor (TGF)-β1, 240-B, TGF-β2, 302-B2, TGF-β3, 243-B3 (R&D Systems, Minneapolis, MN); mibolerone, W-300 (BIOMOL Biomolecular Research Laboratories, Plymouth Meeting, PA).

Extracellular matrix (ECM)

Phenol red-free Matrigel, 4023C and growth factor reduced Matrigel, 40230, (Collaborative Scientific, Lincoln Park, NJ); collagen type IV, 3410-01 and laminin, 3400-10 (Trevigen.; Gaithersburg, MD); human fibronectin, 40008A and gelatin, G1890 (Sigma; St. Louis, MO).

Other materials

HEMA-3 Stain Set, 67-56-1 (Curtin-Matheson, Wood Dale, IL); 24 well plates (Falcon), 08-7721; 12 mm circle coverslips, 12-545-80 and cell strainers (Falcon), 40μ pore size, 2340 (Fisher Scientific, Itasca, IL); Nuclepore membrane, 8μ pore size, 1550446, 96 well plates, 3596 (Costar, Cambridge, MA).

Methods

Cell lines

RWPE-1, RWPE-2, WPE1-NA22, WPE1-NB11, WPE1-NB14, WPE1-NB26, WPE1-NB27 and DU-145 cells lines were used in this study. The RWPE-1 cell line was derived from non-neoplastic human prostatic epithelial cells by immortalization with human papilloma virus 18. These cells mimic normal prostatic cell behavior in their response to growth factors and androgen and in their expression of PSA and androgen receptor in response to androgen stimulation. They do not form tumors in nude mice nor are they invasive (Bello et al., 1997). RWPE-1 cells were transformed by the addition of a Ki-*ras* oncogene to produce the tumorigenic RWPE-2 cell line. These cells show altered response to growth factors and androgen as compared to the parent RWPE-1 cell lines, but do express PSA and androgen receptor in response to androgen stimulation. They form tumors in nude mice and are anchorage independent and invasive (Rhim et al., 1994 and Bello et al., 1997). WPE1-NA22, WPE1-NB11, WPE1-NB14, WPE1-NB26 and WPE1-NB27 were derived in the following manner: RWPE-1 cells were treated with the chemical carcinogen, *N*-nitroso-*N*-methlyurea (NMU) and injected into nude mice (Webber, personal communiation). Tumors were harvested, cells were isolated and grown in culture. These cells were then grown in agar, colonies were isolated and placed back into culture. After a second round through nude mice and agar, the WPE1-NA22, WPE1-NB11, WPE1-NB14, WPE1-NB26 and WPE1-NB27 cell lines were established. Collectively, these cell lines represent different stages of neoplastic transformation from initiated non-invasive, non-tumorigenic to highly invasive, tumorigenic cells.

DU-145 cell line is a commercially available prostate cancer cell line derived from a human brain metastasis. It was used as a negative control.

Cell Culture

RWPE-1, RWPE-2, WPE1-NA22, WPE1-NB11, WPE1-NB14, WPE1-NB26 and WPE1-NB27 cells were maintained in basal Keratinocyte-Serum Free Medium (K-SFM) supplemented with 50 μg/ml bovine pituitary extract (BPE), 5ng/ml epidermal growth factor (EGF), and 1% antibiotic/antimycotic mixture(PSF) which will be referred to as complete K-SFM. Basal K-SFM medium refers to K-SFM medium which lacks BPE and EGF. The DU-145 human prostate carcinoma cell line was maintained in RPMI-1640 medium containing 5% donor calf serum (DCS) and 1% PSF. Cells were passaged upon confluence and seeded at 2 x 10^6 cells/T-75 flask. For passaging, cells were washed with Ca^{++}/Mg-free PBS, then incubated with 3 ml of trypsin: EDTA mixture (0.05% trypsin, 0.02 mM EDTA) diluted 1:1 with Ca^{++}/Mg-free PBS for 7 min. Cells were dislodged by tapping, neutralized by equal part addition of 2 % DCS in Ca^{++}/Mg-free PBS, and recovered by centrifugation (2000 rpm) for 5 min. Cell counts were preformed with a Coulter Counter.

Cell Proliferation in Monolayer Cultures

EGF and mibolerone combined dose response assay: The combined effect of EGF and the synthetic androgen, mibolerone, on growth of RWPE-1 cells, was investigated. EGF was dissolved in sterile distilled water. EGF stocks were stored at -70°C until use. Mibolerone was dissolved in ethanol and was protected from light and stocks were stored at -70°C until used. The final concentration of vehicle in the medium was 0.1% which was not growth inhibitory. 5,000 cells were plated/well in triplicate, in 96 well plates in complete K-SFM Forty-eight hours after plating, medium was changed to basal K-SFM supplemented with BPE containing either 0 or 5 nM mibolerone and a range of EGF concentrations (0, 1.25, 2.5 and 10 ng/ml). Plates were fixed at 5 days after treatment and prepared for absorbance as follows: 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 for 45 min. During this time the dye is taken up by the fixed cells. The cells were then washed twice with distilled water and 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. Becuase of the direct relationship between cell number and absorbance, the absorbance data were plotted against time, analyzed and cell doubling times were determined for low and high cell densities (Webber et al., 1996). All experiments were conducted in triplicate.

Transforming growth factor (TGF)-β1, β2 & β3 dose response assay: The effects of growth factors TGF-$ß_1$, TGF-$ß_2$ and TGF-$ß_3$ on growth of RWPE-1, WPE1-NA22 and WPE1-NB26 cells were investigated. All three TGF-ß isoforms were dissolved in 4 mM HCl containing 1 mg/ml BSA. The final concentration of vehicle in the medium was 0.1% which was not growth inhibitory. 10,000 cells were plated/well in triplicate, in 96 well plates in complete K-SFM. Forty-eight hours after plating, medium was changed to complete K-SFM medium containing TGF-$ß_1$, TGF-$ß_2$ or TGF-$ß_3$ at concentrations ranging from 0 to 10 ng/ml. Plates were fixed at 5 days after treatment and prepared for absorbance as described above. All experiments were conducted in duplicate.

Immunocytochemical analysis of using monolayer cultures: RWPE-1, WPE1-NA22 and WPE1-NB26 cells were grown on sterile coverslips in 24 well plates at a density of 20,000 cells/400 μl in complete K-SFM medium/well. Upon sub-confluence, cells were rinsed twice with PBS and fixed in a 1:1 solution of methanol and acetone for 2 min with gentle agitation. The coverslips were then either processed that day or stored in a -20^{0}C freezer. Immunocytochemistry was performed using an indirect immuno-peroxidase method. Immunostaining experiments were done in duplicate following a modified Vector protocol (Webber et al., 1996). All of the following steps were carried out at room temperature unless otherwise indicated. Cells were blocked in normal horse serum for 1 hr at room temperature and incubated with the appropriate monoclonal antibody (mAb) diluted in normal horse serum. Table 2-1 shows specific antibody dilutions, incubation times and temperature for each antibody used. Cells were stained using either fluoresceine-tagged anti-IgG secondary Ab (1:200/1 h) or biotinylated

Table 1. Antibody and conditions for immunocytochemistry of monolayer cultures

Antibody	Dilution	Incubation time	Temperature
TGF-β1 [1,3]	1:100	2 h	21°C
TGF-β2 [1,3]	1:100	2 h	21°C
TGF-β3 [1,3]	1:100	2 h	21°C
TGF-β receptor II (RII) [3,4]	1:50	2 h	21°C
TGF-β receptor III (RIII) [3,4]	1:50	2 h	21°C

1, Mouse
2, Rabbit

anti-goat/rabbit IgG secondary Ab (1:200/ 1 h) followed by an avidin-fluoresceine (1:200/ 1h). Cells were washed twice in PBS over 10 min between all successive steps after primary Ab application. Cells were washed with in PBS and mounted in Vectashield mounting medium onto alcohol washed slides. Using a laser scanning confocal microscope cells were determined to be either + or - for a given antigen as compared to controls that received non-specific IgG as a control instead of primary antibody. All experiments were conducted in duplicate.

Laser scanning microscopy

Immunocytochemical and i*n vitro* 3-D culture assays were analyzed using a Ziess 10 Laser Scanning Confocal Microscope housed in the laser scanning microscopy (LSM) laboratory at Michigan State University. The LSM laboratory is a university wide facility providing state-of-the-art light microscopy technology to the MSU research community. Confocal microscopes allow for the examination with minimal distortion to the 3-D architecture of the specimen. The outstanding feature of the confocal microscopy is its ability to make "optical sections", images of thin, horizontal slices of fluorescing specimens. In conventional microscopy, light reaches the observer both from the narrow plane, which is in focus (the focal plane image), and from the out-of-focus regions above and below it. The out of focus light severely degrades the quality of the focal plane image and limits the depth to which a specimen can be examined. Consequently, details such as lumens, are not readily seen or easily discriminated. The classical method for overcoming this problem is to paraffin-embed the specimen and slice it mechanically into thin serial sections for conventional light microscope viewing. In contrast, with the confocal

microscope, the specimen is scanned point-to-point with a finely focused laser beam. A pinhole aperture is placed directly in front of a photomultiplier detector which results in the exclusion of the out-of-focus light from the final image. This produces an "optical section", which is directly comparable to what would be seen if the specimen had been mechanically cut at that exact position. However, in this case the specimen remains intact with no disruption of its 3-D architecture and it is available for subsequent optical sectioning in different planes. Additionally, light transmitted (non-confocal) images collected, using a laser scanning microscope are sharper, clearer and crisper than images captured with a conventional Tungsten lamp microscope.

In Vitro 3-Dimension Culture Assays

Acinar morphogenesis assay: Phenol red-free Matrigel, referred to as Matrigel, was used. To the wells of 96-well plates, 50 μl of Matrigel (0.3 mg/50 ml) kept on ice, were added and the plates were placed in a 37^{0}C incubator for 1 h to allow the matrix to gel. RWPE-1, RWPE-2, WPE1-NA22, WPE1-NB11, WPE1-NB14, WPE1-NB26, WPE1-NB27 and Du-145 cells were plated at 10,000 cells/well in complete K-SFM with 6 wells/cell lines. Cells plated on plastic served as monolayer controls. Medium was changed every 48 h. At 4 days, cultures were washed gently with PBS, fixed in 2.5% buffered paraformaldehyde for 15 min, rinsed twice with PBS and then either processed that day or stored in PBS at 4^{0}C (Webber et al., 1997). Acinar formation was examined by confocal microscopy of propidium iodide (1.5 μg/ml) stained cultures using the criteria of four or more spherically arranged polarized cells with lumens. For staining, cultures

were permeabilize using a 1:1 solution of methanol and acetone (3 min) and aqueously mounted with Vectashield mounting medium with propidium iodide or stained with acridine orange (15 min.) and aqueously mounted with Vectashield on alcohol washed slides. Acinar counts were performed at low magnification using a laser scanning microscope (LSM). Lumen formation was verified by randomly visualizing counted acini using laser scanning confocal microscopy. The number of acini formed was expressed as percent of control using RWPE-1 acinar formation as 100%. All experiments were conducted in triplicate.

Immunocytochemical analysis of Matrigel cultures: Immunocytochemistry was performed using an indirect immuno-fluorescence staining following a modified Vector protocol. All of the following steps were carried out at room temperature unless otherwise indicated. Cultures were blocked in 1.5% normal horse serum/PBS for 1 hr at room temperature and incubated with the appropriate monoclonal antibody (MAb) diluted in blocking solution. Table 2-2 shows specific antibody dilutions and incubation times for each antibody used. Cultures were then stained using either fluorescence tagged anti-mouse/rat IgG secondary Ab (1:200/ 1 h) or biotinylated anti-goat or rabbit IgG secondary Ab (1:200/ 1 h) followed by an avidin-fluoresceine (1:200/ 1 h). Cultures were washed

Table 2. Antibody and conditions for immunocytochemistry of Matrigel cultures

Antibody	Dilution	Incubation time	Temperature
Anti-α2 integrin [1,2]	1:100	2 h	21°C
Anti-α6 integrin [1,3]	1:100	2 h	21°C
Anti-β1 integrin [3,4]	1:100	2 h	21°C
Anti-β4 integrin [2,5]	1:100	2 h	21°C
Anti-Laminin [1,2]	1:200	2 h	21°C
Prostate specific antigen (PSA) [1,2]	1:20	24 h	4°C

1, Mouse
2, RWPE-1
3, RWPW-1, WPE1-7, WPE1-10, WPE1-NA22, WPE1-NB27 and WPE1-NB26
4, Rat
5, Rabbit

twice in PBS over 10 min between all successive steps after primary Ab application. Cultures were washed in PBS and mounted in Vectashield mounting medium onto alcohol washed slides. Using a laser scanning confocal microscope, acini were determined to be either + or - for a given antigen as compared to controls that received non-specific IgG instead of the primary antibody. All experiments were done in duplicate.

Induction of functional differentiation by androgen in Matrigel cultures: Experiments to examine the effects of androgens on morphogenesis and expression of differentiated function employed mibolerone treatment using the acinar morphogenesis assay described earlier. Twenty hours after plating RWPE-1 cells on Matrigel, medium was changed to complete K-SFM medium containing 5 nM mibolerone. Medium was subsequently changed every 48 h. After 6 days of mibolerone treatment, prostate specific antigen (PSA) expression was detected by a modified avidin-biotin immunoperoxidase Vector protocol, using monoclonal Ab. Matrigel cultures were washed 2X with PBS, fixed in 2% paraformaldehyde, paraffin embedded, sectioned at 5μ, mounted on poly-L-lysine coated slides and re-hydrated for immunocytochemical analysis. Control sections lacked primary antibody. All sections were washed twice in 500 μl PBS over 10 min between all successive steps after primary Ab application. The following sequential steps were conducted at room temperature unless otherwise noted: sections were blocked with 1.5% normal horse serum dissolved in PBS for 1 h, incubated with PSA Ab was diluted 1:20 in blocking solution for 24 h, followed by biotinylated secondary Ab (1:200) for 30 min, treated with 3% H_2O_2 for 3 min to quench endogenous peroxidase activity, incubated with the avidin-biotin-peroxidase complex for 30 min, developed with diaminobenzidine-nickel

chloride (DAB) substrate for 4-6 min, dehydrated and mounted on acid/alcohol washed slides. Using a light microscope, acini were determined to be either + or - for PSA expression as compared to controls that received no primary antibody. All experiments were done in duplicate.

Analysis of individual basement membrane components: To the wells of 96-well plates, 50 μl of laminin (1 mg/ml) kept on ice, were added and the plates were placed in a 37°C incubator for 1 h to allow the matrix to gel. Due to the liquid state of fibronectin and Collagen type IV a gelatin base was need in order to form a gel. Forty microliters (40 μl) of gelatin were added to each well and the plates were placed in a 37°C incubator for 15 min to allow the gelatin to set. Twenty microliters (20 μl) of Collagen type IV (0.5 mg/ml) or fibronectin (5 mg/ml) diluted 1:1 in gelatin was then added to the gelatin base and placed in a 37°C incubator for 15 min to allow the mixture to gel. RWPE-1 cells were plated at 10,000 cells/well in complete K-SFM with 6 wells representing each test matrix component. Cells plated on plastic and on Matrigel served as the controls. Medium was changed every 48 h. At 4 days, cultures were washed gently with PBS, fixed in 2.5% buffered paraformaldehyde for 15 min, rinsed with PBS and either processed that day or stored in PBS at 4°C. Cultures were permeabilized using a 1:1 solution of methanol and acetone (3 min) and mounted in Vectashield mounting medium containing propidium iodide (1.5 μg/ml) on alcohol washed slides. Acinar formation was examined by laser scanning confocal microscopy using the criteria of four or more spherically arranged polarized cells with lumens. Acinar counts were performed at low magnification using the LSM. The presence of a lumen was verified by randomly visualizing counted acini using

confocal microscopy. The number of acini formed was expressed as percent of control using RWPE-1 cells grown on Matrigel as 100%. All experiments were done in duplicate.

Induction of acinar formation assay: The ability of TGF-$ß_1$, TGF-$ß_2$ and TGF-$ß_3$ to induce acinar formation in tumorigenic WPE1-NA22 cell line was investigated. Non-tumorigenic RWPE-1 cells were used as controls. All three TGF-ß isoforms were dissolved in 4 mM HCl containing 1 mg/ml BSA. The final concentration of vehicle in the medium was 0.1% which was not growth inhibitory. To the wells of 96-well plates, 50 μl of growth factor reduced Matrigel (0.3 mg/50 µl), kept on ice, were added and the plates were placed in a 37^{0}C incubator for 1 h to allow the matrix to gel. Cells were plated at 10,000 cells/well in complete K-SFM with 6 wells representing each concentration. Forty-eight hours after plating, medium was changed to complete K-SFM medium containing TGF-$ß_1$, TGF-$ß_2$ or TGF-$ß_3$ at concentrations ranging from 0 to 10 ng/ml. Medium was changed every 48 h. At 4 days of treatment, cultures were washed gently with PBS, fixed in 2.5% buffered paraformaldehyde for 15 min, rinsed twice with PBS and then either processed that day or stored in PBS at 4^{0}C. Cultures were permeabilize using a 1:1 solution of methanol and acetone (3 min) and mounted in Vectashield mounting medium, containing propidium iodide (1.5 µg/ml), on alcohol washed slides. Acinar counts were performed at low magnification using the LSM. The presence of a lumen was verified by randomly visualizing counted acini using confocal microscopy. The number of acini formed was expressed as percent of control using non-treated control wells as 100%. All experiments were done in duplicate.

Inhibition of acinar formation assay: The ability neutralizing anti-bodies (Ab) against TGF-$ß_1$, TGF-$ß_2$, TGF-$ß_3$, TGF-ßRII, TGF-ßRIII, α6 integrin subunit, β1 integrin subunit or laminin, respectively, to inhibit acinar formation in non-tumorigenic RWPE-1 cells was investigated. To the wells of 96-well plates, 50 μl of growth factor-reduced Matrigel (0.3 mg/50 ml) kept on ice, were added and the plates were placed in a 37^{0}C incubator for 1 h to allow the matrix to gel. Cells were passaged and resuspended in media containing the appropriate neutralizing Ab concentration (Table 2-3) and incubated at room temperature for 45 min. Cells were plated at 10,000 cells/well in triplicate. Forty-eight hours after plating, cultures were washed gently with PBS, fixed in 2.5% buffered paraformaldehyde for 15 min, rinsed twice with PBS and then either processed that day or stored in PBS at 4^{0}C. For staining, cultures were permeabilize using a 1:1 solution of methanol and acetone (3 min) and mounted with Vectashield mounting medium containing propidium iodide (1.5 µg/ml). Acinar counts were performed at low magnification using the LSM. The presence of a lumen was verified by randomly visualizing counted acini using confocal microscopy. The number of acini formed was expressed as percent of control using non-treated control wells as 100%. All experiments were done in duplicate.

EGF effects on acinar morphogenesis: The effects of EGF on acinar formation in non-tumorigenic RWPE-1 cell line was investigated. EGF was dissolved in sterile distilled water. EGF stocks were stored at -70°C until use. The final concentration of vehicle in the medium was 0.1% which was not growth inhibitory. To the wells of 96-well plates, 50 μl of growth factor reduced Matrigel (0.3 mg/50 ml) kept on ice, were added and the plates were placed in a 37^{0}C incubator for 1 h to allow the matrix to gel. Cells were plated at

Table 3. Neutralizing antibody and conditions for inhibition of acinar and ductal formation assay.

Antibody	Dilution	Incubation time	Temperature
Anti-α6 integrin [1]	1:5	45 min.	21°C
Anti-β1 integrin [2]	1:33	45 min.	21°C
Anti-Laminin [1]	Full strength, 1:10 and 1:20	45 min.	21°C
TGF-β1 [1]	1:42	45 min.	21°C
TGF-β2 [1]	1:62	45 min.	21°C
TGF-β3 [1]	1:16	45 min.	21°C
TGF-β receptor II (RII) [3]	1: 16	45 min.	21°C
TGF-β receptor III (RIII) [3]	1: 16	45 min.	21°C
EGF [1]	1:42 and 1:62	45 min.	21°C
IgG [1,2,3]	Full stength[1], 1:33 and 1:2000	45 min.	21°C

1, Mouse
2, Rat
3, Goat

10,000 cells/well in complete K-SFM with 6 wells representing each concentration. Forty-eight hours after plating, medium was changed to basal K-SFM supplemented with BPE medium containing EGF at concentrations ranging from 0 to 10 ng/ml. Medium was changed every 48 h. At 4 days of treatment, cultures were washed gently with PBS, fixed in 2.5% buffered paraformaldehyde for 15 min, rinsed twice with PBS and then either processed that day or stored in PBS at 4^0C to be used later for analysis. Cultures were permeabilize using a 1:1 solution of methanol and acetone (3 min),and mounted with Vectashield mounting medium containing propidium iodide (1.5 μg/ml) on alcohol washed slides. Acinar counts were performed at low magnification using the LSM. The presence of a lumen was verified by randomly visualizing counted acini using confocal microscopy. The number of acini formed was expressed as percent of control using non-treated control wells as 100%. All experiments were done in duplicate.

Invasion assay

The ability RWPE-1, RWPE-2, WPE1-NA22, WPE1-NB11, WPE1-NB14, WPE1-NB26 and WPE1-NB27 cells to invade through Matrigel, a reconstituted basement membrane, was examined using a Boyden chamber *in vitro* invasion assay modified in our laboratory (Bello, 1997). DU-145 cells, which show high invasive ability in this assay, were used as the positive control. The modified assay was conducted as follows: on the day prior to running the invasion assay, the following cells were plated in T-75 flasks in 10 ml of the appropriate medium. RWPE-1, RWPE-2, WPE1-NA22, WPE1-NB11, WPE1-NB14, WPE1-NB26 and WPE1-NB27 cells were plated in complete K-SFM at $3X10^6$/flask and DU-145 cells in RPMI-1640 with 5% DCS at $2X10^6$ cells/flask. Matrigel

was diluted 1:20 with cold (4^{0}C), sterile distilled water on ice for a final concentration of 500 μg/ml. Each Nuclepore filter was coated with 25 μg Matrigel in 50μl and left to dry overnight at room temperature under sterile conditions. Cells were rinsed with D-PBS 24 h after plating and incubated with 3 ml of 1 mM EDTA for 8-10 min, dislodged by tapping and recovered by centrifugation. Pellets were resuspended to obtain 2 million cells per ml. RWPE-1, RWPE-2, WPE1-NA22, WPE1-NB11, WPE1-NB14, WPE1-NB26 or WPE1-NB27 cells were re- suspended in 3 ml basal K-SFM containing 0.1% BSA. DU-145 cells were re-suspended in serum-free RPMI containing 0.1% BSA. The bottom chamber contained 220 μl of NIH/3T3 cell conditioned medium which served as the chemoattractant. For preparing conditioned medium, subconfluent NIH/3T3 cultures in 100 mm plates were fed with 7 ml of serum-free DMEM containing 50 μg/ ml ascorbic acid. Conditioned medium was collected 24 h later, centrifuged to remove cell debri and stored at -20^{0}C. A cell suspension containing 400,000 cells in 200 μl medium was added to the top chamber and allowed to remain undisturbed for 5 min before overlaying with 650 μl of K-SFM for RWPE-1, RWPE-2, WPE1-NA22, WPE1-NB11, WPE1-NB14, WPE1-NB26 or WPE1-NB27 cells or serum-free RPMI medium for DU-145 cells, containing 0.1% BSA. Controls consisted of K-SFM medium containing 0.1% BSA or serum-free RPMI medium. Cells were allowed to invade for 24 h at 37^{0}C at which time the filters were prepared for absorbance reading. The migrated cells were fixed, stained with HEMA-3 and allowed to hydrate in distilled water. Nuclear stain was extracted by placing filters individually in wells of a 24-well plate containing 300 μl of 0.1 N HCl for 15 min and 200 μl from each well were then placed in a 96 well plate and the absorbance measured at 620 nm using a Titertek microplate reader. In addition to evaluating the

number of migrated cells on the filter, cells that had migrated to the bottom chamber were also counted. Medium in the bottom chambers was triturated five times and 100 μl were taken from each of the three chambers, pooled into cuvettes containing 10 ml Isoton, triturated ten times and counted. 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, WPE1-NA22, WPE1-NB11, WPE1-NB14, WPE1-NB26 and WPE1-NB27 cells were calculated using DU-145 as 100%. Three replicate invasion chambers were prepared per treatment. All experiments were done in triplicate.

References

Bello D, Webber MM, Kleinman HK, Wartinger DD and Rhim JS: Androgen responsive adult human prostatic epithelial cell lines immortalized by human papillomavirus 18. Carcinogenesis 18: 1212-1223, 1997.

Rhim JS, Webber MM, Bello D, Lee MS, Arnstein P, Chen L and Jay G: Stepwise immortalization and transformation of adult human prostate epithelial cells by a combination of HPV18 and v-Ki-ras. Proc. Nat. Acad. Sci. USA, 91:11874-11878, 1994.

Webber MM, Bello D, Kleinman HK, Wartinger DD, Williams DE and Rhim JS: Prostate specific antigen and androgen receptor induction and characterization of an immortalized adult human prostatic epithelial cell line. Carcinogenesis 17: 1641-1646, 1996.

Webber MM, Bello D, Kleinman HK and Hoffman MP: Acinar differentiation by non-malignant immortalized human prostatic epithelial cells and its loss by malignant cells. Carcinogenesis 18:1225-1231, 1997.

Chapter Three

Extracellular Matrix and Integrins

Background

Background

In prostatic development, the terms acinar and ductal morphogenesis refer to the process by which inducers alter the shape and outgrowth (branching) of epithelial cells into the mesenchyme for the ultimate establishment of acini and associated ducts (Hay, 1981). These subsequently undergo functional differentiation, by inducer influence, to secrete prostate specific proteins, a major component of semen. The creation (morphogenesis) of acini and ducts from undifferentiated epithelial cells involves coordinated interactions which all work toward the proper establishment of epithelial cell polarization and acinar formation. In order to discuss this morphogenic process, one must have a basic understanding of epithelial polarization, the extracellular matrix (ECM), integrins, growth factors and hormones because all of these are involved in normal glandular morphogenesis. Additionally, this review will briefly discuss prostatic epithelial cell makers such as intermediate filament cytokeratin proteins and prostate specific antigen.

Epithelial Cell Polarity

Epithelial cells are organized into sheets, glands and tubules (ducts) that form the interface between tissue compartments in higher organisms. They function to vectorially transport ions, sugars and amino acids against a transmembrane concentration gradient. Thus, by their functions of secretion and/or absorption, they are able to alter the tissue compartments which they separate (Alberts et al., 1994). In the prostate, epithelial cells produce secretions which are a major component of semen. Prostatic epithelial cells form glandular compartments in which epithelial cells apically secrete their products into a lumen for transport through a ductal network. In this manner, the epithelial cells physically

separate the muscular stroma from the glandular lumen and associated ducts. In order to accomplish this apical secretion and physical separation, the epithelial cells must be polarized.

The induction and maintenance of epithelial cells polarity is a multistage process which depends, in part, on cell to cell and cell to ECM interactions (Hay, 1981). These interactions are made possible by surface adhesion molecules located in the plasma membranes. The plasma membrane is divided into three distinct regions: apical, lateral and basal (Alberts et al., 1994). Each region has a distinct protein and lipid composition. The apical region, which borders the glandular and tubule lumen, is characterized by microvilli and cilia involved in absorption and /or secretion. The lateral surface is involved in cell to cell adhesion and cell communication. It is characterized by tight junctions that separate it from the apical surface and by adhesion molecules, such as integrins and cadherins, that seal them to neighboring cells. The basal surface is typically anchored by integrins to the basement membrane. Epithelial cells polarize in response to contact with neighboring cells and the basement membrane, which is a specific part of the ECM.

Extracellular Matrix (ECM)

The extracelluar matrix (ECM) is that structurally stable non-cellular material which lies under the epithelial cells and surrounds connective tissue cells (Hay, 1981). It may be viewed as the ground substance in the tissues of multicellular organisms. The ECM was once

thought of as a cellular product which served as a mere scaffold onto which cells attached via cellular receptors. Today we know the ECM to be an active and vital player in cell migration, proliferation and differentiation, in addition to its role in establishing and maintaining cell shape (polarization) (Hay, 1981). The macromolecules that constitute the ECM are products of cells which reside in it. These cells take an active role in the patterning of the ECM in that their cytoskeleton structure influences the ECM's orientation (Alberts et al., 1994). During embryonic development the ECM plays an important role in organogenesis and epithelial cell polarization and differentiation. The ECM is composed of three major classes of macromolecules secreted by cells: collagens, proteoglycans and glycoproteins (Alberts et al., 1994).

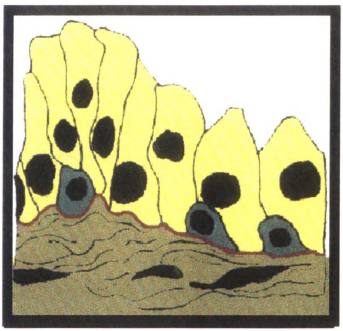


Figure 3-1. Pseudo-stratified prostatic epithelial cell layer. Basal cells and luminal cells are illustrated attached to the basement membrane of the extracellular matrix.

In the prostate, the basement membrane separates the epithelial cells of the glands and ducts from the underlying stroma (ECM proper) (Figure 3-1). The basement membrane (basal lamina) is a structurally distinct zone of the ECM composed of laminin, collagen type IV, nidogen/entactin and heparan sulfate binding proteoglycans (Figure 3-2, Table 3-1). Basement membranes are relatively thin, ranging from 20-300 nm in thickness. They either surround cells as in muscle, fat and nerve axons or they separate cells form the underlying stroma as

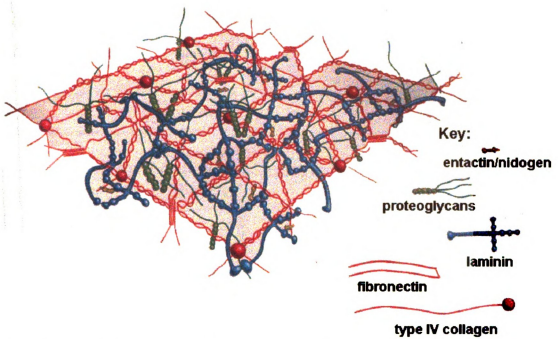


Figure 3-2. A current model of the molecular structure of the basement membrane. The basement membrane is formed by specific interactions between the protiens type IV collagen, laminin, entactin and proteoglylcans (Based on P.D. Yurchenco and J.C. Schittny, 1990).

Table 3-1. Properties of basement membrane components with potential for interaction with laminin. (Timpl et al., 1987)

Component	Ultracentrifugal data		Chain constituents	Domain structure
	$s_{20,w}$	M_r ($\times 10^3$)		
Collagen type IV	4.4 S	550	Two α1(IV) and one α2(IV) chain, each about 180 kDa	Four hundred-nanometer-long triple helix terminating in globular domain
Laminin	11.5 S	950	B1, B2 (each about 230 kDa), and A chain (~450 kDa)	Cross-shaped (115 × 75 nm) with several globular and rodlike domains
Heparan sulfate LD proteoglycan	7.0 S	150	Single-core protein chain (480 kDa) with three HS chains (36 kDa)	Compact, rodlike core (80 nm) HS chains (90 nm) at one pole
HD proteoglycan	4.5 S	130	Small-core protein (5–12 kDa) with four (3–5) HS chains (29 kDa)	Starlike structure with 30-nm HS chains
Nidogen	6.1 S	150	Single chain	Dumbbell (~25 nm long) with central rod

[a] Abbreviations: LD, low-density form; HD, high-density form; HS, heparan sulfate.

in epithelium and endothelium (Timpl and Aumailley, 1989). This section will briefly review ECM components: collagen type IV, laminin/entactin(nidogen), heparan sulfate proteoglycans and fibronectin.

Collagen Type IV: Collagen IV is the major structural component of the basement membrane and functions to anchor other ECM component, most notably, the laminin:cell complex (Timple and Aumailley, 1989). Collagen IV may comprise between 30 to 80% of the total basement membrane mass in different basement membranes. Individual collagen IV chains (1,700 amino acids) assemble to form a 400 nm long triple helix. Although collagen IV forms the scaffold onto which laminin and heparin sulfate proteoglycans bind (Figure 2), it can directly bind to cells via specific collagen IV integrin receptors.

Laminin: Laminins are a family of large trimeric glycoproteins comprised of three disulfide bonded chains. They are found, exclusively, in basement membranes (Malinda and Kleinman, 1996). Developmentally, laminin is one of the first synthesized ECM proteins. In fact, the basement membrane of developing embryos consist mainly of laminin with little or no collagen type IV. In 1979, laminin was first isolated from a tumor and believed to be a third chain of type four collagen. Subsequent, antibody localization and chemical analysis found it to be present in the basement membrane of most tissues (Alberts et al, 1984, Malinda and Kleinman, 1996).

Laminin is a large (~850 kDa) glycoprotein which exist as a cruciform-like structure formed by three very long polypeptide chains (α, β, and γ) which are held together by disulfide bonds (Figure 3-3) (Alberts et al, 1984, Malinda and Kleinman, 1996). The α, β, and γ chains are structurally homologous but lack homology in their amino acid sequence. There are various isoforms of each chain (ie $\alpha 1$, $\alpha 2$, $\alpha 3$), which

assemble to form the different laminin isoform. At the amino terminus, the α chain has three globular domains separate by EGF-like repeats. At the carboxy terminus, it has a coiled-coil domain and a large globular domain. The β and γ chains, being smaller than the α chain, possess only two globular domains and two EGF repeats at the amino terminus and lack a large globular domain at the carboxy terminus. The three chains assemble at the carboxy end. This assembly is mediated by the coiled-coil domain of the α chain, which is critical for chain-specific assembly of the laminin molecule. The assembled cruciform laminin molecule possesses a number of functional domains which bind type IV collagen, heparan sulfate entactin/nidogen and cell surface receptors such as integrins.

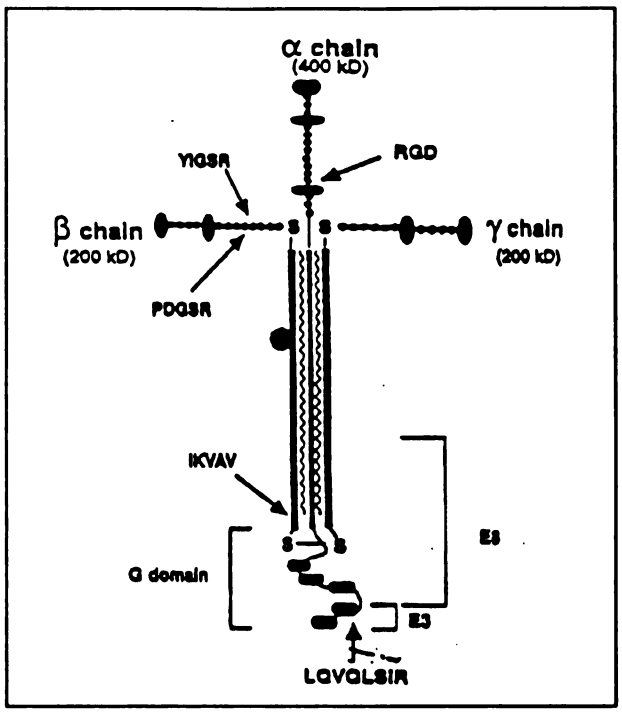


Figure 3-3. Schematic model of Laminin-1 (Malinda and Kleinman, 1996).

Interestingly, entactin/nidogen, a small (150 kDa) sulfated polypeptide molecule, binds through one of its globular domains to the center of the laminin cross (Figure 3-4) and usually co-precipitates with laminin. Although its exact biological function is not known, entactin/nidogen is thought to modulate the activity of a cell binding site in laminin 1 fragment. Entactin/nidogen is able to independently bind to collagen IV, thus, contributing to the intricate linkage pattern necessary for the stabilization of the basement membrane (Malinda and Kleinman, 1996, Martin and Timpl, 1987).

As the key component of the basement membrane, laminins are important both structurally and biologically (Malinda and Kleinman, 1996). Structurally, laminin is thought to initiate the formation of the basement membrane because of its ability to bind collagen IV, entactin and proteoglycans and as well as other laminin molecules. This claim is supported by the observation that it is the first extracellular macromolecule to be expressed by the developing embryo. Biologically, laminins are involved in promoting cell growth, migration, epithelial cell polarity and differentiation, nerve regeneration, wound repair and tumor growth and metastasis. The first biological activity elucidated for laminin was its binding to both normal and neoplastic epithelial cells. Currently, specific adhesion-promoting peptide sequences in laminin have been identified. Laminins affect cellular function by binding to the cells adhesion receptors such as integrins, thereby inducing both mechanical and chemical transduction pathways, which alter gene expression and cell function.

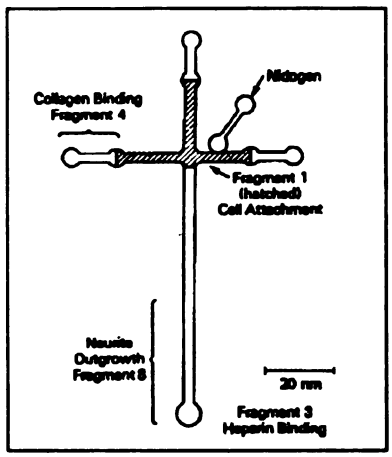


Figure 3-4. A model of laminin-entactin/nidogen complex showing protease fragment indentified by arabic numbers and the lacation of some biological acitivies (Timpl, 1989).

***Heparan sulfate proteoglycans*:** Heparan sulfate proteoglycans are divided into high and low density molecules. The high density molecules (600-700 kDa) are small and star-like in shape and have a low protein content. The low density molecules (130 kDa) have a multi-domain core which is associated with heparan sulfate side chains attached at one end (Figure 3-2). Heparan sulfate proteoglycans are considered to function as filler molecules and serve as selectively charged barriers which make the basement membrane

impermeable to some proteins. Although both types of proteoglycans can bind laminin directly, high-density proteoglycans bind it weakly, while low density proteoglycans bind it strongly.

Fibronectin: Although fibronectin is not usually found in the basement membrane, it is, nevertheless, an important ECM component (Figure 3-2). Fibronectin is a large glycoprotein (210-260 kDa) which is found ubiquitously throughout the body tissues and fluids. Fibronectin functions as a bridge between cells and other ECM molecules, as an attachment factor for cells or as a structural component of ECM. There are two major forms of fibronectin: plasma fibronectin and cellular fibronectin. Cellular fibronectin has large polypeptide chains which exist as monomers, disulfide-bonded dimers and multimeres (Akiyama and Yamada, 1987). The disulfide bonds are unique in that they are usually confined to the carboxy terminus. The odd placement of the disulfide bonds may be to facilitate the ability of the molecule to stretch between distant points while 'mediating adhesive interactions' (Yamada, 1983). These polypeptide chains are composed of discrete globular functional domains. Fibronectin possesses a collagen binding domain, a heparan binding domain, a hyaluronic acid-binding domain, an actin and DNA binding domain and a plasma membrane binding domain (Akiyama and Yamada, 1983, Yamada, 1984). The plasma membrane-binding domain contains an RGD (arginine, glycine, aspartic acid) sequence which is recognized by fibronectin integrin receptors of cells (Garrat and Humpheries, 1995, Sonnenberg 1993).

One aim of the present study is to examine the expression of laminin integrins in human prostatic epithelial cells and their role in human prostatic epithelial polarization and acinar morphogenesis. A second aim is to examine the changes in integrin expression in

human prostate cancer cells and the effects these changes may have on the ability of cancer cells to polarize. Therefore, a brief review of integrin structure and function follows.

Integrins

Integrins are a family of cell adhesion receptors, located in the cell membrane, and are involved in the coupling of cells to the ECM and neighboring cells. Integrins modulate cell adhesion by mechanical transduction and function as signal transduction receptors. Thus, integrins have been shown to play a critical role in embryogenesis, organogenesis, wound repair, cytoskeletal organization, motility, cell growth and differentiation (Geiger et al., 1995, Vinatier, 1995). In addition to their role in establishing cell polarity, integrins are involved in the differentiation of epithelial cells into specialized secretory cells (Sheppard, 1996). Integrins connect cells to their ligands which include laminin, collagens and fibronectin.

Structure

Integrins are heterodimeric transmembrane glycoproteins which consist of an α (120 - 180 kDa) and a β (90-110 kDa) subunit (Figure 3-5). Each subunit has three domains; a large extracelluar domain which binds to the ligand, a transmembrane domain and a short cytoplasmic domain which is associated with the cytoskeleton (Sonnesberg,

1993, (Vinatier, 1995). At present there are 22 known integrin receptors which are the products of different combinations of 16 different α-subunits with 8 different β-subunits (Figure 3-6). The β subunits are promiscuous and can complex with multiple α subunits, while the α subunits are generally loyal to their β subunit partner with the exception of the αv, α4 and α6 subunits (Garret and Humphries, 1995, Vinatier, 1995).

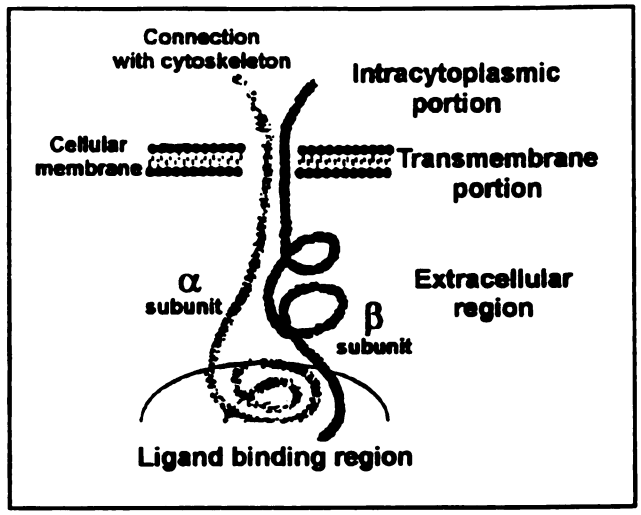


Figure 3-5. A schematic structure of integrins (Vinatier, 1995).

Classification

Integrins are generally classified by their mode of adhesion (Vinatier, 1995). The three binding modes are: cell to ECM adhesion, cell to cell adhesion, and platelet to ECM to platelet adhesion. Integrins are best known for their role in cell to ECM adhesion. Cell to ECM-binding integrins may be loosely divided into two groups. Group one consists of receptors for collagen and laminin and group two consist of receptors for fibronectin and vitronectin. Generally, epithelial cells express integrins

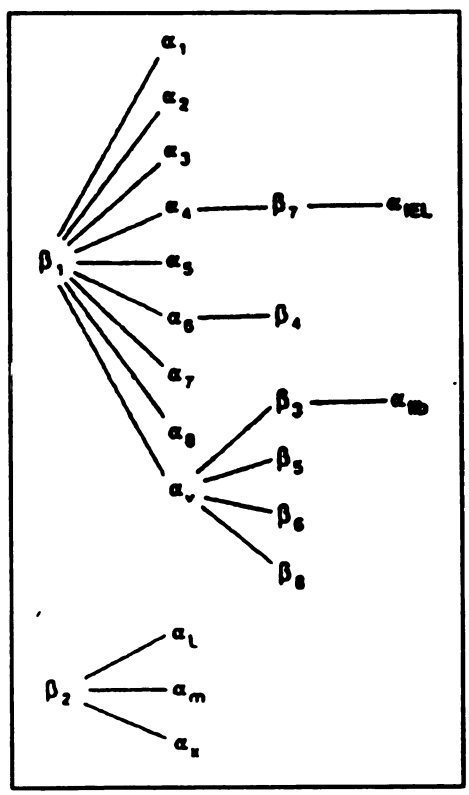


Figure 3-6. The integrin family (Sonnesberg, 1993).

from the collagen/laminin group, while mesenchymal and endothelial cells express integrins from the fibronectin/vitronectin group (Vinatier, 1995) (Figure 3-7).

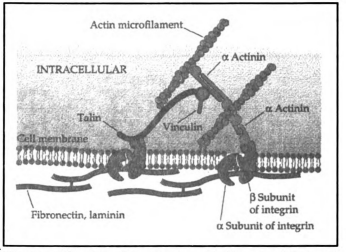


Figure 3-7. Integrin receptor binding to basement membrane. (Gilbert, 1994).

Ligand Specificity and Affinity

Ligand specificity: The different α/β subunit combinations specify the ligand binding ability of integrins. Although integrins are generally considered to be promiscuous receptors because they may bind more than one type of ligand, there are seven integrins (α5β1, αvβ1, αvβ6, αvβ5, α6β1, α5β4 and α7β1) that demonstrate single ligand binding specificity (Table 3-2) (Sonnenberg, 1993). Ligand specificity is also dependant on the cell type, due to differences in post-translational modification of individual integrin subunits (Sonnenberg, 1993). An example of cell-type specificity is seen with the α2β1 integrin. When α2β1 is expressed by platelet cells, it functions as a receptor for collagen, but when it is expressed on endothelial cells, it acts as a receptor for collagen and laminin (Sonnenberg, 1993; Vinatier, 1995).

Table 3-2. Integrins and their ECM binding sites (Sonnenberg, 1993).

Receptor	Alternative name	Ligands
β_1 integrins		
$\alpha_1\beta_1$	VLA-1	Coll (I, IV), Ln
$\alpha_2\beta_1$	VLA-2, GPIa-IIa, ECMRII	Coll (I-V, VI), Ln, Fn?
$\alpha_3\beta_1$	VLA-3, VCA-2, ECMRI, Gaph 3 (hamster)	Ep, Ln, Nd/En, Fn, Coll(I)
$\alpha_4\beta_1$	VLA-4, LPAM-2 (mouse)	Fn.alt, VCAM-1/INCAM110
$\alpha_5\beta_1$	VLA-5, FNR, GPIc-IIa, ECMRVI	Fn
$\alpha_6\beta_1$	VLA-6, GPIc-IIa	Ln
$\alpha_7\beta_1$	VLA-7	Ln
$\alpha_8\beta_1$		?
$\alpha_v\beta_1$		Fn
β_2 integrins		
$\alpha_L\beta_2$	LFA-1	ICAM-1, ICAM-2, ICAM-3
$\alpha_M\beta_2$	Mac-1, CR-3	C3bi, factor X, Fb, ICAM-1
$\alpha_X\beta_2$	p150, 95	Fb, C3bi?
β_3 integrins		
$\alpha_{IIb}\beta_3$	GPIIb-IIIa	Fb, Fn, vWF, Vn
$\alpha_v\beta_3$	VNR	Fb, Fn, vWF, Vn, Tsp, Osp and Bsp1
β_7 integrins		
$\alpha_{IEL}\beta_7$	M290 IEL (mouse)	?
$\alpha_4\beta_7$	LPAM-1 (mouse), $\alpha_4\beta_p$	Fn.alt, VCAM-1
Other β subunit containing integrins		
$\alpha_6\beta_4$	GPIc-IcBP, TSP-180, A9 and EA-1	Ln
$\alpha_v\beta_5$	$\alpha_v\beta_e$, $\alpha_v\beta_{3E}$	Vn
$\alpha_v\beta_6$		Fn
$\alpha_v\beta_8$		?

Ligand affinity: Cells can regulate the binding affinity of integrins to their ligand by either up or down regulating its expression or by direct modulation of the integrin receptor (Vinatier, 1995). Growth factors have been shown to modulate the synthesis and expression of integrin subunits. The first growth factor to be identified as a regulator of integrin expression was transforming growth factor-β1 (TGF–β1) (Garratt and Humphries, 1995). TGF-β, an important negative regulator of epithelial growth, is expressed during embryogenesis. TGF-β1 regulates integrin expression at the level of transcription and by affecting the rate of maturation of the β subunits (Garratt and Humphries, 1995). Specifically, TGF-β1 up regulates the expression of α1 to α6, αv, β1, β3, β5 and β6 integrin subunits. Other growth factors known to regulate integrin expression are nerve

growth factor, interferon, interleukine-1β and tumor necrosis factor-α (Vinatier, 1995).

Binding affinity is also regulated by intracellular events via modification of integrins cytoplasmic domains as well as by extracellular factors (Garratt and Humphries, 1995). Modification of the intracellular, cytoplasmic, domain alters the ability of the extracellular domain to bind to its ligand. This is seen with the expression of α6β1 in macrophages. α6β1 is a laminin receptor which can not bind laminin unless it is first phosphorylated. Phosphorylation occurs as a consequence of macrophage activation (Garratt and Humphries, 1995; Vinatier, 1995). Extracelluarly, integrin affinity is regulated by lipids or by the type of cations present at the ligand binding site. For example, the collagen binding α2β1 integrin will bind collagen in the presence of magnesium but not in the presence of calcium (Garratt and Humphries, 1995; Vinatier, 1995).

Ligand and Receptor Active Sites

Ligand active site: Integrins bind at specific peptide sequences within the ligand called the ligand-receptor binding site. In 1984, the first ligand-receptor binding site was described within the central cell-binding domain of fibronectin as an RGD (Arg-Gly-Asp) tripeptide sequence (Garratt and Humphries, 1995, Sonnenberg, 1993). Although the RGD motif has been shown to be present in most matrix proteins, in some instances, it is masked, thus, becoming unavailable for binding. This is observed in the case of laminin and the αvβ3 integrin. The RGD motif in the laminin is not recognized by αvβ3 integrin because it is hidden within the molecule. Upon proteolytic digestion of laminin, the RGD sequence is exposed and binding by αvβ3 integrin occurs (Sonnenberg, 1993). This could

be one mechanism by which integrins modulate cell motility.

Because all matrix proteins do not contain RGD motifs, and even if they do, it may be masked, this suggests that other receptor binding sites must also exist. At present not all of the receptor binding sites have been discovered but all that have, seem to share one common characteristic. They all have short peptide motifs which contain an acidic amino acid such as aspartic acid and glutamine. Currently, the laminin receptor binding sites that have been identified are fragment p1 (e1-4) for $\alpha 1\beta 1$, fragment E8 for $\alpha 6\beta 1$ and fragment E1 for $\alpha 3\beta 1$. Table 3-2 shows all the receptor binding sites that are presently known.

Receptor active site: The site on the integrin receptor that interacts with the ligand-receptor site is composed of three distinct ligand binding site, as shown in figure 3-8 (Garratt and Humphries, 1995). The first two sites are locates in the α subunit. The first site, called the αA domain, is a 200 amino acid polypeptide segment. The second site is unnamed and is thought to play a key role in maintaining structural integrity. The third ligand-receptor site is located on the β subunit and contains more than one interacting peptide sequence (Garratt and Humphries, 1995).

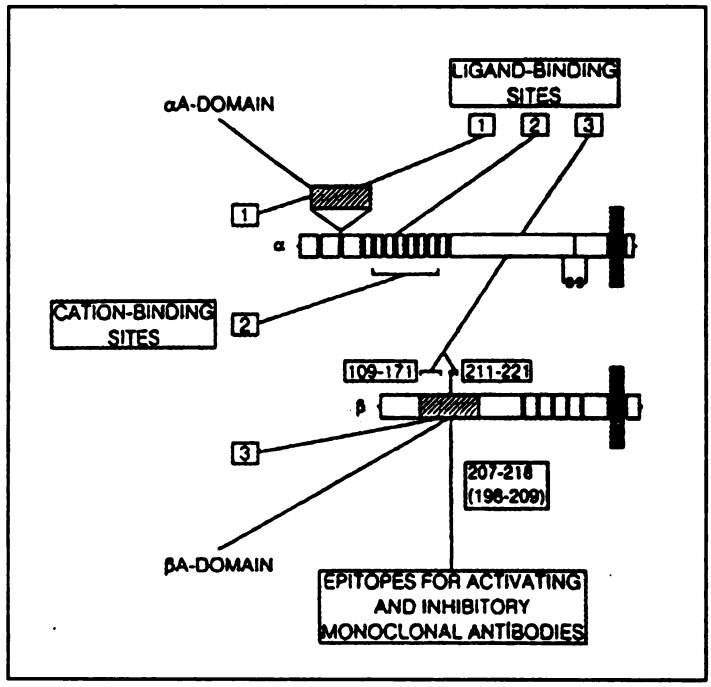


Figure 3-8. Active sites for integrins (Garratt and Humphries, 1995).

Interestingly, three cation binding sites co-localize with the three active binding sites (Figure 3-8). The binding of divalent cations to integrins is required for receptor activation. Generally, the binding of Mg^{2+} or Mn^{2+} to the cation binding sites, along with ligand binding, is stimulatory, while Ca^{2+} binding in inhibitory. Physiologically, the role of cation binding is to mediate ligand binding and regulate the activation state of the integrins (Garratt and Humphries, 1995).

Intracellular Interaction

The intracellular domain of the integrin receptor plays an important role in connecting the plasma membrane to the cytoskeleton either at adhesion junctions called focal contacts, where they are indirectly linked to actin filaments, or at hemidesmosomes which indirectly link them to cytokeratin intermediate filaments (Figure 3-7). Focal contacts are cell:matrix adhesion junctions that contain adhesion-associated proteins such as talin, α-actinin, vinculin, tensin, paxillin, zyxin and cysteine-rich proteins molecules. (Geiger et al., 1996, Sonnenberg, 1993). Prior to activation, the integrin α subunit prevents the integrin receptor from localizing to focal contacts in the plasma membrane. It is upon activation of an integrin receptor that receptors are recruited into a focal contact. Here, the β subunits bind to the cytoskeletal components, while the α subunits are primarily associated with the ligand (Geiger et al., 1996, Sonnenberg, 1993). Within the focal contacts, the cytoplasmic portion of the β integrin subunit is connected to actin filaments via talin or α-actinin. Talin and α-actinin are, in turn, connected to the actin filaments of the cytoskeleton. Additionally, α-actinin along with a molecule, called fimbrin, connect neighboring actin filaments to one another. Tensin also binds different

actin filaments to each other and binds to vinculin. Zyxin and cysteine-rich proteins are associated with the α-actinin molecules. Vinculin binds talin and possibly α-actinin, while at the same time interacting with actin filaments. Finally, paxillin binds to vinculin, which binds to talin that binds to both the β subunit of integrin and the actin filament of the cytoskeleton. Paxillin is also associated with focal adhesion kinase (FAK), that is involved in signal transduction (Geiger et al., 1996). All these connections play an important role in how integrins exert their mechanical effect on the cytoskeleton which, in turn, alter cellular function.

Intracellular signaling

Intracellular signaling by integrins may be accomplished by either mechanical transduction or by signal transduction through secondary messenger pathways. It is believed that the biochemical effects, observed as a result of integrin binding, are predominantly controlled through signal transduction rather than a mechanical transduction (Rosales et al., 1995).

Mechanical transduction: Integrins provide mechanical support by linking the cytoskeleton to the ECM via the plasma membrane (Garratt and Humphries, 1995). Thus, the ability of cells to sense position and shape is determined by the number and distribution of its integrins and integrin-mediated adhesion. Integrins, by virtue of physically linking the ECM to the cytoskeleton via focal contact proteins, create a cytoskeletal organization and mechanical tension that directly influences events in the cytoplasm and the nucleus (Garratt and Humphries, 1995, Rosales et al., 1995). In non-motile cells, such as epithelial cells, the mechanical stress state is important for the maintenance of polarity and cell shape

and for cellular movement in cases of wound healing and inflamation (Garratt and Humphries, 1995). Additionally, mechanical transduction works synergistically with integrin-induced signal transduction events which modulate differentiation and specific gene expression.

Signal transduction by integrins: In recent years, it has become clear that integrins not only function to mediate cellular adhesion to the ECM, but also to trigger biological responses in a manner similar to those transduced by growth factors and hormones (Ruoslahti and Reed, 1994, Streuli et. al., 1995). There is a strong possibility that integrin-initiated signal pathways may impinge pathways involving other type of receptor systems. Although not much is known about the signal transduction pathways associated with integrins some key factors have been identified. A 125 kDa FAK has been shown to phosphorylate tyrosine and autophosphorylate in response to integrin activation and clustering in focal contacts (Geiger et al., 1995, Rosales et al., 1995). The FAK kinase has been associated with PI-3 kinase and GRB-2 which are involved in the tyrosine pathway and G-protein/inositol-lipid pathway (Rosales et al., 1995). Thus, suggesting that integrin FAK activation include proteins and cascades used in the other pathways such as the receptor tyrosine and the G-protein pathway. Unfortunately, the mechanism by which FAK works is unclear at present.

Integrin-Mediated Gene Expression

Integrin-mediated gene expression interacts with and is, thereby, regulated by signals from other receptor system pathways such as hormone and growth factors. For

example, in monocytes, β1 integrin activation induces cytokine and metalloproteinase gene expression, which are dependant on tyrosine phosphorylation (see Lin et al. 1994 in Garratt and Humphries, 1995, Yurochko et al. 1992). In epithelial cells, control of gene expression is best illustrated with the expression of β-casein in mammary epithelial cells (Roskelley et al., 1994). β-casein is an abundant milk protein the expression of which is regulated by prolactin and basement membrane via integrin binding. Basement membrane-dependant expression of β-casein requires that mammary epithelial cells have functional β1 integrin. β-casein gene expression is regulated transcriptionally by an ECM response element located upstream of the β-casein gene (Roskelley et al., 1994).

Roskelley et al. (1994) set out to determine if the β-casein expression, elicited by integrin binding to the laminin rich ECM, was solely based on physical clustering or on biochemical signals. Mammary cells were grown on non-adhesive substratum polyHEMA to cause cell clustering. The cell clusters were then treated with prolactin and assayed for β-casein expression but none was found. Thus, clustering was not sufficient to induce β-casein expression even in the presence of prolactin. When cells plated on the polyHEMA coated plates were overlaid with ECM and treated with prolactin, β-casein expression was seen within 8 hours. They also showed that within 30 minutes of overlay with laminin, tyrosine phosphorylation increased. Therefore, it was concluded that β1 integrin regulates β-casein expression by a biochemical component involving tyrosine phosphorylation associated with integrin activation. Because the prolactin signal transduction pathway is not clear, at present, it is not known how it interacts with the integrin stimulated signal transduction pathway (Roskelley et al., 1994).

In summary, it can be stated that integrins have been shown to play a critical role in embryogenesis, organogenesis, wound repair, cytoskeletal organization, motility, cell growth and differentiation by their ability to trigger signal transduction as well as by mechanical transduction (Geiger et al., 1995, Vinatier, 1995).

In order to establish the validity of an *in vitro* human prostatic epithelial cell model, it is necessary to demonstrate that the cells express epithelial cell marker protiens as well as prostate-specific marker protien. We have previously demonstrated that cell lines used in this study express the epithelial cell marker cytokeratins, as well as prostate epithelial markers androgen receptor and prostate specific antigen (PSA) (Bello et al., 1997). A brief description of these markers follows.

Prostate Epithelial Markers

Prostate Specific Antigen

Prostatic secretory epithelial cells synthesize and secrete prostatic specific antigen *in vivo* (Fong et al., 1991). Prostate specific antigen is a major component of the semen. Its function is to liquefy the semen after ejaculation (Lija et al., 1987, Webber et al., 1995). 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 (Lundwall and Lima, 1987, Webber et al., 1995).

The effects of androgen on prostate specific antigen production are clearly demonstrated by the prostatic cancer cell line LNCaP (Young et al., 1991, Webber et al, 1996). 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 (Fong et al., 1992, Young et al., 1991). 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 (Henttu and Vinkop, 1993). Clinically, serum prostate specific antigen levels, which are elevated in association with prostate cancer, are used for the diagnosis of prostate cancer and for early detection (Garde et al., 1993, Webber et al., 1995). 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 (Webber et al., 1995).

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 (Fong et al., 1991, Webber et al., 1995, Webber et al., 1996). 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.

References

Alberts B, Bray D, Lewis J, Raff M, Roberts K and Wastson JD (eds.): Cell junctions, cell adesion and the extracellular matrix. In: Molecular biology of the cells, third edition. New York, Garland Plublishing Inc., pp. 949-1009, 1994.

Akiyama SK and Yamada KM: Fibronectin. Adv. Enzymol. 59:1-57, 1987.

Bello D, Webber MM, Kleinman HK, Wartinger DD and Rhim JS: Androgen responsive adult human prostatic epithelial cell lines immortalized by human papillomavirus 18. Carcinogenesis 18: 1212-1223, 1997.

Fong CJ, Sherwood ER, Braun EJ, Berg LA, Lee C and Kozlowski JM: Regulation of prostatic carcinoma cell proliferation and secretory activity by extracellular matrix and stromal secretions. Prostate, 21: 121-131, 1992.

Garde SV, Sheth AR, Porter AT and Pienta KJ: A comparative study on expression of prostatic inhibin peptide, prostate acid phosphatase and prostate specific antigen in androgen independent human and rat prostate carcinoma cell lines. Cancer Lett., 70: 159-166, 1993.

Garratt AN and Humphries MJ: Recent insights into ligan binding, activation and signalling by integrin adhesion receptors. Acta Anat. 154, 34-45, 1995.

Geiger B, Yehunda-Levenberg S and Bershadsky AD: Molecular interactions in the submembrane plaque of cell-cell and cell-matrix adhesions. Acta Anat. 154, 46-62, 1995.

Gilbert SF: Developmental biology, fourth edition. Massachusetts, Sinauer Associates, Inc. Publishers, pg. 111, 1994.

Hay E: Collagen and embryonic development. In: Cell biology of extracellular matrix. Hay E (ed.), New York, Plenum Press, pp.379-405, 1981.

Henttu P and Vinkop P: Growth factor regulation of gene expression in the human prostatic carcinoma cell line LNCaP. Cancer Res., 53: 1051-1058, 1993.

Lija, H. Oldbring, J., Rannevik, G. and Laurell, C. Seminal vesicle-secreted proteins. J. Clin. Invest., 80: 281-285, 1987.

Lin TH, Yurochko A, Kornberg L, Morris J, Walker JJ, Haskill S and Juliano RL: The role of protein tyrosine phosphorylation in integrin-mediated gene induction in monocytes. J. Cell. Biol. 126, 1585-1593, 1994.

Lundwall, A. and Lima, H. Molecular cloning of the human prostate specific antigen cDNA. FEBS Lett., 214: 317-322, 1987.

Malinda KM and Kleinman HK: Molecules in focus: the laminins. Int. J. Biochem. Cell Biol. 28:957-959, 1996.

Martin GR: Laminin and other basement membrane components. Ann. Rev. Cell Biol. 3:57-85, 1987.

Rosales C, O'Brien V, Kornberg L and Juliano R: Signal transduction by cell adhesion receptors. Biochim. Biophys. Acta 1242, 77-98, 1995.

Roskelley CD, Desprez PY and Bissel MJ: Extracellular matrix-dependent tissue specific gene expression on mammary epithelial cells requires both physical and biochemical signal transduciton. Proc. Natl. Acad. Sci. USA 91, 12378-12382, 1994.

Ruoslahti E and Reed JC: Anchorage dependence, integrins, and apoptosis. Cell 77:477-478, 1994.

Sheppard D: Epithelial integrins. Bioessays 18:655-660, 1996.

Sonnenberg,A. (1993) Integrins and their ligands. Curr. Top. Microbiol. Immunol. 184, 7-35.

Streuli CH, Schmidhauser C, Bailey N, Yurchenco P, Skubitz AP, Roskelley C and Bissell MJ: Laminin mediates tissue-specific gene expression in mammary epithelia. J. Cell Biol. 129: 591-603, 1995.

Timpl R: Structure and bilogical activity of basement membrane protiens. Eur.J.Biochem. 180, 487-502, 1989.

Timpl R and Aumailley M: Biochemistry of basement mambranes. Adv. Nephrol. 18:59-76, 1989.

Vinatier D: Integrins and reproduction. Euro. J. Obs. Gyn. Reprod. Bio. 59, 71-81, 1995.

Webber MM, Waghray A and Bello D: Prostate-specific antigen, a serine protease, facilities human prostate cancer cell invasion. Clin. Cancer Res., 1: 1089-1094, 1995.

Webber, MM, Bello D, and Quader S: Immortalized and tumorigenic adult human prostatic epithelial cell lines. Characteristics and applications. Part. 1. Cell markers and immortalized, non-tumorigenic cell lines. Prostate 29:386- 394, 1996.

Yamada KM: Cell surface interactions with extracellular materials. Ann. Rev. Biochem. 52:761-99, 1983.

Young CY, Montgomery BT, Andrews PE, Qui SD, Bilhartz DL and Tindall DJ: Hormonal regulation of prostate-specific antigen messenger RNA in human prostatic adenocarcinoma cell line LNCaP. Cancer. Res., 51: 3748-3752, 1991.

Yurochko KM, Liu DY, Eierman D and Hakill S: Integrins as a primary signal transduction molecule regulating monocyte immediate-early gene induction. Proc. Natl. Acad. Sci. USA 89, 9034-9038, 1992.

Yurchenco PD and Schittny JC: Molecular architecture of basement membranes. FASEB J. 4:1577-90, 1990.

Chapter Four

Acinar Differentiation by Non-malignant Immortalized Human Prostatic Epithelial Cells and Its Loss by Malignant Cells

Work presented in Chapter 3 has been published:

Webber MM, Bello D, Kleinman HK and Hoffman MP: Acinar differentiation by non-malignant immortalized human prostatic epithelial cells and its loss by malignant cells. Carcinogenesis 18:1225-1231, 1997.

Other related work:

Bello D, Webber MM, Kleinman HK, Wartinger DD and Rhim JS: Androgen responsive adult human prostatic epithelial cell lines immortalized by human papillomavirus 18. Carcinogenesis 18: 1212-1223, 1997.

Abstract

Invasive prostatic carcinomas and prostatic intraepithelial neoplasia (PIN) are characterized by a loss of normal cell organization, cell polarity, and cell:cell and cell:basement membrane adhesion. The objective of this study was to establish *in vitro* three-dimensional (3-D) cell models which can be used to investigate mechanisms involved in acinar morphogenesis and differentiation in normal prostatic epithelium and their abnormalities in cancer cells. The process of acinar morphogenesis, including structural and functional differentiation, was investigated by culture on basement membrane gels (Matrigel). The human papillomavirus 18 immortalized, non-tumorigenic cell line RWPE-1, the v-Ki-*ras* transformed, tumorigenic RWPE-2 cell line derived from RWPE-1 cells and the human prostatic carcinoma cell line DU-145 were used. When cultured on Matrigel, RWPE-2 cells remain as single cells or form small aggregates and DU-145 cells form large amorphous cell aggregates without any organization or lumen. In contrast, RWPE-1 cells form acini of polarized epithelium with a distinct lumen, show a distinct laminin basement membrane, and express $\alpha 6\beta 1$ integrins at their basal end. Exposure to conditioned medium from NIH 3T3 cultures accelerates glandular morphogenesis. Parallel cultures maintained as monolayers on plastic remain as monolayers. In the presence of the synthetic androgen mibolerone, acinar cells express prostate specific antigen (PSA) as determined by immunostaining. We conclude that normal prostate cells can undergo acinar morphogenesis while tumorigenic cells have lost this ability. The 3-D cultures provide physiologically relevant *in vitro* models for elucidating regulation of growth, morphogenesis and differentiation in the normal human prostate, for defining heterotypic interactions in benign prostatic hyperplasia and for establishing the basis for the loss of normal cell organization in early neoplastic lesions such

as PIN as well as during tumor progression in prostate cancer.

Introduction

Loss of normal cell organization, cell polarity, and cell:cell and cell:basement membrane (BM) adhesion has been observed in many carcinomas including invasive prostatic carcinomas and advanced prostatic intraepithelial neoplasia (PIN) (1,2). These abnormalities are associated with a loss, decrease or altered expression of the basement membrane protein laminin, cell adhesion molecules such as cadherins and integrin receptors for extracellular matrix (ECM) proteins. Normal expression of these proteins is critical for normal morphogenesis. The aim of this study was to establish three-dimensional (3-D) *in vitro* cell models, derived from non-tumorigenic and tumorigenic human prostatic epithelial cells, which could be used to investigate mechanisms involved in acinar morphogenesis and differentiation in both normal and malignant prostatic epithelium.

The ultimate goal of an *in vitro* model, using normal prostatic epithelium, is to mimic the *in vivo* cell architecture and retain differentiated function. Monolayer cultures lack the 3-dimensional cell organization of tissues *in vivo* and a rapid loss of cell function is often observed. Three-dimensional cultures simulate the *in vivo* architecture more realistically than monolayer cultures. Such a model for the human prostate has not previously been available. A necessary prerequisite for such a model is to develop cell lines that retain morphology and function similar to primary cells, and remain responsive to hormones and to other growth regulators. We have developed and characterized several immortalized, non-tumorigenic and tumorigenic human prostatic epithelial cell lines which express functional characteristics of

normal cells (3-7). These cell lines now make it possible to study morphogenesis and differentiation in both normal and malignant prostate cells using standardized and reproducible cell types. It is recognized that immortalization may alter some cell characteristics, however, we have demonstrated that the RWPE-1 cell line, used in the present study, has retained the Y chromosome and normal cytokeratin expression and the cells mimic normal epithelium in their response to growth factors as well as androgens by the expression of PSA and up-regulation of androgen receptor (3).

Although much work has been done on factors that stimulate the growth of prostatic epithelial cells, little is known about ECM-mediated regulation of morphogenesis and differentiation. ECM is necessary for epithelial cell proliferative response to growth factors as well as differentiation which are both necessary for maintaining homeostasis (8). In the normal adult prostate in homeostasis, there is little net growth because a balance between cell loss and cell gain is maintained among and between different cell types. One objective of this study was to develop a 3-D *in vitro* model for human prostate morphogenesis to mimic the *in vivo* acini and to use this for comparison with malignant cell behavior. Freshly isolated acini from benign prostatic hyperplasia (BPH) tissue embedded in collagen and then maintained as xenografts in nude mice have been used for short term cultures to examine epithelial-stromal interactions but these cells only have a finite lifespan (9). Salivary and mammary gland epithelial culture models using culture on extracellular matrix have been described (10,11). However, a 3-D matrix culture model for human prostatic epithelium using well characterized cell lines was not available prior to the present study. This study includes results on polarization and organization of non-malignant prostatic epithelial RWPE-1 cells

into acini when maintained in Matrigel, and androgen-induced functional differentiation, resulting in PSA expression. The behavior of RWPE-1 cells in 3-D cultures, the assembly of laminin in the basement membrane, and the expression of integrins was examined. The ability of the non-tumorigenic RWPE-1 cells to undergo acinar morphogenesis was compared with that of the tumorigenic RWPE-2 and DU-145 cells along with their invasive ability.

Epithelial cell polarization requires both cell-cell and cell-matrix adhesion (12,13). Laminin, an essential component of epithelial cell basement membrane (BM), is the key BM glycoprotein in cell:ECM adhesion and polarization. Little is known about the role of laminin and its integrin receptors in prostate morphogenesis and differentiation. Laminin is a large ~800 kDa glycoprotein consisting of three chains, α, β, and γ arranged in a cruciform-like structure (13). It is the first ECM protein to be synthesized during embryogenesis, which points to its crucial role in morphogenesis and organogenesis (8,13,14). Laminin is a major (60%) component of Matrigel, a re-constituted BM (8), used in this study for the 3-D cultures. Since integrin receptors mediate epithelial cell polarization, integrin expression was examined. Integrins, a complex family of heterodimeric transmembrane receptors for ECM proteins, consist of α and β subunits and provide the link between the ECM and the cytoskeleton. Complexing of α and β subunits in different combinations results in their binding specificity for different ECM proteins. The $\alpha 6\beta 1$ receptor is considered to be the specific receptor for the E8 cell-binding domain of the α laminin chain, and is essential for cell polarization, for example, in developing kidney tubules (13-16). While cell: basement membrane interactions lead to structural morphogenesis of normal epithelial cells, androgens are necessary for functional morphogenesis of prostatic epithelium and for the maintenance

of a differentiated phenotype. Therefore, the expression of PSA was used in this study as a marker of functional differentiation in response to androgen exposure.

Materials and methods

Materials

Keratinocyte-serum free medium (K-SFM) no.17005-042, antibiotic/antimycotic mixture (PSF) no. 15240-013 (Gibco-BRL, Grand Island, N.Y.); Dulbecco's modified Eagle's medium (DMEM) no. CC293, RPMI-1640 no.CC261 (Celox Laboratories, Hopkins, MN); Dulbecco's phosphate buffered saline (D-PBS Ca^{2+}/Mg^{2+}-free) no. 28374 (Pierce, Rockford, IL); donor calf serum (DCS) no. 1110-90 (Intergen, Purchase, N.Y.); mibolerone no. W-300 (BIOMOL Biomolecular Research Laboratories, Plymouth Meeting, PA); monoclonal antibody to α6 integrin, clone GoH3 (AMAC Inc., Westbrook, ME); monoclonal antibody to β1 integrin clone m13 (gift of Dr. S.K. Akiyama, NIDR, NIH); monoclonal antibody to laminin no. c-8271(Sigma;, St. Louis, MO); monoclonal antibody to prostate specific antigen no. M-0750 (DAKO, Carpinteria, CA); Vectastain Elite ABC Peroxidase Kit no. PK-6102 and 3,3-diamino-benzidine (DAB) Substrate Kit no. SK-4100 (Vector Laboratories, Burlingame, CA); 24 well plates (Falcon) no. 08-7721; 12 mm circle coverslips no. 12-545-80 (Fisher Scientific, Itasca, IL); Matrigel was prepared as described (17).

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, plus

antibiotic/antimycotic mixture PSF (Penicillin, 100 U/ml, Streptomycin 100 μg/ml and Fungizone, 25 μg/ml). DU-145 human prostate carcinoma cell line was maintained in RPMI-1640 medium containing 5% donor calf serum (DCS) and 1% PSF. Cells were passaged upon confluence and seeded at 2 x 10^6 cells/ T-75 flask.

Acinar morphogenesis; 3-D cultures on Matrigel: Phenol red-free Matrigel was used. To the wells of 24-well plates, 200 µl of Matrigel kept on ice, were added and the plates were placed in a 37^{0}C incubator for 1 h to allow the matrix to gel. RWPE-1 cells were plated at 100,000 cells/ well in complete K-SFM. The wells were divided, 24 h later, into treatment groups in triplicate and the medium was replaced with test medium. Cells plated on plastic served as the monolayer controls. Medium was changed every 48 h. Acinar formation was followed and recorded photographically. At the appropriate time intervals, cultures were washed gently with PBS, fixed in 2.5% buffered paraformaldehyde for 15 min, rinsed twice with PBS and either stained as whole mounts with Hematoxylin and Eosin (H&E) for gross morphology or paraffin embedded and sectioned for immunostaining.

Androgen treatment: Experiments to examine the effects of androgen on morphogenesis and expression of differentiated function employed mibolerone, a non-metabolizable, synthetic androgen to ensure continuous androgen presence during the experimental period. Stock solutions and cultures were protected from light. Mibolerone was dissolved in absolute ethanol and was added to test media at 5 nM concentration, on the basis of previously observed effects on human prostate epithelium *in vitro* (3,5). Medium was changed every 48 h. Androgen effects on differentiation were evaluated by the expression of PSA using immunostaining.

Conditioned medium (CM) for effects on morphogenesis: NIH 3T3 cells were maintained in DMEM with 5% DCS. When cultures in 100 mm plates reached ~80% confluence, they were rinsed twice with PBS and placed on 7 ml serum-free DMEM. Conditioned medium was collected 24 h later, centrifuged to remove cell debris, aliquoted and frozen at -20^0 C until needed. For CM experiments, K-SFM medium and CM were mixed in a 1:1 ratio for RWPE-1 and RWPE-2 cells or RPMI with 5% DCS and CM in a 1:1 ratio for DU-145 cells.

Immunocytochemical analysis: Protein expression was detected by a modified avidin-biotin immunoperoxidase Vector protocol, using monoclonal antibodies (3,5). Matrigel cultures were washed 2X with PBS, fixed in paraformaldehyde, paraffin embedded, sectioned at 5μ, mounted on poly-L-lysine coated slides and re-hydrated for immunocytochemical analysis. Immunocytochemistry was performed using an indirect immuno-peroxidase modified Vector protocol (3,5). Control sections lacked primary antibody. Antibody dilutions were made in normal horse serum. All sections were washed twice in 500 μl PBS over 10 min between all successive steps after primary Ab application. The following sequential steps were conducted at room temperature and cells were rinsed twice with PBS between steps after application of the primary Ab: sections were blocked with normal horse serum for 1 h, incubated with the appropriate specific antibody for the indicated time followed by biotinylated secondary Ab (1:200) for 30 min, treated with 3% H_2O_2 for 3 min to quench endogenous peroxidase activity, incubated with the avidin-biotin-peroxidase complex for 30 min, developed with diaminobenzidine-nickel chloride (DAB) substrate for 4-6 min, dehydrated and mounted on acid/alcohol washed slides. For immunostaining of PSA, cultures

were treated in K-SFM medium containing 5 nM mibolerone, a non-metabolizable androgen, beginning 48 h after plating. Cultures were processed as described above. The primary antibody dilutions were: for laminin 1:200, α6 integrin 1:100, β1 integrin 1:100, and PSA 1:20, and incubation time was 24 h at 4^0 C.

Invasion assay: The method for the invasion assay is described in detail in the accompanying paper (3). Briefly, cells were plated at a density of 400,000 cells/well, in triplicate, on Matrigel-coated filters in serum-free medium containing 0.1% bovine serum albumin. Cells were allowed to invade for 24 h.

Results

Morphogenesis in 3-Dimensional cultures of RWPE-1, non-tumorigenic cells and immunostaining for laminin, α6 and β1 integrins, and PSA: The effects of a basement membrane substratum (Matrigel) on RWPE-1 cell morphology under different media conditions were first examined. Twenty-four hours after plating, wells were divided into several groups and were maintained on: 1) complete K-SFM; 2) complete K-SFM containing 5 nM mibolerone; 3) NIH 3T3 conditioned medium (CM) in a 1:1 ratio with K-SFM and 4) CM in a 1:1 ratio with K-SFM containing 5 nM mibolerone. Within 6 h after plating, cells attached and they began to migrate and organize by forming cytoplasmic connections with neighboring cells and by 24 h, a web-like pattern was seen in all cultures (Figure 4-1). With time, cells plated on Matrigel migrate into the gel. Morphogenesis by RWPE-1 cells was a little more rapid in CM treated-cultures and by day 6, distinct duct-like structures or tubes

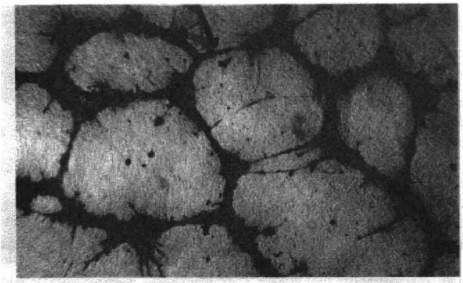


Figure 4-1. Appearance of RWPE-1 cells on Matrigel. RWPE-1 cells (10^5 cells) when plated on Matrigel show a web-like organization 24 h after plating in complete K-SFM medium. (X20).

and acini were seen. Extensive branching and budding from tubes into acini-like structures was seen in greater numbers in the CM treated cells than the controls by day 12 (Figures 4-2a and 4-2b). This budding is also apparent in hematoxylin and eosine stained sections (Figure 4-2c). Figure 4-2d shows the *in vivo* branching morphogenesis in the developing rat prostate (18) while Figure 4-2e shows the *in vitro* morphogenesis of RWPE-1 cells in Matrigel at day 16. It is clear that the *in vitro* morphogenesis and the formation of acini is similar to that seen *in vivo*. Whole acini in live cultures of RWPE-1 cells appear as blebs (Figure 4-2f). In sections, the acini show a well organized, polarized epithelium with a distinct lumen (Figure 4-2g), similar to that seen *in vivo* in a histological section of the human prostate (Figure 4-2h) from which RWPE-1 cells were originally derived.

The organization of laminin in the basement membrane and the expression of α6 and β1 integrins, and PSA were examined by indirect immunoperoxidase staining. Cells organized into acini show a distinct basement membrane consisting of laminin surrounding the acini (Figure 4-2i). Strong positive staining for α6 and β1 integrins was observed at the basal end of the acinar cells (Figures 4-2j, 4-2k) as compared to the control (4-2*l*). Most importantly, cells in acini from mibolerone treated cultures show strong positive staining for PSA (Figure 2m) as compared to the control lacking primary antibody (Figure 4-2n). The secreted material in the lumen of the acini also stained positive for PSA (Figure 4-2m). With time, the acini became distended with secretion and contained dead cells as seen in Figures 4-2k and 4-2m. Cells grown on plastic did not show acinar organization and only formed a monolayer (Fig.4-2*o*).

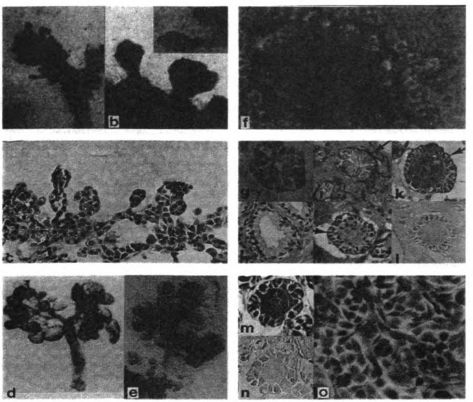
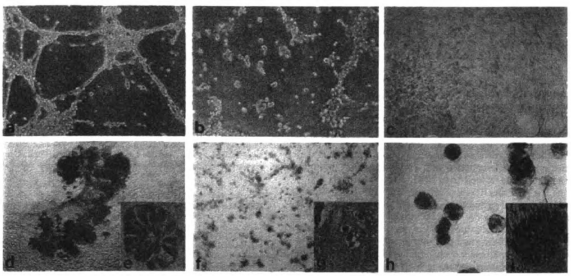


Figure 4-2. 3-D cultures. Acinar formation *in vitro* and *in vivo*. Photographs were taken between 12-17 days after growth on Matrigel. **a**. RWPE-1 culture on day 12 after plating on Matrigel. Extensive branching and budding from tubes into acini-like structures was seen in cultures containing CM and 5nM mibolerone (X50); **b.** budding acini at higher magnification.(X198); **c.** an H&E stained section showing branching and budding, day 12 (X105); **d.** branching morphogenesis in neonatal rat prostate (from Yamashita et al. 1996, Ref. 18); **e.** branching and acinar morphogenesis in a 3-D *in vitro* Matrigel culture of RWPE-1 cells, day 16 (X105) mimics that *in vivo* shown in Figure 2d; **f.** surface architecture of an *in vitro* culture on Matrigel showing several blebs which represent the acini, day 12 (X220); **g.** cross sections (H & E) of an acinus in an *in vitro* Matrigel culture showing polarized organization of cells around a central lumen, day 12 (X525); **h.** a gland in a histological section of a normal human prostate, from which RWPE-1 cells were derived, is shown for comparison (X230); **I-n.** immunoperoxidase staining of acini **I.** laminin shows a well defined basement membrane, day 12 (X390); **j & k.** $\alpha 6$ and $\beta 1$ integrin subunits shows strong staining at the basal end of cells, day 17 (X250); ***l*** **.** a negative control, day 17 (X250); **m.** positive staining for PSA, day 17 (X250); **n.** a serial section negative control for PSA (X250); ***o.*** RWPE-1 cells in a monolayer culture on plastic, day 12 (X55).

Comparison of the ability for structural morphogenesis by the non-tumorigenic RWPE-1 cells with that of the tumorigenic RWPE-2 and DU-145 cells in 3-D cultures: When plated on Matrigel, RWPE-1 cells rapidly begin to organize and within 24 h form a network of cells (Figure 4-3a). However, RWPE-2 cells failed to show such organization and remained as single cells or formed small clumps (Figure 4-3b), and DU-145 cells remained as a monolayer (Figure 4-3c). After culture for 12 days, the RWPE-1 cells showed well developed branching with branches terminating in acini (Figure 4-3d), whereas, the RWPE-2 cells remained either as single cells or formed small clumps without any organization with evidence of cell death (Figure 3f), while DU-145 cells formed amorphous balls (Figure 4-3h). In cross sections, the RWPE-1 acini show distinct lumens lined by a single layer of cells (Figure 4-3e). The RWPE-2 cells showed no such organization (Figure 4-3g) and the DU-145 cells formed amorphous solid balls without lumens (Figure 4-3i). These results demonstrate that RWPE-1 cells retain the ability to organize into acini, similar to those seen *in vivo* but the malignant cells have lost this ability.

The invasive ability shows an inverse relationship with the ability to undergo acinar morphogenesis: The invasive ability of RWPE-1 cells through Matrigel was compared with that of RWPE-2 and DU-145 cells, where DU-145 invasion was taken as 100%. In comparison to DU-145 cells, the RWPE-1 cells were not invasive while the RWPE-2 cells showed 49% invasion (Figure 4-3j).



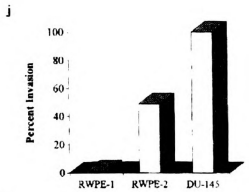


Figure 4-3. Cell organizaton on Matrigel and relationship with invasion. **a, b, c.** Gross morphology of cells plated on Matrigel as seen after 24 h, (X50). **a.** RWPE-1; **b.** RWPE-2; **c.** DU-145 cells. **d,f,h.** cells plated in Matrigel as seen after 16 days (X50). **d.** non-tumorigenic RWPE-1 cells undergo branching and acinar morphogenesis; **f.** tumorigenic RWPE-2 cells remain as single cells or form small clumps; **h.** DU-145 cells form amorphous balls. **e,g,i.** cross sections (H & E) of cultures representing those shown in 2f, 2h and 2e. **e.** RWPE-1 cells are polarized and organized into an acinus containing a lumen (X525); **g.** RWPE-2 cells do not show any organization, (X250); **I.** DU-145 cells form solid balls without a lumen (X250); **j.** The invasive ability of non-tumorigenic RWPE-1 cells was compared with that of the tumorigenic cell lines RWPE-2 and DU-145 using a modified Boyden chamber assay (see Ref. #3). Cells were plated on Matrigel-coated filters at 400,000 cells per chamber and were allowed to invade for 24 h. Invasion by DU-145 cells was taken as 100%.

Discussion

In prostatic tissue homeostasis, a balance is maintained between growth stimulation and inhibition, where the latter involves a decrease in cell proliferation with terminal differentiation into secretory cells which are eventually sloughed off. In this context then, morphogenesis includes two steps: 1) structural differentiation involving acinar organization and 2) functional differentiation involving expression of secretory products. Shifting the balance in favor of functional differentiation should result in a net decrease in growth, a desirable effect for counteracting growth of cancer cells and re-initiation of growth in middle age men which results in BPH. Our results demonstrate that RWPE-1, immortalized but non-tumorigenic cells, retain the ability to undergo acinar differentiation as a result of their interaction with ECM, whereas, the *v-Ki-ras*-transformed RWPE-2 tumorigenic cells and DU-145 human prostatic carcinoma cells have lost the ability to organize in the same manner. Similar observations have been made when normal and malignant human breast epithelial cells were grown on ECM (11,19).

Laminin plays a critical role in cell-ECM adhesion, polarization, morphogenesis and differentiation and the response of cells to laminin *in vitro* resembles the differentiated phenotype of cells *in vivo* (8,13,14). Laminin, a major (60%) component of Matrigel, has been used to study human endothelial and salivary gland morphogenesis (10,20). Our results show that RWPE-1 cells are able to assemble a well defined, laminin-containing BM at the periphery of the acini on the basal cell surface. Current evidence suggests that the α chain of laminin is necessary for basement membrane assembly, morphogenesis, polarization and differentiation *in vivo* (2,16,21). While the induction of kidney tubulogenesis correlates with the appearance of laminin in the mesenchyme, the expression of α chain correlates with

polarization. Inhibition of polarization in organ culture by antibody against α chain suggests that α chain is needed for establishing epithelial cell polarity (2,16,21).

Because of the apparent role of α chain in cell organization, we examined the integrin receptor which bind specifically to the E8 fragment of laminin α chain. Integrin receptors mediate epithelial cells polarization. Amongst the known integrin receptors for laminin, α6β1 is considered to be the specific receptor for the E8 cell-binding domain of the α laminin chain and is essential for cell polarization (13,15,16). Laminin α chain and α6 integrin are coordinately expressed and are co-localized in regions where epithelial cell morphogenesis occurs and polarization begins in kidney tubule formation (16). Antibodies to α6 integrin subunit selectively block binding to the E8 domain and inhibit cell polarization in kidney tubule formation (15,16). The β1 subunit is also an essential part of the laminin receptor and antibody to β1 interferes with morphogenesis (10,16,22). A loss, decrease or diffuse expression of integrin receptors is associated with loss of normal cell organization in cancer cells (11). RWPE-1 cells show strong expression of both α6β1 integrins at the basal end of cells forming the acini and thus, this immortalized cell line maintains the ability to undergo structural morphogenesis similar to normal prostatic epithelial cells.

Information about integrin receptors in the human prostate is very limited and is based primarily on cancer cell lines. Little is known about integrin expression in normal human prostatic epithelial cells. In one study, α6, β1 and β4 were all expressed at the basal end of epithelial cells in the normal human prostate, whereas, α6 and β1 showed a diffused distribution and β4 was not detectable in prostate cancer (23). Human prostate carcinoma cell lines DU-145 and PC-3 were shown to have an unstable, heterogeneous and diffuse expression of α6β1 and α6β4 integrins while in LNCaP cells α6β4 was absent (24,25). On

the basis of current knowledge from the prostate and other tissues as well as the present study, $\alpha 6\beta 1$ may emerge as an important laminin integrin receptor involved in prostate morphogenesis.

Having established structural differentiation in our 3-D prostate epithelium model, the second step in morphogenesis is to accomplish functional differentiation involving expression of secretory products. Androgens regulate growth and functional differentiation of prostatic epithelium (3,5,6,26). PSA is an important marker of the differentiated function of prostatic epithelial cells and its expression is androgen-dependent. We have previously shown that, depending on the duration of treatment and concentration, RWPE-1 and RWPE-2 cells as well as our PWR-1E cell line in monolayer cultures show heterogeneous expression of PSA, upregulate AR and show a growth response when exposed to mibolerone (3,5). PWR-1E is a human prostate epithelial cell line immortalized by an adenovirus-12/simian virus-40 (Ad12-SV40) hybrid virus (5,6). DU-145 cells are considered to be negative for PSA (7). An increase in PSA secretion was observed when freshly isolated prostatic epithelial cells were plated on matrix-coated plates as compared to those on plastic (27). In the acini formed by RWPE-1 cells in Matrigel, PSA expression is strong in acinar cells and the secretion sequestered in the acini also shows strong positive staining for PSA. These results demonstrate that the acini formed by RWPE-1 cells *in vitro* are able not only to synthesize but to also secrete PSA into the lumen when exposed to the synthetic androgen mibolerone and that the process of secretion is polarized, similar to that in normal prostatic acini *in vivo*.

Bissell and collaborators have developed an elegant model of hormone responsive, secretory mouse mammary epithelium to study differentiation and morphogenesis *in vitro* (11,28). In this model, laminin in combination with lactogenic hormones, regulates tissue-

specific gene expression resulting in cell differentiation and the expression of mammary specific β-casein synthesis in mammary epithelial cells *in vitro* (28). Similarly, our 3-dimensional *in vitro* model, employing immortalized prostatic epithelial cells in the presence of androgens, mimics characteristics of the human prostate during puberty and gland remodeling. The processes of branching morphogenesis, acinar formation and differentiation with secretion of PSA occur in the adult prostate as well as in BPH.

The RWPE-1 and RWPE-2 cells provide fully characterized, standardized, and reproducible cell lines. When grown in 3-D cultures, physiologically relevant models are obtained for studies on the relationship between growth, morphogenesis, and functional differentiation in normal cells and abnormalities associated with malignant transformation. In the normal adult prostate in homeostasis, there is little net growth because a balance between cell loss and cell gain is maintained among and between different cell types. This balance can be modulated not only by the level of a specific growth inducer or inhibitor but also by the number of its receptors. Since the expression of growth factors and receptors can be mutually regulated by androgens and growth factors, the picture becomes very complex. The paracrine interactions with stromal cells add another level of complexity, making it difficult to separate the various interactions *in vivo*. Such interactions have been studied in the developing rat prostate (18). The *in vitro* acinar morphogenesis that we have observed, using human prostatic epithelial cells, resembles that seen *in vivo* in the rat prostate (see Figures 4-2d and 4-2e).

The 3-D *in vitro* models for human prostate epithelial cells make it possible to dissect the complex actions and interactions between cells, ECM proteins, growth factors, androgens and their receptors in normal and neoplastic prostate growth, differentiation and

morphogenesis. Since laminin deposition must precede cell organization even in the commonly used collagen gel raft cultures, the 3-D cultures in and on Matrigel, provide a better model for studying the mechanisms of growth regulation involving morphogenesis and differentiation in normal and neoplastic cells. These models also provide the opportunity to study heterotypic cell interactions and changes in homeostasis during initiation of BPH. The 3-D cultures are especially useful for examining changes in cell organization that occur in early neoplastic lesions such as PIN. The comparison of the ability to undergo acinar morphogenesis by the immortalized RWPE-1 cells to the inability of the v-Ki-*ras*-transformed RWPE-2 cells and the formation of amorphous cellular masses by DU-145 prostatic carcinoma cells may suggest a stepwise progression from immortalized to less and then to a more aggressive and invasive carcinoma cells phenotype. We have shown earlier that the invasive ability of RWPE-2 and DU-145 cells is associated with a marked increase in the secretion of urokinase and/or gelatinases (29). DU-145 cells, which are more invasive than RWPE-2 cells, secrete higher levels of urokinase than RWPE-2 cells (29). A loss of the ability to undergo morphogenesis appears to be related to increased ability to invade and the more undifferentiated the cell type the more aggressive the cancer. Many aspects of this aberrant behavior remain to be investigated and further studies to elucidate the basis for the inability to undergo structural morphogenesis are in progress.

References

1. Bostwick, D.G. (1992) Prostatic intrapeithelial neoplasia (PIN): Current concepts. J Cell. Molec. Biol. suppl. 16H10-19.

2. Rodriguez-Boulan, E., and Nelson, W.J. (1989) Morphogenesis of the polarized epithelial cell phenotype. Science 245:718-725.

3. Bello, D., Webber, M.M., Kleinman, H.K., Wartinger, D.D. and Rhim, J.S. (1997) Androgen responsive adult human prostatic epithelial cell lines immortalized by human papillomavirus 18. Carcinogenesis (accepted for publication).

4. Rhim, J.S., Webber, M.M., Bello, D., Lee, M.S., Arnstein, P., Chen, L.and Jay. G. (1994) Stepwise immortalization and transformation of adult human prostate epithelial cells by a combination of HPV-18 and v-Ki-*ras*. Proc. Natl. Acad. Sci. USA 91:11874-11878.

5. Webber, M.M., Bello, D., Kleinman, H.K., Wartinger, D.D., Williams, D.E. and Rhim, J.S. (1996) Prostate specific antigen and androgen receptor induction and characterization of an immortalized adult human prostatic epithelial cell line. Carcinogenesis 17: 1641-1646.

6. Webber, M.M., Bello, D. and Quader, S. (1996) Immortalized and tumorigenic adult human prostatic epithelial cell lines. Characteristics and applications. Part 1. Cell markers and immortalized, non-tumorigenic cell lines. Prostate 29:386-394.

7. Webber, M.M., Bello, D. and Quader, S. (1997) Immortalized and tumorigenic adult human prostatic epithelial cell lines. Characteristics and applications. Part 2. Tumorigenic cell lines. Prostate 30:58-64.

8. Kleinman, H.K., Graf, J., Iwamoto, Y., Kitten, G.T., Ogle, R.C., Sasaki, M., Yamada, Y., Martin, G.R. and Luckenbill-Edds, L. (1987) Role of basement membranes in cell differentiation. Ann. N.Y. Acad. Sci. 513:134-145.

9. Hayward, S.W., Del Buono, R.D., Deshpande, N. and Hall, P.A. (1992) A functional model of adult human prostate epithelium: The role of androgens and stroma in architectural organization and the maintenance of differentiated secretory function. J. Cell Sci. 102:361-372.

10. Hoffman, M.P., Kibbey, M.C., Letterio, J.J. and Kleinman, H.K. (1996) Role of laminin-1 and TGF-β3 in acinar differentiation of a human submandibular gland cell line (HSG). J. Cell Sci. 109:2013-2021.

11. Howlett, A.R., Bailey, N., Damsky, C., Petersen, O.W. and Bissell, M.J. (1995) Cellular growth and survival are mediated by β1 integrins in normal human breast epithelium but not in breast carcinoma. J. Cell Sci. 108, 1945-1957.

12. Drubin, D.G. and Nelson, J.W. (1996) Origins of cell polarity. Cell 84:335-344.

13. Malinda, K.M. and Kleinman, H.K. (1996) Molecules in focus: the laminins. Int. J. Biochem. Cell Biol. 28:957-959.

14. Klein, G., Langegger, M., Timpl, R. and Ekblom, P. (1988) Role of laminin A chain in the development of epithelial cell polarity. Cell 55:331-341.

15. Aumailley, M., Timpl, R. and Sonnenberg, A. (1990) Antibody to integrin α6 subunit specifically inhibits cell-binding to laminin fragment 8. Exp. Cell. Res. 188:55-60.

16. Sorokin, L., Sonnenberg, A., Aumailley, M., Timpl, R. and Ekblom, P. (1990) Recognition of the laminin E8 cell-binding site by an integrin possessing the α_6 subunit is essential for epithelial polarization in developing kidney tubules. J. Cell Biol. 111:1265-1273.

17. Kleinman, H.K., McGarvey, M.L., Hassell, J.R., Star, V.L., Cannon, F.B., Laurie, G.W. and Martin, G.R. (1986) Basement membrane complexes with biological activity. Biochemistry 25:312-318.

18. Yamashita, A., Hayashi, N., Sugimura, Y., Cunha, G.R. and Kawamura, J. (1996) Influence of diethylstilbestrol, leuprolelin (a luteinizing hormone-releasing hormone analog), finasteride (a 5α-reductase inhibitor), and castration on the lobar subdivisions of the rat prostate. Prostate 29:1-14.

19. Petersen, O.W., Ronnov-Jessen, L., Howlett, A.R. and Bissell, M.J. (1992) Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells. Proc. Natl. Acad. Sci. USA 89:9064-9068.

20. Kubota, Y., Kleinman, H.K., Martin, G.R. and Lawley, T.J. (1988) Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. J. Cell Biol. 107:1589-1598.

21. Nomizu, M., Kim, W.H., Yamamura, K., Utani, A., Song, S., Otaka, A., Roller, P.R., Kleinman, H.K. and Yamada, Y. (1995) Identification of cell binding sites in the laminin α1 chain carboxyl-terminal globular domain by systematic screening of synthetic peptides. J. Biol. Chem. 270:20583-20590.

22. Kim, W.H., Jun, S.H., Kibbey, M.C., Thompson, E.W. and Kleinman, H.K. (1994-1995) Expression of β1 integrin in laminin-adhesion-selected human colon cancer cell lines of varying tumorigenicity. Invasion Metastasis 14:147-155.

23. Knox, J.D., Cress, A.E., Clark, V., Manriquez, L., Affinito, K., Dalkin, B.L. and Nagle, R.B. (1994) Differential expression of extracellular matrix molecules and the α6-integrins in the normal and neoplastic prostate. Am. J. Pathol. 145:167-174.

24. Rabinovitz, I., Nagle, R.B. and Cress, A.E. (1995) Integrin α6 expression in human prostate carcinoma cell is associated with a migratory and invasive phenotype *in vitro* and *in vivo*. Clin. Exp. Met. 13:481-491.

25. Rokhlin, O.W. and Cohen, M.B. (1995) Expression of cellular adhesion molecules on human prostate tumor cell lines. Prostate 26:205-212.

26. Tindall, D.J. (1996) Androgen regulation of gene expression in the prostate. International Symposium on Biology of the Prostate, NIDDK, NIH, Washington D.C. p. 17.

27. Fong, C., Sherwood, E.R., Sutkowski, D.M., Abu-Jawdeh, G.M., Yokoo, H., Bauer, K.D., Kozlowski, J.M. and Lee, C. (1991) Reconstituted basement membrane promotes morphological and functional differentiation of primary human prostatic epithelial cells. Prostate 19:221-235.

28. Streuli, C.H., Bailey, N. and Bissell, M.J. (1991) Control of mammary epithelial differentiation: Basement membrane induces tissue-specific gene expression in the absence of cell-cell interaction and morphological polarity. J. Cell Biol. 115:1383-1395.

29. Webber, M.M., Waghray, A., Bello, D. and Rhim, J.S. (1996) Proteases and invasion in human prostate epithelial cell lines. Implications in prostate cancer prevention and intervention. Radiat. Oncol. Invest. 3:358-362.

Chapter Five

Extracellular Matrix and Integrins Modulate Acinar Morphogenesis by RWPE-1 Cells

Abstract

Interactions between cells and the extracellular matrix (ECM) play a critical role in acinar and branching morphogenesis during prostate development. Using HPV-18 immortalized cell line, RWPE-1, a 3-dimensional (3-D) cell culture model was developed and used to identify the specific extracellular matrix protein and its integrin receptor, required for acinar morphogenesis *in vitro*. When cells were plated on, laminin, type IV collagen or fibronectin, laminin was the only ECM protein that was able to induce cell polarization and formation of Acini with a distinct lumen. Cells plated on collagen or fibronectin remained as a monolayer. The number of acini formed in laminin was similar to that formed in Matrigel. Blocking the binding of cells to laminin by anti-laminin antibody caused a decrease in acinar formation in a dose dependent manner. These results show that laminin is required for cell polarization and acinar formation.

The expression of α6 and β1 integrin subunits was examined by confocal immunofluorescence microscopy. RWPE-1 cells show strong expression of α6 and β1 integrin subunits, which are expressed primarily at the basal end of polarized cells. Treatment of cells with anti-functional antibody to β1 reduced acinar formation to 10% of the IgG-treated control which was taken as 100%. However, similar treatment with anti-functional α6 antibody only reduced acinar formation to 75% of control. These results demonstrate that while both α6 and β1 are important, acinar formation does not occur in the absence of the β1 integrin. It can, therefore, be stated that laminin and β1 integrin are the two key factors required for acinar morphogenesis to occur. This is the first report of acinar formation *in vitro* for human prostatic epithelial cells. This work provides the basic understanding of cell:matrix interactions in prostate acinar morphogenesis. The 3-D cell

culture model can not only be used to examine normal development of the human prostate, but also for examining early changes in prostate carcinogenesis. This provides the opportunity to identify agents which may be used to prevent the development and progression of prostate cancer.

Introduction

Prostatic epithelial cells, *in vivo,* exist in a 3-dimensional (3-D) environment in which the extracellular matrix (ECM), androgens, growth factors, receptors and stroma influence cellular growth and differentiation (Fong et al. 1991, Webber et al., 1996 Bello et al., 1997). In 2-dimensional cell cultures, human prostatic epithelial cells undergo a few rounds of cell division, lose differentiated function and are then lost. This makes their *in vitro* behavior, in many instances, of little relevance to their *in vivo* condition. Therefore, it is proposed that, to effectively study prostatic epithelial cell biology, a cell systems which mimics the 3-D micro-environment and allows for the expression of normal prostatic functional characteristics, is needed. The use of fully characterized immortalized human prostatic epithelial cells in an *in vitro* 3-D culture system, described in chapter 2, has adequately fulfilled this need.

The ECM is essential for the morphogenesis of tissues. It is involved in cell migration, proliferation, differentiation and in establishing and maintaining cell polarity and cell architecture (Hay, 1981). During embryonic development, the ECM plays an important role in organogenesis and epithelial cell differentiation. In the developing prostate, both the budding urogenital epithelial cells and the mesenchymal cells secrete ECM proteins which the epithelial cells then use to "move", as a train would use railroad

tracks, as they branch into the mesenchyme to form acini and ducts. The basement membrane is a structurally distinct zone of the ECM. In the prostate, it separates the epithelial cells of the acini and ducts from the underlying stroma.

Although work has been done in lung, kidney, breast and salivary gland morphogenesis, not much is known about ECM-regulated acinar morphogenesis in the prostate (Hoffman et al., 1996, Schuger L, 1990, Weaver et al., 1996, Wu and Santoro, 1996). This chapter, will focus on the use of the human prostatic 3-D culture model to identify the key ECM components and integrins essential for epithelial polarization and acinar formation. Because the progression from a normal to a malignant phenotype is characterized by a loss of normal cell organization, cell polarity, cell:cell and cell:ECM adhesion and appropriate integrin receptor expression, the data describe in this chapter provide the necessary baseline for further study into how these factors are altered in the process of carcinogenesis.

As the key component of the basement membrane, laminins are important in cell:ECM adhesion and epithelial polarization. Laminins are ~800 kDa, large glycoproteins which are composed of three chains, α, β, and γ, held together by disulfide bonds and arranged in a cruciform-like structure (Malinda and Kleinman, 1996). Laminins are the first ECM proteins synthesized during embryonic development, and therefore, play a crucial role in morphogenesis and organogenesis (Kleinman, 1987, Malinda and Kleinman, 1996). Type IV collagen is also a major structural component in the basement membrane and functions to anchor other ECM components (Timple and Aumailley, 1989). Individual type IV collagen chains (567 bp) assemble to form 400 nm long triple helices which aggregate to form a loose but continuous network which serves as the "scaffold" of the

basement membrane. Although type IV collagen may bind to cells directly, via cell matrix receptors, epithelial cells attach to collagen indirectly through laminin. Fibronectin, a large glycoprotein (210-260 kDa), found ubiquitously throughout the body tissues and fluids, functions as bridges between cells and other ECM molecules. Although fibronectin is not found in the basement membrane in adults, it is present in most embryonic basement membranes (Kleinman et al., 1982), which suggests a role in the initial stages of prostatic acinar and ductal morphogenesis. It is, therefore, worthy of investigation.

ECM proteins bind to the cell surface via specific receptors. The first family of receptors identified to do so, was the integrin family. Integrins are known to play an important role in embryogenesis, organogenesis, wound repair, cytoskeletal organization, motility, cell growth and differentiation (Geiger et al., 1995, Vinatier, 1995). Because of their critical role in epithelial cell polarization, the identification of key integrins involved in prostatic acinar morphogenesis was examined (Webber et al., 1997). Integrins are heterodimeric transmembrane glycoproteins which consist of an α (120 - 180 kDa) and a β (90-110 kDa) subunit both of which are transmembrane proteins. The β subunits are promiscuous and can complex with multiple α subunits, while the α subunits are generally loyal to their β subunits partner (Garratt and Humphries, 1995, Vinatier, 1995).

The human prostatic epithelial cell line RWPE-1, which mimics normal prostatic epithelial cells *in vivo*, was used in this study. In order to identify the key basement membrane proteins and integrin receptors, involved in prostatic epithelial cell polarization and acinar morphogenesis, a 3-D cell culture model was used. The ability of single ECM components, laminin, type IV collagen and fibronectin, to induce RWPE-1 cells to undergo acinar morphogenesis, was examined. Further, the expression of integrin

receptors α6β1 and α6β4, which bind laminin exclusively, and α2β1, which binds laminin as well as type IV collagen and fibronectin, was examined to elucidate their role in cell polarization. We have already established that RWPE-1 cells undergo acinar morphogenesis in Matrigel 3-D cultures (Webber et al 1997). Therefore, the Matrigel cultures were used as a positive control while those in gelatin were used as a negative control.

Results

Laminin is required for acinar morphogenesis

The ability of individual basement membrane glycoproteins, laminin (laminin-1 was used in this study), type IV collagen or fibronectin, to induce RWPE-1 acinar morphogenesis was first examined to determine which component was essential for acinar formation. RWPE-1 cells were plated onto either laminin, type IV collagen or fibronectin-coated wells. Matrigel and gelatin-coated wells served as positive and negative controls, respectively. Within 24 h after plating, cells plated on all matrices attached but only those cells on Matrigel formed cytoplasmic connections with neighboring cells. At four days, cells plated on Matrigel had formed distinct acini with associated ducts which gave a "bunch of grapes" appearance (Figure 5-1 a). When the "grape bunch" structure was stained with anti-laminin antibody (Ab) and optically sectioned by laser scanning confocal microscopy, laminin expression was found to be most intense at areas immediately surrounding individual acini and weaker at areas of duct-matrix contact (Figure 5-1 b). Optical serial sections through a single propidium iodide-stained acinus demonstrated that the acinus was a 3-D spherical gland consisting of polarized cells with a distinct central lumen (Figure 5-2 a-f).

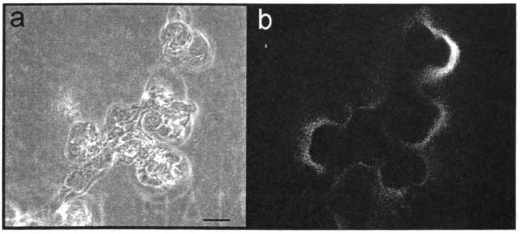


Figure 5-1. Acinar morphogenesis by RWPE-1 cells in Matrigel. Localization of laminin in four day 3-D Matrigel cultures using anti-laminin antibodies. a. LSM transmitted image of acini and associated duct network. b. optical confocal section of laminin expression. Note strong staining surrounding base of acini (Bar 25 μ).

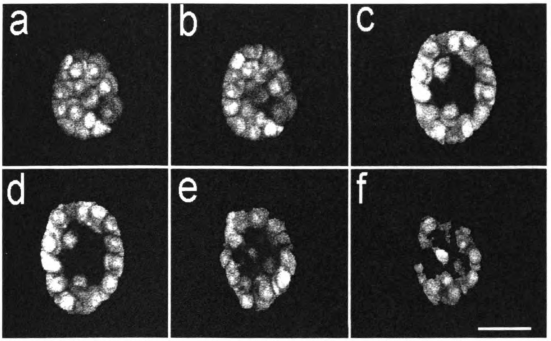


Figure 5-2. Optical serial sections of a propidium-iodide stained RWPE-1 acinus grown on Matrigel for 4 days. Sections are arrange in a top to bottom configuration with (a) being the top, (c)and (d) more middle and (f) the bottom of the acinus (Bar 25μ).

Most importantly, cells plated on laminin formed distinct spherical acini consisting of polarized cells with a distinct lumen (Figure 5-3 a-f). This clearly demonstrates the ability of laminin-1 to induce acinar formation. A comparison of acini formed on Matrigel and laminin-1 (Figure 5-4 a and b, respectively), shows that both consist of well polarized cells around a central lumen.

When cells were plated on fibronectin and type IV collagen acinar formation did not occur. Cells merely attached and spread (Figure 5-4 c and d, respectively). Cells grown on gelatin did not form acini or spread but formed amorphous clumps (Figure 5-4 e). Cells grown on plastic did not show acinar organization and only formed a monolayer (Figure 4-2o, Chapter 4 page 87).

When the number of acini formed on the different matrices was compared, no significant difference between Matrigel (100%) and laminin-1 (98%) was observed (Figure 5-5). No acini (0%) formed on either type IV collagen or fibronectin.

If laminin is necessary for acinar formation, then blocking the binding of laminin to the cells should result in the inhibition of acinar formation. This was tested using anti-functional (neutralizing) antibody directed against laminin. The anti-laminin Ab binds to the laminin in Matrigel and physically prevents the cells from binding to laminin. The neutralizing laminin Ab was diluted 1:20, 1:10 and 1:5 and added to Matrigel-coated wells 1 h before plating cells. Results showed that acinar formation was decreased to 23%, 4% and 2.5% when compared to IgG treated controls (100%) (Figure 5-6). These result indicate that cell must bind to laminin in order for acinar formation to occur.

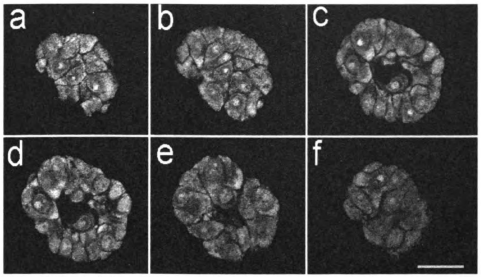


Figure 5-3. Optical serial sections of a propidium iodide-stained RWPE-1 acinus grown on laminin for 4 days. Sections are arrange in a top-bottom configuration with (a) being cranial, (c)and (d) medial and (f) posterior (Bar 25μ).

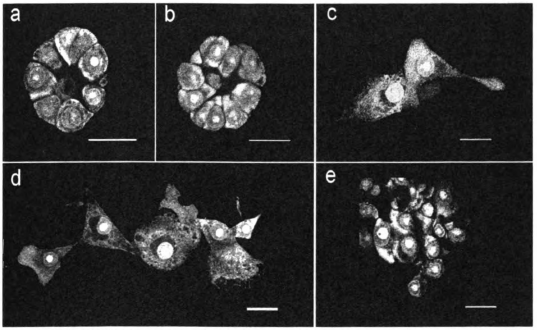


Figure 5-4. RWPE-1 cells on ECM Matrices. Optical sections of propidium iodide-stained RWPE-1 cells at 4 days of growth on (a) Matrigel (positive control), (b) laminin, (c)fibronectin, (d) type IV collagen and (e) gelatin (negative control). Acinar formation was only observed on (a) Matrigel and (b) laminin. Cells spread, but did not form acini, on (c)fibronectin and (d) type IV collagen. (e) Cells formed amorphous clumps on gelatin (Bar 25μ).

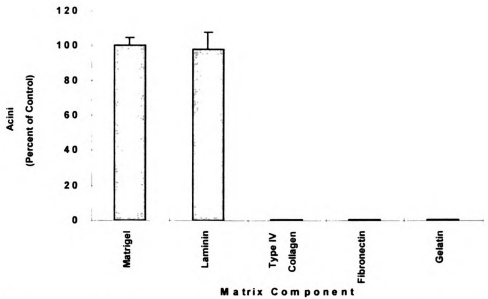


Figure 5-5. Acinar Formation on Matrix Components. RWPE-1 cells were grown on Matrigel, laminin type IV collagen, fibronectin or gelatin. Acini-forming ability was determined by staining cultures with propidium iodide and counting individual acini using the LSM transmitted light at low power. Counts are expressed as a percent of the control. Matrigel control was set at 100% (Bar=+/-SE).

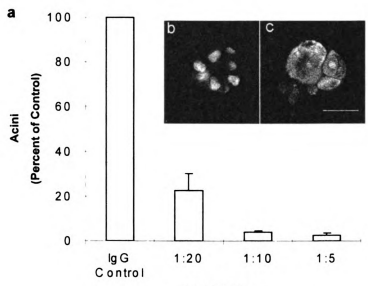


Figure 5-6. Inhibition of acinar formation by anti-laminin neutralizing antibody. RWPE-1 cells were grown on Matrigel and pre-treated with laminin anti-functional antibody. (a) Acini-forming ability was determined by staining cultures with propidium iodide and counting individual acini using the LSM transmitted light at low power. Counts are expressed as a percent of the IgG control which was set at 100% (Bar= +/-SE). (b) Optical confocal section of a control acinus.(c) Optical confocal section of 1:20 antibody-treated cells which form aggregates but lack acinar formation (Bar 25 μ).

β1 integrin is essential for acinar formation

Epithelial cells bind laminin via integrin receptors. Therefore, α6β1, α6β4 and α2β1 laminin integrin receptor expression was examined, using antibodies directed against α2, α6, β1 or β4 integrin subunits, by indirect immunofluorescence staining of intact acini. Integrin expression was visualized by optical sectioning using a laser scanning confocal microscope.

Strong positive staining for α6 (Figures 5-7) was observed at the basal end of the acinar cells as compared to the IgG control (Figure 5-8). Strong staining was observed also for the β1 integrin subunit (Figure 5-9). β1 integrin expression was located at the basal end of the acinar cells in comparison to the IgG control. These results demonstrate that RWPE-1 cells show strong basal expression of the α6β1 laminin integrin. In comparison to α6 or β1 staining, only weak staining for α2 was observed as compared to the IgG control. Staining appeared focally at the basal end of the acinar cells (Figures 5-10). Integrin β4 staining was also observed. Staining appeared weak but continuous at the basal and lateral edge of acinar cells (Figures 5-11) as compared to the IgG control. Taken together, the data show that RWPE-1 cells weakly express α6β4 and α2β1 laminin integrins, while the α6β1 laminin integrin expression is predominant.

In order to demonstrate that the α6 and β1 laminin integrin subunits are the key integrins involved in epithelial polarization and acinar morphogenesis, anti-functional antibodies against β1 or α6 integrins were employed. β1 or α6 integrin antibody, diluted 1:33 and 1:5, respectively, was added to the cell suspension and allowed to incubate for 1 h before cells were plated onto Matrigel-coated wells. Anti-β1 Ab decreased acinar

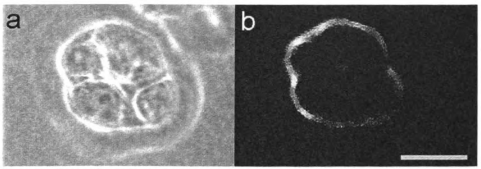


Figure 5-7. α 6 Integrin Expression. RWPE-1 cells, grown on Matrigel, were fixed at 4 days and stained for α6 integrin expression by immunofluorescence using anti-α6 antibody. (a) LSM transmitted image of an acinus. (b) Optical confocal section of the same acinus showing α6 integrin expression. Note the strong staining at the basal end of polarized cells forming the acinus (Bar 25 μ).

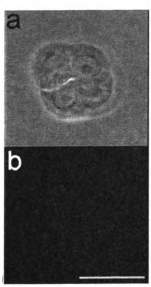


Figure 5-8. IgG Control for integrin expression. RWPE-1 cells, grown on Matrigel, were fixed at 4 days and stained using a non-specific IgG instead of the primary antibody. (a) LSM transmitted image of an acinus. (b) No non-specific staining was observed when IgG was used (Bar 25 μ).

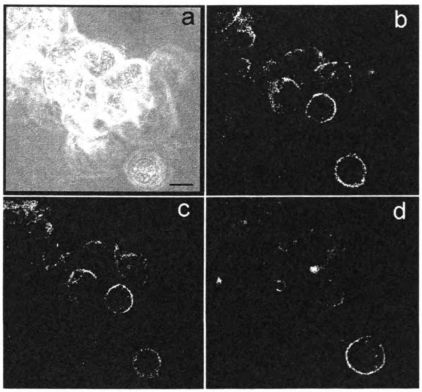


Figure 5-9. β1 Integrin Expression. RWPE-1 cells, grown on Matrigel, were fixed at 4 days and stained for β1 integrin expression by immunofluorescence using anti-β1 antibody. (a) LSM transmitted image of acini. (b, c and d) β1 integrin expression as seen in optical serial confocal sections. Note the strong staining at the basal end of polarized cells forming acini and that different acini come into view at different optical depth (Bar 25 μ).

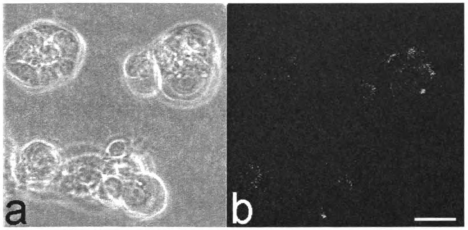


Figure 5-10. α2 Integrin Expression. RWPE-1 cells, grown on Matrigel, were fixed at 4 days and stained for α2 integrin expression by immunofluorescence using anti-α2 antibody. (a) LSM transmitted image of acini. (b) Optical confocal section of acini showing α2 expression. Note focal staining at the basal end of polarized cells forming acini (Bar 25 μ).

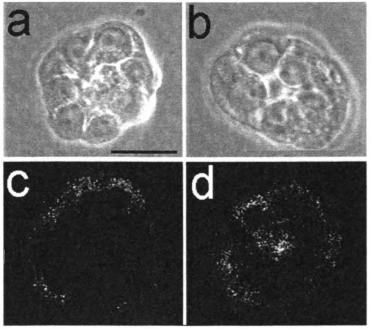


Figure 5-11. β4 Integrin Expression. RWPE-1 cells, grown on Matrigel, were fixed at 4 days and stained for β4 integrin expression by immunofluorescence using ant-β4 antibody. (a,b) LSM transmitted image of acini. (c,d) Optical confocal section of acini showing β4 integrin expression. Note weak staining at the basal and lateral surface of polarized cells forming the acini (Bar 25 μ).

formation to 10% of the IgG control which was set at 100% (Figure 5-12). However, anti-functional α6 integrin Ab only caused a decrease to 75% of control in acinar formation (Figure 5-12). These results show that binding of cells through the β1 integrins receptor is essential for cell polarization and acinar morphogenesis.

Discussion

The process of prostatic acinar morphogenesis is poorly understood. Animal models, although useful, intrinsically posses a plethora of unknown variables which can not be fully controlled. Additionally, such *in vivo* animal systems primarily use rodent prostatic cell lines which may be injected at various sites into animals, producing data which must then be extrapolated to the human condition. Monolayer 2-dimensional cell cultures, utilizing primary human prostatic epithelial cells are ideal, but isolated cells typically undergo a few rounds of cell division, lose differentiated function, undergo senescence and are lost. In many instances, the information gathered from these 2-D cell culture systems is of little relevance to the *in vivo* condition. In order to overcome the limitations of using primary cultures of human prostatic epithelial cells, our laboratory has developed the RWPE-1 cell line. The RWPE-1 cell line was derived from non-neoplastic human prostatic epithelial cells by immortalization with human papilloma virus 18. These cells mimic normal prostatic cell behavior in their response to growth factors and androgen and in their expression of PSA and androgen receptor in response to androgen stimulation. They do not form tumors in nude mice nor are they invasive (Bello et al., 1997). Because these cells are immortalized but have retained their normal functional ability, they provide a uniform, standardized and reproducible cell model for use in studying prostate

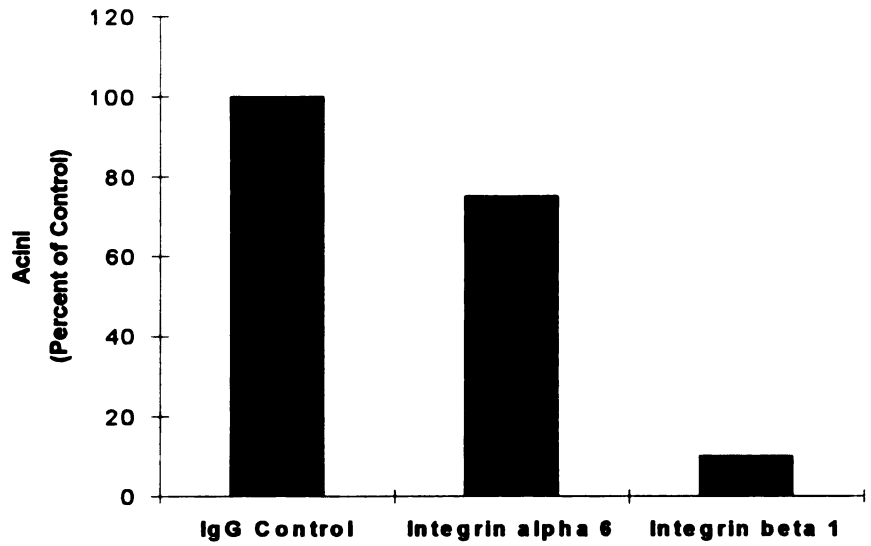


Figure 5-12. Inhibition of acinar formation by anti-integrin neutralizing antibodies. RWPE-1 cells were grown on Matrigel, pre-treated with anti-functional integrin α6 or β1 integrin antibody for 1 h. Acini-forming ability was determined by staining cultures with propidium iodide and counting individual acini using the LSM transmitted light at low power. Counts are expressed as a percent of the IgG control which was set at 100%.

development, prostate cell biology and the neoplastic transformation which occurs in the process of carcinogenesis. I have described a 3-D cell culture system, using RWPE-1 human prostatic epithelial cells, which mimics the *in vivo* micro-environment and allows for a reproducible model system. This model system can be used to study the factors involved in prostatic acinar morphogenesis and how these factors are altered in the progression through prostatic intraepithelial neoplasia (PIN) and carcinogenesis.

It should be stated at the outset that when performing 3-D cultures, it is important to use strict criteria for evaluation of acini. The best definition for an acinus is: a spherical arrangement of polarized cells which form a distinct central lumen. In order to confirm acinus formation under this definition, an investigator may examine cultures by either 2 μm paraffin sections or by optically sectioning acini using a confocal microscope. Light microscopy and phase contrast techniques alone are not sufficient for evaluation of acini formation. I have found that preperation of samples for confocal microscopy causes the least distortion of the 3-D architecture, as compared to the embedding process, and the loss of material due to processing is minimal. Recently, other investigators have reported gland-like structures being formed when mouse prostatic cancer cells were grown on Matrigel. Unfortunately, this study lacks clear illustration of polarized cells with distinct lumen. The phase contrast pictures presented were not sufficient for confirmation of acini formation (Jorcyk, et al., 1998). Results reported in the present study are the first to show acinar formation *in vitro* for human prostatic epithelial cells.

The focus of this chapter is to elucidate the role of ECM components and the associated integrin receptors in acinar morphogenesis using this 3-D cell culture system. In the developing prostate, urogenital epithelial cells bud out of the urogenital sinus to

invade the surrounding mesenchyme where they terminate and differentiate into acini. It is known that the ECM, that is deposited by both the budding epithelial cells and the fibroblasts in the mesenchyme, plays an important role in acinar formation. It is, however, not known as to which ECM component is essential for acinar formation. When grown on the reconstituted basement membrane Matrigel, RWPE-1 cells form distinct acini and ducts (Figure 5-1 a and Figure 5-2). Therefore, the ability to form acini on laminin-1, type IV collagen and fibronectin was examined. Laminin-1 is the predominant component of the basement membrane and Matrigel (Hoffman et al., 1996, Malinda and Kleinman, 1996). Type IV collagen is another major component of the basement membrane and acts as the scaffold onto which laminin and cells may bind (Hay, 1981). Although fibronectin is not found in adult basement membranes, it is present in embryonic basement membranes when initial epithelial budding and branching is occurring (Kleinman et al., 1982). Results presented here show that laminin-1 (Figure 5-4 b), and not type IV collagen or fibronectin (Figure 4-4 c and d), was able to induce acinar formation and the number of acini formed in laminin was comparable to that observed in Matrigel (Figure 5-5). These results demonstrate the ability of laminin-1 to induce acinar formation in the same manner and frequency as Matrigel. The ability of RWPE-1 prostate epithelial cells to undergo acinar formation in laminin is supported by similar acinar organization seen by others who have utilized mammary and submandibular epithelial cells (Hoffman et al., 1996, Weaver et al., 1996).

If laminin truly plays an essential role in acinar formation as implicated above, then blocking cell binding to laminin should disrupt acinar formation. Results show that the addition of anti-functional Ab to laminin, which blocks cell-to-laminin binding, reduced

the ability of RWPE-1 cells to form acini by 97.5 % at the lowest Ab dilution (1:5) and 77% at the highest dilution (1:20) (Figure 5-6). Taken together, these results demonstrate that the laminin component of the basement membrane is necessary for prostatic acinar morphogenesis.

Cells attach to the ECM via integrin cell adhesion receptors. Integrins are critical for establishing cell polarity and differentiation of epithelial cells into specialized secretory cells (Sheppard, 1996, Webber et al., 1997). Specifically, the $\alpha 6\beta 1$ is the key integrin receptor for laminin-1 and is essential for cell polarization (Sorokin, 1990, Webber et al., 1997). As shown earlier, $\alpha 6$ and $\beta 1$ integrin subunits are strongly expressed by RWPE-1 cells, suggesting strong expression of the $\alpha 6\beta 1$ integrin receptor (Figure 5-7 and 5-8). RWPE-1 acini also express $\alpha 2$ and $\beta 4$ integrins. This suggests that $\alpha 6\beta 4$ and $\alpha 2\beta 1$ laminin integrins may also be formed, but their expression is substantially weaker than $\alpha 6\beta 1$ expression, indicating that their role may be a supporting one (Figure 5-10 and 5-11). The presented data indicate that the primary laminin integrin expressed by RWPE-1 cells is the $\alpha 6\beta 1$ integrin.

If $\alpha 6\beta 1$ integrin is the key integrin involved in cell polarization and acinar organization, then the disruption of binding of the $\alpha 6\beta 1$ integrin receptor to laminin should result in inhibition of epithelial cells polarization, required for acinar formation. The importance of $\alpha 6\beta 1$ integrin in prostatic epithelial cells polarization and acinar morphogenesis was further elucidated by blocking $\alpha 6$ and $\beta 1$ function using neutralizing Abs. Anti-functional Ab to $\beta 1$ showed a dramatic 90% decrease in acinar forming ability (Figure 5-12). While, inhibition of $\alpha 6$ function resulted in only a 25% decrease. Although studies on submandibular gland epithelial cells have shown that disruption of either $\alpha 6$ or

β1 integrin significantly affects acinar formation (Hoffman et al., 1996), our findings indicate that in the prostate, the β1 and not the α6 integrin subunit is critical for epithelial polarization and acinar morphogenesis.

Normal prostatic epithelial cells and epithelial cells in prostatic intraepithelial neoplasia (PIN) express α6β1, α6β4 and α2β1 laminin receptors, but the expression of α2 and β4 are lost in cancer cells (Cress et al., 1995). This is not surprising since cancer is characterized by a loss of normal cell organization, cell polarity, cell:cell and cell:ECM adhesion which would result from a loss of appropriate integrin receptor expression. Although expression of α6β1 is present in prostate cancer cells, its expression is not restricted to the basal aspects of the cell, as in normal cells, but is diffuse throughout the cell membrane (Cress et al., 1995, Hao et al., 1996, Rabinovitz et al., 1995, Rokhlin and Cohen, 1995). In RWPE-1 cells, the expression of α6β1 integrin is restricted to the basal cell surface. RWPE-1 cells also express α2β1 and α6β4 integrins but their expression is much weaker than α6β1 expression.

In summary, the data presented here show that laminin-1 is essential for acinar morphogenesis and that cell polarization and organization occurs in the presence of functional α6β1 integrin binding. Absence of type IV collagen and fibronectin does not inhibit prostatic epithelial cells polarization and cell:matrix and cell:cell interactions. RWPE-1 prostatic epithelial cells undergo epithelial cell polarization and acinar formation when grown on the reconstituted basement membrane, Matrigel, as a result of their interaction with laminin via the α6β1 integrin. This work has provided the basic understanding of cell:matrix interactions necessary for further study of changes in cell:matrix interactions that occur during the process of carcinogenesis. It is hoped that

further studies, utilizing the 3-D cell model system, will shed light on the role of laminin-1, $\alpha 6\beta 1$ integrins and other factors in the progression from normal to malignant prostatic epithelial cells.

References

Bello D, Webber MM, Kleinman HK, Wartinger DD and Rhim JS: Androgen responsive adult human prostatic epithelial cell lines immortalized by human papillomavirus 18. Carcinogenesis 18: 1212-1223, 1997.

Cress AE, Rabinovitz I, Zhu W and Nagle RB: The alpha 6 beta 1 and alpha 6 beta 4 integrins in human prostate cancer progression. Cancer Metastasis Rev. 14:219-28, 1995.

Fong CJ, Sherwood ER, Sutkowski DM, Abu-Jawdeh GM, Yokoo H, Bauer KD, Kozlowski JM and Lee C: Reconstituted basement membrane promotes morphological and functional differentiation of primary human prostatic epithelial cells. Prostate 19: 221-35, 1991.

Garratt AN and Humphries MJ: Recent insights into ligan binding, activation and signalling by integrin adhesion receptors. Acta Anat. 154, 34-45, 1995.

Geiger B, Yehunda-Levenberg S and Bershadsky AD: Molecular interactions in the submembrane plaque of cell-cell and cell-matrix adhesions. Acta Anat. 154:46-62, 1995.

Hao J, Yang,Y, McDaniel, KM, Dalkin BL, Cress AE and NagleRB: Differential expression of laminin 5 (alpha 3 beta 3 gamma 2) by human malignant and normal prostate. Am. J. Pathol.149:1341-9, 1996.

Hay E: Collagen and embryonic development. In: Cell biology of extracellular matrix. Hay E (ed.), New York, Plenum Press, pp.379-405, 1981.

Hoffman MP, Kibbey MC, Letterio JJ and Kleinman HK: Role of laminin-1 and TGF-β3 in acinar differentiation of a human submandibular gland cell line (HSG). J. Cell Sci. 109:2013-2021, 1996.

Jorcyk C, Liu-ML, Shibata MA, Maroulakou IG, Komschlies KL, McPhaul MJ, Resau JH and GreenJE: Development and characterization of a mouse prostate adenocarcinoma cell line: ductal formation determined by extracellular matrix. Prostate 34:10-22, 1998.

Kleinman HK, Woodley DT, McGarvey ML, Robey PG, Hassell JR and Martin GR: Interaction and assembly of basement membrane components. In extracellular matrix eds. Hawkes S and Wang JL, Academic Press, 1982.

Malinda KM and Kleinman HK: Molecules in focus: the laminins. Int. J. Biochem. Cell Biol. 28:957-959, 1996.

Orian-Rousseau V, Aberdam D, Rousselle P, Messent A, Gavrilovic J, Meneguzzi G; Kedinger M and Simon-Assmann P: Human colonic cancer cells synthesize and adhere to laminin-5. J. Cell Sci. 30(Pt 14):1993-2004, 1998.

Rabinovitz I, Nagle RB and Cress AE: Integrin alpha 6 expression in human prostate carcinoma cells is associated with a migratory and invasive phenotype in vitro and in vivo. Clin. Exp. Metastasis. 13: 481-91, 1995.

Rokhlin OW and Cohen MB: Expression of cellular adhesion molecules on human prostate tumor cell lines. Prostate. 26: 205-12, 1995.

Schuger L, O'Shea KS, Nelson BB and Varani J: Organotypic arrangement of mouse embryonic lung cells on a basement membrane extract: involvement of laminin. Development. 110:1091-9, 1990.

Timpl R and Aumailley M: Biochemistry of basement mambranes. Adv. Nephrol. 18:59-76, 1989.

Vinatier,D. Integrins and reproduction. Euro. J. Obs. Gyn. Reprod. Bio. 59, 71-81,1995.

Weaver VM, Fischer AH, Peterson OW and Bissell MJ: The importance of the microenvironment in breast cancer progression: recapitulation of mammary tumorigenesis using a unique human mammary epithelial cell model and a three-dimensional culture assay. Biochem. Cell Biol. 74:6,: 833-51, 1996.

Webber MM, Bello D, Kleinman HK and Hoffman MP: Acinar differentiation by non-malignant immortalized human prostatic epithelial cells and its loss by malignant cells. Carcinogenesis 18:1225-1231, 1997.

Wu JE and Santoro SA: Differential expression of integrin alpha subunits supports distinct roles during lung branching morphogenesis. Dev. Dyn. 206:2, 169-81, 1996.

Chapter Six

Malignant Human Prostatic Epithelial Cells Lose the Ability to Undergo Acinar Morphogenesis

Abstract

Acinar morphogenesis occurs as a result of complex multiple interactions between cells and the surrounding ECM, via their integrin receptors, as well as growth factors present in the micro-environment and growth factor receptors expressed by cells. Alterations in these interactions may occur during carcinogenesis, as cells progress from a normal to a malignant, invasive phenotype. A family of cell lines, which apparently represent the multiple steps in carcinogenesis, similar to PIN, as well as subsequent tumor progression, were used to determine whether the degree of loss of ability of cancer cells to undergo acinar morphogenesis, in the 3-D cell culture model, has any relationship with changes in integrin expression, their invasive ability, and their response to EGF. The cell lines used include: RWPE-1, which represents the non-tumorigenic but immortalized state, and the tumorigenic cell lines, arranged in order of their increasing malignant characteristics, WPE1-NA22, WPE1-NB27, WPE1-NB14; WPE1-NB11, and WPE1-NB26, all of which were derived from RWPE-1 by transformation with NMU. The DU-145, malignant and invasive prostate cancer cell line was used as a control. Results show that an inverse relationship exists between the degree of ability to form acini and the degree of invasive ability, where RWPE-1 cells and WPE1-NA22 show high acinar-forming ability but no or little invasive ability, while the more invasive cell lines WPE1-NB26 and DU-145 cells show no acinar-forming ability. In addition, while RWPE-1 and WPE1-NA22 cells show basal expression of $\alpha 6\beta 1$ integrin, which correlates with their ability to polarize and form acini, a loss $\alpha 6$ and abnormal, diffused expression of $\beta 1$ integrin in WPE1-NB26 and DU-145 cells, was observed. Further, exposure of RWPE-1 cells to EGF causes a dose dependent decrease in acinar formation, at 10 ng/ml

EGF, to 56% of untreated control. Addition of EGF anti-functional antibody caused an enhancement of acinar formation. These results suggest that malignant cells lose the ability to undergo acinar morphogenesis and that the degree of loss is directly related to their invasive ability and loss or abnormal expression of laminin-specific integrins. Another contributing factor to loss of acinar morphogenesis appears to be increase in cell proliferation. These results show that the *in vitro* 3-D cell culture model may mimic carcinogenesis and tumor progression *in vivo*. This study provides basis for exploring the potential for identifying agents which may normalize acinar formation, and thus, control cancer development.

Introduction

Homeostasis in the prostate is maintained by an interplay of multiple factors which directly or indirectly regulate cell growth and cell death. Factors involved in this regulation of homeostasis include, laminin and its integrin receptors, hormones, and growth factors. Prostate cancer arises when the balance between the cell proliferation and cell death is altered in favor of proliferation (Sensibar, 1995). Prostatic intraepithelial neoplasia (PIN) is considered to be the first step in prostate carcinogenesis. Late PIN is considered to be equivalent to carcinoma *in situ* (Webber et al., 1996). Established primary tumors are heterogeneous and consist of invasive and non-invasive cell populations. The next step in prostate cancer development is invasion of the basement membrane by cancer cells and subsequent metastasis to secondary sites, which is the primary cause of death from prostate cancer (Flug and Kopf-Maier, 1995, Webber et al., 1996).

Recently, it has been recognized that any scientific study of prostate cancer necessitates a knowledge of morphogenesis (Bonkhoff and Remberger, 1998, Webber et al, 1997). Therefore, the overall aim of this chapter is to describe a 3-D *in vitro* cell model with which to study mechanisms involved in acinar morphogenesis and its loss in the progression from normal epithelium, to PIN and then to invasive carcinoma. Such a model system requires well characterized cells line that represent different stages in the multi-step process of carcinogenesis. Such cells lines have been developed and fully characterized in Dr. Webber's laboratory. Cell lines, which represent different stages of carcinogenesis, were developed by transfection or exposure of RWPE-1 cells to a carcinogen. RWPE-1 cells were transformed by the addition of a Ki-*ras* oncogene to produce the tumorigenic RWPE-2 cell line (Bello et al., 1997, Rhim, 1994). Other RWPE-1 cells were treated with the chemical carcinogen, *N*-nitroso-*N*-methlyurea (NMU), to establish cell lines of varying tumorigenicity including: WPE1-NA22, WPE1-NB27, WPE1-NB14, WPE1-NB11 and WPE1-NB26 (Webber, personal communication). Collectively, these cells lines represent different stages of neoplastic transformation from low tumorigenic to highly tumorigenic cells.

Late stages of PIN and prostate cancer are characterized by a loss of normal cell organization, cellular polarization and cell:cell and cell:basement membrane adhesion, which may involve alterations in integrin receptors expression (Webber et al., 1997). Proper expression of $\alpha 6\beta 1$ integrin and the presence of laminin are necessary for normal acinar morphogenesis (see Chapter 5). Alterations in integrin expression play a role in the course of invasion where tumor cells must first attach to the basement membrane before degrading it and then detach and move into the ECM to intravasate into nearby blood

vessels to travel and metastasize to a secondary site.

Growth factors enable cells to maintain local homeostasis and adjust to their biological environment. Epidermal growth factor (EGF) is involved in the regulation of growth of the normal human prostatic epithelium (Bello et al., 1997, Webber at al., 1996). During malignant transformation, initiated cells may accomplish selective growth by autocrine over-expression of EGF (Jarrard et al., 1994, Nakamoto, 1992), which has been implicated in neoplastic transformation in numerous types of cancers such as gastric, esophageal, breast and prostate cancers (Jarrard et al., 1994).

Studies reported in this chapter will examine the relationship between the degree of loss of the ability to undergo acinar morphogenesis by cancer cells and their invasive ability. Further, possible relationships with $\alpha 6\beta 1$ integrin expression as well as response of various cells to exogenous EGF will be examined.

Results

Loss of acinar morphogenesis in 3-D cultures by malignant cell lines

WPE1-NA22, WPE1-NB27, WPE1-NB14, RWPE-2, WPE1-NB11 and WPE1-NB26 cell lines represent different stages of neoplastic transformation and tumor progression from low-tumorigenic to highly tumorigenic cells. The RWPE-1 cell line served as the positive control, while, the DU-145 human prostate cancer cell line, which originated from a brain metastasis, served as the negative control. All cell lines were grown on the reconstituted basement membrane, Matrigel, to determine their ability to undergo acinar morphogenesis. Within 24 h after plating, cells attached and all, except DU-145 cells, formed cytoplasmic connections with neighboring cells. Only DU-145 cells

formed a monolayer. At four days, cultures were fixed, stained with propidium iodide and counts of acini were performed at low magnification using laser scanning confocal microscopy. Acini-forming ability of the different cell lines was determined by counting individual acini using the LSM transmitted light at low power. Marked differences between the cell lines were observed. The most notable difference was observed between the non-tumorigenic RWPE-1 cells and the tumorigenic WPE1-NB26 and DU-145 cells, where the highly malignant WPE1-NB26 and DU-145 cells did not organize at all into acini (0%) (Figure 6-1), while the non-tumorigenic RWPE-1 cells used as the positive control, formed acini at a high frequency, and this number was set at 100%. The highest frequency of acini formation amongst the tumorigenic cells lines was seen in WPE1-NA22 and WPE1-NB27 cells with 69% and 51% of control, respectively. Lower acini formation was seen in WPE1-NB14 at 21%, RWPE-2 at 10% and WPE1-NB11 at 9% of the RWPE-1 control. These data demonstrates that as cells increase in tumorigenicity, their ability to polarize and undergo acinar morphogenesis is decreased.

Acinar morphology was examined by optically sectioning propidium iodide-stained acini using a laser scanning confocal microscope. RWPE-1 cells formed acini consisting of polarized cells forming a distinct central lumen (Figure 6-2 a *i*). WPE1-NA22 cells formed acini morphologically comparable to those formed by RWPE-1 (Figure 6-2 b). WPE1-NA22 acini consisted of polarized epithelial cells forming a distinct central lumen with an average area of ~4000 μm^2. WPE1-NB27 cells also formed small acini with well polarized cells and a central lumen (Figure 6-2 c *i*). While mitotic cells were occasionally observed in RWPE-1 and WPE1-NA22 acini, WPE1-NB27 acinar cells tended to be undergoing mitosis at a higher rate than cells in RWPE-1 or WPE1-NA22 acini

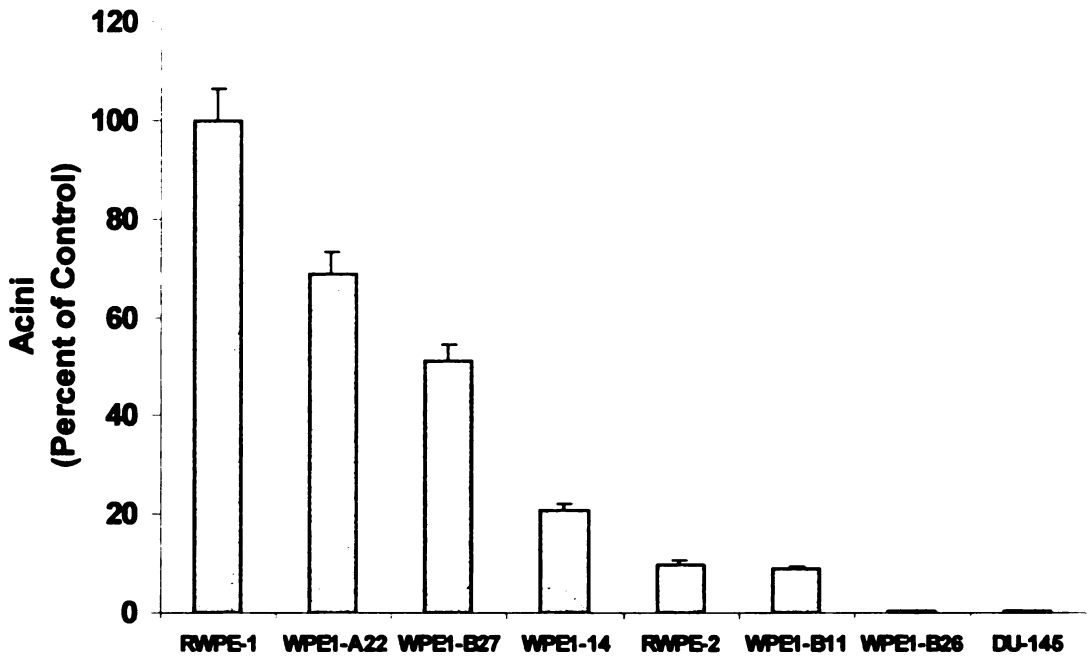


Figure 6-1. Acinar Formation of immortalized RWPE-1 cell line and malignant prostate epithelial cell lines. Cells were grown on Matrigel for 4 days. Acini-forming ability was determined by staining cultures with propidium iodide and counting individual acini using the LSM transmitted light at low power. Counts are expressed as percent of the RWPE-1 which was set at 100% (Bars = +/-SE).

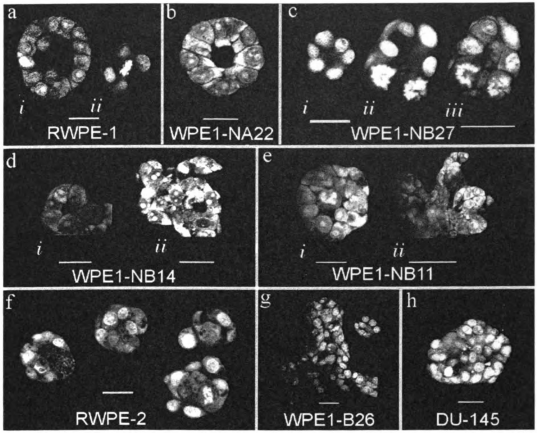


Figure 6-2. Acinar formation by immortalized non-tumorigenic and tumorigenic prostatic epithelial cell lines grown in 3-D Matrigel cultures. Optical confocal sections of propidium iodide-stained cells at 4 days of growth. (a*i*) RWPE-1 (positive control) and (b) WPE1-NA22 cells form acini with well polarized cells and a distinct lumen. (a*ii*) Cell which escape into the lumen undergo apoptosis. (c)WPE1-NB27 cells polarize and form some small acini (i), but as the acini increase in size, the lumen become obstructed by cells growing into it (*ii* and *iii*). (d) WPE1-NB14 cells form half acini attached to large masses of cells (d*i*) and psuedo-acini structures that appear to have lumens but lack polarized cells (d *ii*). (e) WPE1-NB11 cells form acini which get "plugged" by cells migrating to the center of the lumen (e*i*) or structures in a "ribbon" formation consisting of sheets of polarized cells folded over themselves (e*ii*). (f) RWPE-2 cells form small amorphous balls who's cells are undergoing apoptosis. (g) WPE1-NB26 cells do not form acini but organize into large solid cylinder-like structures and amorphous balls. (h) Du-145 cells, which served as a negative control, form large amorphous balls (Bar 25μ).

(Figure 6-2 c *ii*). Additionally, WPE1-NB27 cells, that migrated into the lumen, did not readily undergo apoptosis (Figure 6-2 c *iii*), however, those in RWPE-1 acini did so (Figure 6-2 a *ii*). WPE1-NB14 cells attached and polarized but tended to form only half acini which were attached to large masses of cells (Figure 6-2 d*i*). Additionally, WPE1-NB14 cells tended to form psuedo-acini (Figure 6-2 d *ii*), structures that appeared to have lumens but lacked polarized cells (Figure 6-2 e) . WPE1-NB11 cells did attach and polarize but few acini with lumens were evident. Acini formed tended to get "plugged" by cells migrating to the center of the lumen (Figure 6-2 e*i*). Although some migrated cells were undergoing apoptosis, most were not. The predominant type of structures observed in WPE1-NB11 cultures were "ribbon" formation consisting of sheets of polarized cells folded over themselves (Figure 6-2 e *ii*). Few RWPE-2 cells attached to the Matrigel and, those that did, tended to undergo apoptosis immediately after polarizing and beginning to form acini (Figure 6-2 f). WPE-NB26 cells did not form any acini but did organized into large solid cylinder structures and solid balls (Figure 6-2 g). DU-145 cells also did not form acini, they formed large solid balls with no apparent organization (Figure 6-2 h). These results show that as cells increase in tumorigenicity, their ability to form morphologically distinct, well polarized, acini deteriorates.

The invasive ability shows an inverse relationship with the ability to undergo acinar morphogenesis

The invasive ability of RWPE-1 cells through Matrigel, using the modified Boyden chamber *in vitro* invasion assay, was compared with that of WPE1-NA22, WPE1-NB27, WPE1-NB14, RWPE-2, WPE1-NB11 and WPE1-NB26 cells, where DU-145 invasion was

taken as 100%. In comparison to DU-145 cells, the RWPE-1 cells were not invasive, while the tumorigenic cells lines displayed varying degree of invasion (Figure 6-3). A comparison of the acinar-forming and invasive ability shows a striking inverse relationship (Figure 6-4), that is, as cells becomes more invasive and tumorigenic, they lose the ability to undergo acinar morphogenesis.

Alterations in integrin α6β1 expression in malignant cell lines

Cell lines representing varying degree of malignancy were selected for an examination of α6β1 integrin expression and include RWPE-1, WPE1-NA22, WPE1-NB27, WPE1-NB26. RWPE-1 cells represent a non-malignant stage. WPE1-NA22 and WPE1-NB27 cells represent low-tumorigenic and low-invasive stage, while WPE-NB26 cells represent a highly tumorigenic, highly invasive stage. α6 or β1 integrin expression was visualized by optical sectioning using a laser scanning confocal microscope.

RWPE-1 acini show strong positive staining for α6 at the basal end of the acinar cells as compared to the IgG control (Figures 6-5). WPE1-NA22 also showed strong basal expression of α6 integrin (Figure 6-5). WPE1-NB27 acini and WPE1-NB26 ball structures did not stain for α6 expression (Figure 6-5). Strong β1 integrin basal expression was observed in RWPE-1 acini (Figure 6-5). WPE1-NA22 also showed strong basal expression of β1 integrin (Figure 6-5). However, WPE1-NB27 acini showed positive, but weaker, staining for β1 at the basal end of the acinar cells (Figure 6-5) as compared to the IgG control (Figures 5-8). The ball structures formed by WPE1-NB26 cells showed strong positive staining for β1 integrin but the expression was not basal but was diffuse

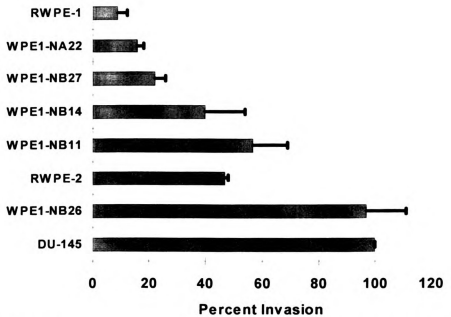


Figure 6-3. Invasive ability of non-tumorigenic immortalized and malignant human prostatic epithelial cells was compared using a modified Boyden chamber assay. Cells were plated on Matrigel-coated filters at 400,000 cells per chamber and were allowed to invade for 24 hours. Invasion by DU-145 cells was taken as 100% (Bars= +/-SE).

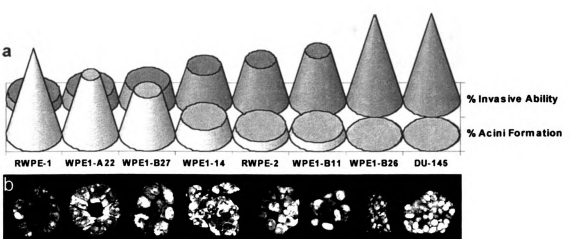


Figure 6-4. (a) Relationship between acini-forming ability and invasive ability. As invasive ability increases, the ability to undergo acinar morphogenesis decreases. (b) Optical confocal sections of propidium iodide-stained cells grown on Matrigel for 4 days. Sections of acini correspond to cell lines shown in the graph above (a).

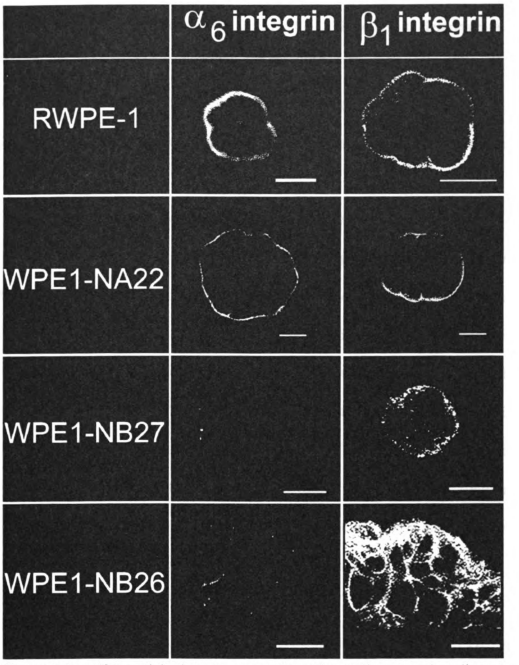


Figure 6-5. α6β1 laminin integrin expression. Non-malignant and malignant prostatic epithelial cells, grown on Matrigel, were fixed at 4 days and stained and examined by confocal microscopy, for α6 or β1 integrin expression by immunofluorescence using monoclonal antibody. Non-malignant RWPE-1 cells show strong basal expression of α6 and β1. WPE1-NA22 cells, representing an early stage of malignance, show basal expression of α6 and β1. WPE1-NB27 cells, representing a later stage of malignancy than WPE1-NA22, lack α6 expression but show basal expression of β1. WPE1-NB26 cells, representing a late invasive stage of malignancy, lack α6 expression and show a diffuse cell membrane expression of β1(Bar 25 μ).

throughout the entire cell membrane (Figures 6-5). These results demonstrate that while α6 integrin expression is decreased or is lost in the malignant transformation in WPE1-NB27 and WPE1-NB26 cells, the β1 expression is altered so that it is no longer confined to the basal cell surface but is diffusely expressed throughout the cell membrane.

Deregulation of growth by EGF inhibits acinar morphogenesis

In the prostate EGF is a positive regulator of prostatic epithelial cell growth and its expression is known to be elevated in prostate cancer cells. Because the prostate is a hormone regulated organ, the effects of EGF and its modulation by androgen were examined in RWPE-1 cells. Cells were treated with or without 5 nM of a non-metabolizing androgen, mibolerone, in the presence of EGF ranging from 0 to 10 ng/ml. EGF alone caused a maximum growth stimulation at 10 ng/ml of 162% of untreated control which was set at 100% (Figure 6-6). In combination with 5 nM mibolerone, 10ng/ml of EGF caused a growth increase to 224% of control (Figure 6-6). These data demonstrate that exogenous EGF causes an increase in cell growth and that this growth is further enhanced in the presence of androgen.

To examine the effects of this growth deregulation by EGF in acinar morphogenesis, RWPE-1 cells were treated with EGF at .01, 0.1, 1 and 10 ng/ml concentrations. Cultures were fixed after four days of treatment. Acini-forming ability for each treatment was determined by staining cultures with propidium iodide and counting individual acini using the LSM transmitted light at low power. EGF caused a dose-dependent decrease in the acinar-forming ability of RWPE-1 cells when compared to the non-treated vehicle control set at 100%. At 0.1 EGF ng/ml, acini formation was decreased

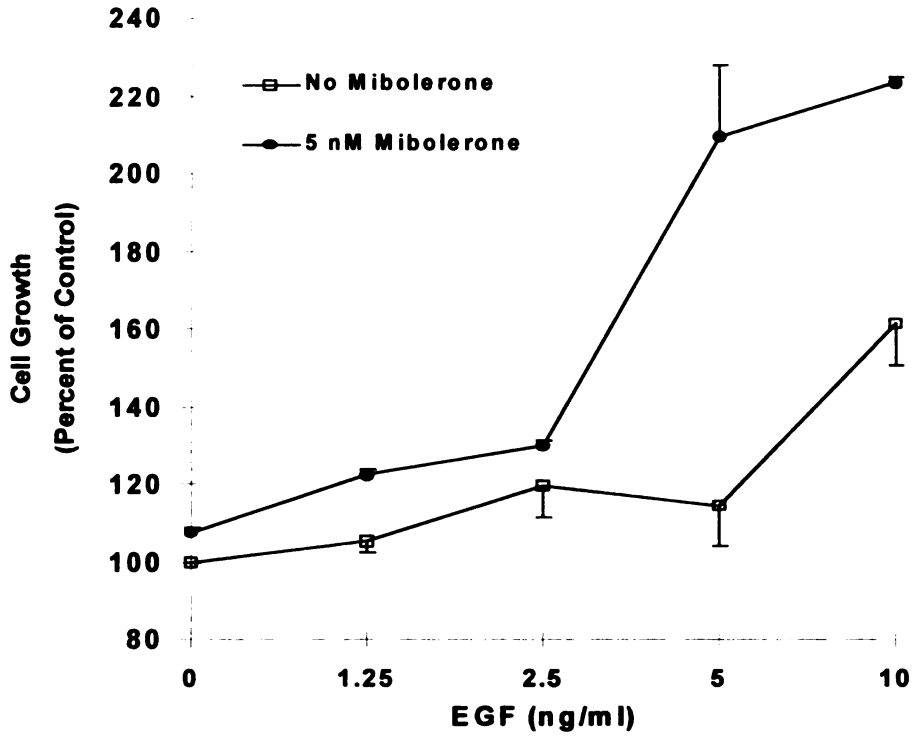


Figure 6-6. Effects of EGF on the growth RWPE-1 cells and its modulation by the synthetic androgen Mibolerone. 10,000 cells/well were plated in 96 well plates. Cells were treated with EGF in the absence and presence of 5 nM Mibolerone for 5 days. RWPE-1 cells showed a dose-dependent growth response to EGF which was accentuated in the presence of Mibolerone (Bars = +/-SE).

to 67% of control and a maximum inhibition of 56% was seen at 10 ng/ml of EGF (Figure 6-7). These results show that exogenous EGF reduces the ability of RWPE-1 cells to undergo acinar morphogenesis.

If the decrease in acinar formation is due to a deregulation, which favors cell proliferation, then blocking EGF activity should induce acinar formation. This was tested using anti-functional (neutralizing) antibody directed against EGF. The anti-EGF Ab binds to the EGF, which is a component of the culture medium, thereby preventing its binding to the cellular EGF receptors and, thus, inhibiting a growth response. The neutralizing EGF antibody, diluted 1:63 and 1:14, was added to the cell suspension and allowed to incubate for 1h before cells were plated onto Matrigel-coated wells. Anti-EGF Ab increased acinar formation to 114% and 120%, respectively as compared to the IgG control which was set at 100% (Figure 6-8). These results show that inhibition of cell proliferation by neutralization of EGF activity enhances acinar morphogenesis.

Discussion

Any scientific study of prostate cancer necessitate a knowledge of morphogenesis. The study of prostate cancer requires the analysis of the various factors which control normal acinar morphogenesis and their alteration in the process of carcinogenesis. Currently, little is known about the mechanisms that control normal acinar morphogenesis or alterations which occur in the pathogenesis of prostate cancer (Bonkhoff and Remberger, 1998, Webber et al, 1997). This is due, in part, to the lack of appropriate *in vitro* cell model systems which mimic the process of prostatic carcinogenesis and still retain the 3-D micro-environment found *in vivo*. To be useful as a consistently

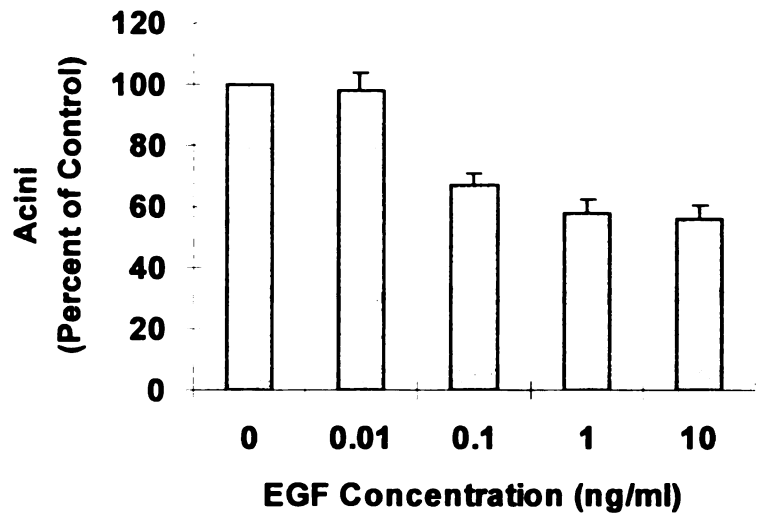


Figure 6-7. Reduction of acinar formation by EGF. RWPE-1 cells were grown on Matrigel and treated for 4 days with EGF. Acini-forming ability was determined by staining cultures with propidium iodide and counting individual acini using the LSM transmitted light at low power. Counts are expressed as a percent of the non-treated control which was set at 100%(Bars = +/-SE).

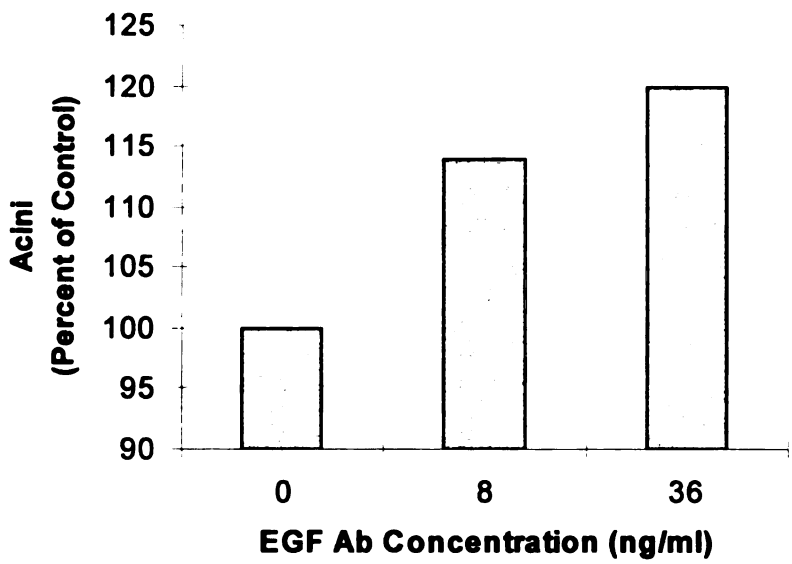


Figure 6-8. Enhancement of acinar formation by EGF neutralizing antibody. RWPE-1 cells were grown on Matrigel and pre-treated with EGF anti-functional antibody. Acini-forming ability was determined by staining cultures with propidium iodide and counting individual acini using the LSM transmitted light at low power. Counts are expressed as a percent of the IgG control which was set at 100%.

reproducible cell system, such model systems must consist of cell lines which are representative of different stages of malignancy and do not undergo senescence. Ideally, these cells line would originate from a common non-neoplastic established parent cell line which could also be used for comparison. Herein is described the use of such immortalized cells lines, in a 3-D *in vitro* cell model, which together with their parent RWPE-1 cell line, represent different stages in the progression from normal epithelium to prostatic intraepithelial neoplasia (PIN) and then to invasive carcinoma. RWPE-1 cells were transformed by the addition of a Ki-*ras* oncogene to produce the tumorigenic RWPE-2 cell line (Rhim et al., 1994 and Bello et al., 1997). Further, RWPE-1 cells were treated with the chemical carcinogen, *N*-nitroso-*N*-methlyurea (NMU) (Webber, personal communication) and injected into nude mice. The WPE1-NA22, WPE1-NB27, WPE1-NB14, WPE1-NB11 and WPE1-NB26 cell lines were established form tumors in nude mice which were subsequently cloned in agar. Unlike the parental RWPE-1 cell line, they show varying degree of tumorigenicity and anchorage independent growth. Additionally, these cell lines show altered phenotypes, in their growth rates and response to growth factors and androgen, in comparison to each other and to the parent cell line. They have retained the ability to express PSA and androgen receptor in response to androgen stimulation (Bello et al., 1997, Webber, personal communication). This chapter has focused on the loss of ability of malignant cells to undergo acinar morphogenesis, and changes in their integrin expression as well as the relationship of these characteristics to their invasive ability. Additionally, the role of epidermal growth factor in acinar formation was also examined.

Homeostasis in the prostate is maintained through an interplay of multiple factors which directly or indirectly regulate cell growth and cell death. Prostate cancer arises when the balance between the cell proliferation and cell death is altered in favor of proliferation (Sensibar, 1995). Carcinogenesis in the prostate involves the presence of PIN, carcinoma *in situ* and invasive carcinoma. During progression to an invasive malignant state, cancer cells are said to de-differentiate and lose normal cell organization, cell polarity, cell:cell and cell:ECM adhesion. It is believed that this happens in a stepwise manner in which cells progress from an immortalized to low tumorigenic to a highly tumorigenic, invasive phenotype (Webber et al., 1997). This phenomenon was examined in prostate epithelial cells using the RWPE-1, WPE1-NA22, WPE1-NB27, WPE1-NB14, RWPE-2, WPE1-NB11 and WPE1-NB26 cell lines.

Results show a loss in acinar-forming ability with increasing malignancy (Figure 6-1). Similar observations have been made in the breast when normal and malignant cells were induced to form acini (Petersen et al., 1992, Howlett et al., 1995). When plated on the reconstituted basement membrane, Matrigel, non-malignant RWPE-1 prostatic epithelial cells underwent acinar morphogenesis forming distinct acini with well polarized cells and lumens (Figure 6-2 a). The low-tumorigenic WPE1-NA22 cells also formed distinct acini with well polarized cells and lumens, which are comparable to those formed by RWPE-1 cells, but at a lower frequency of 69% as compared to the RWPE-1 controls (Figure 6-1 and 6-2 a and b, respectively). WPE1-NA22 seem to represent a stage in PIN, in that they retain the ability to form acini, have a growth rate similar to RWPE-1, have a very low invasive ability and form very small tumors. WPE1-NB27 cells, with a reduced acini-forming ability of 51%, formed small acini with well polarized cells and a central

lumen (Figure 6-1 and 6-2 c*i*). Although mitotic cells were occasionally observed in RWPE-1 and WPE1-NA22 acini, WPE1-NB27 acinar cells tended to be undergoing mitosis at higher rate suggesting a growth de-regulation in favor of cell proliferation (Figure 6-2 c *ii*). This is in agreement with WPE1-NB27 growth rate which show a doubling time twice that of RWPE-1 and WPE1-NA22 (Webber, personal communication). Additionally, cells that migrated into the lumen did not readily undergo apoptosis upon loss of attachment to the matrix as is seen in RWPE-1 cells (Figure 6-1 c *iii* and a *ii*, respectively). Both WPE1-NB14 and WPE1-NB11 cells appear to polarize but are unable to properly regulated their cell proliferation, thus, forming fewer acini, at 21% and 10% of control, respectively. The acini that do form consist of polarized cells with half lumens or lumens that tended to "get plugged" by cells migrating into it (Figure 6-2 d*i and* e*i*). Only a few RWPE-2 cells attach to the Matrigel and, those that do, tend to undergo apoptosis immediately after attachment (Figure 6-2 f). WPE-NB26 cells did not form acini but did organized into large solid cylinder structures and solid balls of cells similar to those formed by DU-145 (Figure 6-1 g and h, respectively). These results suggest that interactions between prostate epithelial cells and the ECM microenvironment are important for regulating normal acinar morphogenesis and that these interactions are disturbed in the process of tumorigenesis.

The results from the *in vitro* invasion assay demonstrate that the invasive ability of the malignant cell lines shows an inverse relationship with their acinar-forming ability (Figure 6-7). This suggests that the factors which promote acinar differentiation inhibit invasion and those which favor invasion deny the cells the ability to undergo differentiation. Taken together, the preceding results, thus, allow the ranking of the cell

lines in order of their increasing malignancy: WPE1-NA22, WPE1-NB27, WPE1-NB14, RWPE-2, WPE1-NB11 to WPE1-NB26 cell lines (see order in Figure 6-4). The non-tumorigenic, non-invasive immortalized RWPE-1 cell line represents a non-malignant cell type.

Extracellular matrix:cell interactions are mediated by integrin receptors. As demonstrated in chapter 5, the laminin component of the basement membrane and its key laminin integrin receptor, $\alpha 6\beta 1$, mediate the ability of prostatic epithelial cells to polarize and undergo acinar morphogenesis. Alterations in the expression of laminin integrins has been observed in a majority of prostate and breast cancers (Howlett et al., 1995, Knox et al., 1994, Rabinovitz et al., 1995, Wewer et al., 1997). Because of the importance of laminin in epithelial polarization and acinar morphogenesis, alterations in $\alpha 6\beta 1$ integrin expression were examined. The non-malignant RWPE-1 cell line formed acini that demonstrated strong basal expression of the $\alpha 6\beta 1$ integrin receptor (Figure 6-5). The WPE1-NA22 cell line, which has low tumorigenecity and low invasive ability, also showed basal expression of the $\alpha 6\beta 1$ integrin receptor (Figure 6-5). This is in agreement with the observation that normal prostatic epithelial cells and PIN show basal expression of $\alpha 6\beta 1$ laminin receptors *in vivo* (Cress et al., 1995, Knox et al., 1994, Rabinovitz et al., 1995). While WPE1-27 cells express $\beta 1$, they do not express the $\alpha 6$ integrin receptor. This may suggest a weaker attachment of cells to the basement membrane. The highly invasive and the most malignant WPE-26 cells lack $\alpha 6$ integrin expression. They show $\beta 1$ expression but it is not restricted to the basal cell surface and is diffuse throughout the cell membrane. This diffuse $\beta 1$ staining pattern was also observed in the malignant and highly invasive Du-145 (data not shown). This is in agreement with findings of other investigators

who have observed a diffuse β1 integrin expression pattern in invasive prostate and breast cancer cells *in vivo* and *in vitro* and have suggested that it may play a role in invasion (Knox et al., 1994, Rabinovitz et al., 1995, Wewer et al., 1997).

However, the lack of α6 integrin expression in WPE1-NB26 and WPE1-NB27 is in conflict with accounts where an increase in α6 expression in prostate cancer cells *in vivo* and in established *in vitro* prostate cancer cell lines was observed (Knox et al., 1994, Rabinovitz et al., 1995, Wewer et al., 1997). To explain this difference, I suggest that integrins may be expressed in a transient manner in prostate carcinogenesis, similar to their expression in the developing prostate. Perhaps WPE1-NB27 and WPE1-NB26 cells represent two different stages in tumor progression in which the expression of α6 is turned off. At the WPE1-NB27 stage, cells lack α6 expression but retain a normal basal expression of β1, suggesting a weak attachment to the basement membrane. This would fit with their low acinar-forming ability and the slightly higher invasive ability as compared to the α6β1 integrin expressing RWPE-1 and WPE1-NA22 cell lines. WPE1-NB26 might represent a more advanced stage, when the cells are most motile and the lack of α6 integrin expression maintains the cells detached from the ECM. This, in combination with the diffuse expression of β1, would facilitate invasion. This is supported by the observation that WPE1-NB26 cells are the most invasive amongst this family of malignant cells lines. Additionally, matrix studies with normal and malignant breast cancer cells show a diffuse staining for β1 and absence of α6 integrin staining in malignant cells, as compared to normal breast cells (Howlett et al., 1995).

In analyzing the morphology of the acini formed by the malignant cell lines and their integrin expression profile, I observed that the loss of the ability to undergo acinar

formation could be divided into two phases. In the first phase, cells lose the ability to control their growth but retain the ability to attach and polarize, as is seen in WPE1-NA22 and WPE1-NB27. In the second phase, in addition to the loss of cell growth, cells also lose proper integrin expression. In this phase, cells attach but do not polarize, instead, they grow in masses which increase in size according to their growth rate as seen by RWPE-2, WPE1-NB26 and DU-145 cells.

Perhaps the decrease in acinar-forming ability seen in the WPE1-NA22 and WPE1-NB27 cells lines, which retain some degree of acinar-forming ability, is due to the deregulation of growth observed in malignant cells. The autocrine over-expression of growth factors, such as EGF, has been implicated in neoplastic transformation (Jarrard et al, 1994). In primary cultures of normal prostate cells exogenous EGF causes an increase in cell proliferation (McKeehan et al., 1984). In prostate cancer cell lines, such as PC-3 and DU-145, exogenous EGF causes little to no effect on cell proliferation. The lack of growth response to EGF may be due to the autocrine production of EGF and, therefore, the cells are not as sensitive to exogenous EGF. EGF dose response studies show that WPE1-NA22, WPE1-NB27 are less sensitive the exogenous EGF treatment than RWPE-1 cells (Webber, personal communication). This, along with the fact that their doubling time is twice that of RWPE-1, supports the idea that the decreased acinar-forming ability is due to a de-regulation of growth in favor of cell proliferation.

In order to determine if a deregulation of growth alone could alter acinar formation, the role of exogenous EGF on RWPE-1 cell growth and acinar-forming ability was examined. Results show that exogenous EGF causes an increase in cell growth (Figure 6-6). Intentional deregulation of RWPE-1 cell growth using exogenous EGF, significantly

reduced the acini-forming ability of RWPE-1 cells when plated on Matrigel (Figure 6-7). Conversely, if EGF activity was blocked using neutralizing antibody, acinar formation was significantly enhanced. Furthermore, the increase in RWPE-1 cell proliferation, in response to EGF, is accentuated by the presence of androgen (Figure 6-6). Androgen is known to causes an up-regulation of the EGF receptor (Carpenter and Wahl, 1990), thereby increasing the sensitivity of cells to EGF. Since the development of prostate cancer is dependant on the presence of androgen, the regulation of EGF responsiveness by androgen presents one manner by which PIN cells are able to escape the confines of acini and expand clonally.

In summary, the data presented in this chapter demonstrate the use of an *in vitro* 3-D cell culture system that mimics different stages in prostate carcinogenesis represented by PIN *in vivo*. Using this system it was demonstrated that malignant cells lose the ability to undergo acinar differentiation and that the degree of loss is correlated with their degree of malignancy. The loss of the ability to form acini showed an inverse relationship to the invasive ability of the cells. Additionally, data show that in the progression through the multi-step process of carcinogenesis, alterations in laminin integrin $\alpha 6\beta 1$ expression and deregulation of growth, involving alteration in growth factors expression and responsiveness, occur early on in the process. It is hoped that further studies, utilizing the 3-D cell culture model system, will shed light on the role of integrin receptors and other factors in the progression from normal to malignant prostatic epithelial cells. This information would facilitate the development of drugs for cancer prevention and treatment.

References

Bello D, Webber MM, Kleinman HK, Wartinger DD and Rhim JS: Androgen responsive adult human prostatic epithelial cell lines immortalized by human papillomavirus 18. Carcinogenesis 18: 1212-1223, 1997.

Bonkhoff H and Remberger K: Morphogenetic concepts of normal and abnormal growth in the human prostate. Virchows Arch. 433: 195-202, 1998.

Carpenter G and Wahl MI: The epidermal growth factor family. In: Peptide growth factors and their receptors I. Sporn, M.B. and Roberts, A.B. (eds), New York, Springer-Verlag, pp. 69-171, 1990.

Cress AE, Rabinovitz I, Zhu W and Nagle RB: The alpha 6 beta 1 and alpha 6 beta 4 integrins in human prostate cancer progression. Cancer Metastasis Rev: 14:219-28, 1995.

Flug M and Kopf-Maier P: The basement membrane and its involvement in carcinoma cell invasion. Acta. Anat. Basel. 152: 69-84, 1995.

Howlett AR, Bailey N, Damsky C, Petersen OW and Bissell MJ: Cellular growth and survival are mediated by beta 1 integrins in normal human breast epithelium but not in breast carcinoma. J. Cell. Sci. 108: 1945-57, 1995.

Jarrard, D.F., Blitz, B.F., Smith, R.C., Patai, B. L. and Rukstalis. Effects of epidermal growth factors on prostate cancer cell line PC3 growth and invasion. Prostate, 24: 46-53, 1994.

Knox JD, Cress AE, Clark V, Manriquez L, Affinito KS, Dalkin Bland Nagle RB: Differential expression of extracellular matrix molecules and the alpha 6-integrins in the normal and neoplastic prostate. Am. J. Pathol. 145:167-74, 1994.

McKeehan, W. L., Adams, P.S. and Rosser, M.P. Direct mitogenic effects of insulin, epidermal growth factor, glucocorticoid, cholera toxin, unknown pituitary factors and possibly prolactin but not androgen, on normal rat prostate cells in serum-free, primary cell cultures. Cancer Res., 44: 1998-2010, 1984.

Nakamoto, T., Chang, C.S., Li, A.K. and Chodak, G.W. Basic fibroblast growth factor in human prostate cancer cell lines. Cancer Res., 52: 571-577, 1992.

Petersen OW, Ronnov-Jessen L, Howlett AR and Bissell MJ: Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells [published erratum appears in Proc. Natl. Acad. Sci. U.S.A. 89: 9064-8, 1992.

Rabinovitz I, Nagle RB and Cress AE: Integrin alpha 6 expression in human prostate carcinoma cells is associated with a migratory and invasive phenotype *in vitro* and *in vivo*. Clin. Exp. Metastasis. 13: 481-91, 1995.

Rhim JS, Webber MM, Bello D, Lee MS, Arnstein P, Chen L and Jay G: Stepwise immortalization and transformation of adult human prostate epithelial cells by a combination of HPV18 and v-Ki-ras. Proc. Nat. Acad. Sci. USA, 91:11874-11878, 1994.

Sensibar JA: Analysis of cell death and cell proliferation in embryonic stages, normal adult, and aging prostates in human and animals. Microsc. Res. Tech. 30: 342-50, 1995.

Webber MM, Bello D, Kleinman HK and Hoffman MP: Acinar differentiation by non-malignant immortalized human prostatic epithelial cells and its loss by malignant cells. Carcinogenesis 18:1225-1231, 1997.

Webber, MM, Bello D, and Quader S: Immortalized and tumorigenic adult human prostatic epithelial cell lines. Characteristics and applications. Part. 1. Cell markers and immortalized, non-tumorigenic cell lines. Prostate 29:386- 394, 1996.

Webber MM, Waghray A, Bello D and Rhim JS: Proteases and invasion in human prostate epithelial cell lines: Implications in prostate cancer prevention and intervention. Radiat. Oncol. Invest. 3:358-362, 1996.

Wewer UM, Shaw LM, Albrechtsen R and Mercurio AM: The integrin alpha 6 beta 1 promotes the survival of metastatic human breast carcinoma cells in mice. Am. J. Pathol. 151: 1191-8, 1997.

Chapter Seven

Regulation of Acinar Morphogenesis by TGF-β in Non-malignant and Malignant Human Prostatic Epithelial Cells

Abstract

An important event in the transformation of normal cells to malignant cells is the loss of normal growth regulation. This occurs as a result of a loss of balance between stimulatory and inhibitory growth factors. Studies described in Chapter 6 showed that excessive stimulation with EGF can cause a decrease in the ability of cells to undergo acinar morphogenesis. This may be one factor responsible for the loss of ability of cancer cells to polarize and forma acini. In the present study, the role of the inhibitory growth factor TGF-β and its receptors in acinar morphogenesis in non-malignant RWPE-1 and progressively more malignant WPE1-NA22 and WPE1-NB26 cells was examined. Results show that TGF-β2 and 3 were more effective in inhibiting growth of RWPE-1 cells that TGF-β3. An examination of the expression of the three TGF-β isoforms by immunostaining showed that they were expressed in RWPE-1 cells in the following order: TGF-β1 > β2 > β3. A strong expression of TGF-β RII and TGF-β RIII was also observed. Exposure of cells to anti-functional TGF-β1 and 2 antibody caused inhibition of cell polarization and reduced acini formation to 42% and 46% of control, respectively, indicating that TGF-β1 and β2 are necessary for cell polarization but TGF-β3 is not essential. The importance of TGF-β function was further demonstrated by treating cells with anti-functional antibody to TGF-β RII and RIII receptors. Anti-TGF-β RII and RIII antibodies decreased acini formation to 26 and 35% of control, respectively, thus, validating the importance of TGF-β binding in acinar morphogenesis.

Exposure of WPE1-NA22 cells to TGF-β1, 2 and 3, in monolayer cultures, reduced growth to 54, 60 and 70% of control, respectively. Similar treatment of

WPE1-NB26 cells reduced growth to 74, 74 and 85% of control, respectively. In comparison to RWPE-1 and WPE1-NA22, WPE1-NB26 cells show reduced sensitivity to TGF-β1. Further, WPE1-NA22 cells showed strong expression of TGF-β1 but that of β2 and β3 was not detected. They do express TGF-β1 RII and RIII receptors and the addition of exogenous TGF-β1 or TGF-β2 induced an increase in acini formation by 134 and 159% of control. However, the more malignant WPE1-NB26 did not show any TGF-β1 expression by immunostaining and only weakly expressed TGF-β1 RII. Taken together, these results demonstrate that TGF-β1 and β2 play a critical role in acinar morphogenesis, that a stepwise loss of TGF-β and/or receptor expression is associated with increasing malignant characteristic of prostate cancer cells and that exogenous TGF-β can restore the ability, at least of cells at the lower end of malignancy, to undergo acinar morphogenesis.

Introduction

During the multi-step process of carcinogenesis, an important event is the loss of normal growth regulation. This deregulation of growth is achieved, in part, by alteration in growth factor expression and in the ability of cells to respond to growth factors. Transforming growth factor-β and epidermal growth factor (EGF) are two major growth factors involved in the regulation of the normal prostatic epithelium (Webber at al. 1996). In normal prostate epithelial cells, TGF-β acts predominantly as a negative growth regulator, while EGF acts as a positive growth regulator. Changes in the level of these growth factors have been observed in malignant prostate epithelial cells (Jarrard et al, 1994, Nakamoto et al., 1992). 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 mitogenic growth factors and the down-regulation or cessation of inhibitory growth factor function (Culig, 1994, Nakamoto et al., 1992,). Data in chapter 6 demonstrated that a deregulation of growth by exogenous EGF inhibits acinar morphogenesis and that the neutralization of EGF activity enhanced acinar-formation and differentiation. This chapter will examine the role of TGF-β in acinar morphogenesis and alterations in TGF-β expression and response in cancer cells, using the 3-D cell culture model.

TGF-βs are members of a superfamily of polypeptides that regulate cell cycle progression, differentiation and chemotaxis of many different types of cells. Their 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 (Franzen et. al., 1993, Roberts and Sporn, 1990, Webber et. al., 1996). In addition to inhibiting epithelial cell growth, TGF-β stimulates the production of extracellular matrix components and protease inhibitors and causes a decrease in the net activity of extracellular matrix-degrading proteolytic enzymes (Franzen et al., 1993, Roberts and Sporn, 1990,). Presently, three form of TGF β (TGF-β1, TGF-β2 and TGF-β3) have been identified in the prostate (Dahiya et al, 1996).

Like most growth factors, TGF-β exerts its biological activity through a specific transmembrane receptor. Thus far, three classes of TGF-β receptor subunits have been recognized: TGF-β type I (RI), TGF-β type II (RII) and TGF-β type III (RIII) (Mckeehan, 1991). RI (60- 70 kDa) and RII (85-110 kDa) are serine-threonine kinase receptors, while RIII (100-140 kDa), with a large extracellular domain and a short cytoplasmic tail, possesses no obvious signaling motif. RIII is the predominantly expressed receptor in comparison to the RI and RII receptors, and has high affinity for TGF-β1 and β2 (Mckeehan, 1991, Moustakas et al., 1993). A functional TGF-β receptor is formed as a heterodimer by a single disulfide bond between different receptor subunits. The heterodimer is formed upon exposure to the ligand. In the presence of TGF-β, RII/ RIII and RI/ RII form heterodimers but RIII/RI heterodimers form rarely (Moustakas et al., 1993). The RI/RII complex binds TGF-β1 and β3 well but binds TGF-β2 poorly, while the RII/RIII complex binds TGF-β2 as well as TGF-β1 with high affinity. The RII/RIII complex also binds TGF-β3. The human prostate epithelial cells express all three TGF-β

receptors (Guo et al., 1997).

Although TGF-β is expressed by both the urogenital mesenchyme and the urogenital epithelium of the fetal mouse, the highest levels are detected in the mesenchyme and not the epithelium. Its exact role in prostatic morphogenesis has remained unclear. Conflicting reports indicate that TGF-β expression follows the path the epithelial cells take as they penetrate into the surrounding mesenchyme, while others find focal loss of TGF-β expression at bud-forming regions (Nemeth, et al. 1997, Silberstein et al, 1992, Timme et al. 1994).

Because of its apparent important role in branching and acinar morphogenesis *in vivo*, the present chapter will focus on the role of a TGF-β 1, 2 and 3, and RII and RIII receptors in normal acinar morphogenesis by RWPE-1 cells and its alteration in the PIN-like WPE1-NA22 cells line and the malignant WPE1-NB26 cells. Additionally, the ability of TGF- β isoforms to restore the ability to undergo acinar morphogenesis will also be examined.

Results

Effects of TGF-β isoforms on the growth of RWPE-1 cells

In the human prostate TGF-β is a negative regulator of normal prostatic epithelial cell growth. In order to elucidate the individual roles of TGF-β1, TGF-β2, and TGF-β3 on cell growth, RWPE-1 cells were treated with either TGF-β1, TGF-β2 or TGF-β3 ranging from 0 to 10 ng/ml in the presence of 5 ng/ml of EGF. At 10 ng/ml, TGF-β1 caused a maximum growth inhibition to 66% of untreated control which was set at 100% (Figure 7-1). TGF-β2 caused a maximum growth inhibition to 76% of untreated control

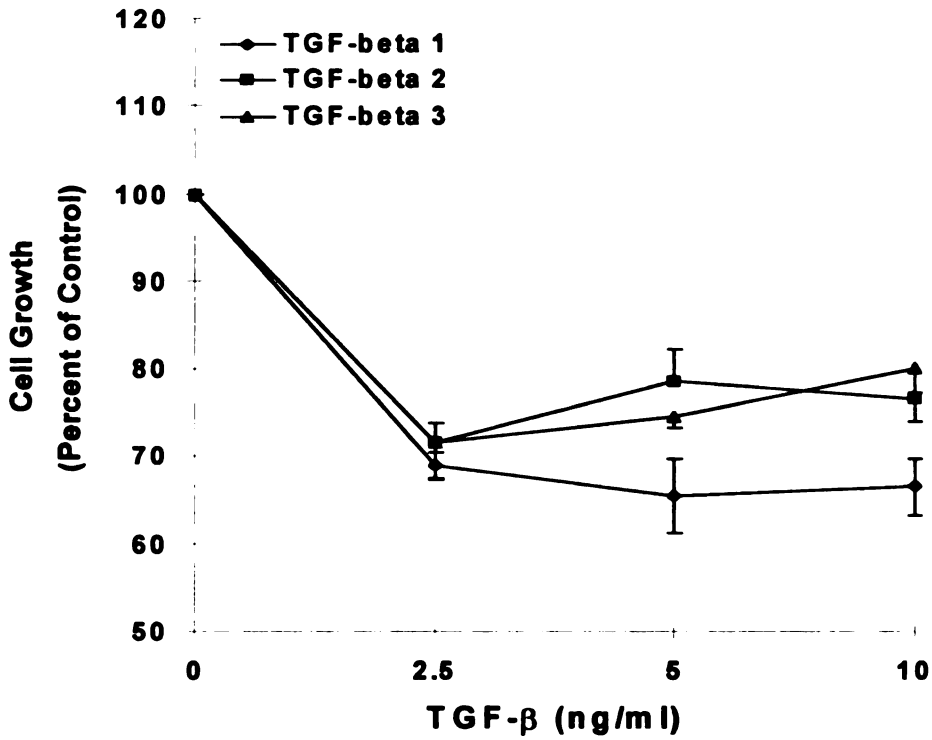


Figure 7-1. Effects of TGF-β1, TGF-β2 and TGF-β3 on the Growth of RWPE-1 cell in monolayer cultures. 10, 000 cells/well were plated in complete K-SFM medium in 96 well plates in triplicate. Cells were treated with TGF-β1, TGF-β2 or TGF-β3 for 5 days. RWPE-1 cells showed a dose-dependent growth inhibition for all three cells line. Note that TGF-β1 was the most effective growth inhibitor. (Bars = +/-SE)

at 10 ng/ml. TGF-β3 show similar response as TGF-β2 with a maximum growth inhibition to 80% of untreated control at 10 ng/ml (Figure 7-1). These data demonstrate that while all three forms elicited growth inhibition, TGF-β1 was most affective.

RWPE-1 cells express TGF-β1, 2 and 3 and the TGF-β RII and RIII receptors

The expression of TGF-β1, TGF-β2 or TGF-β3 in RWPE-1 cells was examined by indirect immunofluorescence using a laser scanning microscope (LSM). Because the TGF-β RII/ RIII receptor complex binds both TGF-β1 and TGF-β2 with high affinity, and also binds TGF-β3, the expression of RII and RIII was examined. RWPE-1 cells show strong positive staining for TGF-β1 as compared to the IgG control (Figures 7-2). TGF-β2 expression was detected but staining was weaker in comparison to TGF-β1 (Figure 7-2). Very weak staining was observed for TGF-β3 as compared TGF-β1 or 2 (Figure 7-2). RWPE-1 showed strong expression for both the TGF-β RII and TGF-β RIII as compared to the IgG control (Figures 7-3). These results demonstrate that TGF-β1 is the predominant type expressed by RWPE-1 cells and that TGF-β2 and TGF-β3 are expressed in decreasing order. Strong expression of TGF-β RII and TGF-β RIII receptors was also observed in these cells.

TGF-β1 and TGF-β2 are necessary for acinar morphogenesis

In order to determine if TGF-β1, TGF-β2 or TGF-β3, expressed by RWPE-1 cells, play a critical role in acinar morphogenesis, anti-functional antibodies against TGF-β1, TGF-β2 or TGF-β3 were employed. TGF-β1, TGF-β2 or TGF-β3 antibody, diluted 1:42,

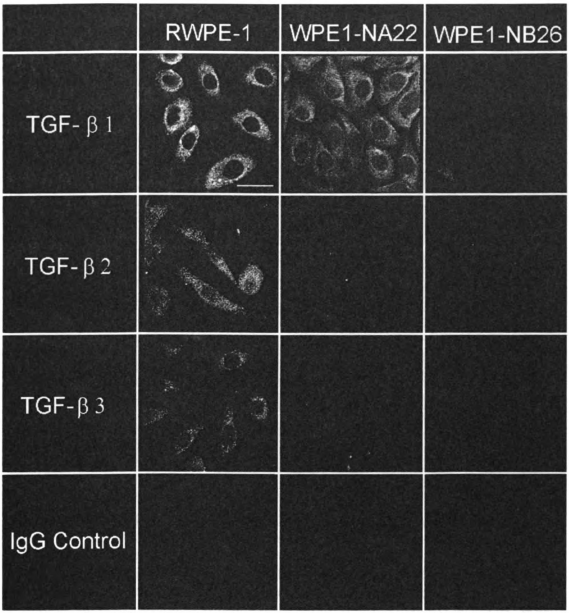


Figure 7-2. TGF-β1, TGF-β2 and TGF-β3 expression in non-malignant RWPE-1 and malignant human prostatic epithelial cells, WPE1-NA22 and WPE1-NB26. Cells were grown on glass coverslips, fixed and examined for TGF-β1, TGF-β2 and TGF-β3 expression by immunofluorescence using monoclonal antibody. Staining was visualized by LSM. Non-malignant RWPE-1 cells show strong expression of TGF-β1, but weaker expression of TGF-β2 and TGF-β3. WPE1-NA22 cells, representing an early stage of malignancy, show positive expression of TGF-β1, but lack expression of TGF-β2 and TGF-β3. WPE1-NB26 cells, representing a later invasive stage of malignancy than WPE1-NA22, lack expression of TGF-β1, TGF-β2 and TGF-β3 (Bar 25 μ).

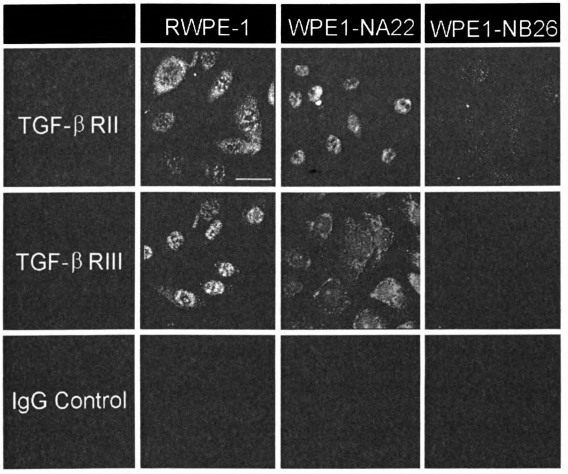


Figure 7-3. TGF-β RII and TGF-β RIII receptor expression in non-malignant RWPE-1 and malignant prostatic epithelial cells, WPE1-NA22 and WPE1-NB26. Cells were grown on Matrigel, fixed at 4 days and examined for TGF-β RII and TGF-β RIII receptor expression by immunofluorescence using monoclonal antibody. Staining was visualized by LSM. Non-malignant RWPE-1 cells show strong expression of TGF-β RII and TGF-β RIII. WPE1-NA22 cells, representing an early stage of malignancy, show positive expression of TGF-β RII and TGF-β RIII. WPE1-NB26 cells, representing a later invasive stage of malignancy than WPE1-NA22, show faint expression of TGF-β RII and lack TGF-β RIII. expression (Bar 25 μ).

1:62 and 1:16, respectively, was added to the cell suspension and allowed to incubate for 1 h before cells were plated onto Matrigel-coated wells. Anti-TGF-β1 neutralizing Ab decreased acinar formation to 42% of the IgG control which was set at 100% (Figure 7-4). Anti-TGF-β2 neutralizing Ab also decreased acinar formation to 46% of the IgG control (Figure 7-4). However, anti-functional TGF-β3 Ab only caused a decrease to 80% of control in acinar formation (Figure 7-4). These results indicate that TGF-β1 and TGF-β2, but not TGF-β3, are essential for cell polarization and acinar morphogenesis.

If TGF-β function is necessary for acinar formation, then blocking the binding of TGF-β to the cells should result in the inhibition of acinar formation. This was tested using anti-functional antibody directed against TGF-β RII or TGF-β RIII. The neutralizing TGF-β RII or TGF-β RIII Ab, diluted 1:16, was added to the cell suspension and allowed to incubate for 1 h before cells were plated onto Matrigel-coated wells. Anti-TGF-β RII neutralizing Ab decreased acinar formation to 26% of the IgG control which was set at 100% (Figure 7-5). Anti-TGF-β RIII neutralizing Ab decreased acinar formation to 35% of the IgG control (Figure 7-5). These results validate the importance of functional TGF-β binding in acinar morphogenesis.

Effects of TGF-β isoforms on the growth of WPE1-NA22 and WPE1-NB26 cells

Because a loss of autocrine TGF−β activity and/or sensitivity to exogenous TGF-β appears to provide cells with a growth advantage in malignant progression, effects of TGF-β 1, 2 and 3, on the growth of WPE1-NA22 and WPE1-NB26 cells, were examined. WPE1-NA22 and WPE1-NB26 cells were treated with either TGF-β1, TGF-β2 or TGF-β3

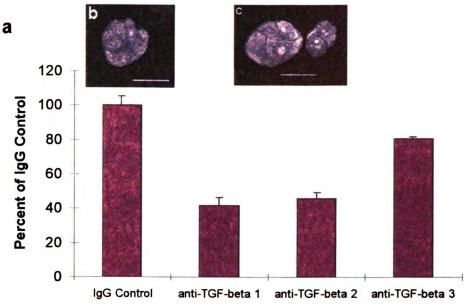


Figure 7-4. Inhibition of acinar formation by anti-TGF-β neutralizing antibodies. RWPE-1 cells were pre-treated with TGF-β1, TGF-β2 or TGF-β anti-functional antibody before plating on Matrigel. (a) Acini-forming ability was determined by staining cultures with propidium iodide and counting individual acini using the LSM transmitted light at low power. Counts are expressed as percent of the IgG control which was set at 100% (Bars = +/- SE). (b) Optical confocal section of a control acinus. (c) Optical confocal section of TGF-β antibody-treated cells which form aggregates but lack polarization and acinar formation (Bar 25 μ).

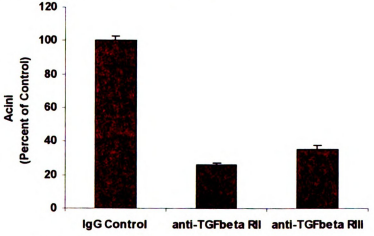


Figure 7-5. Inhibition of acinar formation by anti-TGF-β receptor neutralizing antibodies. RWPE-1 cells were and pre-treated with TGF-β RII or TGF-β RIII anti-functional antibody before plating on Matrigel. Acini-forming ability was determined by staining cultures with propidium iodide and counting individual acini using the LSM transmitted light at low power. Counts are expressed as a percent of the IgG control which was set at 100% (Bars= +/- SE).

at concentrations ranging from 0 to 10 ng/ml. In WPE1-NA22 cells, TGF-β1 caused a maximum growth inhibition to 54% of untreated control at 10 ng/ml (Figure 7-6). Untreated control was set at 100%. TGF-β2 caused a maximum growth inhibition to 60% of untreated control at 10 ng/ml (Figure 7-6). TGF-β3 was the least effective at eliciting an inhibitory response, with a initial growth inhibition to 70% of untreated control at 2.5 ng/ml but at 10 ng/ml concentration growth was inhibited only to 82% (Figure 7-6).

In WPE1-NB26 cells, TGF-β1 caused a maximum growth inhibition to 74% of untreated control at 10 ng/ml (Figure 7-7). TGF-β2 caused a maximum growth inhibition at 10 ng/ml to 74% of untreated control (Figure 7-7). TGF-β3 was the least effective at eliciting an inhibitory response, with a initial growth inhibition to 74% of untreated control at 2.5 ng/ml but at 10 ng/ml growth was inhibited only to 85% (Figure 7-7). These data demonstrate that while all three forms elicited a growth inhibition, TGF-β1 and TGF-β2 were most effective. In comparison to RWPE-1 and WPE1-NA22, WPE1-NB26 cells show reduced sensitivity to TGF-β1 (Figure 7-8).

Alteration in TGF-β1, 2 and 3 and TGF-β RII and TGF-β RIII receptor expression in malignant WPE1-NA22 and WPE1-NB26 cells

The escape from the negative growth control imposed by TGF-β may be due to either down-regulation of growth factor expression or a decrease or loss of receptor expression. Therefore, the expression of TGF-β1, TGF-β2, and TGF-β3 and TGF-β RII and RIII in WPE1-NA22 and WPE1-NB26 cells was examined by indirect immunofluorescence using the LSM. WPE1-NA22 cells show strong positive staining for

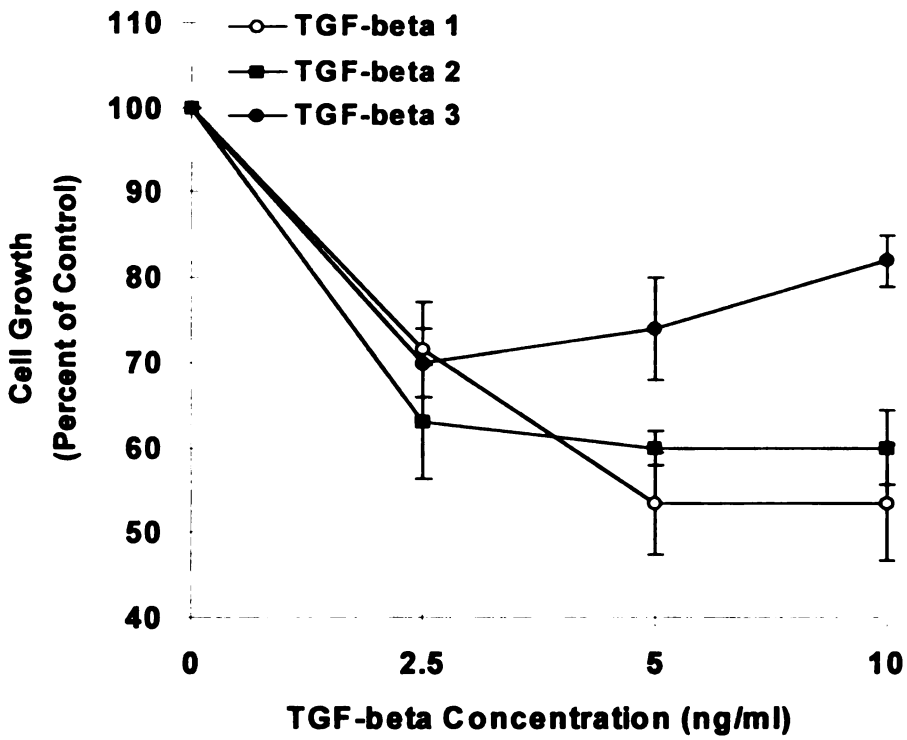


Figure 7-6. Effects of TGF-β1, TGF-β2 and TGF-β3 on the growth of WPE1-NA22 cells. 10, 000 cells/well were plated in complete K-SFM medium in 96 well plates. Cells were treated with TGF-β1, TGF-β2 or TGF-β3 for 5 days. WPE1-NA22 cells showed a dose-dependent growth inhibition for all three isoforms. Note that TGF-β1 and TGF-β2 were more inhibitory than TGF-β3 (Bars = +/- SE).

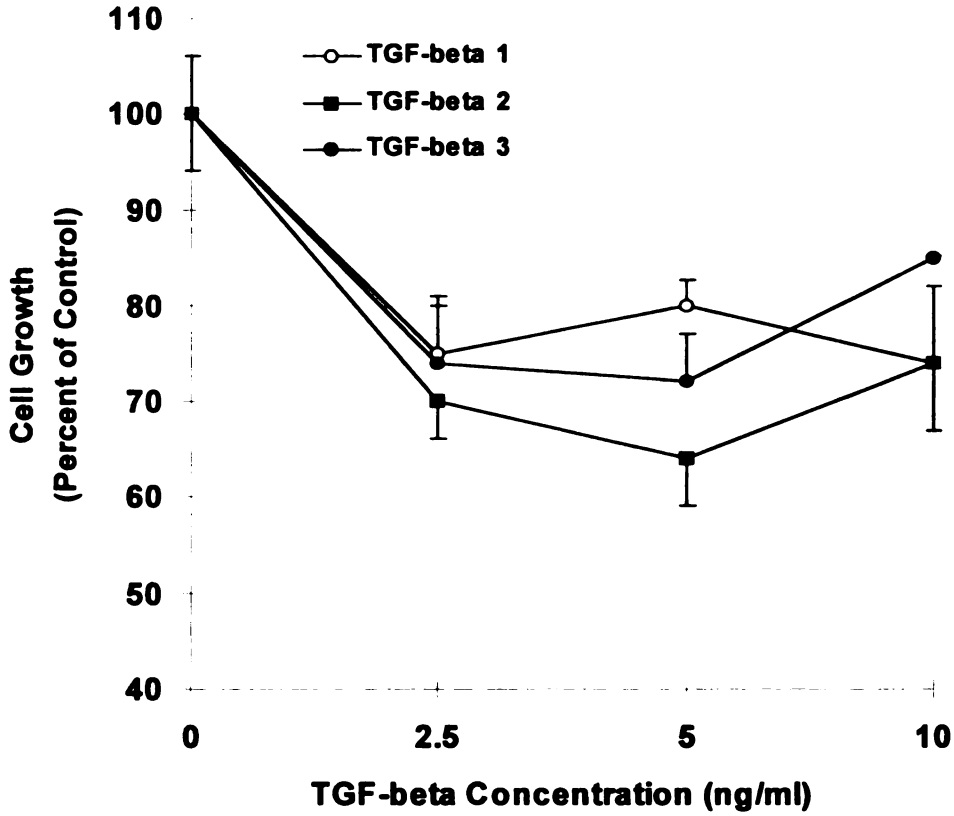


Figure 7-7. Effects of TGF-β1, TGF-β2 and TGF-β3 on the growth of WPE1-NB26 cells. 10, 000 cells/well were plated in complete K-SFM medium in 96 well plates. Cells were treated with TGF-β1, TGF-β2 or TGF-β3 for 5 days. WPE1-NB26 cells showed a growth inhibition for all three isoforms. Note that TGF-β2 was the most inhibitory isoform (Bars = +/-SE).

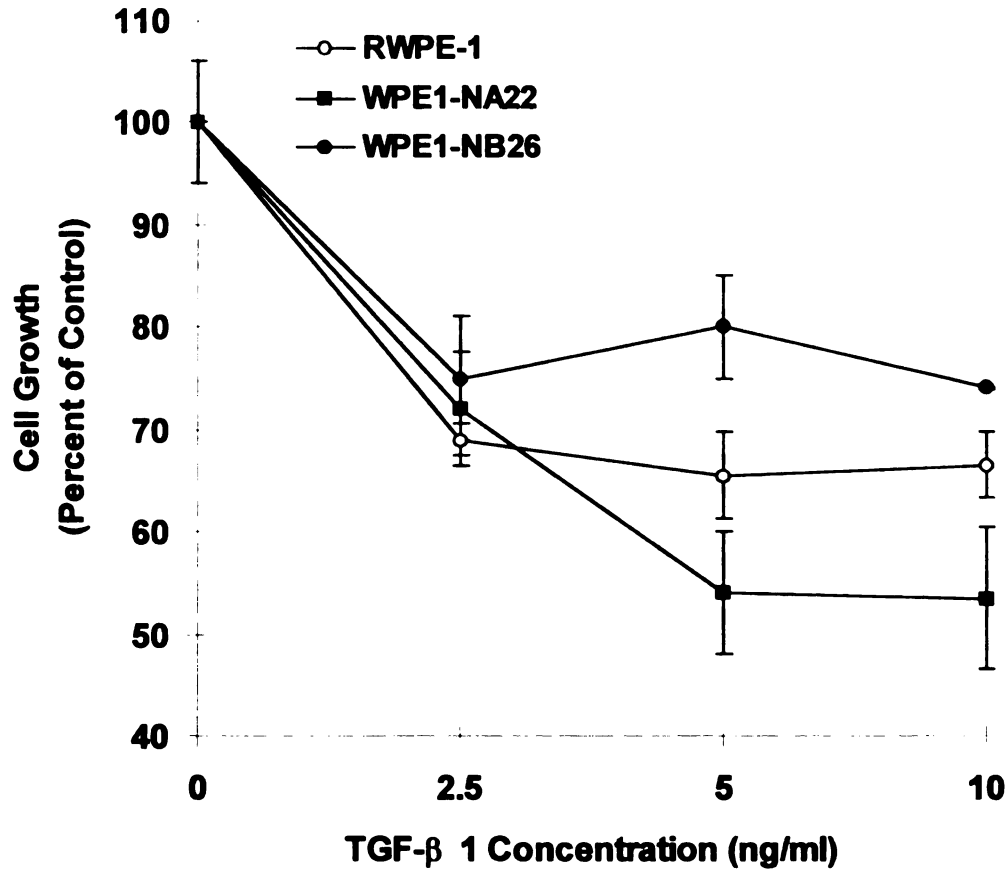


Figure 7-8. Effects of TGF-β1 on the growth of RWPE-1, WPE1-NA22 cells and WPE1-NB26. 10, 000 cells/well were plated in complete K-SFM medium in 96 well plates for each cell line. Cells were treated with TGF-β1 for 5 days. WPE1-NA22 showed the strongest inhibition by TGF–β1. WPE1-NB26 cells showed the least inhibition when compared to RWPE-1 cells (Bars = +/-SE).

TGF-β1 as compared to the IgG control (Figures 7-2). TGF-β2 and TGF-β3 expression was not detected by immunofluorescence staining as compared to the IgG control (Figures 7-2). WPE1-NB26 cells show no positive staining for TGF-β1, TGF-β2 or TGF-β3 expression by immunofluorescence staining, as compared to the IgG control (Figures 7-2). WPE1-NA22 show positive expression for both the TGF-β RII and TGF-β RIII receptors as compared to the IgG control (Figures 7-3). WPE1-NB26 show weakly positive expression for TGF-β RII receptor but do not show TGF-β RIII receptor expression as compared to the IgG control (Figures 7-3). These results demonstrate that WPE1-NA22 cells express TGF-β1 but lack TGF-β2 and TGF-β3 expression and they do express TGF-β RII and RIII receptors. WPE1-NB26 cells show a lack TGF-β1, TGF-β2, TGF-β3. WPE1-NB26 cells show only a weak expression of TGF-β RII and lack TGF-β RIII receptor expression.

Restoration of acinar-forming ability by TGF-β1 and TGF-β2

Because WPE1-NA22 cells show some acinar-forming ability (69%, see chapter 6 Figure 6-1), it was selected for use to determine if exogenous TGF-β1 and TGF-β2 could induce acinar formation. TGF-β1 and TGF-β2 were chosen because of their ability to inhibit cell proliferation of WPE1-NA22. WPE1-NA22 cells were treated with TGF-β1 or TGF-β2 at concentration ranging from 0 to 10 ng/ml. Cultures were fixed after four days of treatment. Acini-forming ability for each treatment was determined by staining cultures with propidium iodide and counting individual acini using the LSM transmitted light at low power. TGF-β1 caused an increase in the acinar-forming ability of

WPE1-NA22 cells to 134% at 10 ng/ml as compared to the non-treated vehicle control which was set at 100% (Figures 7-9). TGF-β2 caused a dose-dependent increase in the acinar-forming ability of WPE1-NA22 cells to 159% at 10 ng/ml (Figures 7-9). These results show that exogenous TGF-β1 and TGF-β2 enhance the ability of WPE1-NA22 cells to undergo acinar morphogenesis.

Discussion

Loss of homeostasis of growth in the prostate is an important event in prostatic carcinogenesis. It results from a shift in the balance between cell proliferation and cell death in favor of proliferation (Sensibar, 1995). This deregulation of growth may result from altered expression and sensitivity to growth factors. EGF and TGF-β are key growth factors which regulate prostate epithelial growth (Webber at al. 1996). TGF-β acts predominantly as a negative growth regulator of prostate epithelial cell growth, while EGF acts as a positive growth regulator. As a part of this growth deregulation, exogenous EGF was shown to inhibit acinar morphogenesis. Further, the neutralization of EGF activity resulted in the enhancement of acinar-formation and differentiation (see chapter 6). The focus of this chapter was to examine the role of the negative growth regulator, TGF-β, in acinar morphogenesis in non-malignant and malignant prostatic epithelial cells.

The importance of TGF-β in prostate function is clearly illustrated by studies in castrated rats. During castration-induced involution, the prostate responds to the ablation of androgens with a decrease in EGF-receptor expression and a sharp increase in TGF-β and TGF-β RII receptor expression. This increase in the TGF-β and receptor expression

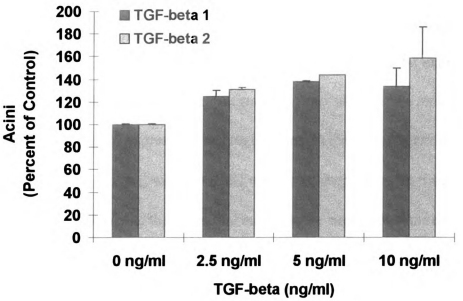


Figure 7-9. Induction of acinar formation by TGF-β. WPE1-NA22 cells were grown on Matrigel and treated with TGF-β1 and TGF-β2 for 4 days. Acini-forming ability was determined by staining cultures with propidium iodide and counting individual acini using the LSM transmitted light at low power. Counts are expressed as a percent of the vehicle treated-control which was set at 100%(Bars= +/-SE).

causes the prostatic epithelial cells to undergo apoptosis (Byrne et al., 1996, Culig et al, 1996, Nishi et al., 1996). In the normal adult prostate, TGF-β functions as a negative growth regulator and maintains growth homeostasis in prostatic epithelium. In fact, TGF-β is the major antagonist of positive growth factors in the prostate (Culig et al, 1996).

Although TGF-β1, TGF-β2 and TGF-β3 are known to be expressed in the fetal prostate, their roles in prostatic morphogenesis are not clear. In the mouse prostate model, TGF-β2 and TGF-β3 expression, although present in the fetal urogenital sinus, is virtually absent in the adult tissue, while TGF-β1 expression increases with time. Fetal expression of TGF-β1 is localized to the urogenital mesenchyme surrounding the developing prostatic buds (Timme et al., 1994). In contrast, work in breast and lung development has shown a spatial and temporal pattern for TGF-β accumulation during acinar morphogenesis with a notable loss in focal TGF-β1 expression at the bud forming regions (Daniel et al., 1989, Heine et al. 1990, Silberstein et al, 1992, Timme et al., 1994).

In order to elucidate the roles of TGF-β1, TGF-β2, and TGF-β3 in human prostatic acinar morphogenesis, their effect on non-malignant immortalized human prostatic RWPE-1 cells was examined. TGF-β1, TGF-β2 and TGF-β3, respectively, inhibits EGF induced RWPE-1 cell growth. This EGF, at 5 ng/ml, is a component of the culture medium. RWPE-1 cells demonstrate a differential sensitivity to TGF-β isoforms, as TGF-β1 > TGF-β2 > TGF-β3, with cells being most sensitive to TGF-β1 (Figure 7-1). RWPE-1 cells show strong endogenous expression of TGF-β1 and weak expression of TGF-β2 and TGF-β3 (Figures 7-2). RWPE-1 cells express TGF-β RII and TGF-β RIII receptors suggesting the formation of RII/RIII TGF-β receptor heterodimer (Figures 7-3).

The RII/RIII receptors binds both TGF-β1 and TGF-β2 with high affinity as compared to their binding of TGF-β3. These data suggest an autocrine growth regulation of RWPE-1 cells by TGF-β.

TGF-β1 and TGF-β2 expression plays a critical role in acinar morphogenesis of RWPE-1 cells. Inhibition of TGF-β1 or TGF-β2 activity by anti-functional antibodies reduced acini-formation by 48% and 44%, respectively. Inhibition of TGF-β3 activity only reduced acinar-formation by 20%. The data clearly indicate that TGF-β1 and TGF-β2 are essential for cell polarization and acinar morphogenesis. The importance of TGF-β in prostate acinar morphogenesis is supported by similar findings in the submandibular gland (Hoffman, 1996).

If TGF-β function is truly necessary for acinar formation, then blocking the binding of TGF-β to the cells should result in the inhibition of acinar formation. Results show that the addition of anti-functional Ab to TGF-β RII or TGF-β RIII, which block the binding of TGF-β, reduced the ability of RWPE-1 cells to form acini by 74% and 65%, respectively. Taken together, these data show that functional TGF-β binding is necessary for acinar morphogenesis and suggest that disruption of either TGF-β or TGF-β receptor function inhibits acinar-forming ability.

As stated earlier, a deregulation of epithelial cell growth is a step in prostate carcinogenesis. Tumor cells at different stages in progression from slow-growing, low-tumorigenic, to fast-growing, invasive and highly tumorigenetic, exhibit distinct alterations in the expression of specific growth factors and their receptors (McKeehan, 1993). Additionally, a decrease in sensitivity to exogenous TGF-β may provide cells with

a growth advantage in malignant progression. Therefore, the effects of TGF-β1, TGF-β2 and TGF-β3 on PIN-like WPE1-NA22 and malignant WPE1-NB26 cells were examined. TGF-β1, TGF-β2 and TGF-β3, respectively, differentially inhibited EGF induced WPE1-NA22 and WPE1-NB26 cell growth. WPE1-NA22 cells show relatively equal sensitivity to TGF-β1 and TGF-β2 (Figure 7-6). WPE1-NB26 cells demonstrate greater sensitivity to TGF-β2 than to TGF-β1 (Figure 7-6). In comparison to RWPE-1 and WPE1-NA22, WPE1-NB26 cells show reduced sensitivity to TGF-β1 (Figure 7-8). Thus, inhibition of cell growth in response to TGF-β decreases as cells progress toward a more malignant and invasive state. This is in agreement with studies which observed that the inhibitory effects of TGF-β on prostate cancer diminish as cells progress toward a less differentiated and more malignant state (Wilding, 1991). In both WPE1-NA22 and WPE1-NB26, TGF-β3 at 2.5 ng/ml initially caused an a 30% and 26%inhibition in growth, respectively, but as the concentration of TGF-β3 increased to 10 ng/ml, cells became less sensitive showing only a 18% and 15% reduction in growth (Figure 7-6). This may be due to a possible biphasic response to TGF-β or simply due to a down regulation of the receptor by the higher level of the growth factor. Additionally, TGF-β1 and TGF-β2 have similar *in vivo* biological effects (Hoffman et al., 1996).

The escape from the negative growth control imposed by autocrine TGF-β expression may be due to either down regulation of growth factor expression and/or a loss of receptor expression (Jakowlew et al, 1997). WPE1-NA22 cells, which may represent an early stage in carcinogenesis, show strong expression of TGF-β1 but TGF-β2 and TGF-β3 expression was not detected (Figures 7-2). On the other hand, WPE1-NB26 cells which

are highly invasive and may represent a late stage in tumor progression, show no TGF-β1, TGF-β2 or TGF-β3 expression (Figures 7-2). There are conflicting reports in the literature as to the expression of TGF-β in cancer (Kostenuik et al, 1997, Merz et al, 1994, Sehgal et al, 1996). Some reports note an increased in TGF-β expression associated with prostate cancer, while others find no difference in TGF-β expression between the normal prostate and prostate cancer (Glynne-Jones et al. 1994). It must be noted that in the latter studies, TGF-β expression was localized in the stroma (Kostenuik et al, 1997, Merz et al, 1994, Sehgal et al., 1996, Thompson et al., 1992,). Another study suggests that autocrine expression of TGF-β functions as a tumor suppressor (Sun and Chen 1997), in which a decrease in TGF-β autocrine expression ultimately results in a deregulation of growth, favoring cell proliferation and carcinogenesis. This is in agreement with my finding that non-malignant RWPE-1 express TGF-β1, 2 and 3, while WPE1-NA22 and WPE-26 cells demonstrates a stepwise loss of TGF-β isoform expression. WPE1-NA22 cells express TGF-β1 but lack TGF-β2 and TGF-β3, while WPE1-NB26 show no expression of any TGF-β isoforms as examined by immunostaining (Figures 7-2). Additionally, the observed loss of TGF-β3 is in agreement with its decrease observed in prostate cancer *in vivo* (Merz et al, 1994)

Another mechanism by which cells may become insensitive to TGF-β regulation is by the loss of TGF-β receptor expression. Result show that WPE1-NA22 express both the TGF-β RII and TGF-β RIII receptors, similar to the parent non-tumorigenic cell line RWPE-1, while WPE1-NB26 only weakly express TGF-β RII receptor and lack TGF-β RIII receptor expression (Figures 7-3). These results suggest that a decrease in

responsiveness of the malignant WPE1-NB26 cell line may be due to a decrease or loss of TGF-β receptor expression. This is agreement with other reports where a decrease or loss of RII receptors in prostate cancer cells has been observed (Gerdes et al, 1998, Guo et al, 1997). In fact, the loss of RII has been correlated with Gleason grade and is associated with poor prognosis (Glynne-Jones et al., 1994). WPE1-NB26 cells do not express TGF-β and show very weak expression of only one receptor but it does exhibit some growth inhibition in response to exogenous TGF-β. This maybe due to the fact that the TGF-β receptor is upregulated in the presence of the growth factor. Additionally, interruption of TGF-β in knock out mice does not cause cancer but makes cells very susceptible to cancer by enhancing cell proliferation during promotion (Schirmacher et al., 1998).

The restoration of acinar-forming ability by reestablishing TGF-β negative growth regulation was examined using WPE1-NA22 cells. Because WPE1-NA22 represent a PIN-like stage in carcinogenesis, it shows some acinar-forming ability (69%, see chapter 6 Figure 6-1). Results show that TGF-β1 and TGF-β2 caused an increase in the acinar-forming ability of WPE1-NA22 cells by 34% and 59%, respectively (Figures 7-9). These results show that exogenous TGF-β1 and TGF-β2 can enhance the ability of WPE1-NA22 cells to undergo acinar morphogenesis.

In summary, results demonstrate the critical role of autocrine TGF-β1 and TGF-β2 expression in human prostatic acinar morphogenesis. The loss of sensitivity apparently results from a decrease in TGF-β growth factor and receptor function and appears to be associated with a loss in the ability to undergo acinar morphogenesis. A stepwise loss in autocrine TGF-β expression and TGF-β receptor expression was seen in malignant WPE1-NA22 and WPE1-NB26 cells. These decreases caused a decrease in sensitivity that was

correlated to their malignant characteristics and their loss of ability to undergo acinar morphogenesis. Additionally, restoration of acini-forming ability was stimulated by exogenous TGF-β treatment which re-established negative growth control. The data in this chapter have enhanced our understanding of the role of TGF-β in prostate acinar morphogenesis and its alteration in the process of carcinogenesis and tumor progression.

References

Byrne RL, Leung H and Neal DE: Peptide growth factors in the prostate as mediators of stromal epithelial interaction. Br. J. Urol. 77: 627-33, 1996.

Culig, A. Androgen receptor activation in prostate tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. Cancer Res., 54: 5474-5478, 1994.

Daniel CW, Silberstein GB, Van-Horn K, Strickland P and Robinson S: TGF-beta 1-induced inhibition of mouse mammary ductal growth: developmental specificity and characterization.Dev-Biol. 135:20-30, 1989.

Dahiya R, Lee C, Haughney PC, Chui R, Ho R and Deng G: Differential gene expression of transforming growth factors alpha and beta, epidermal growth factor, keratinocyte growth factor, and their receptors in fetal and adult human prostatic tissues and cancer cell lines Urology. 48: 963-70, 1996.

Franzen, P., Ichijo, H. and Miyazono. Different signals mediate transforming growth factor-b 1-induced growth inhibition and extracellular matrix production in prostate carcinoma cells. Exp. Cell Res., 207: 1-7, 1993.

Gerde MJ, Larsen M, McBride L, Dang TD, Lu B and Rowley DR: Localization of transforming growth factor-beta1 and type II receptor in developing normal human prostate and carcinoma tissues .J. Histochem. Cytochem. 46:379-88, 1998.

Glynne-Jones E, Harper ME, Goddard L, Eaton CL, Matthews PN and Griffiths K: Transforming growth factor beta 1 expression in benign and malignant prostatic tumors. Prostate. 25: 210-8, 1994.

Guo Y, Jacobs SC and Kyprianou N: Down-regulation of protein and mRNA expression for transforming growth factor-beta (TGF-beta1) type I and type II receptors in human prostate cancer. Int. J. Cancer. 71:573-9, 1997.

Heine UI, Munoz EF, Flanders KC, Roberts AB and Sporn MB: Colocalization of TGF-beta 1 and collagen I and III, fibronectin and glycosaminoglycans during lung branching morphogenesis. Development. 109:29-36, 1990.

Hoffman MP, Kibbey MC, Letterio JJ and Kleinman HK: Role of laminin-1 and TGF-β3 in acinar differentiation of a human submandibular gland cell line (HSG). J. Cell Sci. 109:2013-2021, 1996.

Jakowlew SB, Moody TW, Mariano JM: Transforming growth factor-beta receptors in human cancer cell lines: analysis of transcript, protein and proliferation. Anticancer Res. 17:1849-60, 1997.

Jarrard DF, Blitz BF, Smith RC, Patai BL and Rukstalis DB: Effect of epidermal growth factor on prostate cancer cell line PC3 growth and invasion. Prostate. 24:46-53, 1994.

Kostenuik PJ, Singh G and Orr FW: Transforming growth factor beta upregulates the integrin-mediated adhesion of human prostatic carcinoma cells to type I collagen. Clin. Exp. Metastasis. 15:41-52, 1997.

McKeehan WL: Growth factor receptors and prostate cell growth. Cancer Surv.11:165-75, 1991.

McKeehan WL, Hou J, Adams P, Wang F, Yan GC and Kan M: Heparin-binding fibroblast growth factors and prostate cancer. Adv. Exp. Med. Biol. 330:203-13, 1993.

Merz VW, Arnold AM and Studer UE: Differential expression of transforming growth factor-beta 1 and beta 3 as well as c-fos mRNA in normal human prostate, benign prostatic hyperplasia and prostatic cancer. World J. Urol. 12:96-8, 1994.

Moustakas A, Lin HY, Henis YI, Plamondon J, O'Connor-McCourt MD and Lodish HF: The transforming growth factor beta receptors types I, II, and III form hetero-oligomeric complexes in the presence of ligand.J-Biol-Chem. 1993 Oct 25; 268(30): 22215-8, 1993.

Nakamoto, T., Chang, C.S., Li, A.K. and Chodak, G.W. Basic fibroblast growth factor in human prostate cancer cell lines. Cancer Res., 52: 571-577, 1992.

Nemeth JA, Sensibar JA, White RR, Zelner DJ, Kim IY, Lee C: Prostatic ductal system in rats: tissue-specific expression and regional variation in stromal distribution of transforming growth factor-beta 1. Prostate. 33: 64-71, 1997.

Nishi N, Oya H, Matsumoto K, Nakamura T, Miyanaka H and Wada F: Changes in gene expression of growth factors and their receptors during castration-induced involution and androgen-induced regrowth of rat prostates. Prostate. 28: 139-52, 1996.

Roberts, A.B. and Sporn, M.B. The transforming growth factor-βs. In: Peptide growth factors and their receptors I. Sporn, M.B. and Roberts, A.B. (eds.), New York, Springer-Verlag, pp. 419-472, 1990.

Schirmacher P, Amendt C , Weber H and Blessing M: Expression of a dominant negative type II TGF-beta receptor in mouse skin results in an increase in carcinoma incidence and an acceleration of carcinoma development. Oncogene. 17: 25-34, 1998.

Sehgal I, Baley PA and Thompson TC: Transforming growth factor beta1 stimulates contrasting responses in metastatic versus primary mouse prostate cancer-derived cell lines in vitro.Cancer-Res. 56:3359-65, 1996.

Sensibar JA: Analysis of cell death and cell proliferation in embryonic stages, normal adult, and aging prostates in human and animals. Microsc. Res. Tech. 30: 342-50, 1995.

Silberstein GB, Flanders KC, Roberts AB and Daniel CW: Regulation of mammary morphogenesis: evidence for extracellular matrix-mediated inhibition of ductal budding by transforming growth factor-beta 1. Dev-Biol. 152: 354-62, 1992.

Sun L and Chen C: Expression of transforming growth factor beta type III receptor suppresses tumorigenicity of human breast cancer MDA-MB-231 cells.J-Biol-Chem. 272:25367-72, 1997.

Thompson TC, Truong LD, Timme TL, Kadmon D, McCune BK, Flanders K, Scardino PT, Park SH: Transforming growth factor beta 1 as a biomarker for prostate cancer. J. Cell Biochem Suppl. 16H:54-61, 1992.

Timme TL, Truong LD, Merz VW, Krebs T, Kadmon D, Flanders KC, Park SH and Thompson TC: Mesenchymal-epithelial interactions and transforming growth factor-beta expression during mouse prostate morphogenesis. Endocrinology. 134: 1039-45, 1994.

Webber, MM, Bello D, and Quader S: Immortalized and tumorigenic adult human prostatic epithelial cell lines. Characteristics and applications. Part. 1. Cell markers and immortalized, non-tumorigenic cell lines. Prostate 29:386- 394, 1996.

Wilding G: Response of prostate cancer cells to peptide growth factors: transforming growth factor-beta. Cancer Surv. 11:147-63, 1991.