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dissertation entitled

UROKINASE-TYPE PLASMINOGEN ACTIVATOR AND ITS INHIBITOR, PLASMINOGEN ACTIVATOT INHIBITOR-1 IN DU-145 HUMAN PROSTATE CARCINOMA CELLS

presented by

ANURADHA WAGHRAY

has been accepted towards fulfillment
of the requirements for

Ph.D degree in ZOOLOGY

Major professor

Date April 6, 1997

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By

ANURADHA WAGHRAY

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Zoology

1997

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ABSTRACT

UROKINASE TYPE PLASMINOGEN ACTIVATOR AND ITS INHIBITOR, PLASMINOGEN ACTIVATOR INHIBITOR-1, IN DU-145 HUMAN PROSTATE CARCINOMA CELLS

By

Anuradha Waghray

Urokinase-type plasminogen activator (u-PA) is associated with tumor progression involving extracellular matrix (ECM) degradation and invasion by cancer cells. Human prostatic epithelial cells normally secrete u-PA. The role of urokinase in invasion by prostate cancer cells and the ability of urokinase inhibitor, plasminogen activator inhibitor-1 (PAI-1) to block urokinase proteolytic activity were examined. Extracellular secreted u-PA activity of the invasive DU-145 human prostate carcinoma cells was compared with that of normal prostatic epithelium by SDS-PAGE zymography and chromogenic substrate assay. DU-145 cells secreted at least five times more u-PA than normal cells. Treatment of cells with 0.1 to 1.0 μM *all trans* retinoic acid (RA) for 48 h caused a decrease in u-PA activity and u-PA protein levels, as demonstrated by SDS-PAGE zymography and Western blot analysis but this short treatment did not inhibit growth. Urokinase alone degraded the ECM glycoprotein fibronectin but not laminin, which was degraded by plasmin, formed by the activation of plasminogen by u-PA. While serum-free conditioned medium from DU-145 cells degraded both fibronectin and laminin, after treatment of cells with 1 μM RA for 48 h, fibronectin and

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laminin degradation was reduced. RA treatment decreased invasion by DU-145 cells in a dose-dependent manner to 34.3% of control at 10 μM RA in an *in vitro* invasion assay. Results show that RA reduces degradation of ECM proteins and invasion by inhibiting extracellular, secreted u-PA activity. Treatment with PAI-1 antigen inhibited invasion by DU-145 cells in a dose-dependent manner. To further asses the role of PAI-1 in prostate cancer progression, DU-145 cells were transfected with PAI-1 sense and antisense cDNA. While the PAI-1 antisense and vector transfectants showed u-PA activity similar to DU-145 cells, the PAI-1 sense transfectants showed a marked decrease in extracellular u-PA activity and an increase in PAI-1 staining by immunostaining technique. In addition, an increase in u-PA/PAI-1 complex formation was also observed in PAI-1 sense transfectants, as demonstrated by Western blot analysis. Further, PAI-1 sense transfected cells showed a decrease in growth and invasive ability of DU-145 cells. Therefore, inhibition of invasion by decreasing extracellular u-PA activity either by treatment with agents such as *all trans* retinoic acid or with u-PA inhibitors may be a promising approach for controlling prostate cancer progression. These studies demonstrate that RA and PAI-1 may have important applications in blocking the progression of prostatic intra-epithelial neoplasia (PIN) to invasive cancer.

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ACKNOWLEDGMENTS

I would like to take this opportunity to thank the individuals who have contributed directly or indirectly to the work described in this dissertation. First and foremost I thank Dr. Mukta M. Webber for her unfailing and enthusiastic support of my endeavors. I also thank the members of my committee. Dr. Will Kopachik, Dr. Nikolay Dimitrov, Dr. Kenneth Schwartz and Dr. Daniel Williams for their advice and encouragement whenever solicited. I particularly thank Dr. W. Kopachik for patiently teaching me molecular biology techniques. Thanks are also due to W. Scott Metcalfe, Diana Bello, Nestor Deocampo, Saalman Quader, William Chu and Melissa Carey who have diligently provided expert technical advice and assistance. I thank Dr. D. Ginsburg, University of Michigan, Ann Arbor for providing PAI-1 cDNA, Dr. Johng. S. Rhim, NIH, for pSV_2-neo plasmid, Dr. W. Kopachik for BS-KS plasmid and Dr. H. Kleinman for a sample of conditioned medium from HUVEC cells.

I would also like to acknowledge the contribution of Beth Roestel, Natasha Nesbitt and Daniel Edwards without whom my Ph.D. would have been harder. I would also like to thank the Zoology office staff for providing me complete information regarding the graduate school requirements.

Finally, but not the least I must recognize the contribution of my family. My husband Ishwar Lal has supported, guided encouraged and loved. My parents have sacrificed to send me far away from home to achieve my goals. Without their unfailing support I could never have done this.

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LIST OF ABBREVIATIONS

BM:	Basement membrane
BPH:	Benign prostatic hyperplasia
BSA:	Bovine serum albumin
CM:	Conditioned medium
Coll-IV:	Collagenase-type IV
DHT:	Dihydro testosterone
5α-DHT:	5- alpha Dihydro testosterone
DEN:	Diethylnitrosamine
DMBA:	7,12-dimethylbenz{a}anthracene
DMSO:	Dimethyl sulfoxide
ECM:	Extracellular matrix
EDTA:	Ethylenediamine tetraacetic acid
EGF:	Epidermal growth factor
ETOH:	Absolute ethanol
FBS:	Fetal bovine serum
4-HPR:	N-(4-hydroxyphenyl) retinamide
IFN-α:	Interferon-alpha
kDa	KiloDalton
MNU:	N-methyl-N-nitrosourea
Ovex:	Ovariectomy
PA:	Plasminogen activator
PAI-1:	Plasminogen activator inhibitor-1
PAI-2:	Plasminogen activator inhibitor-2
PAI (S):	PAI-1 sense DNA
PAI(AS):	PAI-1 antisense DNA
PAI:	Plasminogen activator inhibitor
PCa:	Prostate cancer
PIN:	Prostatic intraepithelial neoplasia
PSA:	Prostate specific antigen
RA:	*all trans* retinoic acid
RAR:	Retinoic acid receptor
RXR:	Retinoid X receptor
SF-CM:	Serum free-conditioned media
Scu-PA:	Single chain urokinase type plasminogen activator
Tam:	Tamoxifen
t-PA:	tissue-type plasminogen activator

tcu-PA:	Two chain urokinase type plasminogen activator
TGF-ß:	Transforming growth factor-ß
u-PA:	Urokinase-type plasminogen activator

INTRODUCTION

Adenocarcinoma of the prostate is the most common cancer, after skin cancer and it is the second leading cause of cancer-related death in American men. The American cancer society has estimated that 317,100 new cases and 41,400 deaths by prostate cancer in 1996 (85). With an increase in the ageing population, prostate cancer incidences will continue to increase in the future, hence it is a major health concern today. The precancerous leisons (latent cancer) referred to as prostatic intraepithelial neoplasia (PIN) or "carcinoma *in situ*" preceed prostate cancer by more than five years (13).

One of the important criteria for diagnosis of prostate cancer is invasion through the basement membrane (BM) and extracellular matrix (ECM). The major ECM degrading enzymes are the serine proteases, especially urokinase (u-PA), and a family of structurally related metalloproteases (75). Urokinase is a protease secreted by prostatic epithelial cells and it plays an important role in liquefaction of semen (93). u-PA activates plasminogen to plasmin, a serine protease with broad subtrate specificity, which has the ability to degrade ECM proteins (3). Thus an increase in u-PA secretion may result in increased plasmin formation, which in turn may cause ECM degradation and invasion.

Studies have demonstrated that human prostate cancer cells produce higher levels of u-PA than normal or benign prostate cells and u-PA expression is associated with invasion and metastasis (1,10,25,55). An increase in the plasma u-PA level has been observed in about 80% of patients with disseminated prostate cancer and, thus,

it was suggested that u-PA may be a reliable marker for the diagnosis of metastatic disease (47). Therefore a decrease in extracellular, secreted u-PA activity and thus, invasion, may be one approach in the treatment of prostate cancer. Inhibition of ECM degradation and invasion has been observed by blocking u-PA activity either by antibody to u-PA (83) or by increasing the production of specific inhibitors to u-PA such as plasminogen activator inhibitors (PAIs) (5,24).

There are two types of PAIs, PAI-1 and PAI-2, both are involved in inhibiting u-PA activity. PAI-1 has a stronger affinity to u-PA than PAI-2 (3). Both form equimolar, stable complex with u-PA rendering it inactive. Under normal physiological conditions such as embryo morphogenesis, tissue remodelling, wound healing and cell migration, u-PA mediated proteolysis is tightly regulated by its inhibitors. However in tumors, the fine balance between u-PA and PAI shifts in favor of proteolysis (116). In addition to high levels of u-PA, tumor cells also produce high levels of plasminogen activator inhibitors (14,87), however, the ratio between u-PA and PAI has not been evaluated. In these tumor cells, production of u-PA may be higher than its inhibitor, thus, favouring proteolysis.

Although a correlation between u-PA activity, invasion and metastatic potential in prostate cancer has been identified, the mechanisms involved in ECM degradation during tumor progression remain unknown. Furthermore, the role of PAI-1 in invasion of prostate cancer cells has also not been studied. In the present study the role of u-PA and PAI-1 in invasion was analyzed by using DU-145 human prostatic carcinoma cells, derived from a metastatic brain lesion of human prostate adenocarcinoma (112). DU-145 cells are a useful cell model for studies on the role of u-PA and its inhibitor in invasion because they secrete high levels of u-PA and undetectable levels of PAI-1

(70,122). In addition, the ability of *all trans* retinoic acid (RA) to inhibit ECM degradation and invasion by DU-145 cells was examined.

Retinoids are a group of natural and synthetic vitamin A analogues. They are known modulators of cell proliferation and differentiation (108) and they also suppress carcinogenesis (68,69), making them potentially useful chemotherapeutic and chemopreventive agents. The anticancer effects of retinoids in prostate cancer development have been recognized in some *in vitro* studies. N-(4-hydroxyphenyl) retinamide (4-HPR), a synthetic retinoid decreases tumor incidence of ras-myc induced carcinomas in mouse prostate reconstituted models (106) and reduces the incidence of metastasis by tumors induced by methylnitrosourea and testosterone in Lobound-Wistar rats (91,92). Therefore, retinoid is considered to be a promising candidate for prostate cancer prevention and therapy. Recent epidemiological studies have also shown an increased risk for prostate cancer in men with low serum vitamin A levels (95).

Objectives:

The objectives of this study are to elucidate the role of urokinase and its inhibitor PAI-1 in human prostate tumor progression and the mechanisms by which RA inhibits invasion. The specific aims are:

1. To compare the levels of extracellular, secreted urokinase activity by DU-145 cells with those by normal human prostatic epithelial cells.
2. To analyze the role of urokinase in extracellular matrix degradation and invasion
3. To examine the effects of *all trans* retinoic acid on:
    a. Extracellular, secreted urokinase activity and protein levels.

b. Degradation of extracellular matrix proteins laminin and fibronectin.

c. Invasive ability of DU-145 cells through a reconstituted basement membrane, Matrigel

4. To analyze the role of PAI-1 in invasion by transfecting DU-145 cells with full length PAI-1 cDNA in sense and antisense orientation.

5. To compare the secreted urokinase activity by DU-145 cells with those of cells transfected with vector only, PAI-1 antisense or sense cDNA.

6. To compare the PAI-1 levels expressed by DU-145 cells with those of cells transfected with vector only, PAI-1 antisense or sense cDNA.

7. To compare the growth of DU-145 cells with those of cells transfected with vector only, PAI-1 antisense or sense cDNA.

8. To compare the invasive ability of DU-145 cells with those of cells transfected with vector only, PAI-1 antisense or sense cDNA.

Hypotheses:

The following hypotheses are proposed:

i) that retinoids inhibit invasion and extracellular matrix degradation by decreasing urokinase production and activity.

ii) that a decrease in secreted, urokinase activity by its natural, specific inhibitor PAI-1 decreases the invasive ability of DU-145 cells.

iii) that transfection of DU-145 cells with PAI-1 sense cDNA decreases extracellular, secreted urokinase activity and thus, decreases the growth and invasive ability of these cells.

CHAPTER 1

LITERATURE REVIEW

Literature Review

The primary cause of death from prostate cancer is invasion and metastasis. An association between increased urokinase (u-PA) activity and metastatic potential has been observed in various cancers including prostate cancer (1,116,63). *In vivo,* urokinase activity is regulated by its specific inhibitor, plasminogen activator inhibitor-1 (PAI-1). Therefore, an analysis of the role of u-PA and PAI-1 in the progression of prostate cancer is proposed. Further, the effect of *all trans* retinoic acid, a chemopreventive agent on u-PA will also be studied. In order to analyze the effect of u-PA and PAI-1 on prostate cancer progression, it is important to understand the structure and function of prostate gland as well as urokinase and its inhibitors. In addition, the functional role of RA should also be understood before studying its effects on u-PA. In this chapter, the structure and functions of prostate gland, u-PA and PAIs will be discussed along with the functions of RA.

The Prostate:

The adult prostate is a tubuloalveolar gland consisting of epithelium lined glands and ducts with smooth muscles and collagenous stroma interspersed between the glands. Prostate surrounds the urethra below the neck of the bladder (Figure.1-1) (72). Adult Prostate is divided into three distinct zones: peripheral zone (PZ), central zone (CZ), and transition zone (TZ). Central Zone constituting 25% of the gland, is thought to be of mesodermal origin and consists primarily of glandular and ductal tissue surrounding the ejaculatory duct. Peripheral Zone, occupying the

majority of the gland, is derived from the endoderm of the urogenital sinus, and is the most common site of origin of adenocarcinoma of the prostate. Transitional Zone, derived from the primitive mesodermal origin surrounds the urethra and it is the site of origin of benign prostatic hyperplasia (86). The normal human prostate gland is the size of a walnut. It slowly increases in size from birth to puberty, at which time a rapid growth spurt occurs. The size attained by the third decade remains stable until around the age of 45. At older age, there may be an increase in the size of the prostate due to benign prostatic hyperplasia (107). By virtue of its location, prostate can impair urinary function if it becomes enlarged.

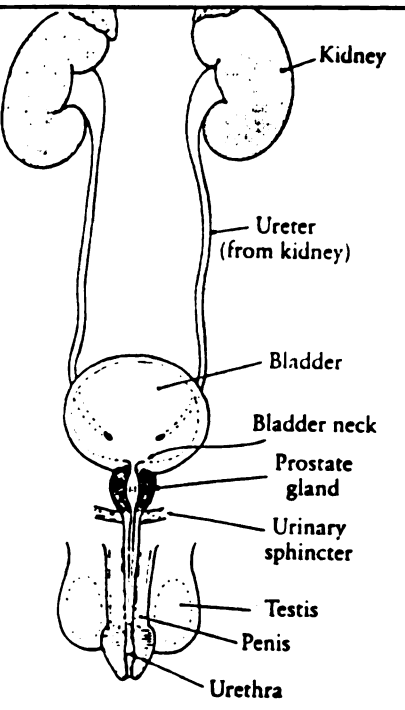


Figure 1-1: A Diagram showing human prostate (Ref #: 72)

The primary function of the human prostate gland is to discharge the fluid into the prostatic urethra during ejaculation. Prostatic fluid constitutes a major component of semen and enhances sperm motility. The liquefaction of the seminal coagulum is mediated by the activity of some of the proteases secreted by the prostate gland. The major proteases involved in this process are prostate specific antigen (PSA) and urokinase type plasminogen activator (61,122,126).

Benign Prostatic Hyperplasia (BPH):

BPH is an age related, nonmalignant enlargement of the prostate gland that may cause voiding dysfunction. It is a characteristic condition of older men and is only occasionally seen in men younger than 40 (53). BPH seems to be hormonally mediated because it is not seen in men castrated before puberty and regresses in adult men after castration or anti-androgen treatment (86). It has been suggested that increased expression of 5α-dihydrotestosterone (5α-DHT), a major androgenic metabolite within the prostate could be correlated with BPH (123). The level of 5α-DHT was observed to be 2-3 times greater in hyperplastic tissues, particularly in periurethral tissue from where BPH originates, than in normal glands (86).

The relationship between BPH and prostatic carcinoma is still unclear. Some studies show a positive relationship with approximately 50% of BPH patients developing prostate cancer, while others suggest lack of association between BPH and prostate cancer. Some suggest that BPH and prostate cancer coexist (53,125). Although, both are age and hormone related, they arise from different locations of the gland. While prostate enlargement may also occur in prostate cancer, carcinomas differ from benign enlargement in that they can be invasive and metastatic.

Prostate Cancer:

Prostate cancer is the most common cancer in adult men in the United States and is the second leading cause of cancer mortality. The incidence of prostate cancer increases with age, it is estimated that 317,100 new cases and 41,400 deaths from prostate cancer will occur in US in 1996 (85). Due to an

increasing age of the U.S. population, more attention has been given to prostate cancer. Prostate cancer has generally been viewed as an "indolent" malignancy, that is one that progresses at a very slow rate. The premalignant lesion of prostate cancer is referred to as prostatic intraepithelial neoplasia (PIN) or "carcinoma *in situ*". PIN predates the onset of carcinoma by more than five years (13,126).

Prostatic intraepithelial neoplasia is characterized by dysplasia of the epithelial cells of ducts and acini (77). Low grade PIN includes PIN I and II with mild dysplasia and an intact basal cell layer. High grade PIN (PIN III) encompasses severe dysplasia where basal cell layer is disrupted and basement membrane degradation occurs. PIN III is associated with invasive carcinoma in 87% of patients (41).

Normal prostate epithelial cells have an organized morphology and exhibit polarized secretion of proteins at their apical ends. However, during progression from PIN III to invasive carcinoma, the general morphology of prostate epithelial cells is altered resulting in a loss of polarized secretion. This leads to some secretion of proteins at the basal end of cells, which may facilitate degradation of the basement membrane and invasion *in vivo* (32).

Prostatic epithelial cells, along with other proteins, secrete urokinase into the lumen of the acini (93). In prostate cancer cells, due to the loss of polarity, elevated levels of secreted u-PA may be found towards the basal end of the cells. This may facilitate extracellular matrix (ECM) and basement membrane (BM) degradation and invasion. Elevated level of urokinase and its association with matrix degradation and invasion has been observed in prostate tumors (128). The invading cells metastasize to distant sites and the frequent sites of metastasis are bones of the pelvic, lumbar, and thoracic spine. Organ involvement (lungs, liver, and kidneys) is seen primarily in

the late stage of terminal disease (86).

Clinical studies as well as *in vitro* studies with appropriate cell models are necessary for better understanding of prostate cancer progression. Well characterized cell lines derived from human prostate epithelial cells can be good research models for studying prostate tumor progression. Several cell lines from malignant human prostatic epithelial cells have been established. The three most commonly used cell lines are DU-145, PC-3 and LNCaP. DU-145 cells that were isolated from a metastatic brain lesion of a human prostate adenocarcinoma, were selected for the proposed studies because they primarily secrete urokinase and are invasive (49,112,125).

Epidemiology of prostate cancer:

In a review of epidemiological studies, Webber and collaborators (124), examined the world distribution of prostate cancer (Table 1-1). From this information it is evident that African American men in the United States have the highest incidence of prostate cancer in the world and their overall mortality rate is rising. Numerous studies have associated prostate cancer to environamental, hormonal and dietary factors (79,95,124).

Environmental factors:

Among the most impressive studies pointing to the role of environmental factors in the development of prostate cancer are the studies on migrant populations. Akazaki and Stemmermann (2), found an increased mortality rate for prostate cancer among first generation Japanese in Hawaii compared to Japanese in Japan (Table 1-2). African American men in the United States showed high incidence of prostate cancer

Table 1-1

United States:

Black men	85.4/100,000
White men	47.0/100,000
North American Indian	31.6/100,000
China:	0.8/100,000
Thailand:	0.13/100,000

Ref: # 124. Webber,M.M. et.al. 1988

Table 1-2

Age-Adjusted Prevalence of Latent Carcinoma of the Prostate

		Prevalence (%)		
Study group	Number of Autopsies	All latent cancers	Proliferative type	Nonproliferative type
Japanese in Japan	239	20.5	8.7	11.8
Japanese in Hawaii	158	26.7	19.1	7.6

Ref: # 2. Akazaki,K. et. al. 1973

as compared to native African men in Africa. Incidence of prostate cancer in African men in Nigeria was observed to be 10.1/100,000, men, which is significantly lower than 85.4/100,000 men reported for African American men in United States (124). This suggests that increased risk for prostate cancer may be associated with environmental factors. Swedish men who migrated to the United States had lower risk than those in Sweden (12). In addition, the incidence of prostate cancer appears to be higher in industrialized countries as compared to non-industrialized countries, however there are some exceptions. For example: Japan, a highly industrialized country, shows a low mortality rate (2.18/100,000 men) as compared to the less industrialized Caribbean islands (14.13/100,000men) (12).

Hormones:

Androgens regulate the growth and development of the prostate. Some evidence suggests that androgenic stimulation promotes prostate carcinogenesis. Prostate cancer is rare in men castrated before puberty. On the other hand exogenous administration of testosterone causes prostate cancer in rats (18). The principal circulating androgen secreted by the testes is testosterone. Testosterone is converted to 5α-DHT by 5α-reductase, an enzyme present in the nuclear membrane. Inhibition of 5α-reductase decreases 5α-DHT formation, the major androgen responsible for growth of the prostate. Suppression of 5α-DHT activity may inhibit prostate tumor growth and development. This hypothesis is supported by the observation that 5α-reductase activity is low in Asian populations who have low risk of prostate cancer compared to American men (19). Finasteride, a competitive inhibitor of 5α-reductase causes a decrease in serum 5α-DHT levels and inhibits growth of prostate tumors (19). Retinoids have also been shown to suppress androgenic stimulation by inhibiting

5α-reductase activity (54).

Anti-androgen therapy is the treatment of choice for prostate cancer patients, however, androgen ablation does not completely inhibit the growth of tumor cells. There may be two reasons for this: One possibility is that initially prostate cancer cells are homogenously androgen dependent for growth. However, following androgen withdrawal by castration or decrease in androgen production by anti-androgen therapy, androgen dependent tumor cells stop proliferating and die. Some cells, however, survive and adapt to grow in the absence of androgens. An alternative explanation is that prostate cancer cells are a heterogenous population, consisting of both androgen dependent and independent cells. Androgen depletion may result in the loss of androgen dependent cells but androgen independent cells continue to grow (52).

Diet:

Several studies have focussed on the association of increased dietary fat, ß-carotene or Vitamin A with prostate cancer. A large number of studies have demonstrated a correlation between prostate cancer incidence and fat intake (57,73). The data associated with ß-carotene and prostate cancer risk are inconsistent. Some studies show decreased risk while others show increased risk for prostate cancer with increased ß-carotene consumption and some have found no association between ß-carotene intake and prostate cancer risk (57,73,82,95,103).

Vitamin A and Prostate Cancer:

Much emphasis is currently being placed on exploring the protective role of Vitamin A against prostate and other cancers. Vitamin A has been shown to have different effects ranging from tumor prevention to tumor enhancement. Vitamin A has a variety of effects on epithelial tissues and it plays a critical role in epithelial cell differentiation in developing organisms and in the maintenance of normal epithelia in adult tissues (8).

Epidemiological evidence suggests that low levels of blood serum retinol may be associated with increased risk for prostate cancer. Evidence gathered from two case studies, suggest that the mean level of serum retinol was significantly lower in prostate cancer patients than in control and there was a statistically significant trend of increased prostate cancer risk associated with decreasing serum retinol levels (50,95).

The diet and life style of Seventh-day adventist men seems to be associated with a low risk for prostate cancer. Their diet is free from red meat and alcohol (74). Further, increased consumption of beans, lentils, peas, tomatoes, raisins, dates, and other dried fruits and vegetables were all associated with significantly decreased risk for prostate cancer, whereas, high consumption of red meat, animal fat, and whole milk increased the risk of prostate cancer (74). This leads one to consider the importance of dietary factors in the risk for prostate cancer. A case control study of 371 prostate cancer patients and comparable control subjects showed a significant protective effect of high levels of ß-carotene intake (73). In a study done by Ohno and collaborators (82), an inverse relationship between prostate cancer and ß-carotene or vitamin A consumption was observed in Japan. These results show a protective effect

of dietary ß-carotene and vitamin A against prostate cancer. In another study, a group of men younger than 70 years of age showed no significant association between vitamin A intake and risk for prostate cancer, however, men 70 years or older showed increased risk for prostate cancer with increased consumption of vitamin A (57). Thus the role of dietary intake of vitamin A and its derivatives in prostate cancer remains controversial. However, more recent studies suggest that increased risk for prostate cancer is associated with long term, low vitamin A consumption in the diet (50,95).

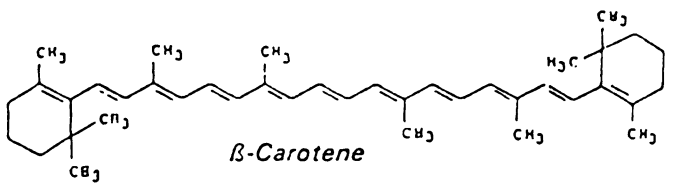
Vitamin A

This brief review deals with the importance of vitamin A in normal development, growth and differentiation and its potential applications in prostate cancer prevention and treatment.

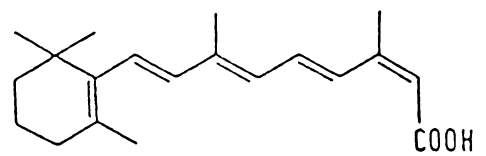
Retinoids, a group of natural and synthetic analogues of Vitamin A, are potent agents that controls both cellular differentiation and proliferation. β-carotene is the precursor of naturally occurring retinoids. The natural retinoids exists in the *all trans*, 9-cis and 13-*cis* configuration (Figure.1-2), with great preponderance of the body's retinoids being present in the *all trans* configuration (11). Because of the successful use of retinoids in the treatment of cancer, synthetic retinoids have been developed. The most commonly used synthetic retinoid for the treatment of cancer is N-(4-hydroxyphenyl) retinamide (4-HPR) (Figure 1- 2). Vitamin A is an important dietary constituent in human nutrition because animals are not capable of *de novo* synthesis. Deficiency of vitamin A contributes to high fatality rates (119).

Functions of retinoids:

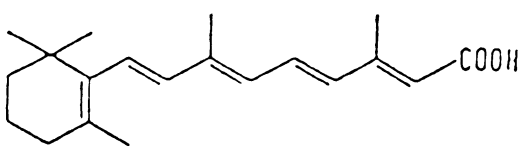
Analogues of retinoids (RA) have pleiotropic effects. They are important in maintaining normal development, cell proliferation and differentiation. Retinoids also exert their effects on neoplastic cells by suppressing growth, angiogenesis and metastasis. These effects of RA are considered to be due to activation of transcriptional factors and alteration of gene expression (67,69,108).



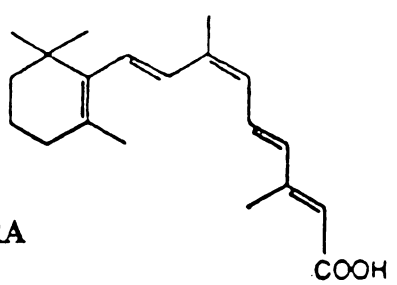
ß-Carotene



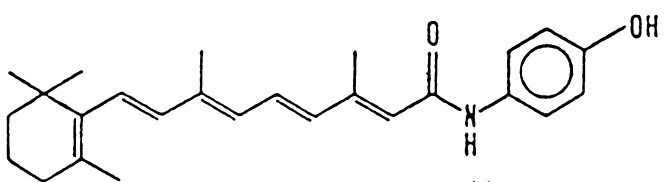
13-Cis RA



All-trans RA



9-*cis* RA



4-Hydroxyphenyl Retinamide

Figure 1-2: Molecular structures of analogs of Retinoic Acid

Effects of RA on cellular growth and differentiation:

Retinoids are known modulators of cell proliferation and differentiation. RA has different effects on different cell types.

Effects on epithelial cells:

An early observation in vitamin A deficient animals was the development of squamous metaplasia in the epithelia of eye, respiratory tract, and salivary glands. Upon the administration of vitamin A, squamous metaplasia was reversed to normal columnar epithelial cells. In vitamin A deficient mice developmental defects particularly, squamous metaplasia of epithelia of seminal vesicles and prostate were also observed (66). In addition to morphological alterations, vitamin A deficient animals also reveal changes in the biosynthesis of proteins and nucleic acids and show excessive cellular proliferation and loss of differentiation. Similar effects were observed *in vitro*, in organ and in cell culture system. In organ culture system, removal of vitamin A from the growth medium caused squamous metaplasia and addition of vitamin A resulted in reversal to normal morphology (67). In primary cultures from human prostatic epithelial cells, RA inhibited cellular proliferation, stimulated differentiation and reversed squamous metaplasia to normal prostatic epithelial morphology (88). RA has also been found to inhibit cellular proliferation and stimulate differentiation in a variety of cancer cell lines including, human myeloid leukemia cells HL-60, human neuroblastoma cells SK-N-MC and in F9 mouse teratocarcinoma cells (20,34,98). This demonstrates that RA has the ability to modulate growth, differentiation and is required for the maintenance of normal epithelial morphology. However, excess of Vitamin A causes a variety of toxic effects (67). Vitamin A deficiency and excess effects are reversible.

Different epithelial cells may respond to retinoids differently. There are contradictory reports on the effects of retinoids on cellular proliferation. In human bladder carcinoma cell lines, 4-HPR and RA both inhibit cell proliferation with 4-HPR being 10 fold more effective. 4-HPR was shown to be converted to RA, suggesting that the antiproliferative activity is mediated by RA (117). In contrast, in cultured human buccal epithelial cells, retinoic acid enhances the growth (113). Further, different levels of RA are required by different cell types for the maintenance of epithelial architecture. For example, in primary cultures of human prostatic epithelial cells, high doses of RA (3 nM or higher) inhibited proliferation while low doses (0.03 nM) were stimulatory (88). In cultures of respiratory tract epithelial cells 1 to 10 nM of vitamin A showed stimulatory effects on growth (130). Thus the response to RA is different for different cells.

Effects on Mesenchymal cells:

Retinoids have significant and selective teratogenic effect on cells of mesenchymal origin during early development. Retinol deficiency or excess causes failure of mesenchymal cells to proliferate and differentiate to form the early vascular system (108). In retinoid deficient embryos, the vascular system fails to develop normally, however, upon treatment with appropriate amounts of retinoids normal development is restored. Thus stringent amounts of retinoids are required for controlling the proliferation and differentiation of mesenchymal precursor cells. Vitamin A deficiency also shows characteristic and reversible changes in the bones and cartilage (67).

Effects on embryonic development:

The most striking teratogenic effects of vitamin A excess that have been described in experimental studies in rodents are the production of various craniofacial abnormalities that resemble from those observed in human embryos. These abnormalities include malformation of many parts of the cartilaginous and bony facial and oral skeleton (8). Administration of excess of Vitamin A to pregnant mice causes limb malformation (67). Vitamin A deficiency is also associated with a number of developmental defects such as retardation of growth, atrophy of certain organs, and gross changes in the eye (66). These observations demonstrate the importance of retinoids during embryogenesis.

Retinoid and Neoplastic cells:

Retinoids are known modulators of cellular growth and differentiation. Since cancer is associated with loss of differentiation and stimulation of growth, retinoids might be involved in reversing proneoplastic changes (68,108). Several epidemiological and experimental studies also provide strong evidence for an inverse relationship between Vitamin A and cancer (76,95). The effects of vitamin A on embryonal carcinoma cells and leukemia cells have been well studied.

Embryonal carcinoma cells:

Differentiation of mouse embryonal carcinoma cells is considered to be a useful model for normal embryogenesis and early mammalian development. RA induces differentiation and inhibits growth in embryonal carcinoma cells. F9 embryonal carcinoma cells can be induced by RA to differentiate (51). Human germ cell tumors

(33) and human teratocarcinoma cell line NT2/D1 (6) show growth inhibition and induction of differentiation when exposed to RA. Accompanying these effects are changes in ECM components where RA induces F9 cells to synthesize type IV collagen and laminin (62).

Leukemia cells:

Retinoids promote terminal differentiation of neoplastic cells to a nonneoplastic phenotype and inhibit growth in human promyelocytic leukemia cells such as HL-60 cells (28). While stimulation of differentiation and inhibition of growth have been reported in some acute myeloid leukemia (AML) cells, growth stimulation or no effect was observed in others (36,60). Thus RA appears to have diverse effects on differentiation and growth of different types of leukemia cells.

In addition to modulation of cellular proliferation and differentiation, retinoids also have the ability to regulate the expression of extracellular matrix proteins and proteases and their inhibitors.

Effect of RA on the extracellular matrix proteins:

Extracellular matrix degradation is the critical step in the process of tumor cell invasion and metastasis. ECM degradation involves binding of tumor cells to the ECM proteins via the cell surface receptors and secretion of proteases, which cause localized matrix degradation and finally invasion and metastasis. Numerous reports indicate that synthesis and degradation of ECM proteins are regulated by retinoids (43,45,68,69). RA shows different effects on the synthesis of ECM proteins in different cell types. In human fibroblasts, RA suppresses collagen synthesis, but topical application of RA

to the skin enhances synthesis of collagen (43). In F9 teratocarcinoma cells RA increases laminin mRNA level (121) and in human melanoma cells, it induces the expression of laminin receptors (45). RA inhibits ECM degradation by modulating the protease activity (45,68). Thus, by regulating the synthesis and degradation of ECM proteins and their receptors, RA alters cellular adhesion and modulates tumor invasion and metastasis (23,45,68). The effect of RA in inhibiting invasion and metastasis has been observed in various experimental animal model systems and in several types of human and rodent tumor cells including, lung carcinoma, mammary carcinoma and melanomas (68,69,76).

Effect of RA on proteases and their inhibitors:

Genes encoding a variety of proteases and protease inhibitors exhibit striking changes in their expression in response to RA. The level of secreted type IV collagenase is decreased by RA in various cell types (43,45,78). Type IV collagenase is an enzyme that specifically degrades type IV collagen a component of the basement membrane. However, the response of plasminogen activators to RA treatment differs in cells of different origin eg., 1 μM RA induces PA production in cells of mesenchymal origin but decreases PA in human normal kidney epithelial cells (127). In F9 mouse teratocarcinoma cells, RA induces differentiation and concomitantly suppresses u-PA expression (114) and increases tissue type-plasminogen activator level (30). Induction of t-PA might be correlated with the induction of differentiation.

Further, RA shows dual effects on PA production in estrogen responsive cells. RA at concentration of 10^{-9} to 10^{-6} M, stimulates PA production in the presence of estrogen and in its absence RA inhibits PA production, whereas estrogen resistant cells

were unresponsive to RA (22). The possible explanation for this is that the receptors for estrogen and retinoic acid belong to the same superfamily of steroid receptors (35) and the DNA binding domain through which the receptor binds to its specific responsive element is highly conserved. The responsive elements of both RAR and estrogen receptors may be closely related, therefore both RARs and estrogen receptors may act simultaneously to regulate the same gene expression (22). However, in estrogen responsive cells, RA treatment increases the level of estrogen receptors (22) and PA production, particularly t-PA (100). Therefore it is possible that, RA increases the estrogen mediated effects by increasing its receptor expression and allowing more estrogen to bind to its receptors.

In addition to decrease in u-PA and collagenase synthesis, RA treatment also increases the expression of their specific inhibitors. Induction by RA of tissue inhibitor of metalloproteinase mRNA (17,129) and plasminogen activator inhibitor (115,118) have been observed in human cultured tumor cell lines. Increase in the expression of protease inhibitors by RA may inhibit the protease activity and thus inhibit ECM degradation and invasion. This suggests that RA may be useful as a chemopreventive agent because it has the ability to modulate cell proliferation and differentiation and simultaneously inhibit invasion and metastasis by regulating protease activity.

Clinical Application:

Several epidemiological studies have shown an association between low dietary intake of vitamin A and the development of prostate cancer (50,95). Studies have also shown that retinoids alone or in combination with other agents can suppress the process of carcinogenesis in experimental models (Table 1-3). Both synthetic and

natural analogues of vitamin A are active in certain premalignant and malignant epithelial disorders. The most useful retinoids for chemoprevention have been *all trans* RA and 4-HPR. This suggests that retinoids are prime candidate agents for cancer prevention and treatment. Several clinical trials have also been conducted using analogues of RA (Table 1-4). Limiting factors in the use of any vitamin A analogue are large pharmacological doses which are usually required to reach therapeutic efficacy. In most individuals, these high doses of retinoids produce significant side effects. 4-HPR, a synthetic retinoid, appears to be the most efficacious agent because it is less toxic than other analogues of Vitamin A (91). In the future, combination therapy seems to be the method of choice for chemoprevention because multiple steps are involved in tumor progression which require more than one agent in order to be effective and this mode of treatment can also circumvent the toxic effects of RA.

Mechanism of action of RA:

Inspite of known pleiotropic effects of RA which, include inhibition of preneoplastic lesions and carcinomas *in vitro* and *in vivo*, its mechanism of action is not well understood. A single mechanism of action seems difficult to reconcile with the multiplicity of effects. Chytil and Ong (26) hypothesized that retinoids, like steroid hormones, act via specific receptors. Several laboratories have identified nuclear retinoic acid receptors (RARs) which belong to a family of receptors that includes receptors for steroid hormone, thyroid hormone and vitamin D3 (35,89). The structural similarity in the family of nuclear receptors, suggests conservation of basic function (35).

Table 1-3

Effectiveness of retinoids alone or in combination with other chemopreventive agents in experimental carcinogenesis

Species	Organ	Carcinogen	Treatment	Effect	Reference
Rat	Mammary	DMBA	4-HPR	Inhibition	76
Rat	Mammary	MNU	*all Trans* RA	Inhibition	76
Hamster	Respiratory tract	Benzo (a) Pyrene	13-cis RA	Inhibition	76
Hamster	Lung	DEN	4-HPR	Inhibition	76
Human	Lung	Nitrosamine	13-Cis RA	Inhibition	76
Rat	Liver	3-methyl-4-dimethyl amino benzene	13-Cis RA	Inhibition	76
Mice	Liver	DEN	4-HPR	Inhibition	76
Rat	Mammary	MNU/DMBA	4-HPR + Ovex	Inhibition	76
Rat	Mammary	MNU	4-HPR + Tam	Inhibition	76
Rat	Prostate	MNU + Testosterone	4-HPR	Inhibition	92
Mouse	Prostate	*ras* + *myc*	4-HPR	Inhibition	106

Table 1-4

Clinical trials with different analogues of Retinoic acid

Type of cancer	Treatment	Effect	References
Skin Cancer	13-cis RA	Prevented skin cancer in Xeroderma patients	65
Lung Cancer	13-cis RA + IFN-α	81% responded and 31% complete remission	4
Head and Neck squamous cell carcinoma	13 Cis RA	Prevention of secondary tumors	48
Breast Cancer	4-HPR + Tamoxifen	Preventive regimen for high risk patients	22
Acute Promyelocystic Leukemia (APL)	45-100 mg/day of all-trans RA	70% remission	36
Cervical Intraepithelial Neoplasia (CIN)	Topical application of all trans RA	Regression of dysplasia	109
Advanced stages of Squamous cell carcinoma of cervix	13-cis RA + IFN-α	15% complete remission	64
Leukoplakia	60mg/day of all trans RA	complete remission and reduction in micronucleated cells, withdrawal resulted in recurrence	111

Three types of retinoic acid receptors have been isolated, they are RAR-α, ß and Γ. All three subgroups show strong homology in DNA binding domain among themselves and also with other steroid receptors (35), but retinoic acid responsive element (RARE), which is essential for RAR binding and which confers RA responsiveness, is specific for each subtype of RAR. This is however, not always true, for example, the Laminin B1 promoter (LamB1) contains RARE that is not selective for a particular RAR subtype (120,121). RA binding domain is highly conserved in all RARs but their ability to bind to RA could be different. RAR-Γ has the highest affinity for RA compared to RAR-α and RAR-ß (39). RAR expression is widespread throughout various developing tissues and organs and they perform different functions. RAR-α is ubiquitously expressed and may play a general function. RAR-ß is expressed in epithelial tissues, implying that this subtype has a particular role in stimulating differentiation of certain epithelia (66,90) and RAR-Γ is predominantly expressed in the skin and also in cartilages and differentiating squamous keratinizing epithelia, suggesting its role in morphogenesis, chondrogenesis and differentiation of squamous epithelia (66,99,133). RAR-Γ mutant mice exhibited squamous metaplasia of the seminal vesicles and prostate (66).

In addition to RARs, a second family of nuclear receptors, the retinoid X receptors (RXR) appear to be involved in mediating cellular responses to retinoids (71). Although RARs and RXRs differ substantially in primary structure, both bind to RA. RXR requires relatively higher concentrations of RA for activation than do RARs suggesting that RXR might be specific for a retinoid other than RA (71). Recently 9-*cis* RA, an isomer of RA has been identified as a likely candidate for the RXR ligand (46). This naturally occurring retinoid binds to RXRs with higher affinity than does *all-trans* RA, the metabolite from which it is derived. Thus *all-trans* RA serves as a

"prohormone" to the isomer 9-*cis* RA, which is a high affinity ligand for human RXR. 9-*cis* RA is a more potent ligand and is 40 fold more active than *all trans* RA (46).

The mechanism by which retinoids modulate the gene expression may be via its receptors and cellular retinoic acid binding proteins (CRABP). RAR's and RXR's form heterodimers and bind to the specific DNA sequence on RARE which is located in the promoter region of the retinoid regulated gene. RA binds to its specific nuclear receptor, activates it to mediate the RA effects at transcriptional level and regulates the gene expression. The RAR-RXR heterodimers have stronger affinity to RARE than the homodimers. However, each member of the heterodimeric complex appears to be able to activate transcription independantly (135). The response is dependant on the relative RAR and RXR concentrations and the intracellular levels of all trans RA and 9-cis RA. The RA mediated effects in the cells may be controlled by CRABP proteins. CRABP may be involved in the RA mediated signal transduction by sequestering RA in the cytoplasm and enhancing its catabolism such that lower level of RA reaches the nucleus to bind to its nuclear receptors thus limiting the RA specific activation of genes (14,15).

Recently it has been reported that the mRNA expression of RAR-ß is decreased in human head and neck squamous cell carcinoma (HNSCC) tissues compared to normal tissues and also in adenocarcinomas of lung and SCC cell lines (131,135). Therefore, RAR-ß may contribute to neoplastic progression of epithelial cells. Since RAR-ß is expressed by epithelial cells and is involved in RA mediated stimulation of differentiation of these cells, decrease in mRNA transcript in cancer cells may decrease RA mediated effects such as, growth, differentiation and maintainance of the normal phenotype. This suggests that the effects of RA depend upon the concentration of its receptors and the ability of RA to bind to its receptor.

The alternate mechanism of action of RA is that it may act via transforming growth factor (TGF-ß). TGF-ß has multiple biological activities on different cell types. It inhibits growth of epithelial cells while stimulates growth of mesenchymal cells such as fibroblasts (31). In addition, TGF-ß induces the mRNA and protein expression of ECM and serine protease inhibitors (110). Since TGF-ß and RA share many biological activities especially on epithelial cells, it has been speculated that the mechanism of action of these molecules are interactive. Studies have shown that RA stimulates TGF-ß production and its receptor expression (7,31). Thus an increase in TGF-ß activity by RA may result in inhibition of proliferation (81) and may induce ECM proteins and plasminogen activator inhibitor expression thereby reducing protease activity and invasion by cancer cells. This suggests that RA and TGF-ß may act synergistically in inhibiting cancer cell growth and the malignant phenotype. Since I am analyzing the effect of RA on urokinase, in the following section of this chapter urokinase and its inhibitors plasminogen activator inhibitors will be discussed.

Plasminogen activators and their inhibitors:

As mentioned earlier, prostatic epithelial cells secrete urokinase, a protease that facilitates in extracellular matrix and basement membrane degradation and invasion. Plasminogen activators, particularly urokinase, will be discussed in this section.

Plasminogen activators:

There are two types of Plasminogen activators (PA's): Tissue type (t-PA) and Urokinase type (u-PA) plasminogen activators. t-PA is mainly involved in intravascular thrombolysis whereas u-PA mediates pericellular proteolysis during embryogenesis, cell migration and tissue remodelling (101). The key role of these PA's is to activate the inactive zymogen plasminogen to plasmin, an enzyme with broad substrate specificity. Plasmin can degrade components of extracellular matrix and the basement membrane (102) and it can also activate collagenases (116). I am studying the role of urokinase in extracellular matrix degradation and invasion by Du-145 human prostatic carcinoma cells.

Structure and Function of urokinase:

Urokinase is synthesized by normal and tumor cells as an enzymatically inactive single chain u-PA (scu-PA) or pro u-PA (Mr 54 kDa). Pro u-PA is activated by serine proteases such as plasmin to enzymatically active high molecular weight, two chain u-PA (HMW, tchu-PA) (Mr 54 kDa). Enzymatically active tcu-PA comprises of two

polypeptide chains linked by disulfide bonds. The A-chain (21 kDa) has receptor binding domain and the B-chain (33 kDa) has the enzymatic active site. The A-chain of HMW u-PA is further cleaved by plasmin to aminoterminal fragment (ATF) which consists of growth factor like domain. Pro u-PA binds to the specific cell surface urokinase receptor (u-PAR) via the growth factor domain and is activated to a HMW two chain u-PA (tcu-PA), HMW u-PA can also bind to u-PAR (102).

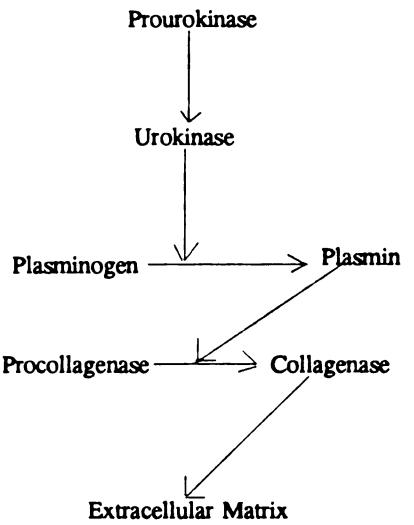


Figure 1-3: Urokinase formation and action

u-PAR is present on both normal and tumor cells. Both pro u-PA and HMW, tchu-PA bind to cell surface receptor with similar affinity and receptor bound tchu-PA retains its activity at cell surface (102). tcu-PA activates plasminogen to plasmin (102), it can also activate procollagenase to collagenase (97) and degrade fibronectin (40) (Figure 1-3). The activity of u-PA is regulated by its specific inhibitors plasminogen activator inhibitors (PAIs). u-PA/u-PAR complex once bound to PAIs is rapidly internalized, resulting in a decrease in u-PA activity (29). Thus the net u-PA activity depends on the amount of enzyme present, its state of activation and the amount of PAIs present. A decrease in u-PA activity, either by decreasing the level of u-PA or by increasing the PAI expression could result in a decrease in plasmin formation, which in turn may lead to a decrease in the degradation of ECM proteins and thus in invasion of cancer cells.

Urokinase in tumors:

A strong correlation between u-PA activity and aggresive tumor cell behavior has been reported both *in vivo* (37,102) and *in vitro* (10,27,44,132). Tissues from primary cancers and metastatic sites of breast, prostate, ovary and lung cancer contain elevated levels of u-PA compared to benign tissues (102) and increased expression of u-PAR has been detected in breast cancer tissues compared to nonmalignant tissues (9). This suggests that receptor bound u-PA is associated with the progression of cancer. The functional role of u-PAR in facilitating u-PA mediated invasion has been well documented by Reiter, et.al., (96) using HT-29 human colon carcinoma cell lines. These cells express urokinase receptors but do not express u-PA and are non invasive. Upon,transfection with human u-PA cDNA, the cells degrade ECM and are invasive (96). However, new studies provide evidence that receptor bound u-PA is not a prerequisite for promoting u-PA mediated activities, but free, secreted u-PA can directly perform its functions. Murine melanoma B16-F1 cells secrete low levels of u-PA and exhibit low metastatic potential. However, on transfection with human urokinase gene, these cells show an increase in their metastatic ability. This occured, inspite of the fact that human urokinase does not bind to murine cell surface urokinase receptor (133). Further,inhibition of urokinase activity either by antibodies against u-PA or u-PA inhibitors resulted in inhibition of ECM degradation, *in vitro* invasion and metastases in various human tumor cell lines (5,21,24,59,83,96). These results provide direct experimental support for the role of increased level of secreted u-PA activity in the process of invasion. Since prostatic epithelial cells secrete u-PA, the role of u-PA in prostate cancer progression is very important and is the topic of my investigation.

Urokinase in prostate cancer:

Elevated level of secreted u-PA activity has been found to be associated with prostate cancer (1,55,56). u-PA activity was found to be higher in prostate cancer patients compared to BPH patients, (25) and in bony metastasis as compared to their corresponding primary lesions (1,47).

The prominant role of u-PA in facilitating prostate tumor cell invasion and metastasis has been observed in studies of human prostate tumor cell lines, conducted both *in vitro* and *in vivo* in nude mice (38,128). The human prostate tumor cell line 1013-L does not express u-PA and is non invasive (10) whereas, the human DU-145 and PC-3 cell lines that have been isolated from metastatic lesions secrete high levels of u-PA and are highly invasive (38,49). Similarly, LNCaP cells that secrete very low levels of u-PA are not very invasive (49,70). The role of u-PA in the invasive behavior of prostate cancer cell lines was also studied in secondary tumors after injecting PC-3 or a subline of PC-3, 1-LN cells into the nude mice. The tumors produced by injecting 1-LN cells expressed higher u-PA and were invasive near the site of injection (128). Thus it appears that induction of u-PA activity may be an important regulatory event in prostate tumor progression. In order to protect ECM degradation and prevent prostate tumor progression, u-PA activity must be regulated. Since PAI-1, a specific plasminogen activator inhibitor regulates u-PA activity, I analyzed the role of PAI-1 in inhibiting the invasive ability of DU-145 cells.

Plasminogen activator inhibitors:

Secreted urokinase activity is efficiently regulated *in vivo* by its specific plasminogen activator inhibitors. In normal cells, there is a balance between urokinase

and its inhibitors. However, in malignant cells, this balance is disturbed resulting in increased extracellular urokinase activity and ECM degradation, which may result in invasion (116). There are two types of PAIs: plasminogen activator inhibitor-1 (PAI-1) and plasminogen activator inhibitor-2 (PAI-2). Both inhibitors are members of the serine protease inhibitors superfamily. PAI-1 and PAI-2 have only 26% of structural homology but their internal sequence, comprising of the PA binding region, shows 68% identity. In addition they also show structural homology with other proteins, eg; PAI-1 shows 34% homology to alpha-chymotrypsin, a trypsin inhibitor (3) and PAI-2 is 31% homologous to MASPIN (Mammary serine proteases inhibitor) (134).

PAI-1 is known as the endothelial type inhibitor because it was first identified in endothelial cells. It is a glycoprotein with the Mr. of 54 kDa and is a major secreted protein in cultures of endothelial cells, a human fibrosarcoma cell line (HT 1080) and a variety of mesothelial cell lines (3). It is synthesized in an active form and binds to vitronectin in the ECM (84). Vitronectin is a adhesive glycoprotein that promotes spreading of cells, PAI-1/VN complex retains both PA inhibitory activity as well as attachment and spreading of cells (3). However, PAI-1 in serum or secreted into conditioned medium of cultures spontaneously loses its activity upon secretion and is unable to inhibit u-PA activity. The secreted, inactive PAI-1 *in vitro* can be activated by denaturants such as SDS and guanidium hydrochloride (3).

PAI-2 is known as the placental type inhibitor because it was first identified in the placenta. It is a glycoprotein with the Mr. of 48 kDa and is synthesized in an unglycosylated active form. Upon secretion it undergoes glycosylation and remains in an active form. PAI-2 is found in the human placenta and in plasma during pregnancy and is secreted by leukocytes and fibrosarcoma cells (3).

Both, PAI-1 and PAI-2 inhibit tcu-PA but not scu-PA and PAI-1 has stronger affinity than PAI-2 (116). The major role of PAIs is to form equimolar and stable complexes of covalent nature with PAs and render them inactive (29). Inactivation of u-PA inhibits plasmin formation and its proteolytic activity such as fibrinolysis and ECM degradation (3). Both PAI-1 and 2 are regulated by a variety of hormones, cytokines and growth factors (Table 1-5). Increased levels of PAI-1 are found in various pathological conditions such as septicemia, obesity, hyperinsulinemia and neoplasia (3).

Plasminogen activator inhibitors in cancer:

PAI-1 and PAI-2 have been found to be elevated in various tumors, where they may regulate u-PA mediated proteolytic activity. The role of PAI-1 in tumors is well demonstrated by Kristenson et al, (58) where they studied the distribution of u-PA and PAI-1 in primary Lewis lung carcinoma in mice. u-PA and PAI-1 were heterogenously distributed and areas that contained u-PA also contained PAI-1, but at focal contact areas a marked increase in u-PA was observed with no or minimal increase in PAI-1. These areas showed signs of tissue destruction and metastasis to distant sites. Thus, increase in u-PA production compared to PAI-1 results in a net increase in u-PA activity, which facilitates tumor progression. Similar results were obtained in colon and lung cancer tissues (42,104). In addition, poorly metastatic rat mammary adenocarcinoma cells exhibit significant increase in secreted protease inhibitor levels as compared to highly metastatic cells (80). The role of PAIs in inhibiting ECM degradation and invasion was analyzed in several *in vitro* studies (5,24,59). Treatment of highly invasive colon cancer cell lines, which express high levels of u-PA, with PAI-2 antigen, reduced the u-PA activity and thus inhibited ECM degradation and invasion (5).

Table 1-5

Regulation of plasminogen activator inhibitors

	PAI-1		PAI-2	
Factors	**Activity**	**mRNA**	**Activity**	**mRNA**
Glucocorticoids	↑	↑	-	-
Dexamethasone	↑	↑	↓	↓
Hormones				
FSH	↓	↓/-	-	-
LH	⇓	⇓	-	-
Insulin	⇑	⇑	-	-
Cholera toxins	-	-	⇑	-
Endotoxin and Cytotoxins				
LPS	⇑	⇑	⇑	⇑
IL-1	⇑	⇑	-	-
TNF-*α*	⇑	⇑	⇑	⇑
Growth Factors				
TGF-ß	⇑	⇑	-	-
EGF	⇑	⇑	-	-
Macrophages	-	-	⇑	-
Phorbol esters	⇑	⇑	⇑	⇑
Thrombin	⇑	⇑	-	-
Activated Protein C	⇓	⇓	-	-

Compiled from Refs: # 3 and 110

Similarly, transfection of HT-1080 fibrosarcoma cells with PAI-1 or PAI-2 cDNA inhibited invasion (24,59).

The role of PAI-1 in the prevention of matrix degradation is further explained by Shirasuna et al (104) using human adenoid cystic carcinoma cells (AdCC). The AdCC cells produce large amount of ECM containing active PAI-1 and they also produce significant amount of u-PA. The ECM of these cells is resistant to degradation by tumor cells as well as by pure u-PA. Removal of PAI-1 from ECM resulted in ECM degradation, suggesting that PAI-1 protects ECM from degradation. This information could be helpful in understanding the biology of cancer and the role of PAI-1 in tumor progression. In contrast, several reports have demnostrated that increased level of PAI-1 or PAI-2 are correlated with poor prognosis in a variety of tumors (14,87) and cell lines (105,116). Since the ratio between PAI and u-PA levels has not been analyzed, it is difficult to determine whether poor prognosis is correlated with high levels of PAI. In tumor cells, PAI levels may increase to balance the increased u-PA activity, but the increase in PAI-1 may not be sufficient to completely block u-PA mediated activity. The ability of PAI to inhibit ECM degradation and invasion allows one to speculate that PAI genes as well as other anti-protease genes may be cancer suppressor genes.

In this study, I analyzed the role of PAI-1 in the invasive ability of DU-145 human prostatic carcinoma cells. Prior to this study, the role of PAI-1 in prostate cancer progression had not been studied, therefore, I analyzed its effect on u-PA activity and invasion by transfecting DU-145 cells with PAI-1 cDNA.

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CHAPTER 2

Retinoic Acid Modulates Extracellular Urokinase-type Plasminogen Activator Activity in DU-145 Human Prostatic Carcinoma Cells

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This Chapter was originally published in Clin.Cancer Res.

Reference: Waghray, A., and Webber, M.M. Retinoic Acid Modulates Extracellular Urokinase-type Plasminogen Activator Activity in DU-145 Human Prostatic Carcinoma Cells. Clin.Cancer Res., 1: 747-753, 1995.

ABSTRACT

Effects of all-*trans* retinoic acid (RA) on the net enzymatic activity of secreted, extracellular urokinase-type plasminogen activator (u-PA) in DU-145 human prostatic carcinoma cells were examined to assess the potential use of retinoids in human prostate cancer prevention and treatment. u-PA is associated with tumor progression involving invasion and metastasis. Based on a chromogenic substrate assay, results show that DU-145 cells secrete five times more u-PA than normal human prostatic epithelium. DU-145 cells were treated with 0.1 to 10 μM RA for 48 h. This short treatment of cells with RA did not inhibit growth. After a 48 h treatment of cultures with RA, serum-free conditioned medium was analyzed for u-PA activity by SDS-PAGE zymography. Two major bands of u-PA with Mr of ~54 kDa (high Mr u-PA) and ~33 kDa (low Mr u-PA) were detected. Plasminogen- dependent catalytic activity of these bands could be specifically inhibited with antibody to u-PA, confirming that these bands represent u-PA. A 48 h treatment with 1.0 μM RA reduced u-PA activity in conditioned medium to 51.6% of control. A 50% reduction in free u-PA antigen level, as compared to control, was further demonstrated at 1.0 μM RA by Western blot analysis and densitometry. These results show that all-*trans* retinoic acid can decrease the net extracellular urokinase activity produced by prostatic carcinoma cells. It is proposed that these effects of RA may have important implications not only in the chemoprevention of prostate cancer, by inhibition of promotion of prostatic intraepithelial neoplasia (PIN) to invasive carcinoma, but also in tumor progression during invasion and metastasis, by decreasing extracellular matrix degradation, as shown in our accompanying article (1).

INTRODUCTION

Prostate cancer, excluding non-melanoma skin cancer, is the most common cancer in adult men in the United States (2,3). The American Cancer Society estimates that 200,000 new cases and 38,000 deaths from prostate cancer will occur in the U.S. in 1994 (2). The incidence of such cancers increases with age and about 80% are diagnosed in men over the age of 60. Both incidence and mortality from prostate cancer are increasing (3). Because of the increasing lifespan and an aging population in the U.S., prostate cancer is a major health concern today (4). Based on autopsy studies, approximately 11 million men in the U.S., older than 45-50 years, have histologically identifiable carcinoma of the prostate and it has been predicted that one in ten men will develop prostate cancer in his lifetime (3,5). A primary cause of death from prostate cancer is invasion and metastasis. One of the first steps in progression to malignancy is degradation of the basement membrane. Proteases intervene at the transition from *in situ* to invasive carcinoma where local dissolution of the basement membrane occurs. The major extracellular matrix degrading enzymes in neoplasia comprise the serine proteases, plasminogen activators (PAs) and plasmin, and a family of structurally related metalloproteases. A correlation between metastatic potential and increased expression of these proteases has been demonstrated (6). The PA-mediated proteolysis involves activation of the zymogen plasminogen by PA to plasmin, a trypsin-like enzyme with broad substrate specificity.

There are two types of PAs, the tissue-type plasminogen activator (t-PA) is found in most normal tissues and in blood where it is involved in fibrinolysis. The

urokinase-type plasminogen activator (u-PA) is involved in the degradative events involving extracellular proteolysis during tissue remodeling, involution, wound healing and cancer. u-PA, a glycoprotein, is secreted as a single-chain pro-enzyme (scu-PA). The active form of u-PA is a two chain (tcu-PA), 54 kDa molecule consisting of a light A chain representing the NH_2-terminus containing the receptor-binding domain and a single kringle unit, and a heavy B chain which contains the catalytic domain (7). The catalytic domain is conserved in all serine proteases including trypsin, plasmin and kallikrein. Two active, two-chain forms of u-PA have been identified, the high Mr ~54 kDa, and the low Mr ~33 kDa, the latter composed primarily of the B chain.

Prostatic epithelium intrinsically secretes both t-PA and u-PA. Prostatic PAs are contributed to the seminal fluid as part of the prostatic secretion, of which u-PA is a major component and is involved in the liquefaction of semen (8). Our hypothesis is that because of this high u-PA expression, prostatic intraepithelial neoplasia and prostatic carcinomas (PCas) have an inherent advantage and predilection for progression to invasion and metastasis. A change in normal homeostasis and regulation of u-PA occurring even in very small foci of malignant cells, such as PIN, could result in invasion. Prostatic carcinomas express more u-PA activity than benign tumors and most of this activity represents the ~54 kDa u-PA (9). u-PA levels also show a correlation with bony metastases (10,11). PA activity in several human prostatic carcinoma cell lines has been correlated with the aggressiveness of the cell type (12).

Although PA activity has been recognized in prostatic tissue, the regulation and role of different PAs in extracellular matrix (ECM) degradation during tumor progression has not been studied extensively in prostate cancer. Hormones, growth factors and tumor promoters can modulate the expression and secretion of PAs. EGF, TGF-ß, bFGF, PDGF and cytokines (IL-1, IL-4, TNF), can modulate u-PA synthesis, however,

the effects of various factors may vary with the cell type (13). Estrogens and prolactin stimulate PA secretion in breast cancer cell lines while hydrocortisone inhibits it (14). A large variety of carcinoma cells express high levels of u-PA and a correlation between u-PA secretion, malignant transformation, and aggressiveness of human and animal cancers has been shown (15).

Our objectives were: i) to compare levels of secreted u-PA by normal and malignant prostate cells, ii) to examine modulation of the net extracellular u-PA activity by all-*trans* retinoic acid, and iii) to determine possible usefulness of RA in inhibition of tumor progression involving invasion and metastasis in prostate cancer. Many retinoids are inhibitors of tumor promotion *in vitro* and *in vivo* (16, 17). Of special interest is the observation that retinoids inhibit invasion and metastasis (1,18). Information is not available on the possible regulatory effects of retinoic acid on the extracellular u-PA activity in prostate cancer. Therefore, the present investigation examines the effects of retinoic acid on the net activity of u-PA secreted by DU-145 human prostatic carcinoma cells.

MATERIALS AND METHODS

Materials: Cells: Human prostatic carcinoma cell line DU-145 #HTB 81 from American type Culture Coillection; RPMI-1640 medium #320-1875AJ; antibiotic/antimycotic mixture #600-5240AG from GIBCO; fetal bovine serum (FBS) from Intergen; KGM serum-free medium #CC-3001 from Clonetics; EGF #40001 from Collaborative Research; all-*trans* retinoic acid #R-2625, human plasminogen #P5661 from Sigma; human urokinase standard #128, polyclonal antibody to u-PA #389, synthetic substrate for u-PA, Spectrozyme UK #244 from American Diagnostica; goat anti-rabbit second antibody #8612-37 from Organon Teknika; gelatin for zymography #170-6537 from BioRad; filter units for concentrating conditioned medium: Centriprep 10, #4304 from Amicon; Avidin-Biotin immunoperoxidase Vectastain ABC kit #AK5001 from Vector; alkaline phosphate substrate 4-nitro blue tetrazolium chloride #1087-479 and 5-bromo-4-chloro-3-indolyl phosphate #760-986 from Boehringer-Mannheim.

METHODS: Cell culture: DU-145 cells were maintained in RPMI-1640 medium containing 2 mM glutamine, 100 U penicillin, 100 μg streptomycin and 0.25 μg fungizone/ml medium, and 5% fetal bovine serum. Cells were subcultured once/week. Normal prostatic epithelial cells were isolated from fresh surgical specimens according to methods developed in this laboratory (19). Cells were maintained in KGM serum-free medium supplemented with 10 ng/ml EGF. Passage 1 and 2 cells were used as normal controls. Absolute ethanol (ETOH) was used as the vehicle for RA. The final concentration of the vehicle in the culture medium was 0.1%.

Microplate assay for establishing effects of RA on the growth of DU-145 cells: 10,000 cells were plated/well in a 96 well plate in 100 μl of RPMI-1640 with 5% FBS. 5 wells/treatment were made. RA at 2X the final concentration in 100 μl medium was added 24 h later. Medium containing RA was changed every 72 h. Test plates were recovered over a 10-day period and stained with the protein-binding dye methylene blue. Bound dye was released with 1% SDS, and absorbance measured at 620 nm with a Titertek microplate reader (20).

Assays for Plasminogen Activators. For the assays listed below for PAs, 500,000 cells were plated/60-mm culture dish in RPMI 1640 and 10% fetal bovine serum. 24 h later, cells were washed twice with PBS, and serum free RPMI 1640 medium containing RA was added. Samples of SF-CM from 48 h treated and untreated cells were collected and centrifuged at 2000 rpm for 5 min to remove cell debris. If not immediately used, medium samples were stored at -75^0 C. For some experiments, the medium was concentrated using filter units with a molecular weight cutoff of 10,000.

Chromogenic Substrate Assay for Plasminogen Activator Activity.

The u-PA activity was analyzed in SF-CM, using a synthetic substrate for u-PA by measuring the increase in absorbance of the free chromophore generated in comparison to the original substrate per unit time at 405 nm. At excess substrate concentration, the rate of absorbance increase, due to the amount of chromophore released, is linearly related to enzyme concentration (21).

Gel Electrophoresis and Zymography: SDS-PAGE zymography was performed as described by Heussen and Dowdle (22), using 10% polyacrylamide gel copolymerized with plasminogen (12μg/ml of protein) and gelatin (1gm%) as substrates. PAs have species specificity for plasminogen, hence human plasminogen was used. The volume of SF-CM loaded per lane in the gel was standardized against a fixed cell number as indicated in the legends to Figures 1, 2 and 4. Minigels were run at 200 V at 4°C for 45 min, then gently rocked in two changes of 2.5% triton X-100 in distilled water for 1h at room temp to remove SDS, and incubated at 37°C for 18 h in Tris-HCl buffer (pH 7.6) to allow re-naturing of the enzyme. The gels were stained with Coomassie blue and destained in methanol:acetic acid:water (3:1:6). The presence of enzyme activity is indicated by specific bands of lysis against a dark background.

Inhibition of u-PA Activity in Zymograms by Specific Antibody. To confirm the presence of u-PA activity, SF-CM from DU-145 cells were incubated for 2 h with undiluted polyclonal antibody to u-PA at 37°C and loaded onto SDS-PAGE Zymography. This antibody has anticatalytic activity. Identical experiment was done by incubating 1:200 diluted SF-CM from Du-145 cells with undiluted antibody to u-PA.

Western Blot Analysis: Effects of RA on the free u-PA antigen levels in the 48h SF-CM were examined by western blots. The volume of concentrated SF-CM loaded per lane in the gel was standardized against a fixed cell number of 12,000 cells. Minigels were run at 200 V at 4°C for 45 min. Following electrophoresis the proteins were electroblotted onto an immobilon-P membrane (Millipore) and stained with the primary polyclonal antibody to u-PA (1:1000). Visualization of the u-PA protein occurred by incubating the blot with biotin conjugated secondary antibody (1:10,000), avidin-biotin

alkaline phosphatase and substrate for alkaline phosphatase, 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate.

RESULTS

u-PA activity in conditioned medium from malignant DU-145 prostatic cells is higher than in SF-CM from normal prostatic epithelium: PA activity in 48 h conditioned medium from primary cultures of normal human prostatic epithelium was compared with that from DU-145 cells. Figure 2-1A shows a control gel without plasminogen, which received a sample of CM from DU-145 cells similar to Lane labelled as DU-145 in Figure 2-1B. Absence of PA-mediated lysis in Figure 2-1A confirms that PA activity is plasminogen-dependent. The very light, thin band (Figure 2-1A) represents gelatinase activity. Results in Figure 2-1B show that DU-145 cells produce considerably higher levels of u-PA activity than the normal cells, as evident from the large zones of lysis at ~54 kDa and ~33 kDa (Figure 2-1B). A clear ~74 kDa t-PA band is shown by normal cells but not by DU-145 cells.

Antibody to u-PA inhibits ~54 kDa and ~33 kDa u-PA bands: In order to establish the identity of the lysis bands, an anti-catalytic antibody to u-PA was used to determine whether the respective PA activity in zymograms could be inhibited. In Figure 2-2A, PA activity in SF-CM from DU-145 cultures is shown in the left lane (-Ab). When an identical sample of conditioned medium was pre-treated (2 h at 37°C) with the

antibody to u-PA (Figure 2-2A, +Ab), the ~54 and ~33 kDa bands were inhibited. Mixing the same amount of antibody with a 1:200 diluted sample of the SF-CM resulted in complete inhibition (Figure 2-2B, +Ab), confirming that the ~54 kDa and ~33 kDa Mr bands represent u-PA.

Effects of retinoic acid on the growth of DU-145 cells: Effects of RA at concentrations from 0.001 nM to 10 μM were tested in dose response and time course experiments over a 10 day period. RA concentrations varying from 0.001 nM to 10 nM did not show any significant effect on growth (data not shown). However, when treated with RA at levels from 0.1 to 10 μM, significant growth inhibition was observed after 10 days of RA treatment (Figure 2-3). It was important to first establish the effects of RA on growth so that samples of conditioned medium for zymography and chromogenic assays could be collected at a time when there was no growth inhibition. Based on these results, SF-CM samples were collected after 48 h of RA treatment (Figure 2-3). Nevertheless, medium samples corresponding to a fixed cell number were used for testing u-PA activity in zymograms, chromogenic assays and western blot analysis.

Retinoic acid causes a decrease in extracellular u-PA activity: Triplicate cultures were placed on Serum-free RPMI-1640 medium (untreated control), or medium containing 0.1% ethanol (vehicle control), 0.1, 0.5 or 1.0 μM retinoic acid. Conditioned medium was collected after a 48 h treatment, and assayed for PA activity by SDS-PAGE zymography, using a 10% polyacrylamide gel (Figure 2-4). In untreated and ethanol treated controls (Lanes Du-145 and ETOH respectively), the large zone of lysis represents the high Mr ~54 kDa u-PA and the smaller ~33 kDa band, the low Mr u-PA. Results show (Figure 2-4) that treatment with RA causes a reduction of both Mr

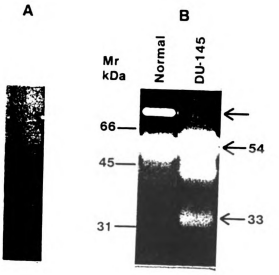

Figure 2-1: Urokinase (u-PA) activity in 48 h conditioned medium (CM) from normal prostatic epithelium and DU-145 cells was analyzed by SDS-PAGE zymography using plasminogen and gelatin as substrates (10% polycrylamide gel). The volume of CM sample loaded per lane was standardized against a fixed cell number of 4,000 cells. Panel A. A CM sample from DU-145 cells was run in a control gel in the absence of plasminogen; Panel B. Normal, CM sample from normal prostatic epithelium which shows a strong t-PA lysis band at ~74,000 (arrow). The ~54,000 and ~33,000 bands represent u-PA. Lane DU-145: CM sample from DU-145 cells. kDa, kilodalton.

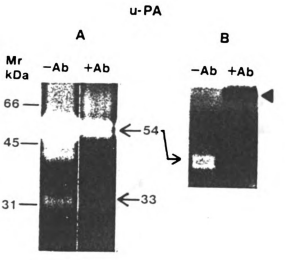

Figure 2-2: SDS-PAGE zymograms (A. 12%, B. 10% polyacrylamide gel) were prepared to demonstrate that the ~33 kDa and ~54 kDa bands represent urokinase (u-PA). Panel A. volume of conditioned medium (CM) sample loaded per lane was standardized against a fixed cell number of 4,000 cells. The control sample (-Ab) consisted of CM from DU-145 cells while a second identical sample of CM was pre-treated with antibody (+Ab) to u-PA for 2 h at 37°C.
Panel B. lane -Ab received a 1:200 diluted sample of CM; lane +Ab received a 1:200 diluted sample of CM pre-treated with the same amount of antibody as in panel A (+Ab). *Arrowhead*, u-PA/Ab complex.

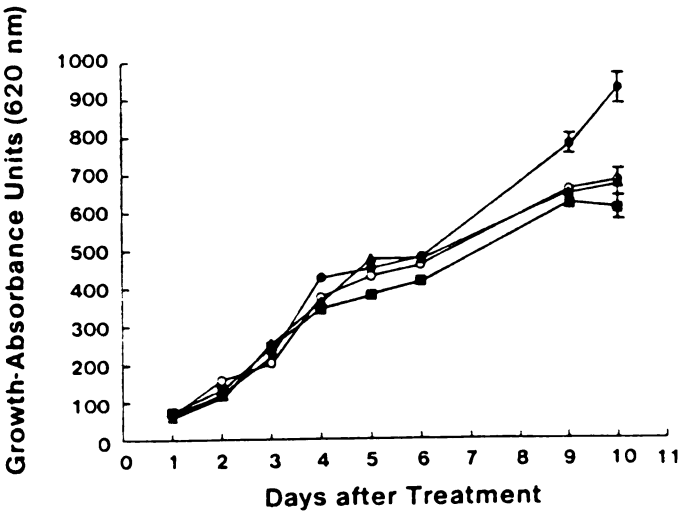

Figure 2-3: Effects of all-*trans* retinoic acid (RA) on the growth of DU-145 cells, a time course study. 10,000 cells/well were plated in 96-well plates. DMSO vehicle control (●); 0.1 μm RA (○); 1.0 μM RA (▲); 10 μM RA (■); verticle bars, SD.

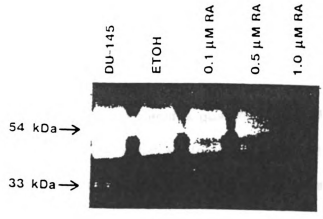

Figure 2-4: SDS-PAGE zymogram (10% polyacrylamide gel) showing the effects of retinoic acid (RA) on secreted urokinase (u-PA) activity in serum-free conditioned medium from DU-145 cells treated for 48 h with ethanol (ETOH), 0.1, 0.5 or 1.0 μM RA. The volume of conditioned medium sample loaded per lane was standardized against a fixed cell number of 5,000 cells. Lane DU-145 is untreated control. The ~54,000 and ~33,000 u-PA bands are shown. kDa, kilodalton.

~54 kDa and ~33 kDa u-PA bands in a dose-dependent manner. The urokinase/plasminogen based zymography is a sensitive assay for detecting low levels of u-PA activity. However, if u-PA activity is very high in a test sample, effects of modulators of u-PA activity can be missed due to excessive lysis in the zymogram. Therefore, dilution of samples of SF-CM may be needed to detect the effect. The RA effect was quantified and confirmed by a chromogenic substrate assay.

Decrease in u-PA activity by retinoic acid is quantified by a chromogenic substrate assay: Using a specific, synthetic substrate for u-PA, u-PA activity was measured in conditioned medium from normal prostatic epithelium and from ethanol, 0.5 or 1.0 μM RA treated Du-145 cell cultures. Results show (Figure 2-5) a low level (21.1% of control) of u-PA activity in conditioned medium from cultures of normal prostatic epithelial cells as compared to a high level (100%) of activity from ETOH treated control cultures. A dose-dependent decrease in u-PA activity to 66% and 51.6% of control was observed in SF-CM from cultures treated with 0.5 μM RA and 1.0 μM RA, respectively.

Retinoic acid also reduces extracellular, free u-PA antigen levels: Effects of RA on u-PA antigen levels in the conditioned medium were investigated. 2.5 million cells were plated/150 mm plate and treated as described earlier, with ethyl alcohol alone or with 0.5 or 1.0 μM RA. Concentrated conditioned medium was used for Western blots, which were stained with antibody to u-PA. Results show (Figure 2-6) a wide band representing the high Mr ~54 kDa u-PA in conditioned medium from untreated and ETOH treated cells and a dose-dependent decrease in free u-PA antigen levels in conditioned medium from cells after treatment with 0.5 or 1.0 μM RA for 48 h. In a

densitometric analysis of these u-PA bands, if ethanol control = 100%, then 0.5 μM RA = 82% and 1.0 μM RA = 49.6% of control.

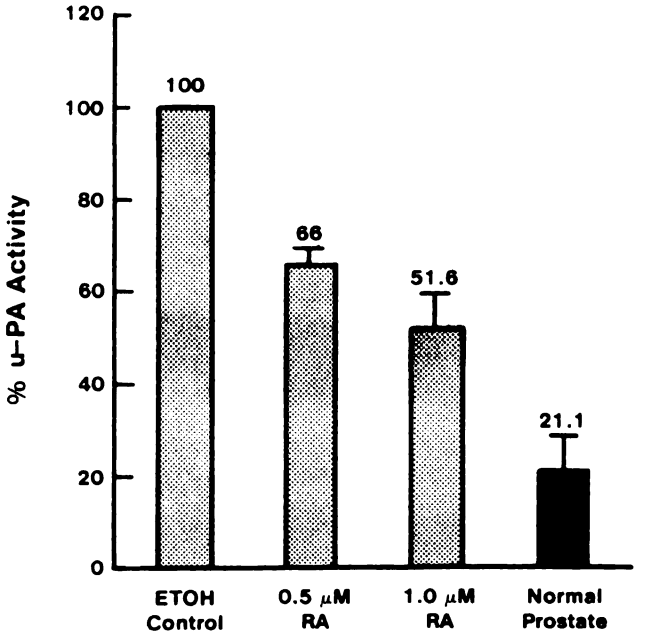

FIGURE 2-5: Urokinase activity in serum-free conditioned medium from normal prostatic epithelial cells (■) and control and retinoic acid (RA) treated DU-145 cells (□) was compared, using a chromogenic substrate assay. DU-145 cells were treated for 48 h with ethanol (ETOH), 0.5 μM or 1.0 μM RA. Data represent a mean of 3 experiments. Bars, SD.

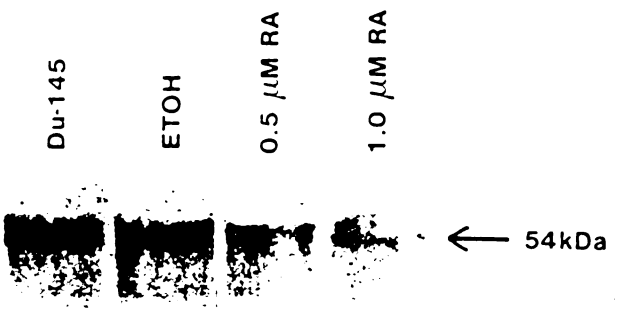

Figure 2-6: Western blot analysis showing the effects of retinoic acid (RA) treatment on the levels of Mr ~54,000 urokinase (u-PA) antigen secreted into the serum-free conditioned medium (SF-CM). Cultures were treated for 48 h with the ETOH, 0.5 μM or 1.0 μM RA. SF-CM samples were concentrated and run on a 10% polyacrylaminde gel. The volume of CM sample loaded per lane was standardized against a fixed cell number of 12,000 cells. The blot was stained with antibody to u-PA. This is a representative of three experiments. kDa, kilodalton.

DISCUSSION

Malignant cells show anchorage independence, reduced intercellular and substrate adhesion, and loss of contact inhibition, which facilitate their increased mobility. However, normal cells in solid tissues are under the restraints of contact inhibition of movement and growth. An increase in extracellular plasminogen activator secretion can remove these constraints, mediate the altered tumor cell behavior and favor the process of invasion and metastasis. *In vivo*, secreted prostatic PAs become a part of the semen and their activity is plasminogen dependent (unpublished data). *In vitro*, normal and malignant prostatic epithelial cells secrete PA into the culture medium. DU-145 cells secrete higher levels of u-PA than normal cells (Figure 2-1). Since normal prostatic epithelium normally secretes urokinase, we propose that malignant prostatic epithelial cells have an inherent advantage and a predilection for progression to invasion and metastasis and that even very small foci of transformed cells have the ability to invade. This argument is supported by the following observations from other tumors. It is generally agreed that carcinoma cells arising from a variety of epithelia express more u-PA and that PA levels are also higher in several chemical carcinogen and virus-induced animal tumors and in human breast, lung, ovarian and colon cancers than their normal tissue of origin (23). A correlation between u-PA secretion, malignant transformation, metastasis and aggressiveness in animal and human breast, bladder, and lung tumors has also been demonstrated (15). Increased skeletal metastasis by rat prostate cancer cells, transfected with u-PA cDNA, is associated with overproduction of u-PA (10).

Retinoids are important in normal epithelial cell proliferation and differentiation but they can also reduce invasive and metastatic potential of human and rodent epithelial tumor cells *in vivo* and *in vitro*, an effect associated with a decrease in PA and collagenase activity (1,18,24). Our objective was to determine whether RA could inhibit pericellular proteolysis and extracellular matrix degradation by reducing the net extracellular urokinase enzymatic activity. Treatment with 1.0 μM retinoic acid for 48 h caused a 50% reduction in extracellular u-PA enzymatic activity and in free u-PA antigen levels (Figures 2-4 to 2-6). However, growth was not inhibited at this time period (Figure 2-3). We propose that since normal prostatic epithelium, and by inference, early invasive prostatic carcinomas would intrinsically secrete u-PA, invasion may begin at an early stage. Our implication is that it may be possible to inhibit these degradative processes *in vivo* by retinoids (1).

The response of PAs to RA treatment differs in cells of different origin, e.g, 1 μM RA induced PA in cells of mesenchymal origin but decreased PA by 50% in human normal kidney epithelial cells (25). In F-9 mouse teratocarcinoma cells, RA induced differentiation and concomitantly suppressed u-PA expression (26). u-PA is involved not only in invasion and metastasis but also in tumor promotion in earlier stages of carcinogenesis. TPA enhanced PA expression in DU-145 human prostate carcinoma cells (data not shown). In mouse skin carcinogenesis, retinoids inhibit phorbol ester mediated tumor promotion (27). Inhibition of these promotional effects by retinoids may be of special significance. The net extracellular u-PA activity is determined not only by the amount of the enzyme itself but also by its state of activation and the presence and levels of specific inhibitors of PA. The mechanisms of RA effects on extracellular u-PA activity and expression in human prostatic carcinoma cells are not known. Our preliminary results suggest that RA may not have a direct

effect on u-PA expression, however, the decrease in extracellular u-PA activity may be the result of changes in the expression of TGF-β and urokinase inhibitors.

A pronounced heterogeneity in the u-PA content in tissue sections from different parts of a tumor has been observed, with most intensive staining being in the areas of invasive growth (28). Similar heterogeneity has been encountered in clonal populations derived from tumor cell lines in culture (29). The picture becomes even more complex when one considers hormone-responsive tissues. For example, estrogen induced u-PA in estrogen-receptor positive breast cancer cells, but estrogen-receptor negative cells intrinsically secreted high u-PA levels and were highly invasive. Therefore, u-PA in breast cancer was considered to be a prognostic marker. This example further illustrates the variation in response to RA in hormone-responsive tissues. In estrogen-responsive cells, RA caused inhibition of PA production in the absence of estrogen, but increased PA production in its presence. On the other hand, the estrogen-resistant clone showed unresponsiveness to RA (23,30). The advantage of using heterogeneous tumor cell populations, such as DU-145 and most other tumor cell lines, is that they reflect the cellular heterogeneity of tumors *in vivo*. However, the disadvantage is that different clones within a cell population may respond differently to the test agents, resulting in variations in response under different culture conditions. Variability in PA secretion and response to RA has also been found amongst rhabdomyosarcoma and glioma cell lines (29,31). We have observed similar variations in u-PA expression and response to RA in the DU-145 cell line, which consists of a very heterogeneous cell population. In some experiments, a decrease in u-PA activity was observed only at 10 μM RA. Therefore, it would not be surprising to find wide variations in secreted u-PA activity (29) and response to RA in different clones and cell lines under different culture conditions (13,30).

The following facts about advanced prostatic carcinoma demonstrate the clinical relevance of our findings. In 80% of patients with disseminated prostatic carcinoma, a significant increase in plasma u-PA levels has been observed and it was suggested that u-PA may be a reliable marker for prostatic metastatic disease (11). PCa patients have a latent tendency to bleed and have higher levels of fibrin-degrading enzymes in the plasma than in benign prostatic hyperplasia patients. The clinical picture is usually one of a patient with bony metastases and generalized bleeding. ϵ-aminocaproic acid, a potent plasmin inhibitor, has been used to treat bleeding associated with PCa (32). In about 20% of prostate cancer patients, fibrinolysis is associated with hemorrhagic manifestations. Extracts of tissue from metastases showed two-fold more fibrinolytic activity than primary tumors (33). Huber et al. (34) have recently reported that plasma u-PA levels were indicative of colon cancer in 75.5% of the cases, as compared to 51.5% for carcinoembryonic antigen, but together they gave the highest sensitivity value of 90.9% for detection of colon cancer. u-PA could serve as a useful prognostic marker also for prostate cancer.

Retinoids can restore normal cell differentiation in a dysplastic epithelium (18). An example of such epithelium are the PIN or carcinoma *in situ*, which represent precancerous, dysplastic lesions, characterized by proliferation and anaplasia of cells (35). PIN also represent a continuum of morphological changes which progress toward increased cell proliferation, crowding, nuclear and nucleolar enlargement and heterogeneity and finally to microinvasion of the basement membrane. There is evidence to suggest that prostatic carcinomas arise in foci of PIN (35). Because an increase in u-PA is associated with tumor promotion, PIN are an excellent target for chemoprevention. The most desirable effect of a chemopreventive agent would be to cause a regression of such preneoplastic lesions. Retinoids and protease inhibitors may

accomplish this. A recent report shows that dietary 4-HPR decreases tumor incidence of *ras-myc*-induced carcinomas in the mouse prostate reconstitution model (36).

Our proposition is that u-PA plays a key role in tumor progression from *in situ* carcinomas such as PIN to invasive tumors. The role of type IV collagenases in invasion and metastasis has been elegantly described by Liotta and Collaborators (6). However, the procollagenases must be first activated to collagenases. This activation can not only be efficiently accomplished by plasmin generated from plasminogen by u-PA action, but a recent study reports that u-PA can directly activate type IV collagenase (37). These observations point to a key role of u-PA in initiating the enzymatic cascade at the cancer cell:ECM interface in the prostate, since we have observed that DU-145 cells express only trace gelatinase activity (Figure 2-1A). Further, *ras* and *src* oncogene expression is associated with increased u-PA production and inhibition of u-PA expression by antisense results in inhibition of lung colonization by NIH-3T3 cells transfected by EJ/vHa-*ras* (15,38). Thus, transformation itself imparts the ability to secrete increased levels of u-PA. This evidence provides further support for our hypothesis that u-PA activity is critical in early invasion and plays a key role in the progression of PIN to invasive carcinoma. Measurement of u-PA along with prostate specific antigen (PSA) levels in serum for early detection of prostate cancer may be very useful. Reduction of extracellular u-PA activity in advanced PIN by retinoids and protease inhibitors is an important area of investigation in cancer prevention and intervention particularly when one considers the following observations. The incidence of histologic prostate carcinoma is high and an estimated 11 million men older than 46-51 may have latent carcinoma (3). PIN predate clinical carcinoma by about five years (35). Also, the incidence of latent carcinoma shows only small differences between different racial groups worldwide, e.g. 20.5% in Japan and 34.6% in the U.S.

white population (4). However, the incidence of clinical cancer is about 8 and 15 times higher in the U.S. white and black men respectively than in native Japanese men, suggesting that latent carcinomas progress to invasive cancer more frequently in American men than in native Japanese (4). Thus, this progression, spanning a period of 20-30 years, is an appropriate target for early intervention and prevention of prostatic carcinoma in American men.

Clonal evolution within epithelial neoplasms begins early in the neoplastic process and regression of early intraepithelial lesions by retinoids has been reported. For example, remission of precancerous lesions such as oral leukoplakia in tobacco chewers occurs by intervention with ß-carotene or vitamin A (17,39). However, cancer prevention by retinoids is not a universal finding. In some cases, retinoids have been shown to promote skin cancer induction in animals (40). Since tumor cells must successfully complete a number of critical, sequential steps before invasion occurs and metastasis can be established, this implies that the inhibition of any one of these steps should lead to a decrease in invasion. We conclude that retinoic acid decreased the net extracellular urokinase activity and free u-PA antigen levels in cultures of DU-145 human prostatic carcinoma cells. The role of urokinase in extracellular matrix degradation and invasion and the effects of RA on these processes are examined in our accompanying paper (1).

ACKNOWLEDGEMENTS

We thank Dr. Jean Burnett for her critical evaluation of this paper and Diana Bello, Daniel Edwards, W. Scott Metcalfe, Beth Roestel, and Salmaan Quader for their invaluable assistance.

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CHAPTER 3

Urokinase-Mediated Extracellular Matrix Degradation by Human Prostatic Carcinoma Cells and its Inhibition by Retinoic Acid

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This Chapter was originally published in Clin.Cancer Res.

Reference: Webber, M.M., and Waghray, A. Urokinase-mediated extracellular matrix degradation by human prostatic carcinoma cells and its inhibition by retinoic acid. 1: 755-761, 1995.

ABSTRACT

Both normal and malignant prostatic epithelial cells in culture secrete urokinase-type plasminogen activator (u-PA) into the culture medium. u-PA has been shown to have a direct association with invasive and metastatic potential of many types of cancers. We propose that prostate cancer has the intrinsic ability to invade and metastasize because of its inherent ability to secrete the serine protease u-PA. We further propose that in prostate cancer, u-PA is the key enzyme which occupies a place at the apex of the proteolytic cascade and initiates the degradative process. Subsequently, collagenases are recruited after activation of pro-collagenases by another serine protease plasmin, formed by the activation of plasminogen by u-PA. Extracellular proteolysis involving plasmin can cause massive degradation of extracellular matrix (ECM). We show that u-PA alone can use fibronectin as a substrate and degrade it but u-PA alone did not degrade laminin. Serum-free conditioned medium (SF-CM) from DU-145 human prostatic carcinoma cells has the ability to degrade both fibronectin and laminin. However, treatment of cultures with 1 μM *all-trans* retinoic acid (RA) for 48 h reduced the ability of SF-CM to cause u-PA-mediated degradation of fibronectin and laminin. Thus, RA had a protective effect on these ECM glycoproteins. Treatment of cells with RA also decreased their ability to invade "Matrigel" in the *in vitro* invasion assay in a dose dependent manner. RA at 0.5, 1 and 10 μM level reduced invasion to 65.7, 46.7 and 34.3% of control, respectively. RA reduced extracellular proteolysis and thus inhibited ECM degradation and invasion. These results may also explain one mechanism by which retinoids inhibit invasion and metastasis *in vitro* and *in vivo*. These studies have important translational value in the chemoprevention of progression of prostatic intraepithelial neoplasia (PIN) to invasive carcinoma.

INTRODUCTION

The primary cause of death from prostate cancer is invasion and metastasis. In the early stages of tumor development, cells with a metastatic phenotype may exist in the heterogeneous tumor cell population in a primary tumor. Prostatic intraepithelial neoplasia are considered to be equivalent to carcinoma *in situ*. During progression from PIN to invasive carcinoma, tumor cells cross tissue boundaries and invade the surrounding stroma. Invasion of the basement membrane (BM) is an active process involving cell adhesion to the BM, degradation of the extracellular matrix and migration (1). Localized degradation of ECM takes place in areas where the ratio of proteolytic enzymes to their natural inhibitors, present in the surrounding ECM, shifts in favor of proteolysis.

Invasion is a critical initial step in the metastatic cascade. Degradative enzymes involved in invasion and metastasis include serine proteases, metalloproteases, cathepsins and heparanases. A cascade including all or some of these is probably involved in the invasion process, however, in different tumors, one type of enzymes may dominate the process. A correlation between invasion and metastatic potential and increased expression of ECM degrading proteases has been demonstrated (1). However, little is known about the interactions of normal and malignant prostatic epithelial cells with their ECM or about the mechanisms involved in the degradation of the BM and ECM during tumor progression, invasion and metastasis in prostate cancer. An understanding of the mechanisms regulating these interactions is essential for a complete understanding of tumor progression and for devising ways by which these

degradative processes could be inhibited while still in the early PIN stage of tumor development.

Retinoids play an important role in the control of normal epithelial cell proliferation and differentiation and they inhibit carcinogenesis, growth, invasion and metastasis of certain tumors both *in vivo* and *in vitro* (2, 3). A reduction in the incidence of primary prostate cancer and metastases induced by an initiation-promotion protocol involving methylnitrosourea and testosterone in Lobund-Wistar rats and inhibition of angiogenesis in such tumors by 4-HPR has been reported (4,5). Further, inhibition of melanoma cell invasion *in vivo* and *in vitro* by retinoic acid has also been shown (6). It is interesting to note that a recent epidemiological study showed an increased risk for prostate cancer in men with low serum vitamin A levels (7).

In the presents study we examined: i) the ability of u-PA alone and that of conditioned medium from DU-145 human prostatic carcinoma cells to degrade extracellular matrix glycoproteins fibronectin and laminin; ii) the effects of retinoic acid on the degradation of ECM in prostatic carcinoma and iii) the effects of RA on *in vitro* invasion by DU-145 cells. In conducting these studies one objective was to identify agents which would decrease or block extracellular activity of degradative proteases and thus inhibit the process of ECM degradation and invasion.

MATERIALS AND METHODS

Materials: Cells: Human prostatic carcinoma cell line DU-145 #HTB 81 from American Type Culture Collection; RPMI 1640 medium #320-1875AJ, antibiotic/antimycotic mixture # 600-5240AG from Gibco; fetal bovine serum (FBS) from Intergen; HEMA-3 stain #122-911 from Curtin Matheson; human fibronectin #4008, mouse laminin #40232 and "Matrigel" #40234, from Collaborative Research; human urokinase #128 from American Diagnostica; *all-trans* retinoic acid #R 2625, human plasminogen #5661, monoclonal antibody to human fibronectin #F-7387 and to mouse laminin #L-8271 from Sigma; polyclonal antibody to human u-PA #389 from American Diagnostica; aprotinin #236-624 from Boehringer-Mannheim; Centriprep 10, #4304 filter units for concentrating conditioned medium from Amicon; 4-15% gradient gels from Joule; for invasion assay, Nuclepore filters #150446, 8μm pore size, from Costar; Immobilon-P transfer membrane # IPUH-304 F0 from Millipore.

Cell culture: Stock cultures of DU-145 cells were maintained in RPMI- 1640 medium containing 2mM glutamine, 100 U penicillin, 100 μg streptomycin and 0.25 μg fungizone/ml medium and 5% fetal bovine serum. Cells were subcultured once/week.

Collection of serum-free conditioned medium (SF-CM): 2.5 million cells were plated in 150 mm culture plates and allowed to grow for 24 h in RPMI-1640 medium containing 10% FBS. Subsequently, the cultures were washed thoroughly with three changes of

PBS and 15 ml serum-free RPMI medium was added per dish. For cultures to be treated with RA, the SF-CM also contained the appropriate concentration of RA dissolved in absolute ethanol. The final concentration of ethanol in the culture medium was 0.1%. Cells were treated with RA for 48 h in all experiments. For assays involving degradation of fibronectin and laminin by SF-CM, the medium was concentrated using filter units with 10,000 Mr cut-off.

Gel electrophoresis and Western blot analysis: SDS-PAGE was performed according to Laemmli (8,9). Samples of pure fibronectin and laminin were incubated with pure urokinase or with conditioned medium from 48 h RA treated and control cultures. Concentrated samples of SF-CM from cultures were standardized for SDS-PAGE on the basis of a fixed cell number (12,000 cells/lane). Samples were run on 4-15% gradient gels at 200 V at 8°C for 45 min to separate fibronectin and laminin fragments after incubation. For Western blots, samples from 4-15% gradient gels were transferred to Immobilon-P membrane and immunoblotted with MoAb to fibronectin or laminin and stained using the Vectastain ABC kit as described (9) to detect FN and LN and their degradation fragments.

Degradation of fibronectin and laminin: Pure samples of human fibronectin (2.5 μg) were mixed with 400 mU of pure urokinase dissolved in water and incubated for 18 h at 37°C. The reaction was stopped with the addition of sample buffer without ß-mercaptoethanol (ß-ME), while the tubes were kept on ice. Non-reduced samples were loaded in 4-15% gradient gels without heating. Only the Mr markers were reduced. The gels were stained with Coomassie blue and destained in methanol:acetic acid:water (3:1:6). For laminin, 2.5 μg samples of pure laminin were mixed with 500

mU of urokinase and incubated as described above. The sample buffer for laminin samples contained ß-ME and these samples were reduced by heating for 5 min at 95°C. Sample mixtures requiring plasminogen contained 1.5 µg plasminogen. Aprotinin, an inhibitor of plasmin, was used to block any plasmin activity in the conditioned medium in order to examine degradation of fibronectin or laminin caused by urokinase alone. 10 units of aprotinin in PBS were added to the conditioned medium sample and incubated for 2 h. Fibronectin or laminin was then added and the mixture was incubated for 18 h at 37°C and processed as described above.

Invasion assay: Cell invasion was assayed in Boyden blind well chambers containing "Matrigel" coated filters, as described by Albini et al. (10). One million cells were plated per 100 mm culture plate. 24 h later, cells were washed 3X with PBS and treated with RA in 10 ml serum-free medium for 48 h and released from culture plates using 1mM EDTA, suspended in RPMI-1640 medium containing 0.1% BSA and counted. Cells were resuspended in medium with BSA at 1 million cells/ml. The lower chamber was loaded with 220 µl of conditioned medium (chemoattractant) from human lung fibroblasts grown for 24 h in serum-free medium containing 50 µg/ml ascorbic acid. In the upper chamber, 200,000 cells were plated in 650 µl of RPMI-1640 with 0.1% BSA on the Nuclepore filter coated with 500 µg/ml "Matrigel." The cells were allowed to migrate for 5 h in the incubator at 37°C. The filters were processed according to a method described by Grotendorst (11). Briefly, The migrated cells were fixed, stained with HEMA-3 and allowed to hydrate in distilled water. Nuclear stain was extracted for 15 min with 0.1N HCl and absorbance measured at 620 nm using a Titertek microplate reader. Three replicate filters were prepared per treatment and the mean values for three such experiments were plotted.

RESULTS

Urokinase degrades fibronectin: In order to determine whether urokinase has the ability to degrade human fibronectin, pure samples of fibronectin and urokinase were incubated for 18 h and analyzed by immunoblotting. Blots treated with MoAb to fibronectin show (Figure 3-1) that u-PA has the ability to degrade fibronectin into smaller fragments with Mr between ~200 and 25 kDa. However, in the presence of plasminogen, which is activated by urokinase to plasmin, further degradation of fibronectin was observed with some fragments having Mr different from those of fragments produced by urokinase alone.

Urokinase does not degrade laminin: Experiments similar to those described above were conducted using laminin as a possible substrate for urokinase. Immunoblots treated with MoAb to laminin show (Figure 3-2) that pure urokinase does not degrade laminin. However, when plasminogen is added to the sample mixture, degradation of laminin occurs with the major band of laminin fragment seen at Mr ~97 kDa and some smaller fragments. The intensity of high Mr laminin bands is concomitantly decreased.

Conditioned medium from DU-145 cells degrades fibronectin: Having established that pure urokinase has the ability to degrade fibronectin, we then examined the ability of conditioned medium from DU-145 cells to degrade fibronectin. Immunoblots treated with MoAb to fibronectin show that CM can degrade fibronectin with the loss of the

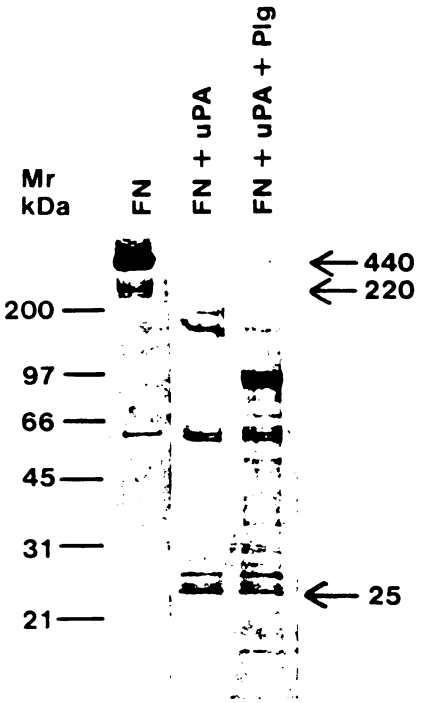

FIGURE 3-1: Western blot analysis of the degradation of fibronectin (FN) by urokinase (u-PA). 2.5 μg FN was used in each sample. Samples were run on a 4-15% gradient gel, transferred to Immobilon-P membrane and immunoblotted with monoclonal antibody to FN. Left, Mr markers. **Lane FN.** Fibronectin alone. The major FN bands are seen at ~440,000 and ~220,000. **Lane FN + u-PA:** FN was mixed with 400 mU of u-PA and incubated at 37° C for 18 h. **Lane FN + u-PA + Plg:** FN was mixed with 400 mU u-PA and 1.5 μg of Plasminogen (Plg). kDa, kilodalton.

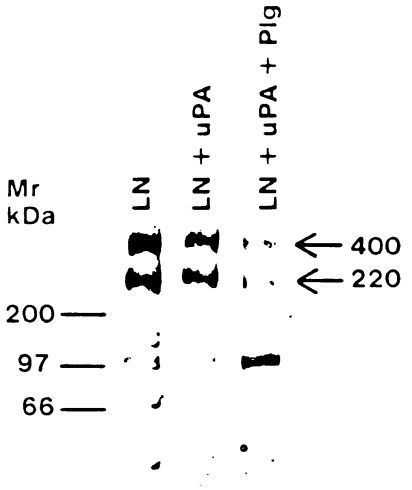

FIGURE 3-2. Western blot analysis of the degradation of laminin (LN) by urokinase (u-PA). 2.5 μg LN was used in each sample. Samples were run on a 4-15% gradient gel, transferred to Immobilon-P membrane and immunoblotted with monoclonal antibody to LN. Left, Mr markers. **Lane LN:** Laminin alone. The major LN bands are seen at ~400,000 and 220,000. **Lane LN + u-PA:** LN was mixed with 500 mU of u-PA and incubated at 37^0 for 18 h. **Lane LN + u-PA + Plg:** LN was mixed with 500 mU u-PA and 1.5 μg of plasminogen (Plg). kDa, kilodalton.

~440 and ~220 kDa bands (Figure 3-3). In order to determine that the observed fibronectin degradation was indeed caused by urokinase, aprotinin was added to the SF-CM mixture to block plasmin activity, before adding fibronectin. In the presence of aprotinin, there was some recovery of the high Mr fibronectin bands (Figure 3-3), indicating that some degradation of fibronectin is attributed to urokinase secreted by DU-145 cells. Inhibition of some degradation by aprotinin indicates the presence of low levels of plasmin activity in the SF-CM.

Conditioned medium from DU-145 cells degrades laminin: The ability of conditioned medium from DU-145 cultures to degrade laminin was then examined. Immunoblots treated with MoAb to laminin show that (Figure 3-4) degradation of laminin occurred resulting in several laminin fragments with Mr between ~200 kDa and ~97 kDa. This degradation could be blocked by the addition of aprotinin, indicating that plasmin but not u-PA degraded laminin.

Degradation of fibronectin is inhibited by conditioned medium from cultures treated with retinoic acid: Cultures of DU-145 cells were treated with RA for 48 h in serum-free medium. Samples of pure fibronectin were incubated with concentrated conditioned medium and run on 4-15% gradient gels. Results show (Figure 3-5) that fibronectin is degraded by CM from untreated or ethanol treated control cultures, with a concomitant decrease in the high Mr FN bands. Conditioned medium from cultures treated with 0.1 μM RA showed the same pattern of fibronectin degradation as the control cultures. However, CM from cells treated with 1.0 μM RA (Figure 3-5) showed inhibition of fibronectin degradation resulting in the recovery of the 220 kDa and 440 kDa fibronectin bands with a concomitant decrease in lower Mr fibronectin fragments.

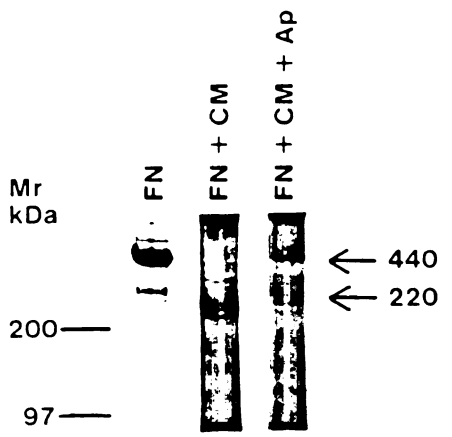

FIGURE 3-3. Western blot analysis of the degradation of fibronectin (FN) by serum-free conditioned medium (SF-CM) from DU-145 cells, and inhibition of degradation by aprotinin. 2.5 μg FN was used in each sample. Samples were run on a 4-15% gradient gel, transferred to Immobilon-P membrane and immunoblotted with monoclonal antibody to FN. Left, Mr markers. **Lane FN:** Fibronectin alone. FN bands at ~440,000 and 220,000 are shown. **Lane FN + CM:** FN was mixed with CM and incubated at 37°C for 18 h. **Lane FN + CM + Ap:** FN was mixed with CM pretreated for 2 h with 10 U of aprotinin (Ap) to block plasmin activity. kDa, kilodalton.

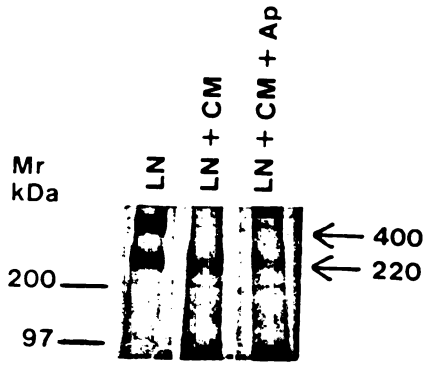

Figure 3-4: Western blot analysis of the degradation of laminin (LN) by serum-free conditioned medium (SF-CM) from DU-145 cells, and inhibition of degradation by aprotinin. 2.5 μg LN was used in each sample. Samples were run on a 4-15% gradient gel, transferred to Immobilon-P membrane and immunoblotted with monoclonal antibody to LN. Left, Mr markers. **Lane LN:** Laminin alone. LN bands at ~400,000 and 220,000 are shown. **Lane LN + CM:** LN was mixed with CM and incubated for 18 h at 37°C. **Lane LN + CM + Ap:** LN was mixed with CM pretreated for 2 h with 10 U aprotinin (Ap) to block plasmin activity.

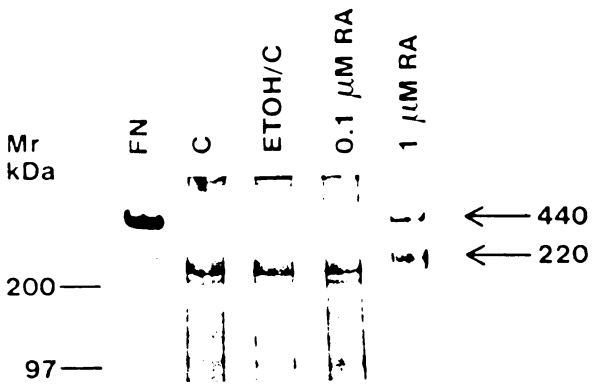

Figure 3-5: SDS-PAGE analysis of the effects of retinoic acid (RA) on the degradation of fibronectin (FN) by serum-free conditioned medium (SF-CM) from DU-145 cells. 2.5 μg of FN was used in each sample. Samples were run on a 4-15% gradient gel. Left, Mr markers. **Lane FN:** Fibronectin alone. FN bands at ~440,000 and 220,000 are shown. **Lane C:** FN was mixed with CM from untreated control cultures. **Lane ETOH/C:** Same as Lane C except that CM from ethanol treated cultures was used. **Lane 0.1 μM RA:** FN was mixed with CM from cells treated with 0.1 μM RA. **Lane 1 μM RA:** FN was mixed with CM from cells treated with 1.0 μM RA. kDa, kilodalton.

Degradation of laminin is inhibited by conditioned medium from cultures treated with retinoic acid: Similar experiments were conducted using laminin as the substrate for degradation by conditioned medium from untreated and RA treated cultures. Results show (Figure 3-6) that laminin is degraded by CM from untreated or ethanol treated control cultures, with a concomitant decrease in the high Mr LN bands. Conditioned medium from cultures treated with 0.1 μM RA showed the same pattern of laminin degradation as the controls. However, CM from cells treated with 1.0 μM RA (Figure 3-6) resulted in the protection from degradation and partial recovery of the 400 kDa laminin band with the concomitant decrease in lower Mr laminin fragments.

Treatment with retinoic acid inhibits invasion of "Matrigel" by DU-145 cells: The ability of DU-145 cells to invade "Matrigel"-coated filters is impaired by a 48 h pretreatment with RA plus RA treatment during the 5 h invasion assay period. Results show (Figure 3-7) that RA treatment reduced invasion of "Matrigel" by DU-145 cells in a dose dependent manner. Invasion, expressed as percentage of control, was reduced to 65.7% at 0.5 μM RA, 46.7% at 1 μM RA and 34.3% at 10 μM RA level. These results represent mean values for three separate experiments.

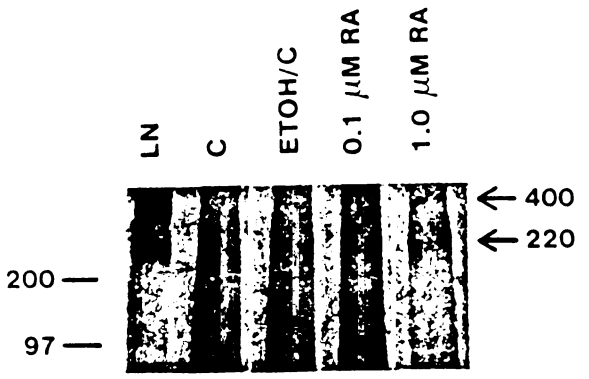

Figure 3-6: SDS-PAGE analysis of the effects of retinoic acid (RA) on the degradation of laminin (LN) by serum-free conditioned medium (SF-CM) from DU-145 cells. 2.5 μg of LN was used in each sample. Samples were run on a 4-15% gradient gel. Left, Mr markers. **Lane LN:** Laminin alone. LN bands at ~400,000 and 220,000 are shown. **Lane C:** LN was mixed with CM from untreated control cultures. **Lane ETOH/C:** Same as Lane C except that CM from ethanol treated cultures was used. **Lane 0.1 μM RA:** LN was mixed with CM from cells treated with 0.1 μM RA. **Lane 1.0 μM RA:** LN was mixed with CM from cells treated with 1.0 μM RA. kDa, kilodalton.

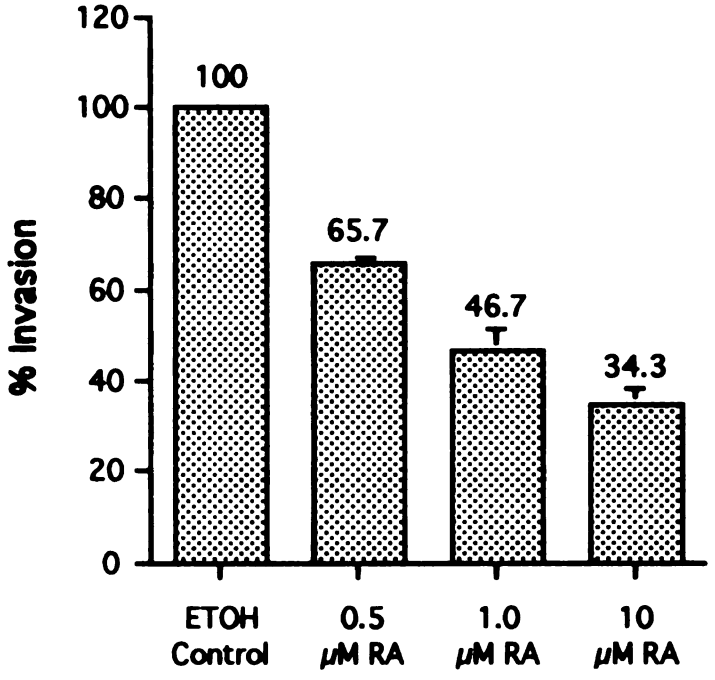

Figure 3-7: Effects of retinoic acid on the invasive ability of DU-145 cells as examined by Boyden blind well *in vitro* invasion assay. Cells were pretreated with RA for 48 h in serum-free medium. 200,000 cells were plated on each "Matrigel" coated filter and further exposed to RA during the 5 h assay period. Data represents a mean+/- S.D. of three experiments. ETOH: Ethanol.

DISCUSSION

Plasminogen activators are considered to play an important role in extracellular proteolysis. Increased u-PA secretion may permit localized proteolysis at the invasive front of the tumor and allow cell detachment, migration and invasion to occur. While a transient, local breakdown of the basement membrane and ECM occurs in a number of normal biological processes, such as wound healing and tissue remodelling, the consequences of uncontrolled matrix degradation can be severe, as in tumor invasion and metastasis. A main difference between a benign and a malignant tumor is the ability of the malignant tumor to invade the surrounding normal tissue and metastasize to distant sites. There is a direct correlation between protease activity and metastatic potential, e.g., high PA and type IV collagenase levels are associated with invasive and metastatic potential (6,9,12,13). Evidence for the association between increased u-PA expression and invasion and metastatic ability of cancer cells is accumulating. For example, EGF increases u-PA expression and invasive ability of PC3 human prostatic carcinoma cells (14). We have shown that DU-145 cells express five times more extracellular, secreted u-PA activity than the tested normal prostatic epithelial cells (9). In human pulmonary adenocarcinomas, more cells in metastases and advanced invasive human lung carcinomas were positive for u-PA than in low grade tumors. Therefore, u-PA was used as a prognostic indicator of tumor growth and metastasis (12). Nude mice inoculated with a human squamous cell carcinoma developed invasive tumors expressing high u-PA, and anticatalytic monoclonal antibodies to u-PA inhibited invasion (15). In human breast cancer, those tumors expressing increased u-PA

showed high risk for early recurrence and poor prognosis (13). In primary breast cancer, metastasis-free survival was best predicted by u-PA levels (16). In bladder cancer patients, tumor recurrence and progression were associated with high u-PA content (17). According to one study, the androgen-responsive, human prostate carcinoma cell line LNCaP does not secrete u-PA, lacks u-PA receptor and shows low metastatic potential while DU-145 and PC-3 cell lines secrete u-PA, express u-PA receptor and are highly invasive in an *in vitro* invasion assay (18). These studies demonstrate a close association between u-PA activity and the ability of tumors to invade and metastasize.

Our results show that u-PA can degrade fibronectin, that its further degradation is accomplished by plasmin and that the ECM components can serve as substrates for u-PA, thereby establishing an important mechanism of localized proteolysis in prostate cancer cell invasion. Thus, u-PA secreting cells in early foci of invasive carcinoma and prostatic carcinomas could cause massive degradation of ECM glycoproteins fibronectin and laminin, mediated by u-PA and plasmin. Fibronectin degradation by u-PA, in the absence of plasminogen, was also observed by Gold et al., (19) and cleavage products of fibronectin differed when u-PA or plasmin were used. Our results confirm this finding. Urokinase has cleavage specificity for arg-val bonds in plasminogen and in synthetic substrates and could similarly cleave such bonds in fibronectin (20). Activation of plasminogen by u-PA is enhanced by the presence of soluble fibronectin (21) and by a laminin peptide (22) which further enhance the degradative proteolytic cascade. It is interesting to note that fibronectin fragments have been found in high concentration in plasma cryoprecipitates from cancer patients (23). We propose that u-PA plays a key role in the proteolytic cascade in prostate cancer. Some evidence for the involvement of a proteolytic cascade independent of metalloproteinases has been

recently presented by Mackay et al. (24). These investigators propose that tumor cells possess a mechanism for the degradation of type IV collagen which is plasminogen-dependent but metalloproteinase-independent and suggest that plasmin may be responsible for this degradation and that type IV collagenase may not be absolutely required. Due to the architecture of the BM and ECM, laminin and fibronectin protect collagen from degradation, so they must be first removed before collagen degradation will occur (24).

Proteases which degrade the BM play an important role in the progression of carcinoma *in situ* to invasive carcinoma. Tumor cells which secrete u-PA are able to recruit the widely distributed proteolytic system involving plasminogen, which is a circulating zymogen present in high concentrations in plasma and extracellular fluids. Since plasmin has a wide substrate specificity and since it can activate pro-collagenases, including type IV collagenase, it is logical to consider that u-PA would play a crucial role in the initial stages of invasion by prostate cancer cells. Even if the amount of u-PA produced by invasive cells is small, the autocatalytic nature of pro-u-PA (25) and the relatively high concentration of plasminogen in ECM would yield high local levels of plasmin, an enzyme with ability to degrade ECM. The observation that invasion and metastasis (15) can be inhibited by antibodies to u-PA provides further credence to the proposition that u-PA has a significant role in invasion and metastasis.

Treatment of DU-145 cells with retinoic acid resulted in the inhibition of fibronectin and laminin degradation by serum-free conditioned medium. This effect is reflected in the marked decrease in the invasive ability of RA-treated DU-145 cells (Figure 3-7). Other evidence also shows that retinoids can inhibit invasion, decrease collagenase and PA activity, reduce invasive and metastatic potential of human and

rodent cells *in vitro* and *in vivo* (3, 6). For example, human melanoma cell lines treated with 10 μM RA secreted lower levels of plasminogen activators and type IV collagenase than untreated cells and showed concomitant decrease in invasion through "Matrigel" (6). Retinoids also stimulate ECM production, alter cell adhesion properties, modulate TGF-ß production and inhibit angiogenesis (2,3,26). Our results show that retinoic acid can decrease the extracellular proteolytic activity, ECM degradation and invasion. Studies are in progress to examine the effects of RA on TGF-β and u-PA inhibitor expression.

As far as the proposition that prostatic cancer cells have an inherent ability and predilection of early invasion is concerned, of special interest are PIN, the precancerous lesions which precede microinvasion and progress to invasive carcinoma (27). PIN show focal proliferation, cellular disorganization and heterogeneity, and disruption of the basal epithelial cell layer followed by disruption of the BM in advanced lesions of high grade PIN. Changes in the polarity of secretion by epithelial cells may take place as a result of dysplasia and anaplasia. In normal prostatic glands *in vivo*, polarized secretion of prostatic proteins including u-PA, takes place at the apical end of glandular epithelium. Cells of a rat prostatic carcinoma cell line grown at low density in medium containing 5% FBS, showed morphology resembling squamous metaplasia and anaplasia. This resulted in a change in polarized secretion so that now cells secreted urokinase equally at the apical and the basal ends (28). Such morphological changes may be comparable to dysplasia and anaplasia in late PIN and their progression to invasive carcinoma (27). Thus, *in vivo*, the disorganization of cellular polarity may result in increased secretion of u-PA at the basal end, resulting in localized proteolysis of the ECM in high grade PIN. Data presented here provide some clues into the mechanisms involved in the intrinsic ability of prostatic cancer cells to degrade the

ECM at the transition from high grade PIN or carcinoma *in situ* to invasive carcinoma. Inhibition of early lesions such as PIN, before the onset of invasion, is especially important in the control of invasive carcinoma. Since retinoids are considered to be important in chemoprevention (29), it is logical to consider the idea that agents such as RA, which can reduce extracellular protease activity and enhance ECM synthesis and integrity, may play an important role in blocking progression of PIN to invasive carcinoma.

The net extracellular matrix proteolysis depends on the ratio between the levels of extracellular or cell-membrane bound, active urokinase secreted by tumor cell and the levels of plasminogen activator inhibitors PAIs, which are u-PA's natural inhibitors. The balance between u-PA and PAI must be in favor of active u-PA in order for proteolysis to occur. It has been shown that some tumor cells also produce high levels of protease inhibitors (30). Thus, it will be necessary to study the balance between proteases and their natural inhibitors. The identification of synthetic serine protease inhibitors has important application in prostate cancer prevention and intervention by inhibition of invasion and tumor progression to a metastatic state.

We propose that urokinase plays a pivotal role in, and occupies a place at the apex of the proteolytic cascade and initiates the degradative process in prostate cancer invasion. This process subsequently recruits collagenases, after the pro-collagenases are activated by plasmin, formed by the activation of plasminogen by u-PA. This proteolytic cascade may include activation of pro-u-PA to active u-PA by autocatalysis and by plasmin, activation of plasminogen to plasmin by u-PA, direct degradation of fibronectin by u-PA, degradation of fibronectin and laminin by plasmin, and activation of collagenases by plasmin. Thus, u-PA-dependent fibronectin and laminin degradation may be a prerequisite for subsequent collagen degradation. Montgomery et al (31) have

shown that a rapid removal of fibronectin and laminin precedes dissolution of collagens. As a result, focal dissolution of the ECM could take place in advanced PIN followed by invasion and metastasis. The autocatalytic activity of u-PA (25) in itself may be sufficient to initiate the enzymatic cascade.

In conclusion, we propose that due to the constitutive property of prostatic epithelium to secrete urokinase, pre-invasive prostatic lesions, such as high grade PIN, have an inherent ability and predilection to invade and metastasize. This may be the reason why 48% of the patients with prostatic carcinoma already have disseminated disease at first diagnosis by rectal examination (32). This also provides suggestive evidence for early metastasis of prostatic carcinomas. Since degradation of endothelial ECM is necessary for angiogenesis, we suggest that u-PA secreted by cancer cells will promote angiogenesis in very small tumors and enhance invasion and metastasis. Our results show that retinoic acid has the ability to decrease the net extracellula proteolytic activity and, thus, decrease ECM degradation and invasion. The mechanisms involved in these RA effects are under investigation. Inhibitors of extracellular proteolyti activity at the cell:matrix interface may have therapeutic value in cancer prevention and inhibition of invasion and metastasis. Our new cell models of immortalized and malignant prostatic epithelial cell lines derived from adult human prostate (33) should facilitate this work.

ACKNOWLEDGEMENTS

We thank Dr. Jean Burnett for her critical evaluation of this paper and Diano Bello, Beth Roestel, Daniel Edwards, Salmaan Quader and W. Scott Metcalfe for their invaluable assistance.

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CHAPTER 4

Inhibition of invasion by Du-145 human prostate carcinoma cells transfected with plasminogen activator inhibitor-1 gene

ABSTRACT

Urokinase (u-PA) plays an important role in the complex processes of tumor invasion and metastasis. Its activity is regulated by specific, plasminogen activator inhibitors. In this study, DU-145 human prostate carcinoma cells, which express high levels of u-PA, were transfected with full length PAI-1 cDNA in an SV_{40} vector (pSV2-PAI-1). Cells transfected with vector (pSV_2-neo) only, with antisense or sense strand of PAI-1 cDNA {PAI-1 (As)}, {PAI-1(S)} were selected. PAI-1 (S) clones expressing low u-PA activity were selected by SDS-PAGE zymography. Untransfected DU-145 cells, Vector and AS clones showed similar u-PA activity, however, 3-2c, 3-2b and 2-2e PAI-1 (S) clones showed decreased but variable levels of u-PA activity, with 3-2b and 2-2e showing the least u-PA activity. Further, DU-145, Vector and AS controls, showed no or only weak staining for PAI-1 antigen as demonstrated by immunostaining, however, PAI-1 (S) clones showed variable level of PAI-1 expression, with 2-2e showing the maximum PAI-1 expression. A correlation between an increase in u-PA/PAI-1 complex and a concomitant extracellular decrease in u-PA activity was observed. Densitometric analysis of Western blots of conditioned medium demonstrated an increase in total PAI-1 including free and complexed PAI-1 in PAI-1 sense transfectants as compared to controls. If the total percent intensity of PAI-1 bands for DU-145 cells is taken as 100%, then, that of Vector and AS was 98.8% and 93.8 % respectively, while PAI-1 sense {PAI-1 (s)} transfected clones express high PAI-1 protein with 3-2c showing, 108.8%, 3-2b, 273.8% and 2-2e, 288.8% of control. Clones 3-2b and 2-2e showed the highest u-PA/PAI-1 complex formation and the least u-PA activity. Further, PAI-1

transfected clones showed a decrease in cell growth as well as in the invasive ability through a reconstituted basement membrane in an *in vitro* invasion assay. The growth of {PAI-1 (S)} clones, 3-2c, 3-2b and 2-2e was reduced to 70.5, 50.5 and 43.5 respectively as compared to DU-145 control cells which were taken as 100%. The invasive ability of clones 3-2c, 3-2b and 2-2e was reduced to 72.2%, 43.4% and 38.7% of DU-145 control respectively, where invasion by DU-145 cells was taken as 100%. Treatment of the invasive DU-145 cells with PAI-1 antigen also resulted in a dose-dependent decrease in invasion. At 100, 500 and 1000 U/ml, PAI-1 reduced invasion to 83%, 58.5% and 47.2% of untreated control, respectively. Taken together, these results demonstrate that an increase in the expression of PAI-1 in DU-145 cells neutralizes the secreted u-PA activity and this is accompanied by inhibition of growth and invasion. It is further shown that a balance between u-PA, a key protease involved in prostate cancer invasion and metastasis, and its inhibitor PAI-1, must be maintained in order to control invasion by prostate cancer cells. A decrease in extracellular u-PA activity and invasive ability occurred as a result of transfection of PAI-1 gene into DU-145 cells. It is proposed that PAI-1 may act as a tumor suppressor gene.

INTRODUCTION

Cancer cells are characterized by their ability to invade and metastasize, which involves localized dissolution of the basement membrane (BM) and extracellular matrix (ECM). The plasminogen activation system seems to play an important role in these processes. In particular, urokinase type plasminogen activator (u-PA) has been associated with the invasive and metastatic potential of various malignant cells, including prostate cancer cell lines (6, 16, 21,30).

Endogenously secreted u-PA catalyzes the activation of cell-associated plasminogen to plasmin, a serine protease which is capable of degrading extracellular matrix components and activating type-IV procollagenase to collagenase (34). Urokinase is a key protease secreted by prostatic epithelial cells and is involved in the liquefaction of semen (36,37). During prostate carcinogenesis, an increase in u-PA expression may lead to localized dissolution of the BM and ECM components. Urokinase activity is tightly regulated, *in vivo*, by specific plasminogen activator inhibitors, which include plasminogen activator inhibitor-1 (PAI-1) and plasminogen activator inhibitor-2 (PAI-2) (4). These inhibitors regulate the activity of u-PA by covalently binding to the active site of receptor bound (9) or secreted u-PA, thus, neutralizing its activity (34). This controlled mechanism is disturbed in cancer cells, resulting in increased plasmin generation due to either increased u-PA or decreased PAI production. Therefore, it is postulated that inhibition of u-PA activity by increasing PAI levels should result in decreased plasmin production, inhibition of ECM degradation and of invasion and metastasis. Some evidence for

this hypothesis is provided by the observations that inhibition of u-PA activity either by u-PA anticatalytic antibodies or exogenously added PAI-1 or PAI-2 results in decreased ECM degradation and inhibition of *in vitro* invasion and *in vivo* metastasis in nude mice (8, 18, 20, 24, 29).

In the present study, the role of PAI-1 in invasion by DU-145 human prostatic carcinoma cells was analyzed. PAI-1 is a 52 kDa glycoprotein, expressed by a variety of cells including endothelial cells, fibroblasts and smooth muscle cells (4). It is bound to ECM in an active form and it controls proteolytic activity at the cell-matrix interface, thus, protecting ECM from degradation (17). The primary objectives of this study were: (i) to transfect DU-145 cells with full length PAI-1 cDNA; (ii) to select clones expressing high PAI-1 levels; (iii) to compare the growth of DU-145 cells with those transfected with PAI-1 (sense), (antisense) and vector only; (iv) to compare the extracellular secreted u-PA activity and PAI-1 protein in cells transfected with PAI-1 sense or antisense DNA and vector alone, with the untransfected DU-145 cells., (iv) to compare the PAI-1 expression by immunostaining technique in DU-145 cells with cells transfected with PAI-1 (sense), (antisense) or vector only., (v) to compare the invasive ability of DU-145 cells with the transfected cells.

MATERIALS AND METHODS

Materials: Cells: Human prostatic carcinoma cell line DU-145 #HTB 81, American type Culture Collection (ATCC); SF-CM from HUVEC cells provided by Dr. H. K. Kleinman, NIH, Bethesda, M.D. RPMI-1640 medium #320-1875AJ; antibiotic/antimycotic mixture #600-5240AG; Gibco; fetal bovine serum (FBS), Intergen; human plasminogen #P5661, Sigma; gelatin for zymography #170-6537; high and low molecular weight standards #161-0303 and 0304, BioRad; monoclonal antibody to PAI-1 #379, American Diagnostica; goat anti-mouse second antibody #8611-3711, Organon Teknika; filter units for concentrating conditioned medium: Centriprep 10 #4304, Amicon; Immobilon-P transfer membrane IPUH-304 FO, Millipore; BCA protein assay kit # 23236, Pierce; Avidin-Biotin immunoperoxidase Vectastain ABC kit #AK5001; Vectastain Elite ABC peroxidase kit # PK-6102 and 3,3'-diaminobenzidine (DAB) substrate kit # SK-4100, Vector; 12 mm circular coverslips # 12-545-80, Fisher Scientific; alkaline phosphatase substrate 4-nitro blue tetrazolium chloride #1087-479 and 5-bromo-4-chloro-3-indolyl phosphate #760-986, Boehringer-Mannheim; for invasion assay, Nuclepore filters # 150446 (8μm pore size), costar; HEMA-3 122-911, Curtin Matheson; for DNA transfection, pSV_2-neo plasmid donated by Dr. Johng. S. Rhim, NIH, Bethesda, M.D.; polybrene #107689, Aldrich Chemicals Co.; DMSO ATCC; geneticin # G-5013, Sigma; restriction enzymes and DNA ligase, Gibco; Wizard Maxi prep kit #A-7270, Promega. BS-KSII plasmid was donated by Dr. W. Kopachik, Michigan State University, East Lansing, MI; and full length PAI-1 cDNA was donated by Dr. Ginsburg, University of Michigan, Ann Arbor, MI (11).

Cell Culture: DU-145 cells were maintained in RPMI-1640 medium containing 2 mM glutamine, 100 U penicillin, 100 μg streptomycin and 0.25 μg fungizone/ml, and 5% fetal bovine serum (FBS).

pSV_2-PAI-1 Plasmid Construction: The pSV_2-PAI-1 plasmid was made from pSV_2-neo vector and 2.0 kb full length PAI-1 cDNA. Full length PAI-1 cDNA was inserted into BS-KS11 plasmid at *Eco*R1 site and allowed to grow. At the time of construction of pSV_2-PAI-1 plasmid, BS-PAI-1 plasmid was digested with *Eco*R-1 enzyme and the 2.0 kb PAI-1 fragment was isolated by electroelution from 1% agarose gel and purified. pSV_2-neo was linearized by digestion with *Eco*R-1. The full length PAI-1 cDNA fragment and the linearized vector (pSV_2-neo) were then ligated overnight at 4°C with 2 units of T_4 DNA ligase. This ligated DNA was used to transform E.Coli DH5. The pSV_2-PAI-1 clones were selected by colony hybridization using a probe to PAI-1. Plasmid DNA of selected clones was screened for pSV_2-PAI-1 in the sense (S) and antisense (AS) orientation by restriction endonuclease digestion with Bgl II. Bgl II was used, first, because it digests PAI-1 cDNA into unequal fragments and second because the vector has only one Bgl II site. The clones with sense and antisense orientation as well as vector only, were amplified and the plasmid DNA was prepared in large scale by using the Wizard maxi prep kit. The final plasmids were confirmed by additional restriction endonuclease digestion.

DNA transfection: Transfection was performed by the polybrene method (30). DU-145 cells were trypsinized, counted and plated at a density of $1X10^6$ cells per 100 mm plate and incubated at 37°C in 5% CO_2. At 70% confluence, the DNA from pSV_2-neo only, pSV_2-PAI-1 in antisense or sense orientation was added to the cells as

follows: The medium was aspirated, cells were washed twice with PBS and replaced with 2.5 ml of culture medium containing 10 μg of specific DNA along with 10 μg/ml of polybrene and incubated for 24 h. The cells were then shocked by exposure for 4 min at room temperature with 5 ml of culture medium containing 30% DMSO. The cells were washed twice with PBS and fed with fresh culture medium. After two days, the medium was changed to the medium containing 250 μg/ml of geneticin for selection of geneticin resistant cells. The pSV_2-neo vector contains the neomycin (neo) gene which confers resistance to geneticin. The cells were re-fed with geneticin-containing medium every three days and were visually inspected for viability every day. At four weeks after the start of geneticin treatment, the surviving clones were expanded for further analysis.

Preparation of serum free conditioned medium (SF-CM): Samples of SF-CM from human umbilical vein endothelial cells (HUVEC) were used as a positive control for PAI-1 expression by Western blot analysis. One million cells were plated/100 mm culture dish in their respective media. DU-145 and PAI-1 clones were maintained in RPMI-1640 medium with 10% FBS. At 70% confluence, the cells were washed twice with PBS, and 8 ml of fresh serum-free RPMI medium was added. The SF-CM was collected after 48 h and centrifuged at 2000 rpm for 5 min to remove cell debris. A sample of SF-CM from HUVEC cells was also provided by Dr. H.K. Kleinman. For Western blot analysis, the SF-CM was concentrated using millipore filter units with a molecular weight cutoff of 10,000. The protein content of SF-CM was determined using the BCA protein assay reagent with bovine serum albumin (BSA) as a standard. All samples were stored at -75^{o}C until used.

MTT assay for comparison of growth in clones and DU-145 cells: The microculture tetrazolium assay was based on the method of Alley et.al. (3). The principle of this assay is, that 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) reacts with viable cells in culture and is reduced to formazan which, following solubilization with DMSO, can be measured using a microtiter plate reader at 540 nm wavelength. The viable cell number/well is directly proportional to the amount of produced formazan. Ten thousand cells per well were plated in a 96 well plate in 200 μl of RPMI-1640 and 5% FBS with five replicates/cell type. Medium was changed every 72 h. After a period of five days, the plate was fixed and stained with MTT. 5mg/ml of MTT stock solution was prepared in PBS, filtered using 0.45 μm filter unit and stored at 4°C for a maximum period of one month. On day five, the culture medium was removed and 50 μl of MTT solution diluted 1:5 in the pre-warmed culture medium was added to each well and incubated for 1 h at 37°C. Following incubation, all but 20 μl of medium was removed from each well and 150 μl of DMSO was added to each well to extract the stain. Following thorough formazan solubilization by triturating each well, the absorbance was measured at 540 nm using a microtiter plate reader.

Gel electrophoresis and Zymography: To compare the u-PA activity of selected clones, SDS-PAGE zymography was performed as previously described (15, 36). The separating gel contained 10% polyacrylamide gel, into which 12 μg of solid plasminogen and 1% gelatin substrates were incorporated. PAs have species specificity for plasminogen, hence human plasminogen was used. The volume of SF-CM samples loaded per lane in the gel was based on a fixed protein concentration (0.1 μg). The low molecular weight marker sample, ranging from Mr. 21,000 to Mr. 97,000 was diluted 1:10 in Laemmli gel loading buffer containing ß-mercaptoethanol, boiled for

3 min and cooled. SF-CM was mixed with 5 μl of Laemmli gel loading buffer without ß-mercaptoethanol and loaded onto a gel. Minigels were run at 200 V at 4°C for 45 min, then gently rocked in two changes of 2.5% triton X-100 in distilled water for 1 h at room temperature to remove SDS, and incubated at 37°C for 18 h in Tris-HCl buffer (pH 7.6) to allow re-naturing of the enzyme. The gels were stained with Coomassie blue and destained in methanol:acetic acid:water (3:1:6). The presence of enzyme activity was indicated by specific bands of lysis against a dark background.

Immunostaining for PAI-1: For detection of PAI-1 protein in cells by immunostaining, 10,000 cells/well were plated on 12 mm circular coverslips in 24 well plates. At 75% confluence, cells were fixed in 50:50 methanol:acetone at room temperature and stored at -80°C, if not immediately used. PAI-1 expression was detected by avidin-biotin immunoperoxidase method (39). Cells were thawed in PBS for 10 min, blocked in normal horse serum for 1 h and washed twice with PBS and between all subsequent steps. Cells were then incubated overnight at 4°C with monoclonal primary antibody to PAI-1 diluted 1:300 in normal horse serum. The following steps were conducted at room temperature. After primary antibody treatment, cells were incubated with biotinylated secondary antibody, diluted 1:200 in horse serum for 30 min; in H_2O_2 for 3 min to quench endogenous peroxidase activity; in avidin-biotin-peroxidase complex for 30 min and finally developed by using DAB-nickel chloride for 5 min. Cells were dehydrated and mounted on acid/alcohol washed slides. Control cells were treated similarly except that they lacked primary antibody treatment.

Western blot analysis for detection of plasminogen activator inhibitor-1: The transfected clones and DU-145 cells were analyzed for production of PAI-1 by Western blot analysis. A fixed amount of protein from SF-CM i.e., 15 μg/lane was separated on 10% SDS-PAGE minigels and electroblotted to immobilon transfer membrane for 1 h (36). The blots were stained using primary monoclonal antibody to PAI-1 diluted to 1:500 in 0.5% Tween-20 in PBS containing 1% BSA, followed by secondary biotinylated goat anti mouse at 1:10,000 dilution. The bands were visualized with the avidin-biotin alkaline phosphatase reaction.

***In Vitro* Invasion Assay:** An *in vitro* invasion assay was performed using boyden blind well chambers (2, 38). Two million cells were plated per 100 mm culture dish. At 80% confluence the cells were released from culture plates using 1mM EDTA, suspended in RPMI-medium containing 0.1% BSA and counted. A suspension of two million cells/ml in RPMI-0.1% BSA was made. The lower chamber was loaded with 220 μl of conditioned medium (chemoattractant) from NIH 3T3 cells. The NIH 3T3 conditioned medium was prepared as follows: At confluence, cells were washed twice with PBS and grown for 24 h in serum free medium containing 50 μg/ml ascorbic acid. The 24 h conditioned medium was collected, centrifuged at 2000 rpm to remove cell debris, aliquoted and stored at -20^{0} C for future use. In the upper chamber 400,000 cells/200 μl were plated on the Nuclepore filters coated with 500 μg/ml of Matrigel. Control consisted of RPMI-1640 medium containing 0.1% BSA only, without cells. The cells were overlaid with 650 μl of RPMI-0.1% BSA and allowed to migrate for 10 h. The migrated cells were fixed and stained with HEMA-3. Nuclear stain was extracted with 0.1 N HCl and the absorbance was measured at 620 nm using Titertek microplate reader, as described previously (14, 38). In addition, the cells in the lower

chamber were counted. The percent absorbance of migrated cells as well as the cells counted in the lower chamber were considered to calculate total invasion. Three replicate filters were prepared per cell type and the mean value from three such experiments was plotted. The total invasion for DU-145 cells was considered as 100% and the total percent of invasion for vector, AS, 3-2c, 3-2b and 2-2e clones was calculated using DU-145 as percent of control.

The ability of PAI-1 antigen to inhibit *in vitro* invasion of DU-145 cells was also examined. PAI-1 antigen at 100, 500 and 1000 U/ml concentration was added to 1×10^6 cells/ml cell suspension and incubated at room temperature for 2 h. These cells were then used for the invasion assay with 200,000 cells/well as described above. Cells were allowed to invade for 6 h.

RESULTS

Plasmid constructs, transfection and selection: The plasmid pSV_2-PAI-1 containing full length human PAI-1 cDNA with SV_{40} promoter and neomycin gene as a selective marker, was prepared and used to transfect DU-145 cells. The 7.6 kb construct is shown in Figure 4-1. The transfected cells were grown and selected in the presence of 250 μg/ml of G-418 for four weeks and the surviving clones were expanded. The plasmids containing PAI-1 gene in sense or antisense orientation were selected by

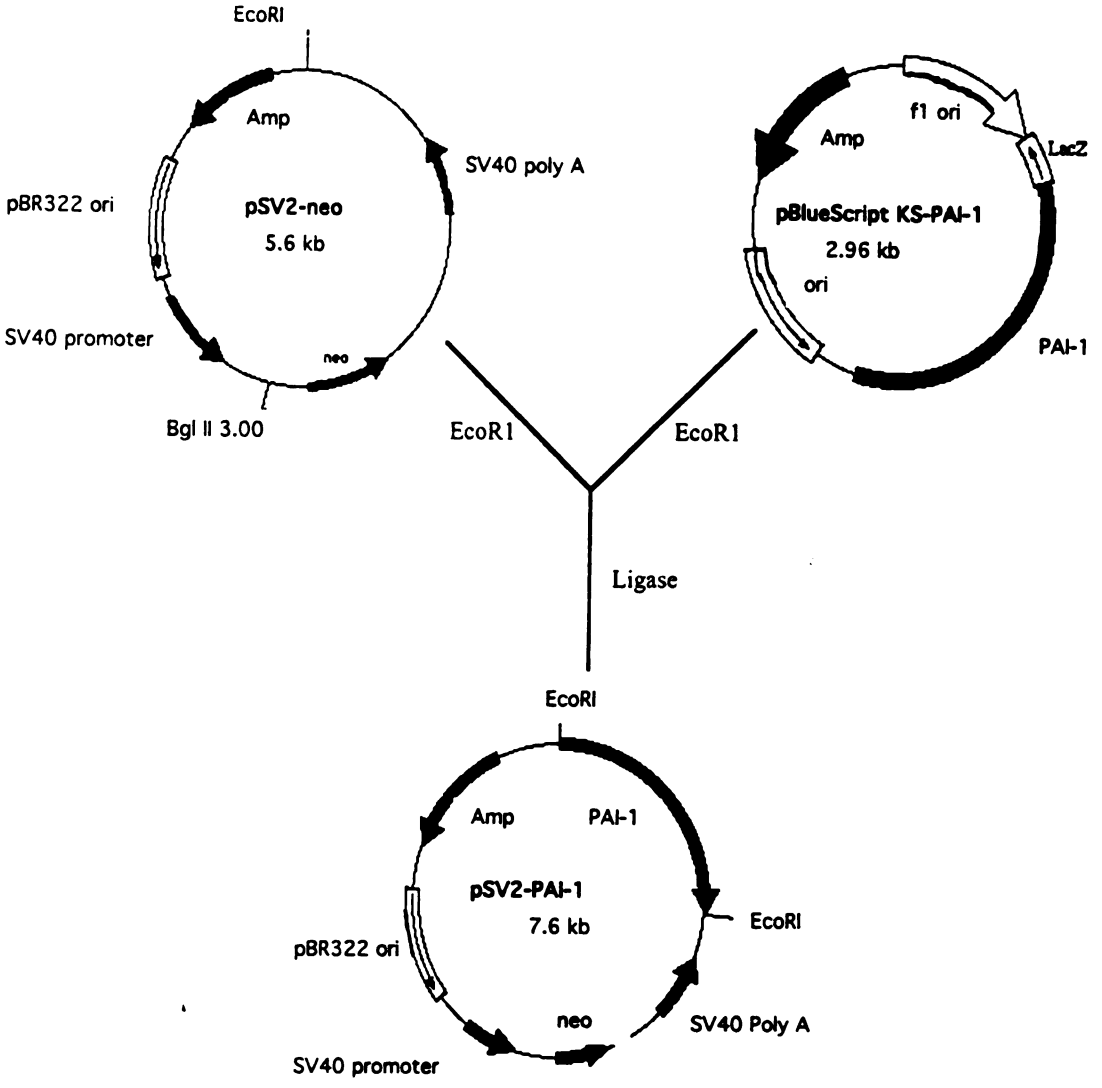

Figure 4-1: Construction of the plasminogen activator inhibitor-1 expression vector pSV2-PAI-1.

The human full length (2.0 kb) PAI-1 cDNA gene was obtained by digestion of plasmid BS-KS II vector with EcoR1. Plasmid pSV_2-neo was linearized by digestion with EcoR1 and PAI-1 cDNA was ligated to it. The plasmid pSV_2-PAI-1 contains SV_{40} promoter, pBR322 origin of replication and neomycin resistant gene.

restriction endonuclease digestion using Bgl II. Cells were then assayed for PAI-1 protein and u-PA activity. Untransfected DU-145 cells and DU-145 cells transfected with the pSV_2-neo plasmid alone or with PAI-1 antisense DNA strand were used as controls.

PAI-1 transfectants show a decrease in growth as compared to controls: The growth of various transfectants was compared with that of DU-145 cells. The assay was perfomed for a peroid of 5 days. The growth of DU-145 untransfected control cells was considered as 100%. The percent of growth for vector (V), antisense (AS), 3-2c, 3-2b and 2-2e clones was 81%, 80.5%, 70.5%, 50.5% and 43.5% of control respectively (Figure 4-2). These results show that vector and AS transfectants have similar growth rate, but were 20% less than DU-145 control cells. However PAI-1 (S) transfected clones 3-2c, 3-2b and 2-2e exhibited a decrease in growth, with 2-2e showing the slowest growth.

Comparison of u-PA activity by Zymography: Extracellular, secreted, u-PA activity in the CM from vector, antisense and sense clones was compared with that of DU-145 cells. Figure 4-3 shows that clones from vector and antisense have u-PA activity similar to that of DU-145 cells, but cells transfected with PAI-1 (S) show a decrease in u-PA activity. Different PAI-1 (S) clones show different levels of u-PA activity. DU-145, Vector and AS clones show a strong ~54,000, high Mr u-PA band as well as ~33,000 low Mr band. However all three PAI-1 (S) clones lack the ~33,000 Mr band and show variable activity at ~54,000 Mr with clone 2-2e exhibiting the least u-PA activity.

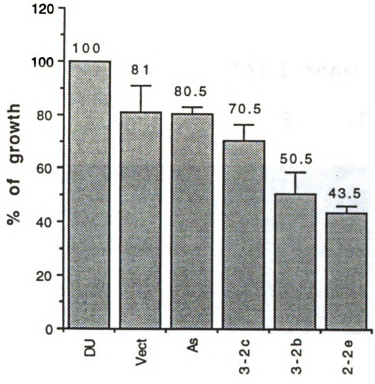

FIGURE 4-2: A comparison of growth of various transfectants
Ten-thousand cells per well were plated in a 96 well plate and the plate was stained on day 5. The viable cell number was measured by using MTT assay and the absorbance was read at 540 nm. Results represent the mean of two experiments with five replicate wells/cell type. DU: DU-145 cells; Vect: pSV_2-neo transfected clone; AS: PAI-1 antisense clone and 3-2c,3-2b and 2-2e PAI-1 sense clones. Bars; SD and the numbers above the bars indicate the % of growth.

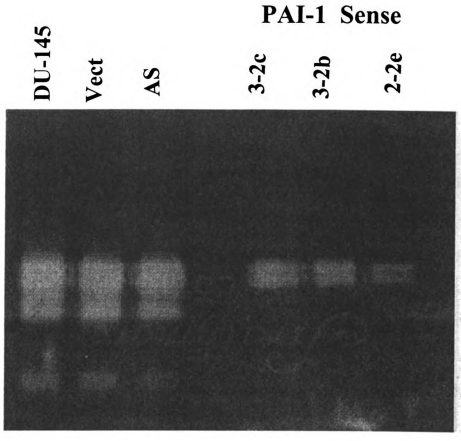

Figure 4-3: SDS-PAGE Zymography for detection of urokinase activity in PAI-1 clones.

urokinase (u-PA) activity in 48h serum free-conditioned medium (SF-CM) from DU-145 control, vector, antisense and PAI-1(sense) transfectants was analyzed by 10% SDS-PAGE zymograms. 0.1μg of proteins were loaded into each lane. Lane DU-145: untransfected control, Lane Vect: pSV_2-neo only transfected clone, Lane AS: PAI-1 (antisense) transfected clone, Lanes 3-2c, 3-2b and 2-2e: PAI-1 (sense) transfected clones. The Mr ~54,000 and ~33,000 bands represent high and low Mr u-PA bands. kDa, kilodaltons

Increased expression of PAI-1 in PAI-1 (S) clones by immunohistochemical analysis: DU-145 and different clones were stained immunohistochemically for PAI-1 using monoclonal antibody to PAI-1. Immunostaining results show (Figure 4-4) that the intensity of PAI-1 staining was strong in all PAI-1 (S) transfected clones as compared to DU-145 cells, vector and antisense transfected clones. The staining in DU-145, Vector and AS cells was similar to that of a control lacking primary antibody, however, in the sense clones PAI-1 staining was variable, with 2-2e showing the highest intensity.

Comparison of PAI-1 antigen levels by Western blot analysis: In this preliminary study, serum free conditioned medium was analyzed by Western blot analysis and PAI-1 levels were quantified by densitometry. The SF-CM from HUVEC cells was used as a positive control for PAI-1 expression. Results show (Figure 4-5) a double band at ~52,000 Mr in all cells which represents free PAI-1. The intensity of free PAI-1 band was in the decreasing order from DU-145 cells to 2-2e cells. The control HUVEC cells also showed a week band. In addition, a high ~106,000 Mr band, representing u-PA/PAI-1 complex was observed in AS, 3-2b and 2-2e PAI-1 (S) clones and in HUVEC cells and a low ~42,000 Mr band was observed only in PAI-1 (S) clones, 3-2c, 3-2b and 2-2e cells. However, in 3-2c PAI-1 (S) clone, a ~42,000 Mr band was very faint and the ~106,000 Mr u-PA/PAI-1 complex band was absent. This shows that PAI-1 (S) clones exhibit a decrease in free PAI-1 and an increase in u-PA/PAI-1 complex. The total PAI-1 which includes free and complexed PAI-1, was compared in cells by densitometric analysis as percent of intensity. If, the total percent intensity of PAI-1 bands for DU-145 is taken as 100%, then that for vector and AS was 98.8% and 93.8% respectively, while PAI-1 sense {PAI-1 (S)} transfected clones express high protein

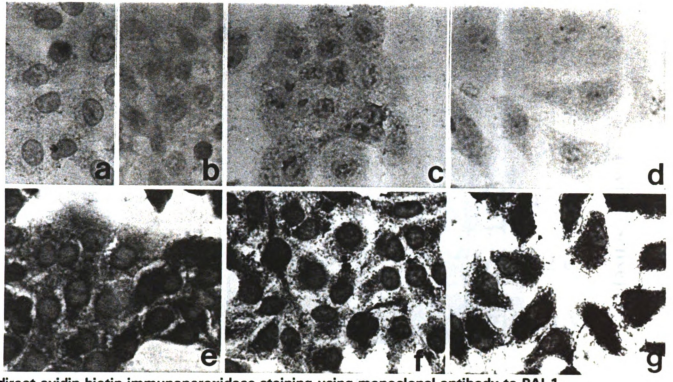

Figure 4-4: Indirect avidin-biotin immunoperoxidase staining using monoclonal antibody to PAI-1.
DU-145 control and all clones were stained with monoclonal antibody to PAI-1. (a) control without primary antibody; (b) DU-145 cells; (c) Vector transfected cells; (d) PAI-1 antisense transfected clone; (e-g) PAI-1 sense transfected clones. 3-2c, 3-2b and 2-2e.

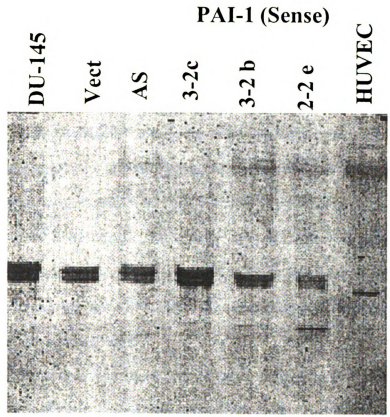

Figure 4-5: Preliminary results of Western blot analysis for PAI-1.
PAI-1 protein levels were analyzed by using concentrated serum free-conditioned medium (SF-CM) (15 μg) from DU-145 cells and different clones. Lane DU-145: DU-145 control, Lane Vect: pSV_2-neo vector only, Lane AS: PAI-1 (antisense), Lanes 3-2c, 3-2b, and 2-2e: PAI-1 (sense) clones and Lane HUVEC: SF-CM from Human umbilical vein endothelial cells (HUVEC). SF-CM from HUVEC cells was used as positive control for PAI-1. The blot was stained with monoclonal antibody to PAI-1. The ~ 52,000 band represents free PAI-1 and ~ 106,000 band is a u-PA/PAI-1 complex.

with 3-2c showing 108.8%, 3-2b, 273.8 and 2-2e, 288.8% of control. Thus, total PAI-1 level is inreased in PAI-1 (S) clones compared to controls, with 3-2b and 2-2e showing the highest PAI-1 level.

Increased expression of PAI-1 decreases *in vitro* invasion: The invasive ability of PAI-1 sense clones was compared with that of antisense, vector only and untransfected DU-145 cells (Figure 4-6). The PAI-1 (S) clones which show high amounts of u-PA/PAI-1 complex formation were less invasive than those producing only free PAI-1. The invasive ability of DU-145 cells was taken as 100% for comparison with the transfectants. In comparison to Du-145 cells, the vector and antisense cells showed 104.7% and 90.8% invasion respectively, whereas, PAI-1 sense clones 3-2c, 3-2b and 2-2e showed reduced invasion to 72.2%, 43.4% and 38.7% of control respectively. These results represent the average of three separate experiments with three chambers per experiment for each clone.

To further examine the effect of PAI-1 antigen on the invasive ability, DU-145 cell suspensions were pre-treated with different concentrations of PAI-1 antigen and allowed to invade. Results show that PAI-1 antigen decreased invasion by DU-145 cells in a dose dependant manner, with a reduction to 83%, 58.6% and 47.2% of control at 100, 500 and 1000 U/ml of PAI-1 concentration, respectively (Figure 4-7). These results suggest that the invasive ability of DU-145 cells can be decreased by increasing extracellular levels of PAI-1 either by transfection or by the addition of exogenous PAI-1.

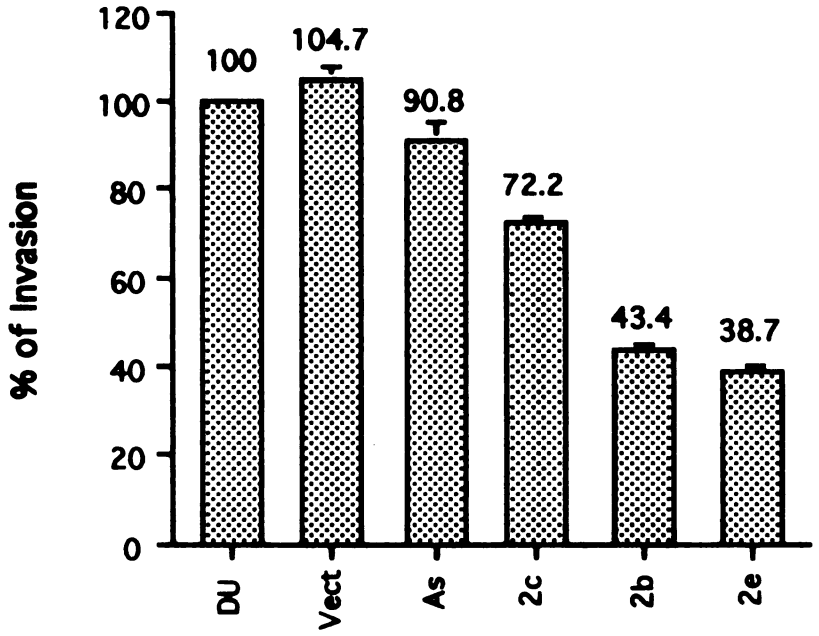

Figure 4-6: PAI-1 transfected cells show a decrease in their invasion as compared to control DU-145 cells.

The invasive ability of DU-145 untransfected cells was compared with various transfected clones. Four hundred thousand cells were overlaid on the matrigel-coated filters and allowed to invade for 10 h at 37^0 C. The filters were stained, the stain was extracted and the absorbance was read at 620 nm. The cells in the lower chamber were also counted. DU: DU-145 untransfected cells; Vect: pSV_2-neo transfected clone; AS: PAI-1 antisense and 3-2c, 3-2b and 2-2e PAI-1 sense transfected clones. Data represent average of three experiments. Bars; S.D.

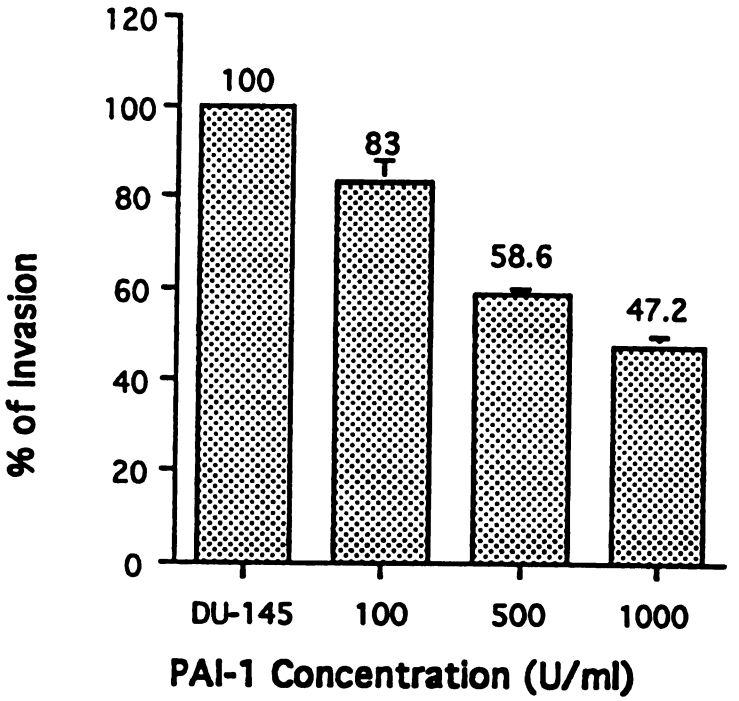

FIGURE 4-7: The effect of PAI-1 antigen on the invasive ability of DU-145 cells. A cell suspension of one million cells was incubated for 2 h with different concentrations of PAI-1 antigen. 100, 500 and 1000 U/ml of PAI-1 antigen concentrations were used. Two hundred thousand cells were overlaid on the matrigel coated filters and allowed to invade for 6 hr. Results represent the average of three experiments. Bars, S.D.

DISCUSSION

There is increased evidence to suggest that elevated levels of urokinase are associated with invasive and metastatic behavior of prostate cancer (1,6,22). We have previously demonstrated that DU-145, human prostate carcinoma cells secrete high levels of u-PA as compared to normal prostate epithelial cells and an increase in u-PA activity resulted in matrix degradation and invasion (36,37).

Since it is well established that increased proteolytic activity is required for ECM degradation and invasion, molecules involved in regulating proteolytic activity are important diagnostic tools. Several investigators have reported that an increase in the level of u-PA inhibitors results in a decrease in extracellular u-PA activity and, thus, a decrease in ECM degradation and invasion (8,20,25). However the role of plasminogen activator inhibitor in the progression of prostate cancer has not been studied. In the present study, the role of PAI-1 by transfecting full length PAI-1 cDNA into DU-145 cells was investigated.

The presence of PAI-1 in PAI-1 (S) transfected cell cultures was detected by immunohistochemical analysis and quantified by densitometric analysis of Western blots (Figures 4-4 and 4-5). In western blot analysis all cells expressed PAI-1 but u-PA/PAI-1 complex formation was observed only in AS, 3-2b and 2-2e clones and in HUVEC cells. In contrast, Lyon, et al., did not detect PAI-1 in DU-145 cells (22). This difference may be because in the present study PAI-1 was analyzed in concentrated conditioned medium whereas, Lyon, et al., analyzed unconcentrated cell lysates (22). An increase in PAI-1 level resulted in a decrease in extracellular secreted u-PA activity

as demonstrated by zymography (Figure 4-3). This decrease in u-PA activity may be attributed to neutralization of u-PA by u-PA/PAI-1 complex formation, as shown in the zymogram and Western blot analysis. Complex formation between u-PA and PAI-1 or PAI-2 resulting in a decrease in u-PA activity has been shown in various cancer cell lines after transfection with PAI cDNA (8,20). The absence of u-PA/PAI-1 complex in control DU-145 cells may be because several fold excess of PAI-1 is required to inhibit the u-PA activity (33). Therefore, PAI-1 (S) transfected clones, which express high levels of PAI-1 can complex with u-PA and inhibit its activity.

Increase in PAI-1 level by transfection resulted in a decrease in growth as well as *in vitro* invasion. In comparison to control cells the PAI-1 (S) transfected clones demonstrated a decrease in growth (Figures 4-2 and 4-6). These findings are in agreement with the previous reports where human prostate cancer cell line 1013-L, which does not express u-PA, shows slow growth of the tumor derived from these cells *in vivo* as compared to tumors derived from DU-145 cells that express high levels of u-PA (6). Mammary tumor cells that express MASPIN gene, a serine protease inhibitor present in mammary cells, also showed a decrease in growth *in vivo* as compared to those that did not express the MASPIN gene (41).

The DU-145 control cells and vector transfected clones, which express high u-PA activity and undetectable u-PA/PAI-1 complex, readily invaded through the reconstituted basement membrane "Matrigel", whereas, 3-2b and 2-2e PAI-1(S) trasfected clones, which express low u-PA activity and high u-PA/PAI-1 complex, showed reduced invasive ability (Figure 4-6). Also, exogenously added recombinant PAI-1 (rPAI-1) decreased invasion of DU-145 cells in a dose dependant manner (Figure 4-7). Similarly rPAI-1, rPAI-2 or antibodies against u-PA inhibited invasion in different cancer cell lines (5,7,18,24). Transfection of PAI-2 cDNA into HT-1080 cells

inhibited *in vitro* invasion and induced a thick peritumoral capsule *in vivo* (20). Additionally, in metastatic colon carcinomas, increased expression of PAI-1 prevented tumor cells from infiltrating by forming a thin collagenous capsule (32). Further, the metastatic ability of murine melanoma cell lines was increased after transfection with u-PA gene (40). These studies imply that increased u-PA activity is a significant factor in the development of a more invasive phenotype, which may be due to an increase in its expression or a decrease in or loss of antiprotease expression. In some cancer cells, loss of antiprotease genes has been observed. A deletion of PAI-2 gene was observed in 73% of colon cancers (35) and loss of MASPIN gene in mammary cancer cell lines resulted in invasion and metastasis. However, transfection with MASPIN gene inhibited their invasive and metastatic ability (41). These observations and the ability of PAI-1 transfected cells to inhibit growth and invasion suggests that PAI-1 or other antiproteases may be cancer suppressor genes.

Although increased expression of PAI-1 regulates the invasive phenotype of cancer cells, several investigators have reported that increased expression of u-PA and PAI-1 or PAI-2 are associated with poor prognosis in cancer patients (10,13,26). However, the net proteolytic balance between u-PA and PAI has not been analyzed. Since excess PAI is required to inactivate u-PA mediated proteolytic activities, it is critical to measure both u-PA and PAI-1 levels as well as u-PA activity in cancer patients. The role of PAI-1 in preventing ECM degradation and invasion was observed by Kristenson, et. al., in murine Lewis lung carcinoma (19). Immunohistochemical analysis showed that at focal contact areas there was a marked increase in u-PA with minimal or no increase in PAI-1 expression and these areas showed signs of ECM degradation and invasion into the surrounding normal tissue. Similarly, in lung tumor tissues, PAI-1 expression was lost in tumors of patients with worst clinical prognosis

but u-PA expression was high (12). In ovarian tumors both u-PA and PAI-1 levels were compared in malignant and benign tissues. u-PA levels were significantly higher in malignant compared to benign tissues, however, the PAI-1 levels were not significantly different. Therefore, the expression of PAI-1 was much lower in malignant tissues than that of u-PA, thus favoring metastasis. This suggests that the balance between u-PA and PAI-1 is important in order to inhibit u-PA mediated invasion and metastasis (27).

In human adenoid cystic carcinoma cells (ACCS) the role of PAI-1 in protecting ECM from degradation was studied. The ECM of these cells was resistant to self proteolysis due to the presence of excess of PAI-1 in the ECM, however, removal of PAI-1 by glycine extraction resulted in matrix degradation (31). Similarly, DU-145 cells in culture, which show high levels of extracellular u-PA activity but undetectable u-PA/PAI-1 complex, could degrade ECM and were invasive (36, 37). However, in the present study, transfection of DU-145 cells with PAI-1 cDNA resulted in increased expression of PAI-1 and a decrease in invasion. These observations suggest that cancer cells may express high levels of PAI-1 to inhibit u-PA mediated proteolytic activities, however, increased expression of PAI-1 may not be sufficient to inhibit these activities. In some malignant tumors PAI-1 expression is localized to fibroblasts and endothelial cells in the stroma suggesting that PAI-1 in such tumors may be involved in inhibiting angiogenesis (23,28).

The present study represents the first evidence that increased expression of PAI-1 by transfection results in a decrease in u-PA activity as well as in growth and invasion of DU-145 human prostatic carcinoma cells. These findings illustrate that u-PA and PAI-1 are produced by DU-145 cells but the level of PAI-1 produced is not enough to inhibit the u-PA mediated proteolytic activities. However, upon transfection with PAI-1 cDNA, extracellular u-PA activity was reduced resulting in a decrease in the

invasive behavior of transfected prostate cancer cells. Further studies need to be conducted to confirm the levels of u-PA/PAI-1 complex formation and to measure the u-PA and PAI-1 levels in these cells. It has been suggested that a plasminogen-activation dependent malignancy index, based on PA and PAI-1 levels, may be a better diagnostic tool in discriminating malignant from benign tumors and metastatic from non-metastatic cancers than by only measuring the u-PA levels (27). Therefore, by comparing the levels of u-PA and PAI-1, the net effect of the plasminogen activation system in prostate tumors could be better determined.

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CONCLUSIONS

The major goal of the research project described in this dissertation was to study the role of urokinase and its inhibitor, plasminogen activator inhibitor-1 in prostate cancer progression. Urokinase is considered to play an important role in extracellular proteolysis. Increased u-PA secretion has been implicated in the degradation of extracellular matrix accompanied by invasion and metastasis in a variety of cancers including prostate cancer.

Human prostatic epithelial cells constitutively secrete u-PA. It is proposed that during progression from prostatic intraepithelial neoplasia (PIN), the early neoplastic lesion, to invasive carcinoma, secretion of u-PA is increased resulting in ECM degradation and invasion. To demonstrate the importance of u-PA in prostate cancer, DU-145 human prostatic carcinoma cell line was used and the secreted u-PA activity in DU-145 cells with normal human prostatic epithelial cells was compared. DU-145 cells express at least five times more extracellular, secreted u-PA activity than normal prostate epithelial cells (Chapter 2). Further, the role of u-PA in ECM degradation, which is one prerequisite for cancer cell invasion, has been studied. It is demonstrated that u-PA alone can degrade fibronectin, but laminin degradation was resistant to u-PA action. However plasmin, produced by the activation of plasminogen by u-PA, was capable of degrading both laminin and fibronectin to smaller fragments (Chapter 3). In addition SF-CM from DU-145 cells, which secrete u-PA, was capable of degrading both FN and LN and these cells were invasive. These results suggest that u-PA plays a key role in prostate cancer invasion. Therefore, by inhibiting u-PA activity one may

control the progression of PIN to invasive carcinoma. The potential use of *all trans* retinoic acid (RA) in human prostate cancer prevention and progression has been assessed because RA plays an important role in normal epithelial cell proliferation and differentiation and can also reduce invasion and metastasis in human and rodent epithelial tumors *in vivo* and *in vitro*. Recent epidemiological studies suggest an increased risk for prostate cancer in men with low serum vitamin A levels.

Treatment of DU-145 cells with different concentrations of *all trans* retinoic acid for 48 h decreased extracellular, secreted u-PA activity and its antigen level by ~ 50% at 1μM RA concentration (Chapter 2). This decrease in u-PA expression resulted in a decrease in ECM degradation and in the invasive ability of DU-145 cells by 1μM RA (Chapter 3). These results show that RA may have important implications in chemoprevention of prostate cancer. The mechanism of RA effects on extracellular u-PA activity and expression is not yet understood. It has been suggested that RA may act via TGF-ß, which increases the expression of plasminogen activator inhibitor, a specific urokinase inhibitor.

Increased expression of PAI-1 results in complex formation with u-PA and thus, a decrease in u-PA activity. Therefore, the role of PAI-1 in prostate cancer progression was also analyzed by transfecting DU-145 cells with full length PAI-1 cDNA (Chapter 4). Results of the present study show that DU-145 cells express PAI-1 but {PAI-1 (S)} transfected clones express higher levels of PAI-1. {PAI-1 (S)} transfected clones showed a decrease in extracellular, secreted u-PA activity. Decrease in u-PA activity was apparently due to an increase in u-PA/PAI-1 complex formation as demonstrated by Western blot analysis which was not observed in DU-145 cells. Further, {PAI-1 (S)} clones showed a decrease in growth and invasion and exogenous addition of PAI-1 antigen also inhibited the invasive ability of Du-145 cells in a dose

dependent manner. These results show that by decreasing u-PA activity either by RA or PAI-1, prostate cancer cell growth, ECM degradation and invasion can be reduced. Thus PAI-1 may act as a tumor suppressor gene.

In conclusion, the work demonstrated in this dissertation provides evidence that by decreasing extracellular, secreted u-PA activity either by RA or by transfection of PAI-1 cDNA, it might be possible to reduce the progression of early lesions such as PIN to invasive carcinoma. In addition, these studies indicate that u-PA plays a key role in prostate cancer progression. These studies have important chemopreventive and therapeutic implications in prevention and treatment of prostate cancer.