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STUDIES ON THE ROLE OF UROKINASE (uPA) AND ITS CELL SURFACE RECEPTOR (uPAR) IN THE INVASION AND METASTASIS OF HORMONE DEPENDENT MALIGNANCIES

by

Rosie Hongmei Xing

A thesis submitted to the faculty of Graduate Studies and Research, McGill University, in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Department of Medicine

Division of Experimental Medicine

McGill University

Montreal, Quebec

Canada

ABSTRACT

Urokinase (uPA), a member of the serine protease family, and its cell surface receptor (uPAR) have been implicated in promoting the progression of various human malignancies including hormone dependent malignancies such as breast and prostate cancer. However, the underlying molecular mechanisms regulating uPA production in breast and prostate cancer progression are poorly understood.

In the current studies, we have examined the role of uPAR in breast cancer progression by developing a homologous model of uPAR overexpression by a rat breast cancer cell line Mat B III. Overexpression of uPAR resulted in increased breast cancer growth, invasion and metastasis *in vitro* and *in vivo*. Development of this syngeneic breast cancer model allowed me to examine the ability of the anti-estrogen, tamoxifen (TAM) and a synthetic active site inhibitor of uPA, 4-iodo benzo[b]thiophene-2-carboxamidine (B-428), to prevent breast cancer progression. TAM and B-428 treatment alone or in combination effectively prevented breast tumor growth, invasion and metastasis *in vitro* and *in vivo*. Morever, TAM and B-428 treatments caused a decrease in uPAR gene expression and protein production. These results underscore the utility of anti-proteolytic agents (B-428) in addition to standard hormone therapy (TAM) in advanced breast cancer patients where the uPA/uPAR system plays a key role in tumor progression. Regulation of uPA production by androgens in prostate cancer was then examined in the androgen insensitive PC-3 cells transfected with the functional human androgen receptor cDNA (PC-3T). Androgens down regulate uPA gene expression and protein production in androgen sensitive PC-3T cells. Furthermore, restoration of androgen responsiveness in PC-3T cells caused a dramatic decrease in tumor growth, invasion and metastasis *in vitro* and *in vivo*. Due to the ability of sex steroids to inhibit uPA gene expression, I have also examined the correlation between hormone sensitivity and uPA expression in several hormone responsive (HR) and hormone insensitive (HI) breast and prostate cancer cell lines. uPA mRNA was expressed only in the highly invasive, HI breast (MDA-231) and prostate (PC-3) cell lines. Failure of uPA mRNA expression in the minimally invasive, HR breast (MCF-7) and prostate (LnCAP) cells was due to transcriptional suppression of uPA gene. Southern blot analysis using methylation sensitive enzymes revealed that the absence of uPA gene transcription in HR breast and prostate cancer cells is due to

hypermethylation of the CpG islands of the uPA gene. These results clearly demonstrate that DNA methylation can differentially regulate uPA gene expression to alter the invasive behavior of tumor cells during breast and prostate cancer progression. Collectively, these results will enhance our knowledge of the mechanisms of regulation of uPA production. Results from these studies will also allow us to develop and evaluate novel therapeutic strategies aimed at improving the management and treatment of breast and prostate cancer.

RÉSUMÉ

L'urokinase (uPA), une sérine-protéase, ainsi que son récepteur de la membrane cellulaire (RuPA) ont été évoqués dans la progression de plusieurs néoplasie chez l'humain, notamment les néoplasies hormono-dépendantes tels les cancers du sein et de la prostate. Cependant, les mécanismes moléculaires régissant la production de l'uPA de ces cancers demeuraient incompris.

Afin d'étudier le role du RuPA dans la progression du cancer du sein, nous avons développé un modèle expérimental en surexprimant le RuPA dans la lignée cellulaire de cancer du sein chez le rat MAT B III. La surexpression du RuPA a été associée à une augmentation de la croissance du cancer du sein tant *in vitro* qu'*in vivo*. Grâce à ce modèle syngénique du cancer du sein, nous avons aussi mesuré l'efficacité du tamoxifène (TAM), un anti-oestrogène, et du 4-iodo benzo[b]thiophène-2-carboxamidine (B-428), un inhibiteur synthétique spécifique au site d'activité de l'uPA, à enrayer la progression du cancer du sein. Les traitements de TAM et le B-428 seuls ou en combinaison ont nettement diminué la croissance, l'invasion et les métastases tumorales *in vitro* et *in vivo*. D'ailleurs, les traitements au TAM et au B-428 ont significativement réduit l'expression génique et la production de protéines du RuPA. Ces résultats soulignent l'importance d'adjoindre des agents anti-protéolytiques (B-428) au traitement courant (TAM) pour les cancers du sein avancés dans lesquels le système uPA/RuPA contribue à la progression de la tumeur.

La régulation androgénique de la production d'uPA dans le cancer de la prostate a été étudiée dans une lignée de cellules humaine insensible aux androgènes PC-3 transfectée avec le récepteur androgénique humain d'ADN complémentaire (cDNA) fonctionnel (PC-3T). Dans cette dernière lignée PC-3T sensible aux androgènes, les androgènes régulent négativement l'expression génique et la production de protéines de l'uPA. De plus, le rétablissement de la réponse aux andrognène dans la lignée cellulaire PC-3T produit à une réduction impressionnante de la croissance, de l'invasion et des métastases tumorales *in vitro* et *in vivo*.

L'inhibition de l'expression génique de l'uPA par les stéroides sexuels nous a amené à étudier la relation entre la sensibilité hormonale et l'expressivité de l'uPA dans des lignées cellulaires humaines hormono-sensibles (HS) et hormono-insensibles (HI) pour les

cancers du sein et de la prostate. L'ARN messager (ARNm) de l'uPA est exprimé dans les lignées hautement envahissantes et HI: MDA-321 (sein) et PC-3 (prostate). L'absence d'expressivité de l'ARNm dans les lignées peu envahissantes et HS, MCF-7 (sein) et LnCAP (prostate), est attribuable à la suppression de la transcription génétique de l'uPA. Le transfert de Southern en présence d'enzymes sensibles à la méthylation a démontré que l'absence de transcription génétique de l'uPA dans les lignées cellulaire HS des cancers du sein et de la prostate est attribuable à l'hyperméthylation des îlots CpG du gène de l'uPA. Ces résultats démontrent, sans équivoque, que la méthylation de l'ADN peut réguler l'expressivité du gène de l'uPA et peut conséquemment moduler le comportment d'envahissement des cellules tumorales durant le développement des cancers du sein et de la prostate.

Les résultats de ces études contribueront à développer et évaluer des stratégies thérapeutiques novatrices afin de diminuer la morbidité et la mortalité des cancers du sein et de la prostate.

FOREWORD

The following except is taken from the Guidelines Concerning Thesis Preparation, Faculty of Graduate Studies and Research, McGill University, and applies to this thesis:

Candidates have the option of including, as part of the thesis, the text of a paper(s) submitted or to be submitted for publication, or the clearly-duplicated text of a published paper(s). These texts must be bound as an integral part of the thesis.

If this option is chosen, **concerning texts that provide logical bridges between the different papers are mandatory**. The thesis must be written in such a way that it is more than a mere collection of manuscripts: in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of "Guildlines for Thesis Preparation". **The thesis must include**: A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient details to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent**. Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers. **Under no circumstances can a co-author of any component of such a thesis serve as an examiner for that thesis.**

PREFACE

The work described in Chapters 2-5 of this thesis has been published in the foollowing journals:

Chapter 2: Xing, R. H., and Rabbani, S. A. Int. J. Cancer. 67: 1-7, 1996.

Chapter 3: Xing, R. H., Mazar, A., Henkin, J., and Rabbani, S. A. Cancer Res., 57: 3585-3593, 1997.

Chapter 4: Xing, R. H., and Rabbani, S. A. J. J. Clin. Invest., submitted, 1998.

Chapter 5: Xing, R. H., and Rabbani, S. A. Cancer Res., submitted, 1998.

The work presented in Chapters 2-5 is my own, except for the contributions of my co-authors as follows: in Chapter 3, Drs. A. Mazar and J. Henkin from Abbott Laboratories (Abbott Park, Illinois) were greatly appreciated for their input in designing the experiment and preparation of the manuscript.

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LIST OF ABBREVIATIONS

Ab:	antibody
AF:	activating factor
AR:	androgen receptor
ATF:	amino terminal fragment
5'-AzaC:	5'-azacytidine
B-428:	4-iodo benzo [b] thiophene-2-carbon-amidine
BCDR:	blood capillary density ratio
bFGF:	basic form of fibroblast growth factor
bp:	base pair
BPH:	benign prostatic hypertrophy
BSA:	bovine serum albumin
CAM:	chorioallantoic membrane
cDNA:	cloned deoxyribonucleic acid
CRE:	cAMP response element
CSF:	colony stimulating factor
DHT:	dihydrotestosterone
DNA:	deoxyribonucleic acid
ECM:	extracellular matrix
EGF:	epidermal growth factor
EGFR:	epidermal growth factor receptor
ELISA:	enzyme-linked immunosorbent assay
ER:	estrogen receptor
ERE:	estrogen response element
ERK:	extracellular signal regulated kinases
FBS:	fetal bovine serum
FGF:	fibroblast growth factor
FITC:	fluorescein isothiocyanate
FLU:	flutamide
GFD:	growth factor domain of uPA
GPI anchor:	glycosyl-phosphatidylinositol anchor

HGF: hepatocyte growth factor

HI: hormone insensitive

HMW uPA: high molecular weight uPA

HR: hormone responsive

HS: hormone sensitive

IGF: insulin-like growth factor

IGF-1, II: insulin-like growth factor I or II

Kb: kilobase

kDa: kilodalton

LMW uPA: low molecular weight uPA

MAPK: mitogen-activated protein kinase

5-mC: 5-methylcytosine

MMP: metalloprotease

mRNA: messenger ribonucleic acid

PA: plasminogen activator

PAI: plasminogen activitor inhibitor

PAI-1: type 1plasminogen activator inhibitor

PAI-2: type 2plasminogen activator inhibitor

PCR: polymerase chain reaction

PI-PLC: phosphoinositol phospholipase C

PKC: protein kinase C

PLCγ: phospholipase Cγ

PLD: phospholipase D

PNH: paroxysmal nocturnal hemoglobinuria

PN-I: protease-nexin

PSA: prostate specific antigen

Rb: retinoblastoma gene

RT: reverse transcriptase

SEM: standard error of mean

SDS: sodium dodecyl sulphate

SHBG: sex hormone binding globulin

TAM:	tamoxifen
TF:	tissue factor
TGF:	transforming growth factor
TGF-α:	transforming growth factor α
TGF-β:	transforming growth factor β
TNF:	tumor necrosis factor
tPA:	tissue-type plasminogen activator
TPE:	triphenylethylenes
uPA:	urinary-type plasminogen activator
uPAR:	urinary-type plasminogen activator inhibitor
VEGF:	vescular endothelial cell growth factor
VN:	vitronectin

CHAPTER 1

INTRODUCTION

1.1. GENERAL INTRODUCTION: TUMOR PROGRESSION IN HORMONE DEPENDENT MALIGNANCIES

Cancer is a systemic and malignant disease which is characterized by un-controlled cell growth and the progression of the initially localized non-invasive tumor to the later highly invasive and metastatic variety that eventually leads to the establishment of distant metastases (Fisher B, 1996). The clinical course of cancer is influenced largely by the process of invasion and metastasis, and the presence of metastatic lesions at distant organs is responsible for the high incidence of mortality associated with cancer. Therefore, a better understanding of the process of tumor progression is essential for the development of new and more effective therapeutic strategies to control and treat these malignant diseases.

Tumor progression is a complex multistep process initiated by a variety of initiation factors to promote the transformation of cells into their malignant phenotype (Figure 1.1). This is rapidly followed by irreversible genetic changes due to germline mutations (Andersen TI, 1996), amplification of oncogenes (Berns EM et al., 1995), mutation of tumor suppression genes (Holmquist GP and Gao S, 1997) which in combination with the growth factors and hormones (estrogen and androgen) rapidly promote this process of tumor progression. All of the steps and the interactions among various factors during tumor initiation have not been fully elucidated. However, a central feature to this process is cell proliferation (Clark R et al., 1994). Once the malignant cellular phenotype is acquired, the process of tumor invasion continues to take place under the influence of a different group of progression factors. During this process, transformed cells acquire additional genetic / epigenetic changes that confer additional growth advantages in specific tumor sub-populations which endow them with the ability of invasion to form metastatic lesions at

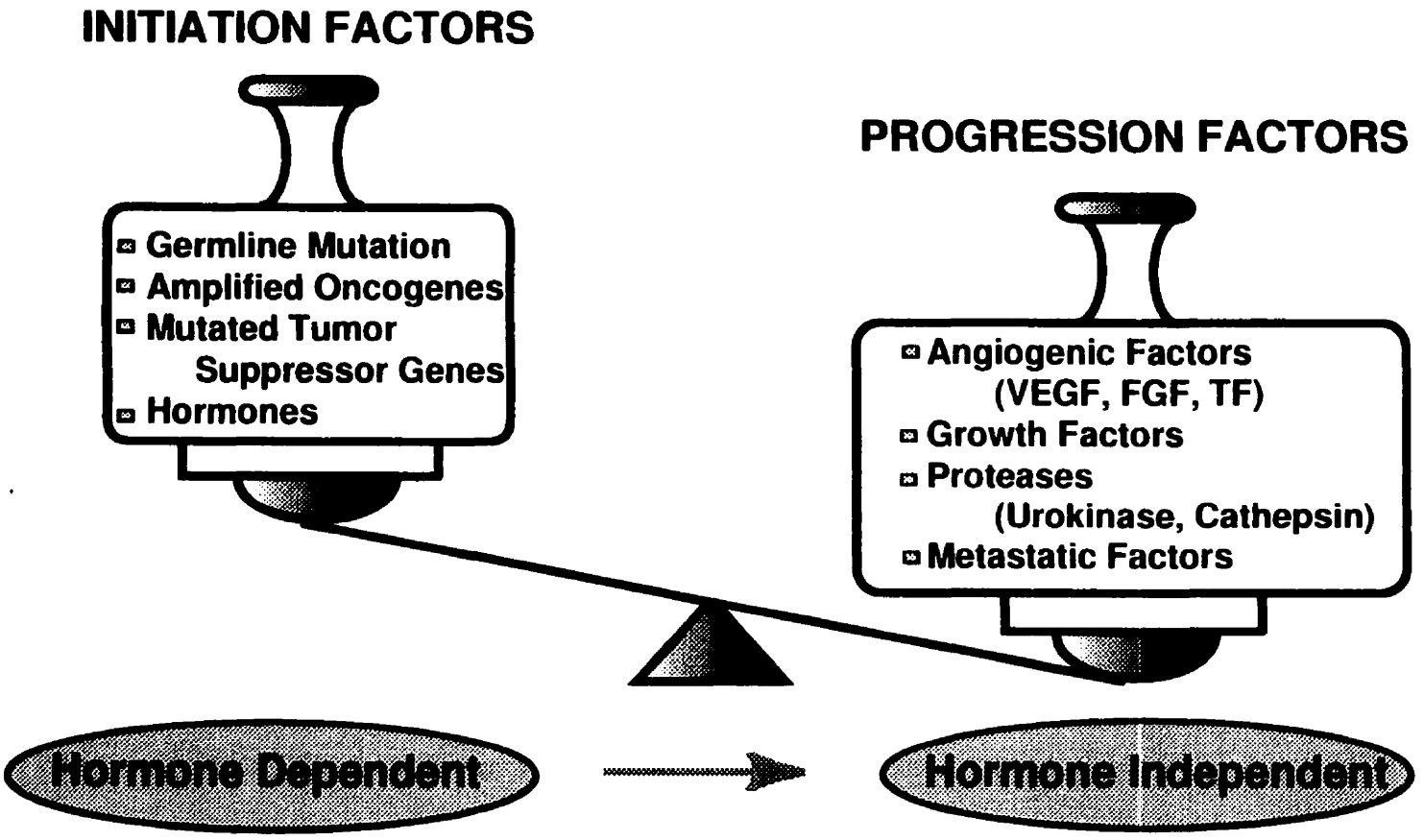


Figure 1.1: **Process of tumor initiation and progression in hormone-dependent malignancies**

secondary sites. These progression factors include various angiogenic factors (Engels K et al., 1997), such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and tissue factor (TF). Additionally growth factors like epidermal growth factor (EGF), transforming growth factors (TGF), and proteases like uPA and cathepsin also participate in this invasive process of the tumor cells promoting tumor metastasis to different organs (Dickson RB et al., 1992; Andreasen PA et al., 1992; Montcourrier P et al., 1990).

Over a century ago, Paget proposed the "seed and soil theory" of tumor metastases stating that metastasis was due to the specific affinity of certain tumor cells, the "seed", for the milieu provided by certain organs, the "soil" (Paget, S, 1889). In contrast to this, the "anatomic-mechanical theory" of metastasis emphasizes the importance of the vascular connections between the primary tumor and the secondary sites of growth (Ewing, J, 1928). Currently, it is recognized that both mechanisms play an equally important role in the establishment of metastases.

Tumor metastasis is a cascade of closely linked sequential steps requiring extensive host-tumor interations. In order to successfully give rise to a metastatic colony, tumor cells must detach from the primary tumor, invade the local host tissue, gain access to the vasculature through neovascularization, arrest at the distant vascular bed, extravasate into the target organ, and finally proliferate in the secondary site (Liotta IA, 1986; Fidler IJ,1990). These events are mediated by a series of molecular interactions resulting from disrupted positive and negative regulatory mechanisms (Liotta, L et al., 1991). Tumor invasion requires disruption of the basement membrane (Bernstein, LR and Liotta, LA, 1994). Both the basement membrane and extracellular matrix (ECM), which are intricate and complex networks of proteinaceous fibers and macromolecules (laminin, fibronectin,

fibrin, proteoglycans and collagens) serve as a physical barrier to confine the movement of cells to specific tissue compartments (Boyd D, 1996). Many biological processes such as cell migration, trophoblast implantation, ovulation, mammary gland involution, wound healing and tissue re-modelling depend on tightly controlled interactions between cells and their surrounding matrix (Saksela O and Rifikin DB, 1988). Similarly, malignant cells escape adhesive restrictions imposed by the matrix network and penetrate the surrounding normal tissues by the modification and breakdown of the ECM. Regulation of molecular events necessary for invasion requires spatial and temporal coordination and cyclic detachment and attachment processes at the level of individual cells. It is a process of motility coupled to regulated adhesion and extracellular proteolysis (Kohn EC and Liotta LA, 1995). The balance of proteolytic and antiproteolytic processes helps to maintain the integrity of the epithelial basement membrane under physiological conditions where degradation of the basement membrane is required. In contrast, during tumor progression, deregulation of extracellular proteolysis favours proteolytic over anti-proteolytic events, resulting in tumor invasion and the establishment of metastases at distant sites (Kohn EC and Liotta LA, 1995).

The extracellular proteolytic events required during tumor metastasis are regulated by various proteases. A positive correlation between tumor aggressiveness and increased protease production has been documented for all four classes of proteases including serine, aspartyl, cysteinyl and metal-dependent proteases (Blasi F, 1993; Liotta LA et al., 1980; Mignatti P et al., 1986; Rochefort H et al., 1990; and Sato H et al., 1994). Cathepsins B and L are the principal representatives of the cysteine class (Sloan BF, 1990; Kane SE and Gottesman MM, 1990) and Cathepsin D is the principal member of the aspartyl protease class (Rochefort H et al., 1990). Collagenases (MMP-2, 9), interstitial collagenases

(MMP-1) and stromelysins (MMP-3, 11) may represent the principal metal-dependent proteases (Birkedal-Hansen H et al., 1993). Finally, urinary plasminogen activator (uPA) and plasminogen which are members of the serine protease family are strongly implicated in breast and prostate cancer (Saksela O and Rifkin DB, 1988). Accumulating evidence from a series of basic and clinical studies has clearly shown the essential role of uPA-mediated plasminogen activation in promoting tumor invasion and metastasis of several malignancies including breast and prostate cancer (Andreasen PA et al., 1997; Bernhard EJ et al., 1994; Dano K et al., 1994). The proteolytic effects of uPA are localized within the tumor cell milieu via a high affinity receptor (uPA receptor) [uPAR] expressed on the tumor cell where it is linked to the cell membrane via a glycophosphatidyl inositol (GPI) anchor (Andreasen PA et al., 1997; Bernhard EJ et al., 1994; Dano K et al., 1994; Ploug M et al., 1991).

Adenocarcinoma of the breast and prostate are two common hormone dependent malignancies affecting women and men respectively, and are associated with high incidence of morbidity and mortality in the late stages (Russo J and Russo IH, 1995; Franks LM, 1973). In these malignancies, the sex steroids estrogen and androgen play a key role in the initiation and progression of cancer (Vessey MP, 1989; Osborne CK et al., 1980). Breast and prostate cancers initiate as hormone sensitive (HS) tumors which eventually progress to a more malignant stage, becoming hormone insensitive (HI) due to the lack of expression of functional estrogen and androgen receptors by tumor cells (McGuire ML, 1978; McGuire WL et al., 1991). Furthermore, this transition from HS to HI variety results in poor therapeutic response to hormonal therapy in patients with breast cancer when tumor metastases are seen in various organs, particularly the skeleton (Kitzenellenbogen BS, 1991; Clark R. et al., 1993; Hortobagyi G, 1991).

In this chapter, I will focus my discussion on the biochemical properties of uPA and uPAR, and their roles in the invasion and metastasis of various malignancies in general and that of hormone dependent malignancies like breast and prostate cancer in particular. The potential molecular mechanisms regulating the expression and activity of the uPA / uPAR system will also be addressed.

1.2. URINARY PLASMINOGEN ACTIVATOR (uPA) IN PERICELLULAR PROTEOLYSIS

1.2.1. Plasminogen Activators

Both the urinary type (uPA) and tissue type (tPA) plasminogen activators (PAs) belong to the family of serine proteases which are capable of catalyzing the conversion of inactive zymogen plasminogen to its active form plasmin (Dano et al., 1985; Mignatti P and Rifkin DB, 1993). Although uPA and tPA share 40% sequence homology, they are encoded by two discrete genes and are immunologically distinct (Belin D et al., 1985; Pennica D et al., 1983; Stoppelli MP et al., 1985). The differences reside in their domain organization resulting in distinct functions of their noncatalytic regions (Ranby M et al., 1982; Collen D, 1987). Additionally, uPA and tPA have distinct targeting determinants in their noncatalytic regions: the "growth factor domain (GFD)"of uPA directs the binding of pro-uPA and uPA to a plasma membrane receptor (Vassalli JD et al., 1985; Blasi F et al., 1990), whereas other structural domains in uPA and tPA (the "finger" region and the "kringles") allow their binding to fibrin and other components of the extracellular matrix (ECM) (Gething MJB et al., 1988). The different extracellular targeting of the two PAs suggests that they play different biological roles. tPA is believed to be primarily

responsible for removal of fibrin from the vascular tree. It has a specific affinity for fibrin and produces clot restricted plasminogen activation (Dano K et al., 1985). uPA, on the other hand, mediates cell migration during various diverse physiological and pathological processes such as tissue remodelling, wound healing, angiogenesis and tumor invasion (Fibbi G et al., 1988; Mignatti P et al., 1986; Ossowski L, 1988; Testa JE and Quigley JP, 1990).

1.2.2. Plasminogen and Plasmin-mediated Cascade of Pericellular Proteolysis

Plasminogen is the preferred substrate for both uPA and tPA. It is produced mainly in the liver and is also present abundantly in blood plasma with concentrations as high as 2 μM (Saksela O and Rifkin DB, 1988; Mignatti P and Rifkin DB, 1993) and in interstitial fluids (Collen D and Verstraete M, 1975). uPA and tPA are the principal activators for converting the inactive zymogen plasminogen to its active form, plasmin, via specific proteolytic cleavage of the internal Arg 560-Val 561 bond of plasminogen (Robbins KC et al., 1967). In addition to uPA and tPA, arginine esterase (Vassalli J et al., 1991) can also convert plasminogen to plasmin *in vitro*. However, only PAs are capable of doing so under physiological conditions.

Plasmin, the active form of plasminogen, is a serine protease consisting of two disulfide bridge linked polypeptide chains. The carboxy terminal B-chain contains a typical serine proteinase domain which is responsible for its catalytic activity and binding to inhibitors. The amino terminal A-chain contains 5 kringle motifs (Andreasen PA et al., 1997). Plasmin is the central component of the PA / plasmin system with a broad spectrum of proteolytic digestion. It is able to hydrolyse many proteins present in the cell

basement membrane and ECM, either directly, or indirectly through the activation of certain latent MMPs (Werb Z et al., 1980 ; Mignatti P and Rifikin DB, 1993; Kleiner DE and Stetler-Stevenson WG, 1993), as well as latent elastase (Chapman HA and Stone OL, 1984) via a cascade of proteolytic events. The abundance of plasminogen in virtually all tissues makes possible the efficient generation of plasmin by very small amounts of PAs, resulting in a dramatic amplification of the proteolytic capacity of PA-producing cells. The proteolytic activity of plasmin is regulated primarily by its specific inhibitor α_2-antiplasmin and by α_2-macroglobulin, both of which are abundant in plasma and interstitial fluids. However, cell surface bound plasmin, in contrast to plasmin in solution, is protected against inhibition by α-antiplasmin (Werb Z et al., 1980).

Although plasminogen and plasmin bind to various cell types (Liotta LA et al., 1979), the cell surface binding sites for plasmin and plasminogen have not been clearly defined. However, it has been shown that binding of plasminogen to the cell surface occurs through lysine binding sites located in the "kringle" domains of the noncatalytic region of its A-chain (Stephens RW et al., 1989). Candidates for plasminogen / plasmin receptors are laminin (Salonen EM et al., 1984), fibronectin (Salonen EM et al., 1985), thrombospondin (Silverstein RL et al., 1984; 1986), tetranectin (Clemmensen I et al., 1991), enolase (Redllitz A et al., 1995), actin (Lind SE and Smith CJ, 1991) and cytokeratin 8 (Hembrough TA et al., 1996). The existence of cell surface binding sites for uPA and plasmin/plasminogen points to the cell surface as the site where the powerful proteolytic system of plasminogen activation is assembled (Stephens RW et al., 1989). At least two steps in the PA / plasmin cascade are positively influenced by cell surface binding of uPA (Pepper MS et al., 1996). First, activation of pro-uPA is markedly increased when it is receptor bound and when plasminogen is simultaneously present on the nearby cell

surface (Meissauer A et al., 1991), this in turn accelerates plasmin formation (Ellis V et al., 1991); and second, when plasmin on the cell surface is protected from its inhibitors α_2-macroglobulin and α-antiplasmin (Plow EF et al., 1986).

1.2.3. Processing of uPA

uPA is synthesized and secreted as an inactive single chain pro-peptide (Pro-uPA) which is converted into its active form uPA through proteolytic cleavage catalyzed *in vitro* by plasmin (Dano K et al., 1985). In addition, plasma kallikrein and blood coagulation factor XIIa (Ichinose A et al., 1986). T-cell associated serine proteases (Brunner G et al., 1990), cathepsin B (Kobayashi H et al., 1991), cathepsin L (Goretzki L et al., 1992), nerve growth factor-γ (Wolf BB et al., 1993), human mast cell tryptase (Stack MS and Johnson DA, 1994) and protease specific antigen (Yoshida E et al., 1995) can also catalyze the activation of pro-uPA *in vitro* via a proteolytic cleavage of uPA at the Lys 158 residue.

The active uPA known as High Molecular Weight uPA (HMW-uPA) consists of a 24 kDa light "A" chain formed from the amino terminal end of the single peptide, and a 30 kDa catalytically active heavy "B" chain, linked together by a disulfide bridge between Cys 148 and Cys 279 (Wun TC et al., 1982). The "A" chain has motifs with homology to different proteins such as plasminogen, fibronectin and prothrombin. In addition, within the amino terminal domain of uPA, there is a cysteine rich growth factor domain (GFD) that shares strong structural homology with the receptor binding domain of epidermal growth factor (Appella E. et al., 1987). HMW-uPA can be further cleaved at Lys158-Ile159 into an Amino Terminal Fragment (ATF) that bears a uPAR binding site (amino acids 12-32), and a Low Molecular Weight uPA (LMW-uPA) that carries the active site for uPA proteolytic activity (Rabbani SA, 1995) (Figure 1.2). The active site

triad of His 204-Asp255-Ser356 in human uPA is identical to that of all serine proteases. The regions surrounding the active site residues are also highly conserved. ATF isolated from the conditioned medium of the human prostate carcinoma PC-3 cells has been shown to have a mitogenic effect on osteoblast cells (Rabbani SA et al., 1992). This finding may be of fundamental importance in understanding the mechanism of development of osteoblastic metastases associated with prostate cancer. Additionally, fucosylation at Thr 18 within the GFD (4-43) serves as a molecular trigger in eliciting the mitogenic response. Removal of fucose at Thr^{18} either by chemical means or by expression of uPA in E. coli where it is not fucosylated resulted in the loss of its mitogenic effects (Rabbani SA et al., 1992). The blood plasma concentration of uPA is around 20 pM, most of which is complexed with its natural inhibitor plasminogen activator inhibitor type I (PAI-I), while another fraction is in the pro-uPA form (Andreasen PA et al., 1994).

Nucleotide sequence encoding uPA of several species including human, baboon, mouse, chicken and rat have been elucidated (Belin D et al., 1985; Verde P et al., 1984; Au YPT et al., 1990; Henderson BR et al., 1992 ; Rabbani SA et al., 1993). These cDNAs have a long 3'-non-translated region and a long 5'-poly A' tail. uPA from these species share a high degree of structural and sequence homology (Verde P et al., 1984; Au YPT et al., 1990; Rabbani SA et al., 1993;Henderson BR et al., 1992). The deduced amino acid sequence from rat uPA cDNA predicts a 431 amino acid protein which is 69% and 85% homologous to its human and mouse counterparts respectively (Rabbani SA et al., 1993; Henderson BR et al., 1992). However, the degree of sequence conservation is lower at the receptor binding region (amino acids 12-32). The interspecies amino acid differences may be responsible for the observed species specific receptor binding (Appella E et al., 1987;

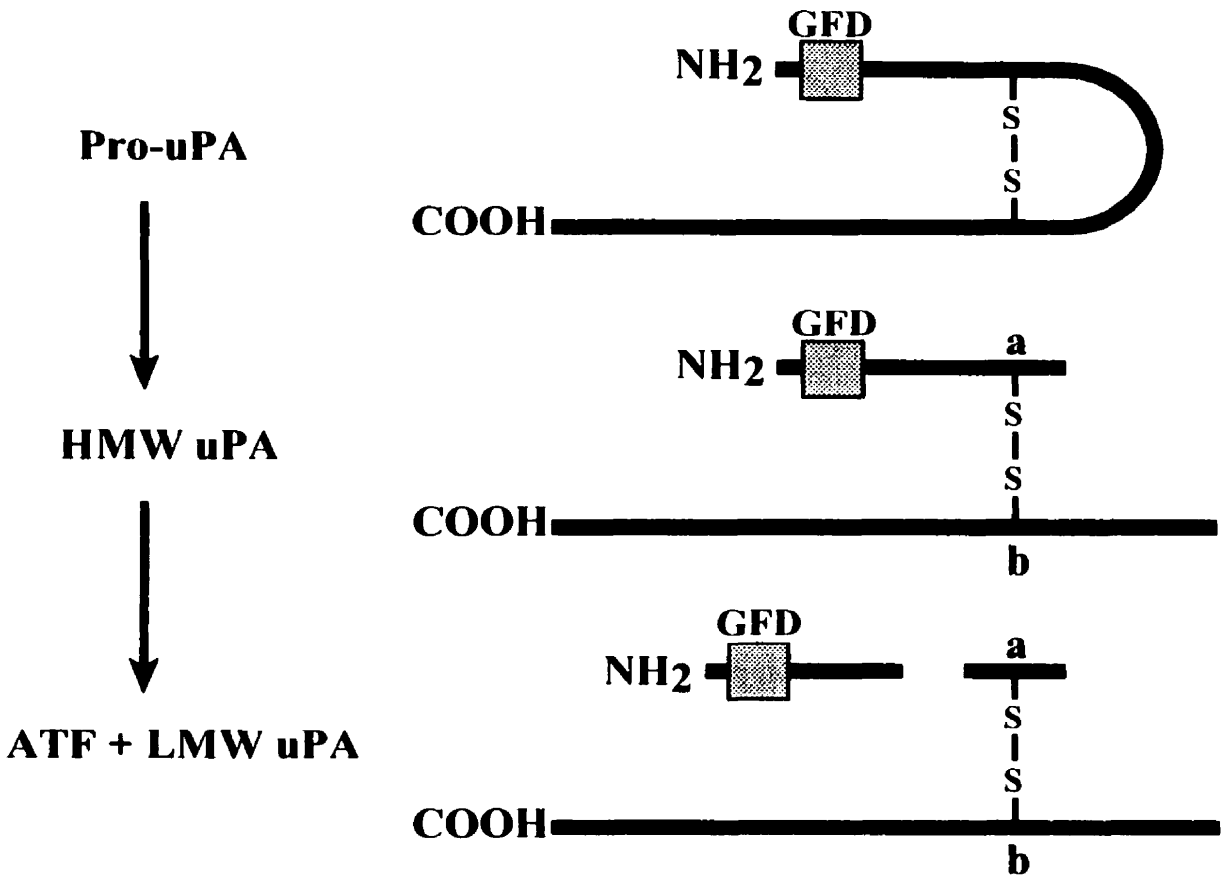


Figure 1.2: Processing of uPA

Rabbani SA et al., 1990). Following its translation from these cDNAs, uPA protein undergoes several post-translational modifications including glycosylation (Buko AM et al., 1991), fucosylation (Rabbani SA et al., 1992) and phosphorylation (Franco P et al., 1992). While fucosylation at Thr18 is essential for the mitogenic effect of uPA, other post-translational modifications may also play critical roles in regulation of uPA enzymatic activity and its plasma clearance characteristics (Rabbani SA, 1995).

1.2.4. Functions of uPA-mediated Pericellular Proteolysis

Following proteolytic processing, different domains of uPA can trigger a host of effects in normal and neoplastic cells. Whereas LMW-uPA is primarily involved in cellular proteolysis, tissue remodelling, embryonic development, zymogen and growth factor activation to promote tumor invasion and metastasis; ATF, following its binding to the cell surface receptor of uPA (uPAR) can promote cell proliferation, migration and adhesion (Figure 1.3). uPAR-mediated effects of uPA, which are independent of the proteolytic activity of uPA, will be reviewed in greater detail later when the functional significance of uPAR is discussed.

uPA is produced by kidney tubule cells, phagocytic cells, keratinocytes, fibroblasts and tumor cells in essentially all tissues (Schmitt M et al., 1992). uPA-dependent pericellular proteolysis plays an important role in both physiological and pathological conditions. It is achieved by converting the inactive zymogen plasminogen to its active form plasmin which may in turn degrade various components of the ECM including laminin, fibronectin, collagens and proteoglycans (Dano K et al., 1985).

Trophoblast cells (Sappino AP et al., 1989) and other migrating embryonic cells such as hemopoietic cells (Valinsky JE et al., 1981) and neural crest cells (Festoff BW,

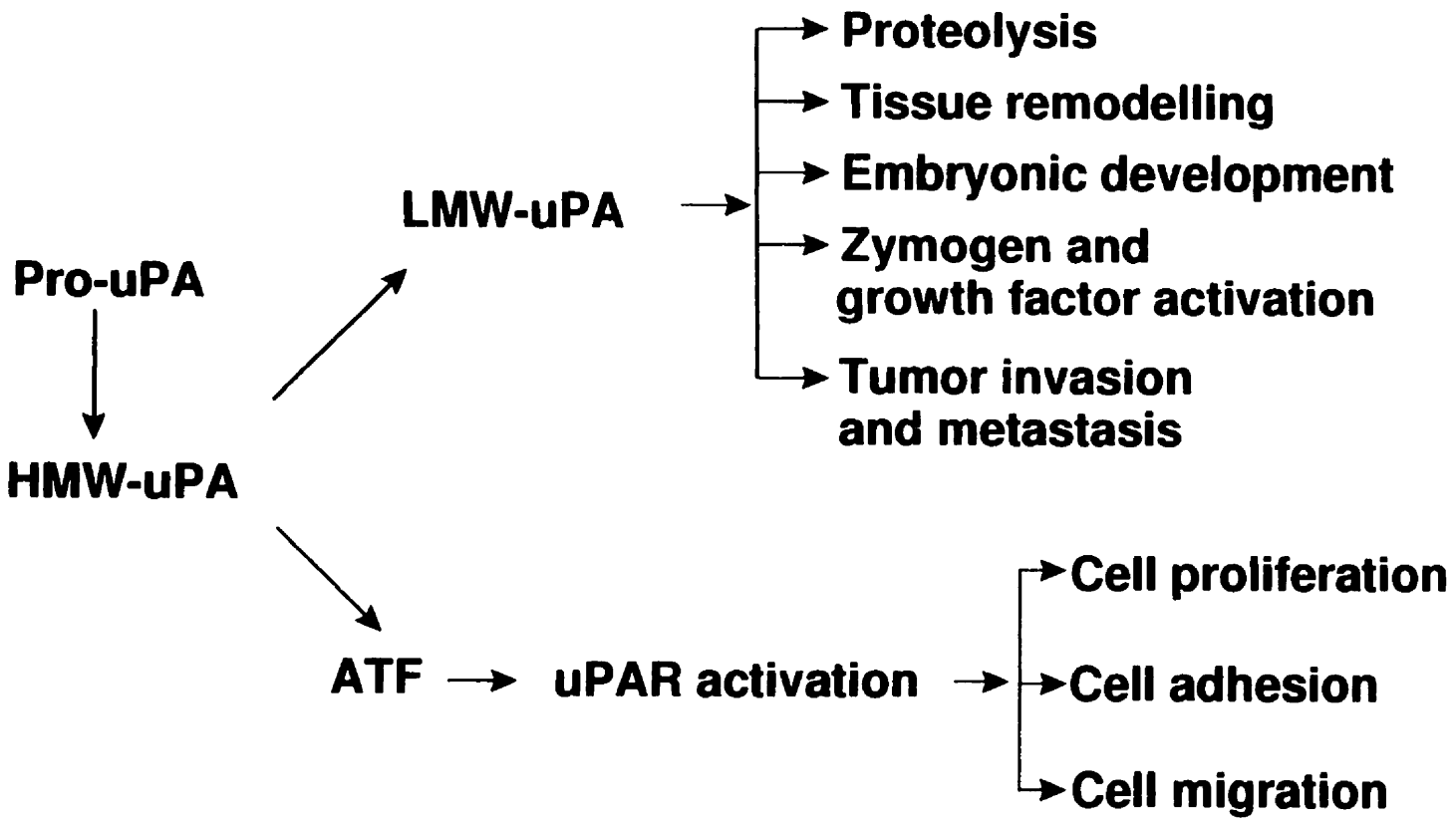


Figure 1.3: Functions of urokinase

1990) all produce high levels of uPA during trophoblast implantation and early growth of the embryo. uPA has been shown to play a role in ovulation (Tsafriri A et al., 1989), sperm migration and fertilization (Huarte J et al., 1987), and embryo implantation (Sappino AP et al., 1989; Strickland S et al., 1976; Menoud PA et al., 1989; Denker HW, 1977).

Additionally, uPA has been implicated in tissue remodelling of the ovary (Sappino AP et al., 1989; Canipari R et al., 1987), prostate (Busso N et al., 1989) and mammary glands (Ossowski L et al., 1979). Furthermore, the presence of uPA in the adult central nervous system (Festoff BW, 1990), as well as in a majority of endocrine glands, suggests that uPA may participate in the proteolytic dependent activation of hormones and growth factors including TGF-ß, basic fibroblast growth factor (bFGF) and hepatocyte growth factors (HGF) (Odekon LE et al., 1994; Salsela O and Rifkin DB, 1990; Gross JL et al., 1983).

In addition to its important functions under physiological conditions, the fibrinolytic system may contribute to several pathological processes, such as inflammation (Pepper MS and Montesano R, 1990), thrombosis (Nilsson IM et al., 1985), atherosclerosis (Juhan-Vague I and Collen D, 1992), glomerulonephritis (Tomooka S et al., 1992), acute respiratory distress syndrome (Idell S et al., 1989), haemangioma formation (Wagner EF, 1990) and tumor invasion and metastasis. The role of uPA and uPAR in tumor invasion and metastasis will be reviewed in greater detail later.

1.2.5. Inhibitors of the PA / plasmin System

The proteolytic activities of the PA / plasmin system are partially regulated by its natural inhibitors. Inhibitors of the PA / plasmin system belong to the serpin superfamily (serine proteinase inhibitor). The formation of a complex between proteinase and inhibitor

takes place in a 1:1 stoichiometry (Stein P and Carrel RW, 1995). Four arginine-specific serine-protease inhibitors which have different targets are of particular relevance to the PA / plasmin system (Andreasen PA et al., 1990).

Type 1 plasminogen activator inhibitor (PAI-1) is secreted as a 45 KDa active antiprotease which is mostly present in the plasma. It is also present in platelets and produced by endothelial cells (Magnatti P and Rofokin DB, 1996). PAI-1 has high affinity for both uPA and tPA. It is often found to be associated with vitronectin in the plasma and ECM which stabilizes it in the active conformation (Seiffert D et al., 1990). In addition, vitronectin and heparin affect the specificity of PIA-1 by enhancing its reactivity toward thrombin (Ehrlich HJ et al., 1991). Type 2 plasminogen activator inhibitor (PIA-2), a 46.6 KDa protein, is expressed most notably by cells of monocyte-macrophage lineage (Kawano T et al., 1970; Astedt B et al., 1985). It inhibits both uPA and tPA, but it is less efficient towards tPA. Receptor bound uPA can react with PAI-1 and PAI-2 (Cubellis MV et al, 1989; Pollanen J et al, 1990), though these reactions may be slightly slower than that between uPA and its inhibitors in the fluid phase (Ellis V et al., 1990). As expected, PAI-1 and PAI-2 are capable of inhibiting ECM degradation mediated by uPAR-bound uPA (Laug WE er al., 1993; Shirasuna K et al., 1993). In addition, PAI-1 also plays an important role in the endocytosis of the uPA / uPAR complex. Cubellis et al. (1990) were the first to demonstrate that the uPAR-bound uPA/PAI-1 complex, in contrast to uPAR-bound uPA alone, is rapidly endocytosed and degraded. Protease-nexin (PN-I), a 45 KDa protein originally purified from fibroblasts (Eaton DL et al., 1984) is capable of inhibiting uPA, plasmin, and thrombin. A fourth inhibitor, called PAI-3 has been identified as a protein C inactivator, but is much less efficient in inhibiting PAs than other inhibitors (Heeb MJ et al., 1987).

α_2-antiplasmin is the primary inhibitor of plasmin in plasma and its inhibition requires unoccupied lysine binding sites of plasmin (Longstaff C and Gaffney PJ, 1991). However, plasmin bound to the cell surface is protected from α_2-antiplasmin probably due to the occupancy of the lysine binding sites by the proposed cell surface receptors (Stephens RW et al., 1989; Duval-Jobe C and Pamely M. J.,1994).

1.2.6. Regulation of uPA Production and Activity

uPA-mediated pericellular proteolysis, under either physiological or pathological conditions, is finely tuned and precisely regulated by a cooperative interplay of different mechanisms including: transcriptional and post-translational controls, spatial restriction of enzymatic activity, regulation of enzyme secretion, zymogen activation, inhibitor-mediated inactivation, and feedback controls (DeClerck YA and Laug WE, 1996). The major differences between normal and pathological states arise from the way in which uPA expression is regulated. It has been widely reported that most tumor cells derived from human solid tumors exhibit enhanced production of uPA. However, the molecular and biochemical mechanisms promoting uPA overexpression in the cancer state are not well understood. It is possible that signals controlling uPA production become constitutively activated during malignant transformation, allowing tumor cells to acquire a high invasive capacity necessary to metastasize. I will focus my discussion on two aspects of the regulation of uPA action in the cancer state: transcriptional regulation of uPA gene expression and spatial restriction of its enzymatic activity.

1.2.6.a. Transcriptional Regulation of uPA Gene Expression.

Among the different potential mechanisms regulating uPA production,

transcriptional regulation of uPA gene expression by various growth factors appears to play a determinant role. The steady state level of uPA mRNA is determined by the expression of the gene which is regulated by the 2.1 Kb 5'-flanking sequence containing binding sites for various transcriptional factors including AP-1, PEA3, NF-kB, NF-1, SP-1 and CREB (Verde P et al., 1988); and by regulating the stability of the uPA mRNA by 3'-untranslated sequence (Nanbu R et al., 1995). It has been widely reported that uPA expression can be up-regulated in tumor cells by various growth factors, cytokines and hormones, including EGF (Laiho M and Keski-Oja J, 1989; Aguirre Ghiso JA et al., 1997), HGF (Pepper MS et al., 1992), IGF I and II (Guerra F et al., 1996), VEGF (Koolwijk P et al., 1996), bFGF (Roghani M et al., 1996), CSF-1 (Chambers C et al., 1995), TGF-ß (Desruisseau S et al., 1996), retinoic acid (Liu DF and Rabbani SA, 1996), cytokines (Panozzo MP et al., 1996) and tumor necrosis factor α (TNF-α) (Koolwijk P et al., 1996). In contrast to these, glucocorticoids are negative regulators of uPA production (Niiya K et al., 1992). The actions of these growth factors and cytokines is mostly at the level of gene transcription.

Since most of these growth promoting factors elicit their actions by activating various intracellular signalling molecules including PLCγ, PKC, PLD, PI-3 kinase, *Ras*, *Raf*, MAPK (KS et al., 1994; Lee M and Severson D, 1994; Blobe GC et al., 1994; Burgering BMT and Bos JL, 1995; Lengyel E et al., 1995), as well as oncogene-encoded protein tyrosine kinases like *v-src*, *v-ros* and *v-yes* (Bell SM et al., 1993), these molecules may act in a cooperative fashion to regulate uPA gene expression. It is thus quite possible that the regulation of uPA production is tightly associated with the mitogenic signalling pathways that control the proliferation of tumor cells. The presence of *cis*-regulatory sequences such as AP-1/PEA-3 transcriptional factors has been shown to confer the

mitogenic signalling pathway mediated up-regulation of uPA production (Rorth P et al., 1990; Reifel-Miller AE et al., 1996; Lengyel E et al., 1996). Therefore, during malignant transformation by oncogenes, tumor cells acquire a highly proliferative state of growth under the influence of various growth promoting factors. At the same time, these growth factors may also up-regulate the production of proteases like uPA in order for tumor cells to acquire an invasive phenotype which is essential for subsequent events related to tumor invasion and metastasis. However, in contrast to this, under normal physiological conditions, the uncoupling of invasive signals from mitogenic signals allows normal cells to respond to the growth factor-mediated proliferative signals in a highly regulated fashion without overexpressing these proteases. The mechanisms underlying the differential regulation of the functions of growth factors remain unanswered questions. It is most likely that differences in transcriptional mechanisms between normal and malignant cells are responsible for a differential regulation of uPA production under physiological and pathological conditions.

1.2.6.b. Spatial Restriction of the Proteolytic Activity

In addition to transcriptional regulation, spatial restriction of the proteolytic activity of uPA is an additional important mechanism regulating cell migration and tumor cell invasion. Spatial localization helps to concentrate proteolysis near the cell surface and to restrict its activity to the immediate pericellular environment. It is achieved by two mechanisms: first, cell surface receptors and extracellular matrix-binding proteins (Vassalli JD and Pepper MS, 1994); and second, co-production of protease inhibitors (Pepper MS and Montesano R, 1990). The proteolytic activity of uPA is tightly controlled by its natural inhibitors (PAIs) present in the tissues and in the circulating system. However, malignant

transformation upsets the balance between active proteases and their inhibitors and appears to shift it in favour of the degradative and invasive phenotype (Cohen RL et al., 1991). The ability of PAIs to bind uPA/uPAR complex leads to the internalization and degradation of uPA / uPAR / PAI complex, and results in the rapid loss of uPA activity and the re-expression of uPAR on the cell surface for more ligand binding (Potempa J et al., 1994).

1.3. BIOCHEMICAL PROPERTIES AND FUNCTIONS OF THE UROKINASE RECEPTOR (uPAR)

The catalytic activity of uPA is mediated by its cell surface binding sites collectively known as uPAR. uPAR was first described by Vassalli et al. (Vassalli JD et al., 1985) and Stoppelli et al (Stoppelli MP et al., 1985) as a cell surface high-affinity binding activity for the A-chain of uPA on the surface of human lymphoma cell line U-937. Subsequently, it was identified in diverse cell types including monocytes, granulocytes, fibroblasts, activated lymphocytes as well as in leukemia cells and tumor cells derived from solid tumors (Chucholowski N et al., 1991; Vassalli JD et al., 1985; Stoppelli MP et al., 1985; Stoppelli MP et al., 1986). uPAR is capable of binding both pro-uPA and HMW uPA with similar affinity (Cubellis MV et al., 1986).The function of uPAR on a given cell type can be regulated by receptor expression (Mignatti P et al., 1991; Pepper MS et al., 1993), binding affinity (Moller LB et al., 1993) and receptor distribution (Pollanen J et al., 1988).

1.3.1. Gene Organization of uPAR

The gene for uPAR is located on the long arm of chromosome 19 (Borglum AD et al., 1992) and it is organized into seven exons spanning 13 Kb to encode a 1.4 Kb mRNA (Roldan AL et al., 1990; Suh, TT et al., 1994). The major functional domains of

the encoded protein include the signal peptide, three cysteine-rich internal repeats and the glycolipid anchor attachment motif which are encoded by separate exons (Pyke C et al., 1993b). The 5'-proximal region of the uPAR gene lacks TATA- or CCAAT-box motifs. Instead, it is flanked by a GC-rich region, which contains a number of potential regulatory elements including SP1 and AP1 binding motifs. The close association of both SP1 and AP1 sites within the proximal promoter region is consistent with the notion that the murine uPAR gene is inducible by phorbol esters, and both sites may contribute to cell type-specific expression (Pyke C et al., 1993b). Recently, cDNAs encoding uPAR mRNA have been isolated and characterized from human, mouse and rat cell lines (Kristensen P et al., 1991; Roldan AL et al., 1990; Rabbani SA et al., 1994).

1.3.2. Protein Structure of uPAR

uPAR is a 50-60 KDa cysteine-rich, hydrophobic and highly glycosylated membrane protein. It is synthesized as a 22 amino acid signal peptide followed by a 313 amino acid mature peptide (Nielsen LS et al., 1988; Estreicher, A et al., 1989; Behrendt N et al., 1990; Solberg H et al., 1992). The 28 cysteine residues of uPAR, forming three homologous repeats with its amino terminal amino acids 1-87 constitutes the ligand binding domain (Behrendt N et al., 1991). The binding of uPA to uPAR has been extensively studied and it has been shown that uPAR functions as a monomeric protein (Behrendt N et al., 1993; Ploug M et al., 1993; Ploug M et al., 1994).

1.3.2.a. Posttranslational Modfications in uPAR

1.3.2.a (i). Glycosylation

The human uPAR sequence contains five potential N-linked glycosylation sites

(Roldan AL et al., 1990) and uPAR is heavily and heterogeneously glycosylated (Behrendt N et al., 1990). The exact function of glycosylation has not been well elucidated. However, site-directed mutagenesis has suggested some modulatory roles of the carbohydrates in the ligand-binding properties of uPAR. This could contribute to the observed differences in affinity for uPAR on different cell types (Picone R et al., 1989), which are known to differ with respect to glycosylation of the uPAR protein (Behrendt N et al., 1990).

1.3.2.a (ii). Glycosylphosphatidylinositol (GPI) anchor

The mature uPAR protein lacks a transmembrane sequence, and is instead anchored to the outer leaflet of the plasma membrane by a glycosylphosphatidylinositol (GPI) moiety. The GPI anchor is added during a post-translational process involving carboxy terminal truncation of the primary translation product at Gly 275 in rat uPAR and Gly 283 in human uPAR. An important consequence of this GPI-anchoring is that no part of uPAR is in direct contact with the cytoplasm. The GPI-mode of membrane anchorage not only provides a mechanism of membrane attachment, but also increases the lateral mobility of uPAR within the plasma membrane, which allows it to more efficiently recruit uPA into areas of the cell surface where an increased proteolytic activity is required (Plough M et al., 1991; Moller LB et al., 1992).

1.3.2.b. Domain Organization of uPAR

A detailed analysis of the primary structure of uPAR revealed the presence of an internal sequence homology due to the presence of three homologous repeats each consisting of approximately 90 amino acid residues (Behrendt N et al., 1991). Similar structural organization of the internal repeat has also been reported in a number of other

GPI-anchored membrane proteins including murine Ly-6 antigens (Friedman S et al., 1990), the membrane inhibitor of reactive lysis (MIRL) or CD 59 (Davies A et al., 1989), the squid brain glycoprotein sqp-2 (Williams AF et al., 1988), a lymphotropic tumor virus protein designated HVS-15 (Albrecht JC et al., 1992) and a family of snake neurotoxins represented by erabutoxin C (Fuse N et al., 1990). This mode of domain organization is determined by a distinct pattern of cysteine residues which is conserved in all members of GPI-anchored protein family including uPAR.

1.3.2.c. Structure-function Relationships in uPAR Ligand Binding

Chemical cross-linking studies have revealed that Domain I (amino acids 1-87) alone is sufficient for the binding of uPA via the GFD of uPA (Appella E et al., 1987; Behrendt N et al., 1991). More specifically, this uPAR binding site is localized within a 7-residue-long loop (amino acids 13-19) of the GFD (Ploug M et al., 1995). Two sub-regions within domain I and Y57 have also been reported to be critical for uPA recognition (Pollanen J , 1993; Ploug M et al., 1995). Although Domain I of uPAR is sufficient for ligand binding (Behrendt N et al., 1991), inter-domain interactions contribute more decisively to the binding since the affinity of uPA to the isolated amino terminal Domain I is 1500-fold lower than that of the binding affinity of the intact uPAR (Ploug M et al., 1994; Behrendt N et al.,1996).

uPAR binds to Pro-uPA, HMW-uPA and ATF (GFD) with similar affinities (Ploug M et al., 1991), and the uPA / uPAR complex remains functionally active on the cell surface with a half life of 4-5 hours until it binds to PAIs and is internalized (Blasi F et al., 1987; Cubellis MV et al., 1990; Estreicher A et al., 1990). The binding of uPAR to uPA is species specific due to their highly specific receptor binding sequences: mouse

receptors do not bind human uPA and vice versa (Vassalli JD et al., 1985; Appella E et al., 1987; Estreicher A et al., 1989).

In addition to uPA, uPAR appears to have other ligands. Recently, a few other proteins have been found to be associated with uPAR. Vitronectin (VN), a 78 KDa ECM glycoprotein can bind uPAR with high affinity (Kd=0.2 to 2 nM) via domains II and III of uPAR (Seiffert D et al., 1991). Binding of uPAR with VN is stimulated by pro-uPA, uPA, ATF and uPA/PAI-1 complex (Wei Y et al., 1994, Deng G et al., 1996; Kanse SM et al., 1996). Moreover, uPAR may also associate with the members of the integrin superfamily including β1- and β2-integrins (Xue W et al., 1994; Bohuslav J et al., 1995; Sitrin RG et al., 1996, Wei Y et al., 1996). Accumulating evidence suggests that the association of VN and integrins with various components of the PA system plays an important role in cancer biology.

1.3.2.d. Alternatively Spliced Forms of uPAR mRNA

Multiple isoforms of uPAR, probably arising from alternative splicing of uPAR mRNA, have been reported (Kristensen P et al., 1991; Roldan AL et al., 1990; Pyke C et al., 1993b; Rabbani SA et al., 1994; Yang JN et al., 1998). Two cDNAs encoding mouse uPAR were first described by Kristensen et al. (Kristensen P et al., 1991). The first (mouse uPAR-1) is homologous to the human uPAR containing a GPI anchor at Gly275. The second mouse uPAR (uPAR-2) encodes a 199 residue protein containing a functional uPA ligand binding domain, and lacking a carboxyl terminal GPI anchor, suggesting that uPAR-2 may represent a secreted uPA binding protein. A specific differential expression of the two mouse uPAR variants have been shown in the gastrointestinal tract. A similar human uPAR variant (uPAR-2) has been identified more recently (Pyke C et al., 1993b).

This variant mRNA was detected in the following human cells and tissues:U937 monocyte-like cells, HepG2 hepatocarcinoma cells, A549 lung carcinoma cells, alveolar macrophages, leukocytes and colon tissue. In addition, Rabbani et al. have isolated three isoforms of rat uPAR from the non-transformed rat osteoblast cell line CFK-1 (Rabbani SA et al., 1994). Rat uPAR-1 and 2 are homologous to the mouse and human uPAR-1 and 2. The third rat uPAR isoform ruPAR-1b is identical to the ruPAR-1, with the exception of a critical single amino acid substituition from Cys to Ser at position 71 in ruPAR-1b. Soluble uPAR variants, without the GPI anchor, have been identified in conditioned medium from some cell lines (Lau HK and Kim M, 1994), in ascites fluid from ovarian cancer patients (Pedersen N et al., 1993) and in biological fluids with inflammatory conditions (Mizukami IF et al., 1995). Such variants may arise by differential splicing (Kristensen P et al., 1991; Pyke C et al., 1993b) or by phospholipase C cleavage of the GPI anchor. The function of the soluble variants of uPAR characterized in different species is presently unclear. Since it retains ligand-binding capacity, it may serve as inhibitors of cell surface proteolysis by inhibition of the binding of uPA to GPI-anchored uPAR. In a most recent study, a novel unspliced mouse uPAR variant was detected in mouse osteoclast and osteoblast cells, as well as in mouse kidney, spleen, and muscle. The function of this unspliced variant is not known. However, its presence on bone cells suggests its potential roles in modulating bone functions under physiological and pathological conditions (Yan JN et al, 1997).

1.3.3. Functional Significance of uPA / uPAR Interaction

1.3.3.a. Localization of uPA Activity on the Cell Surface

The primary role of uPAR is to provide a convenient mechanism for the

confinement of uPA activity to the cell surface, as well as for the localization of uPA to discrete areas of the cell membrane. The interaction between uPA and uPAR leads to the focal distribution of uPA/uPAR complexes to areas of the cell surface where there is an increased requirement for uPA/uPAR action (Dano K et al., 1994). There is increasing evidence implicating uPAR in tumor invasion and metastasis. It is now believed that uPAR expressed on the tumor cell surface serves to localize and focus the proteolytic activity of uPA produced by tumor cells or their surrounding stromal cells within the tumor cell environment to provide a mechanism for the coordination of cell adhesion, proteolysis and migration (Dano K et al, 1994).

1.3.3.b. Activation of Latent Growth Factors

It has been widely reported that uPA and plasmin are involved in proteolytic activation of growth factors, including HGS/SF, TGF-β, and bFGF (Andreasen PA et al., 1997; Baillie CT et al., 1995; Odekon LE et al., 1994; Salsela O and Rifkin DB, 1990). However, in the presence of uPAR, plasmin-dependent activation of TGF-β is greatly enhanced (Odekon LE et al., 1994). Therefore, uPAR could initiate localized activation of growth factors to restrict the activity of PA/plasmin system to a limited population of cells, or even to the sub-cellular domains of each individual cell.

1.3.3.c. Signal transduction by uPAR

Although uPAR lacks a cytoplasmic domain, overwhelming evidence has suggested that occupancy of GPI-anchored uPAR may be directly involved in the activation of various intracellular signal transduction pathways. The potential role of uPAR in signal transduction is supported by several of the uPAR-mediated effects of uPA which

are independent of its proteolytic activity. These include actions on intracellular phosphorylation events or other cellular functions which can be initiated with ATF alone. uPA and ATF can serve as a mitogenic growth factor for various cells (Rabbani SA et al., 1990; Rabbani SA et al., 1992; He CJ et al., 1991, Anichini E et al., 1994). Binding of uPA to uPAR was reported to stimulate tyrosine phosphorylation of an uncharacterized protein with a MW of 38 kDa in U937 cells (Dumler I et al., 1993). Both ATF and GFD have been shown to induce the mRNA expression of early response oncogenes including *c-myc*, *c-jun* and *c-fos* in osteoblast derived human osteosarcoma cells SaSO2 (Rabbani SA et al., 1997). Similar effects of uPA on *c-fos* gene expression were reported in the human ovarian cancer cell line OC-7 by a mechanism involving tyrosine phosphorylation (Dumler I et al., 1994). In addition, Resnati et al. (Resnati M et al.,1996) reported that proteolytically inactivated uPA is able to increase the activity of the tyrosine kinase p56 / p59 *hck*. Busso et al. (Busso N et al., 1994) found increased serine phosphorylation of cytokeratins 8 and 18 after exposure of the human epithelial cell line WISH to pro-uPA. Li et al. (Li C et al., 1995) concluded that the cytokine-induced, uPA-stimulated adhesion of monocytic cells, ascribed to uPAR/VN binding by others, was in fact due to uPA induced uPAR-mediated signal transduction.

At the present time, the molecular mechanisms underlying uPAR-mediated signal transduction events are not known. It has been suggested that the mitogenic effect of uPA on osteoblasts depends on an unusual fucosylation of Thr 18 within the GFD of uPA (Rabbani SA et al., 1992). Busso et al (Busso N et al., 1994) tentatively assigned protein kinase Cε as being responsible for the observed tyrosine phosphorylation of cytokeratins. Del Rosso et al. (Del Rosso M et al., 1993) and Anichini et al. (Anichini E et al., 1994) implicated the formation of diacylglycerol in uPAR signal transduction, whereas Li et al (Li

C et al., 1995) suggested that uPA signalling involves the cAMP pathway. Resnati et al. (Resnati M et al., 1996) showed co-immunoprecipitation of uPAR and p56 / p59 *hck*, and presented evidence that signal transduction initiated by uPA/uPAR binding may be mimicked by proteolytic cleavage of uPAR between domains 1 and 2. Since the binding of ligands to integrins initiates a signal transduction cascade (Clark EA and Brugge JS, 1995), it could be speculated that the reported binding of uPAR to the integrin ligand VN and interactions between uPAR and β1- and β2 integrins are involved in initiation of a signal transduction cascade which regulates cell migration (Cao D et al., 1995; Nip J et al., 1995).

1.4. Messages from the Knockout Studies

The recent advent of uPA-, uPAR-, tPA-, PAI-1- and plasminogen deficient mice has significantly enhanced our knowledge of the components of the PA-plasmin system with respect to their functions in physiological conditions. Furthermore, limited results of studies on tumorigenesis and tumor progression in these animals which are deficient of the components of the PA-plasmin system have supported the notion that PA-plasmin system plays an essential role in tumor progression by promoting tumor invasion and metastasis.

1.4.1. Phenotypic Characteristics of Mice Deficient of the Genes of the PA-plasmin System

Mice deficient of the genes of the PA-plasmin system have been generated by homologous recombination techniques. There was no obvious lethal effect observed in all knock-out mice including uPA-/-, tPA-/-, uPAR-/-, plasminogen-/- and uPA-/-:tPA-/-. They develop normally in utero and are fertile. However, due to a severe systemic defect in fibrinolysis, starting at 2-3 months of age, uPA-/-:tPA-/- mice develop multiple organ

failure as a result of extensive extra-vascular fibrin accumulation as well as generalized microvascular thrombosis (Carmeliet P et al., 1994). A similar phenotype is observed in plasminogen deficient mice (Bugge TH et al., 1995; Ploplis VA et al., 1995). No overt phenotype alterations have been observed in uPAR-/- mice with respect to development, fertility and hemostasis (Bugge TH et al., 1995). Similarly, development and fertility are unaffected in PAI-1deficient mice (Carmeliet P et al., 1993a, b). These findings indicate that PAs and plasminogen are not essential for mouse development, postnatal growth or fertility. However, these studies have helped to address some issues with respect to the physiological significance and function of the PA-plasmin system, as well as the dynamic interactions among the components of this system.

1.4.2. Conversion of Pro-uPA to Active uPA

The fact that targeted disruption of the plasminogen gene does not affect the level of active uPA in urine of plasminogen-/- mice (Bugge TH et al., 1995a) indicates that plasmin-dependent activation of pro-uPA seems to act only as a positive feedback mechanism. Available experimental evidence strongly suggests that binding of uPAR to pro-uPA is a requirement for the plasmin-mediated activation of pro-uPA (Ellis V et al., 1989; Stephens RW et al., 1989). However, the urine of uPAR-/- mice contains active uPA (Bugge TH et al., 1995b), demonstrating that activation of pro-uPA can, at least to some extent, proceed via an uPAR-independent mechanism.

1.4.3. Pericellular Enzyme Activity of uPA and Plasmin

Several experiments have shown that generation of the active plasmin by uPAR-bound uPA occurs on the cell surface (Ellis V et al., 1989; Stephens RW et al.,

1989; Bugge TH et al., 1995b). Although these studies emphasize the importance of uPAR for uPA-catalyzed plasminogen activation, there is evidence from knock-out studies suggesting that uPA-mediated plasminogen activation may also occur via a uPAR-independent mechanism. The common phenotypic characteristic of plasminogen-/- mice and mice deficient in both uPA and tPA (uPA-/-:tPA-/-) is the development of pervasive multi-organ fibrin deposits. This characteristic is seen in a much milder form in nude mice deficient in either uPA or tPA (Carmeliet P et al., 1994; Bugge TH et al., 1995a). These results indicate that uPA and tPA can substitute for each other in the generation of plasmin for fibrinolysis. Importantly, such abnormalities are also much milder in mice deficient in uPAR and tPA (uPAR-/-:tPA-/-), which exhibit a phenotype similar to that of mice deficient in t-PA alone (Bugge TH et al., 1996). These observations suggest that uPA can act independently of uPAR, at least in fibrinolysis where uPA can substitute for tPA.

1.4.4. uPA-mediated Plasminogen Activation in Tumor Progression

Results obtained from animal model systems collectively point to uPA / uPAR-mediated proteolysis at the surface of cancer cells being causally related to the metastatic process (Ossowski L et al., 1988; Quax PHA et al., 1991). The role of uPA and uPAR in tumor invasion and metastasis will be discussed in detail later as a separate issue.

Chemical-induced tumorigenesis was studied in uPA-/- mice. No obvious difference of tumorigenesis was seen in control and uPA deficient mice. However, local invasion was significantly reduced in uPA-/- mice. Moreover, uPA-/- mice exhibited a drastically reduced progression to a more malignant stage (Shapiro RL et al., 1996). The incidence of lung metastases from mammary tumors induced by mouse mammary tumor

virus and polyoma middle T antigen was up to 10-fold lower in plasminogen deficient mice than in control mice, while tumorigenesis and primary tumor progression was unaffected. Thus, the results from mice deficient of uPA and plasminogen are in agreement with the idea that uPA-catalyzed plasminogen activation is rate-limiting for tumor progression, local tumor invasion and / or formation of distant metastasis.

1.4.5. Redundancy of the PA / plasmin System

Although studies on nude mice deficient of the components of the PA/plasmin system have provided important information about the function of this system, there is a lack of similar information on the role of PA/plasmin system in other species; and to date, homozygous plasminogen deficiency has not been reported in humans. One explanation for this might be redundancy, which may point to the importance of extracellular proteolysis for survival. Redundancy may make it difficult to perceive a phenotype if multiple pathways of extracellular proteolysis operating in parallel share the same function, and if removal of one of these pathways is insufficient to perturb developmental and physiological functions.

The lack of a more severe phenotype in the knockout mice might also indicate the existence of compensatory mechanisms i.e. for proteins with related functions, in the absence of regulatory feedback mechanisms, removal of one protein would result in the up-regulation of the other. However, at the present time, there is no persuasive evidence for compensation within the PA/plasmin system, or between the PA/plasmin system and other proteolytic pathways. However, it is also possible that the lack of a lethal phenotype in null mice could simply be a reflection of our inability to measure, in a laboratory setting a survival advantage conferred by the presence of the gene in question. It should also be

noted that deficiencies in uPA and tPA, as well as homozygous plasminogen deficiency, have not been reported in humans. This may point to important differences in the requirements for fibrinolysis in mouse and man, which may be related to differences in life span, the length of gestation and the size of the organism. Deletions in the PA/plasmin system may therefore be better tolerated in mouse than in man.

1.5. MOLECULAR BIOLOGY OF BREAST AND PROSTATE CANCER

1.5.1. Pathophysiology

Breast cancer is a hormone dependent malignancy and it is the most common cancer among women in North America. One of eight women will develop benign or malignant breast lesions before she reaches age of eighty (Cannon-Albright LA and Skolnick MH, 1996). The factors that correlate most with risk of breast cancer are age, genetic and reproductive history (Townsend CM, 1997). Similar to breast cancer, prostate cancer is another common hormone dependent malignancy affecting men, resulting in a high incidence of morbidity and mortality (Franks LM, 1973). Due to the similar nature of tumor initiation and tumor progression in breast and prostate cancer, this discussion will be focused on different aspects of breast cancer. Prostate cancer wil be discussed in areas where it differs significantly from breast cancer.

Breast cancer is known to metastasize early after tumor inception (Wilson MA and Calhoun FW, 1981; Koscielny S et al., 1984). At the time of diagnosis, although the disease is clinically localized, most patients have preexisting subclinical disseminated diseases who will go on to develop metastatic lesions (Lee YTN, 1983). Invasive tumors spread rapidly by infiltrating through tissue spaces and can intravasate both lymphatic and

blood vessels. There is a high incidence of distant metastases associated with breast cancer. The most common sites of breast cancer metastasis are bone (35%), soft tissues (35%), lung (20%) and pleura (12%), followed by liver (10%) and brain (2%) (Townsend CM, 1997). Critical phenotypic changes that occur during the progression of breast cancer include the loss of hormone dependence, acquisition of an invasive and metastatic phenotype and acquisition of a multi-hormonal resistant and a multi-drug resistant phenotype (Clarke R et al., 1994). Prostate cancer progression exhibits a similar pattern as compared to that of breast cancer. However, unlike breast cancer, androgen independent growth is not due to the loss of expression of functional androgen receptors in tumor progression. Rather, the lack of androgen receptor function is mostly due to various point mutations in the androgen receptor (Newmark JR et al., 1992). The curative potential of current therapies for breast and prostate cancer is restricted by the disseminated nature of the disease and the progression of the majority of tumors to a phenotype characterized by resistance to both cytotoxic drugs and hormonal therapies.

1.5.2. The Genetics of Familial Breast and Prostate Cancer

Breast cancer has been recognized to be in part, a familial disease (Cannon-Albright LA and Skolnick MH, 1996). Genetic factors contribute to an estimated 5 to 10% of all cases, and up to 25% of all cases diagnosed before age 30 (Claus EB et al., 1991). Familial breast cancer is associated with other cancers such as Li-Fraumeni syndrome (Thoriacius S et al., 1995), breast-ovarian cancer syndrome (Cannon-Albright LA et al., 1995), breast cancer associated with colon cancer (Wooster R et al., 1995) and with endometrial cancer (Tavtigian SV et al., 1996).

A number of genetic factors signal a higher risk of developing early onset breast

cancer. A breast cancer susceptibility gene, BRCA1, was isolated and localized to chromosome 17q (Hall JM et al., 1990; Miki Y et al., 1994). The estimated cumulative risk of breast cancer and ovarian cancer in BRCA gene carriers is 87% and 44% respectively by the age of 70 years. Germline mutations of BRCA1 tumor suppressor gene are involved in a significant fraction of hereditary breast and ovarian cancers (Ford D et al., 1994).

At about the same time, a second locus, BRCA2, was mapped to chromosome 13q (Wooster R et al., 1994). BRCA2 appears to account for a proportion of early onset breast cancer, which is roughly equal to that resulting from BRCA1. BRCA2, however, appears to be much less of an indication of ovarian cancer risk than BRCA1. BRCA2 gene is associated with a high incidence of male breast cancer. The profile of BRCA2 mutation has not been fully established.

In breast cancer, inactivation of tumor suppressor genes is an early event. Germline mutation-mediated inactivation of one copy of tumor suppressor genes followed by a somatic mutation or deletion of the second copy leads to the loss of functional tumor suppressor genes. Germline mutation in tumor suppressor gene p53 (located on chromosome 17) is a prominent risk factor in breast cancer (Malkin D et al., 1990). In the case of prostate cancer, loss of heterozygosity (LOH) due to chromosomal deletion, may inactivate a tumor suppressor gene. Specific allelic loss on chromosome 8, 10 and 16 are reported in prostate cancer (Carter BS et al., 1990; Bergerheim USR et al., 1991). In addition, allelic deletions on chromosome 13, 17 and 18, which harbour the tumor suppressor genes Rb, P53 and DCC respectively, occurs in 20% of prostate cancer (Isaacs WB et al., 1994). In contrast to breast cancer, p53 mutations are late events in the progression of prostate cancer (Isaacs WB et al., 1991; Visakorpi T et al., 1992; Bookstein

R et al., 1993).

Unlike breast cancer, which has a high incidence among pre-menopausal women, prostate cancer is known for its unusual latency (Gittes RF, 1991). The genetic or epigenetic basis for this cancer latency is unknown. Although prostate cancer predisposition and time to acquire full metastatic potential can be accelerated by inheritance (Carter BS et al., 1992), genes involved in the familial inheritance of prostate cancer which may help unlock the deregulated growth factor pathways involved in prostatic progression for sporadic cases, have not yet been identified.

The identification of inherited susceptibilities for breast and prostate cancer opens new possibilities for the development of novel detection, screening, and treatment strategies. Characterization of these genes will greatly enhance our understanding of breast and prostate cancer, the interaction of genes and environment, and tumorigenesis. Genetic screening for both BRCA1 and BRCA2 will eventually disclose the mystery of inherited susceptibility to develop breast cancer.

1.5.3. Steroid Hormones in Breast Cancer

The development of normal breast tissue is the result of complex interactions between a number of hormones and growth factors including steroids, insulin and various pituitary factors. Breast cancer is characterized by hormonal control of its growth, and estrogen is the hormone most closely associated with the control of neoplastic breast tissue (Clarke R et al., 1992). Clinical observations that support the role of estrogen in the growth of breast cancer are the marked decrease in tumor growth following ovariectomy and remission following treatment with anti-estrogens (Lippman ME, 1985). The presence of specific, high affinity, and low capacity binding sites are obligatory for cells to respond to

hormonal stimulation. Primary breast cancer appears to arise from within the estrogen receptor (ER)-positive epithelial cell populations (6-7%) of normal breast tissue (Petersen OW et al., 1987; Jacquemier JD et al., 1990; Clarke R et al., 1990). Approximately two-thirds of all breast tumors express detectable levels of ER protein in their early state (Clarke R et al., 1992).

The action of estrogen is mediated by its nuclear receptor (ER) which is a complex ligand-activated transcription factor that is a member of the steroid and thyroid hormone nuclear receptor superfamily. A general model of sex steroid action is as follows: the steroid enters the cell by simple diffusion through the plasma membrane and binds to the hormone-binding domain of its intracellular receptor. The ER (King WJ and Greene GL, 1984) is a nuclear protein containing multi-functional domains for DNA binding, transactivation, ligand binding and dimerization (Martin MB et al., 1994). The coupling of receptor to ligand results in the dissociation of HSP90s and allows for homodimerization of the receptor. The dimerized form of the receptor then binds with high affinity to a palindromic sequence in the 5'-upstream region of estrogen-inducible genes called the estrogen responsive element (ERE). The activation of the ER by estrogen leads to conformational changes that enhance the activity of constitutive activating factor AF-1 and ligand-activating factor AF-2. AF-2 acts synergistically with AF-1, leading to enhanced induction of estrogen responsive genes (Clarke R et al., 1994). The multiple actions of estrogen in combination with the estrogen receptor protein in the cell nucleus include stimulation of cell growth; increases in levels of growth factors (e.g. TGF-α) and other secreted proteins, and a decrease in the level of TGF-β, an inhibitory growth factor for breast cancer cells (Dickson RB and Lippman ME, 1987; Knabbe C et al., 1987).

1.5.4. Growth Factors and Stroma Interaction in Breast Cancer

Both stimulatory and inhibitory growth factors are elaborated by breast cancer cells in response to hormone action. Breast cancer cells express a variety of receptors to various growth factors and hormones which results in a wide range of complex autocrine or paracrine interactions that regulate their growth and differentiation (Clarke R et al., 1992). Growth factors that stimulate cell proliferation include members of transforming growth factors α (TGFα), epidermal growth factor (EGF), insulin-like growth factors (IGF), basic fibroblast factor (bFGF) and hepatocyte growth factor (HGF) families. Members of the TGF-β family inhibit cell growth (Kambly C, 1990). The steroid hormone estrogen may play a role in this process via regulation of both growth factors and growth factor receptor gene expression.

The stromal responses to tumors represent a fluid and dynamic equilibrium which reflect the changing biological properties of the tumor cell populations and their interactions with adjacent stromal cells. It has been demonstrated that the paracrine interaction between tumor cells and their surrounding stroma plays a crucial role in tumor progression (Clarke R et al., 1992). Tumor stroma may produce growth factors, thereby providing both inhibitory and stimulatory effects on adjacent tumor cells. On the other hand, growth factors secreted by tumor cells may stimulate the proliferation of stromal cells (Yee D et al., 1989; Bronzert DA et al., 1987; Ewton DZ et al., 1987).

In summary, steroid and polypeptide hormones play multifaceted roles in the control of breast cancer growth and progression.The concerted action of many growth factors, acting by both autocrine and paracrine pathways, may be required to induce the full range of biological responses observed in human breast tissue.

1.5.5. Prognostic Markers

The natural history of breast and prostate cancer is variable and the survival of treated patients presenting at the same clinical stage of disease varies from a few months to several decades. Many effective treatment modalities have been developed, however, each treatment strategy is effective for only some individuals with breast and prostate cancer (Dhingra K and Hortobagyi GN, 1996). Therefore, it is important to identify biological markers that can predict the natural history of these diseases, allow determination of an optimal treatment strategy for each individual and predict the outcome following such interventions. The most important issue in the management of primary breast and prostate cancer is the identification of individuals who harbour micro metastatic disease. Therefore, characterization of these prognostic factors will help to improve prediction of the presence of micro-metastases, prediction of the site of and time to manifestation of metastases and prediction of sensitivity of micro-metastases to planned therapy.

1.5.5.a. Morphological Prognostic Markers

Conventionally, the presence of tumor cells in the draining lymph nodes has been the best predictor of systemic micro-metastases (Dhingra K and Hortobagyi GN, 1996).Tumor size and histopathological features (tubular, mucinous, papillary) have been shown to have predictive value for relapse among node-negative patients (Carter CL et al., 1989). Quantitative pathological features, ie, nuclear morphology (Fischer ER et al., 1993), DNA content (Hedley DW et al., 1993), and proliferative activity (Biesterfeld S et al., 1995), may further demarcate tumors that have a high likelihood of micro-metastases. Although using gross and microscopic pathological features for prognostic assessment offers easy usage and interpretation, they do not allow a precise prediction of outcome in

any given individual. Such arguments have led to the investigation of specific molecular changes as indicators of prognosis.

1.5.5.b. Molecular Prognostic Markers

Tumor metastasis is a complex process in which specific molecular genetic changes may contribute to each of the processes involved in this cascade. Overexpression/activation of oncogenes that promote cellular transformation, tumor growth and / or dissemination has been detected in a significant portion of breast cancer patients (Berns EMJJ et al., 1992). These oncogenes include HER2 / *neu* oncogene (Slamon DJ et al., 1987), *c-myc* oncogene (Berns EMJ et al., 1992), and an amplified locus consisting a group of closely linked genes on chromosome 11q whose protein products are cell cycle-related proteins (Berns EMJJ et al., 1992). A complementary pathway for deregulation of cellular growth is the loss of critical tumor suppressor genes such as p53 (Allred DC et al., 1993).

In addition to fixed genetic lesions, a large variety of proteins involved in cellular differentiation, proliferation, and invasion are differentially expressed in neoplastic and normal breast epithelium. The most widely recognized among these are the hormone receptors, ie, estrogen receptor (ER) and androgen receptor (AR) which are directly correlated with the state of differentiation of the breast and prostate tumor cells and are important predictors of efficacy of adjuvant endocrine therapy (Pichon MF et al., 1992; Esteban JM et al., 1994). Proteolytic enzymes are a key component of the invasive process, and therefore, they are ideal markers for predicting the presence of micro-metastases. The components of the PA system, uPA, uPAR and PAI-1 have been shown to be independent prognostic markers for a number of human malignancies including

breast and prostate cancer (Duffy MJ et al., 1994; Foekens JA et al., 1992; 1994; Janicke F et al., 1993). High levels of uPA, uPAR and PAI-1 are associated with poor prognosis, short disease-free survival and short overall survival. Growth of metastases beyond a size of 0.2 cm requires angiogenesis. Blood capillary density ratio (BCDR) which is an index for angiogenesis has been shown to predict clinical progression of breast and prostate cancer to establish systemic metastases (Wakui S et al., 1992; Pepper MS et al., 1996). In addition, E-cadherin, a calcium-dependent cell adhesion molecule emerges as a potential molecular marker for prostate cancer. Inactivation of the E-cadherin gene has been reported in over 30% of prostate cancer patients (Bergerhein USR et al., 1991). Furthermore, serum prostate specific antigen (PSA), which is significantly elevated in a large percentage of benign prostatic hypertrophy (BPH) and prostate carcinomas is a potential marker for diseases of the prostate (Wang MC et al., 1981). PSA is a protease produced by the epithelial cells lining the acini and ducts of the prostate gland (Hsieh WS and Simons JW, 1993). The presence of an upstream DNA binding sequence for the androgen receptor in PSA gene makes it an androgen-inducible gene (Reigman PHJ et al., 1991). However, the role of PSA as a prognostic marker has not been fully elucidated.

1.5.6. Hormonal Approaches to Breast and Prostate Cancer Treatment: Actions of Anti-estrogens and Anti-androgens

Since the initial therapeutic use of ovarian ablation by Beatson in 1896 (Beatson GT, 1896), and androgen ablation (orchiectomy) by Huggins and Hodges in 1941 (Huggins C and Hodges CV, 1941), hormonal manipulation has been the mainstay of the palliative management of metastatic breast and prostate cancer. With the development of new pharmaceuticals with favourable risk-benefit profiles, hormonal therapies have now

become firmly entrenched as adjuvant therapies to surgery and radiation therapy for breast cancer and prostate cancer (Hsieh WS and Simons JW, 1993; Vogel CL, 1996).

From the 1940s until 1970, available hormonal therapies included medical castration using pharmacologic doses of estrogens, corticosteroids, progestins and LHRH analogues, or surgical castration achieved by oophorectomy, orchiectomy, hypophysectomy, and andrenalectomy (Hsieh WS and Simons JW, 1993; Vogel CL, 1996). Two major developments during the early 70s revolutionized hormonal therapy for treating breast cancer. The first was refinement of assay procedures for ER and PR (McGuire WL and Chamness GC, 1973; Englesman E et al., 1973). The second development was the discovery of the anti-estrogen, tamoxifen (Litherland S and Jackson IM, 1988). Since then, tamoxifen has become the first line of hormonal therapy. Until radically new therapeutic strategies emerge (eg, anti-angiogenic drugs, differentiating agents, anti-growth factor strategies, or anti-oncogene therapies, etc), refinement of the preexisting adjuvant hormonal approaches will continue to remain as the main strategy for the treatment of metastatic breast cancer.

The marked heterogeneity of breast and prostate tumors is a major restriction of the curative potential of single modality treatments. Even with tumors which express high levels of ER, there are areas which do not possess ER which are not responsive to hormone intervention (Van Netten JP et al., 1988). The selective pressure applied by endocrine manipulation would be expected to remove these sensitive populations (ER positive) and could facilitate the emergence of tumors comprised of predominantly endocrine-resistant cells (ER-negative). Therefore, a combination of cytotoxic chemotherapy with hormonal manipulation could result in the cytotoxic drugs killing the ER-negative sub-populations and the hormonal agents eliminating the hormone dependent

cells (Clarke R et al., 1992). Although combinational therapy begins to emerge as an effective approach to treat breast cancer, there remains a continued need to perform preclinical studies to explore this venue in order to establish the validity of these strategies over the traditional therapeutic strategies.

1.5.6.a. Anti-estrogens and Anti-androgens

Since it was first described in 1966 (Harper MJK and Walpole AL, 1967) and introduced into clinical use in the1970s, the synthetic antiestrogen tamoxifen citrate has been shown to contribute to the management of various hormone dependent malignancies, especially breast cancer (Jordan VC, 1994). The tamoxifen therapy, either alone or in conjunction with surgery, significantly improves the prognosis of breast cancer patients. It has a response rate up to 75% in ER-positive tumors. Tamoxifen is the preferred adjuvant treatment for post-menopausal women with nodal involvement and ER-positive tumors. It is also being tested in combination with chemotherapy in patients with ER-negative tumors. Tamoxifen is now also under evaluation in the clinical trials in an attempt to prevent breast cancer in high-risk groups (Townsend CM, 1997).

Tamoxifen is the most widely studied member of a class of molecules called the triphenylethylenes (TPEs). The ubiquitous applications of antiestrogen therapy for breast cancer treatment and the reported increased risk of various forms of cancers including the cancers of endometrium, liver, stomach and colerectum (van Leeuwen FE et al., 1994; Rutquist LE et al., 1995; Grainger DJ and Metcalfe JC, 1996) have encouraged the development of new anti-estrogens, with potentially more favourable pharmacological properties and lower toxicity. Nevertheless, the use of tamoxifen is associated with remarkably few other acute or chronic side effects. It has been well established that the

benefit of tamoxifen therapy will substantially outweigh the increased risks. The carcinogenic effects of tamoxifen could be partially due to its partial estrogen agonist actions, and could also be due to the presence of the long-lived major metabolite of tamoxifen, 4-hydroxytamoxifen, which is known to form DNA adducts. More recent analogues of tamoxifen such as toremifene, droloxifen, idoxifene and raloxifene, which are generated by modifying the chemical structure of tamoxifen, have no estrogenic effects on tamoxifen-targeted tissues, and do not form DNA adducts. They are expected to have a reduced carcinogenic profile during clinical use (Potter GA et al., 1994; Grainger DJ and Metcalfe JC, 1996) (Figure 1.4).

In addition to their wide applications in the treatment and prevention of breast cancer, recent evidence suggests that tamoxifen and its new analogues may be useful in the treatment of a much wider range of diseases including atherosclerosis, osteoporosis and autoimmune diseases like rheumatoid arthritis and multiple sclerosis. Tamoxifen has also been shown to have significant therapeutic benefits for the prevention of coronary heart diseases (Grainger DJ and Metcalfe JC, 1996). The mechanisms underlying the actions of tamoxifen in treating these diseases have not been well elucidated. Since tamoxifen treatment results in the up-regulation of TGF-β which has diverse effects on regulating various cellular functions (Betta A et al., 1992; Mauviel A et al., 1993; Grainger DJ et al., 1995; MacDonald CC et al., 1995), it is an intriguing possibility that up-regulation of TGF-β production, rather than the direct antagonism of estrogen function, is the molecular mechanism underlying the beneficial effects of tamoxifen and its analogues in treating these diseases. However, more studies await to address this issue which could result in the further expansion of the therapeutic applications of these agents.

Similarly, anti-androgens are employed in the treatment of prostate cancer

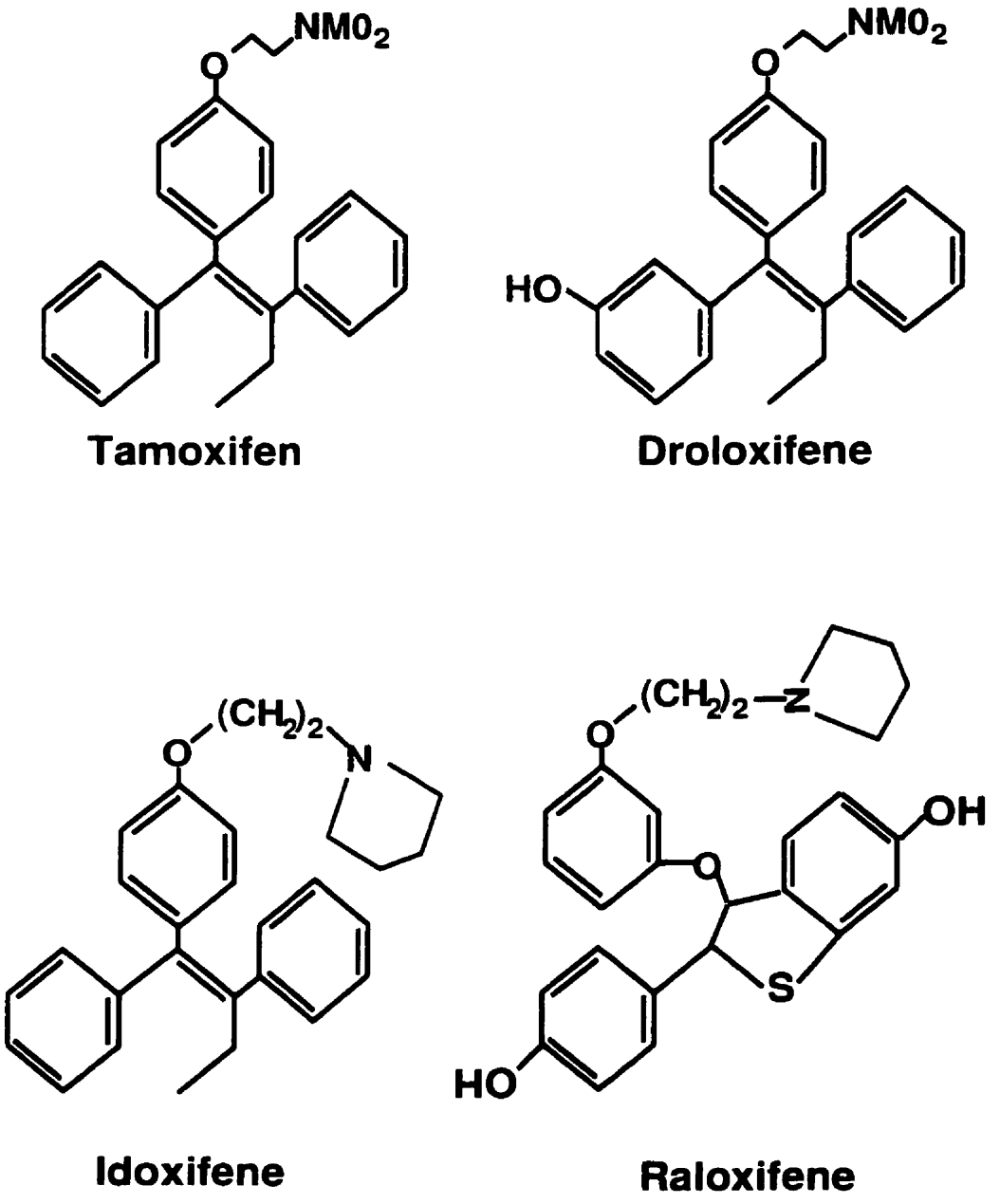


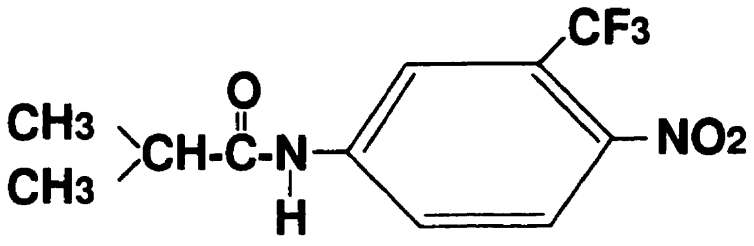
Figure 1.4: Chemical structures of anti-estrogens

presented at different clinical stages as a first line hormone therapy, or in conjunction with surgery and/or chemotherapy. Figure 1.5 shows the chemical structure of two most commonly prescribed anti-androgens flutamide and cyproterone acetate. Flutamide acts as a competitive inhibitor for dihydrotestosterone (DHT) and cyproterone acetate is an inhibitor for androgen synthesis (Vogelzang NJ and Kennealey GT, 1992; Hsieh WS and Simons JW, 1993). However, anti-androgen therapy is less effective in treating prostate cancer than antiestrogen therapy for breast cancer. The molecular mechanisms underlying the actions of anti-androgens are poorly elucidated. Therefore, total androgen ablation achieved through orchiectomy, medical castration and inhibitors of androgen synthesis remains the most effective regime for the control of prostate cancer progression. Anti-androgens are mostly used in conjunction with surgery or chemotherapy (Vogelzang NJ and Kennealey GT, 1992).

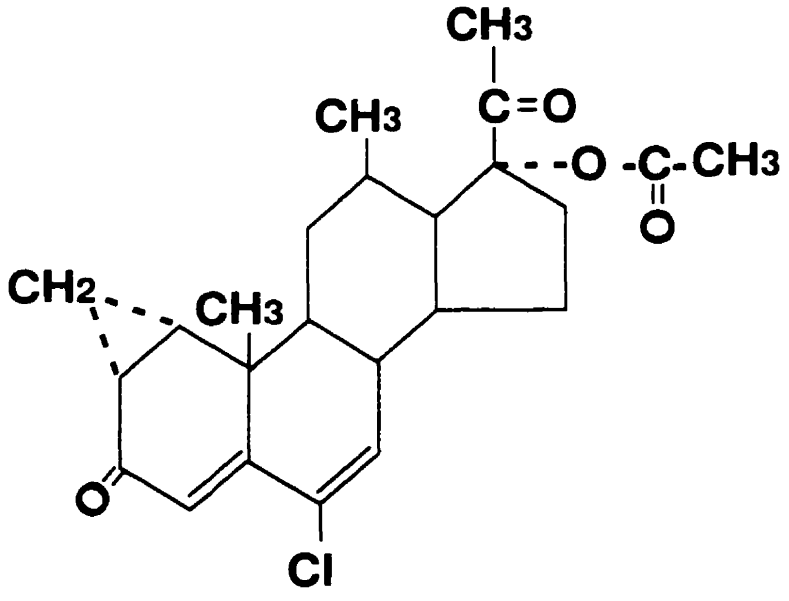
Due to the similar nature and mechanisms of the actions of anti-estrogens and anti-androgens, only the actions of the antiestrogen tamoxifen will be used as an example for further discussion.

1.5.6.b. Anti-estrogenic Actions of Tamoxifen

Anti-estrogens can act at several sites in the chain of events of estrogen action. The major action of tamoxifen results from the fact that the compound and its metabolites bind to the estrogen receptor (Sutherland RL and Jordan VC, 1981; Jordan VC, 1986). Structural differences in the shape of estrogen and anti-estrogen receptor complexes indicate that the steroid binding domain could envelop an estrogen to produce a fully activated complex. In contrast, an anti-estrogen would bind to the ER, but the alkylaminoethoxy side chain would prevent folding of part of the steroid binding domain



Flutamide



Cyproterone acetate

Figure 1.5: Chemical structures of anti-androgens

referred to as the "anti-estrogen region". Part of the molecule would remain exposed resulting in the impairment of full estrogen activation (Lieberman ME et al., 1983; Jordan VC et al., 1984; Jordan VC and Lieberman ME, 1984; Jordan VC et al., 1988). Conventional wisdom has hypothesized that anti-estrogens competitively bind to ER at or near the estrogen binding site, thus restricting access to estrogen, causing decreased transcription of estrogen-regulated genes. The end result is cytostasis with cell cycle arrest in G0/G1 and a decrease of cells in S phase. Therefore, tamoxifen is cytostatic in its anti-tumour action (Jordan VC, 1988; Osborne CK et al., 1983; Sutherland RN et al., 1983). Animal and clinical data suggest that tamoxifen therapy, once initiated, should be given indefinitely because the main action of this drug is as a cytostatic agent (Sutherland RL and Jordan VC, 1981).

However, many receptor independent effects of tamoxifen have also been defined and these non-estrogen receptor-mediated actions of tamoxifen may be equally or more important. Additionally, tamoxifen appears to stimulate the production of the progesterone receptor (Horowitz KB et al., 1978) and liver synthesized proteins such as sex hormone binding globulin (SHBG) (Jordan VC et al., 1987). SHBG is the major blood transport protein for estrogenic steroids and, therefore, an increase in its binding capacity may ultimately decrease the concentration of the free steroid in plasma. This could result in less steroid being available to the breast cancer cells. Tamoxifen has been reported to have a wide variety of pharmacological activities. These include inhibition of protein kinase C, acting as a calmodulin antagonist and the blockade of various choloride channels (O'Brain CA et al., 1985; Gulino A et al., 1986; Benry J et al., 1987). However, perhaps its most interesting action is the regulation of the cytokine, TGF-β 1. Tamoxifen has been shown to increase the production of TGF-β 1 by breast tumor cells in culture and *in vivo* (Grainger

DJ and Metcalfe JC, 1996). TGF-β 1 has bifunctional effects on tumor development. During early tumor initiation and growth, direct inhibition of tumor cell proliferation mediated by tumor-derived and circulating TGF-β 1 is the dominant effect. Later, during advanced tumor growth and metastasis, TGF-β 1 may promote tumor growth and spread by reducing immune surveillance and increasing angiogenesis. Anti-estrogens may also block the actions of stimulatory paracrine growth factors on ER-positive cells (Freiss G et al., 1990) and reduce the availability of IGF-1 both in the circulation (Pollak MN et al., 1990) and locally in normal tissues (Huynh HT et al., 1993). The inhibitory effect of anti-estrogens on tumor growth may contribute to their ability to inhibit angiogenesis (Jordam VC, 1994).

1.6. ROLE OF uPA AND ITS RECEPTOR (uPAR) IN INVASION AND METASTASIS OF HORMONEDEPENDENT MALIGNANCIES

There is convicing evidence for the involvement of the uPA system in tumor invasion and metastasis. It is now established beyond reasonable doubt that uPA/uPAR-mediated events are central to this process. First, high levels of uPA, uPAR and PAI-1 in tumors are associated with poor prognosis in various human malignancies. Secondly, many preclinical model systems have provided evidence for a causal role of the uPA system in the progression of hormone dependent malignancies like breast and prostate cancer.

1.6.1. uPA and uPAR in Cell Migration

Cell migration is the coordinated locomotion of a cell over an ECM substratum,

and it is an important event in tumor invasion and metastasis. It involves precisely regulated attachment and detachment of cells from the ECM. This process is mediated by adhesion events via adhesion molecules including integrins (Hynes RO, 1992; Lauffenburger DA and Horwitz AF, 1996).

Considerable evidence, obtained from *in vitro* migration assays, supports the notion that the uPA/uPAR system plays an essential role in regulating cell migration (Estreicher A et al., 1990; Odekon LE et al., 1992; Morimoto K et al., 1993; Quax PHA et al., 1994; Busso N et al., 1994; Stahl A and Mueller BM, 1994). The effects of uPA / uPAR on cell migration may be mediated by a proteolytic as well as a non-proteolytic mechanism. A proteolytic mechanism of uPA enhancement of cell migration would involve plasmin generation at focal adhesion sites, catalyzed by uPAR-bound uPA, which would then lead to ECM degradation and thus facilitate detachment of the trailing edge. With a non-proteolytic mechanism, uPA would stimulate cell migration by enhancing adhesion at the leading edge, through stimulation of binding of uPAR to VN, modulation of uPAR / integrin interactions and / or by initiation of signal transduction cascades. Both mechanisms of action could be operating simultaneously in each migrating cell. The relative importance of the proteolytic and the non-proteolytic elements of uPA action depends on the expression of uPA, uPAR, PAI-1, and integrins by the individual migrating cells and on the composition of the ECM and the plasminogen concentration. The fact that uPA-/-, uPAR-/- and PAI-1-/- mice develop to term, grow to adulthood and are fertile shows that any actions of the uPA system in cell migration during development, and therefore, possibly at other times, can be substituted by the actions of other proteins (Carmeliet P et al., 1994; Bugge TH et al., 1995; Carmeliet P et al., 1993a, b).

1.6.2. uPA and uPAR in Tumor Cell Invasion

When progressing from cell migration to cell invasion, an additional factor of complexity is added as invasion requires not only cell locomotion but also the active penetration of cells into the ECM. The role of the uPA system in cell invasion has been extensively investigated using several different *in vitro* assays, including assays with invasion through isolated human amniotic membrane (Mignatti P et al., 1986; Bruchner A et al., 1992), into Matrigel (Reich R et al., 1988, Kobayashi H et al., 1993; Xing RH and Rabbani SA, 1996) and into a fibrin gel (Montesano R et al., 1987; Kramer MD et al., 1994). From these studies, it has been demonstrated that maximum invasive activity requires uPA-catalyzed plasminogen activation (Mignatti, P et al., 1986; Crowley CW et al., 1993; Kobayashi H et al., 1994). Inhibition of the proteolytic activity of uPA with antibodies, anti-sense sequences and uPA inhibitors were found to efficiently inhibit cell invasion (Reich R et al., 1988; Kramer MD et al., 1994; Wilhelm O et al., 1995; Xing RH and Rabbani SA, 1996). Addition of uPA to cells with high uPAR and low uPA expression increased invasion (Hollas W et al., 1991). Inhibition of uPA / uPAR binding with anti-uPA or anti-uPAR antibodies, or other specific uPA / uPAR-binding antagonists inhibited cell invasion (Hollas W et al., 1991; Kobayashi H et al., 1993; Stahl A and Mueller BM, 1994; Luparello C and Del Rosso M, 1996; Xing RH et al., 1997). Overexpression of uPAR made recipient cells more invasive (Kariko K et al., 1993; Xing RH and Rabbani SA, 1996). In contrast, down-regulation of uPAR by transfection with anti-sense sequences significantly decreased the invasive capacity of recipient cells (Quattrone A et al., 1995). Soluble recombinant uPAR inhibited invasion by scavenging uPA (Wilhelm O et al., 1994). Collectively, these experiments strongly suggest that plasmin generation on the cell surface by uPAR-bound uPA is rate limiting for invasion of

the cell into the ECM.

1.6.3. uPA and uPAR in Tumor Angiogenesis

The process of tumor-associated angiogenesis is central to the growth and metastasis of malignancies (Hanahan D and Folkman J, 1996; Weidner N and Folkman J, 1996; Folkman J, 1997). Angiogenesis and tumor invasion appear to be related. Both events involve the dissolution of basement membranes and the migration of proliferating cells into the interstitial stroma (Liotta LA et al., 1991). In addition, the shedding of tumor cells into the circulation occurs at the onset of angiogenesis and is quantitatively related to the surface area of tumor vessels. Moreover, the proliferating capillaries of tumor neovasculature are leaky and contain fragmented basement membranes, thereby increasing the entry of tumor cells into the circulation (Dvorak HF et al., 1988).

The process of angiogenesis requires the direct interaction of endothelial cells with their surrounding matrix and is dependent on a number of precisely controlled functions of endothelial cells which can be modulated by proteases like uPA (Vassalli JD and Pepper MS, 1994). First, proteases like uPA and plasmin can mediate cell-matrix interaction during angiogenesis by promoting basement membrane degradation and ECM invasion. This allows endothelial cells to breach the mechanical barriers of the ECM (Pepper MS and Montesano R, 1990). Second, PA / plasmin system may contribute to the process of angiogenesis by activating latent cytokines, releasing matrix-bound cytokines, or by releasing membrane-anchored cytokine precursors which are modulators of angiogenesis (Flaumenhaft R and Rifkin DB, 1992). Third, the role of uPA in angiogenesis is not only mediated by its catalytic activity, but also by a paracrine / autocrine effect on endothelial cell migration. This is related to the ability of uPAR to modulate integrin functions (Pepper MS

et al., 1992; Pepper MS et al., 1993; Odekno LE et al., 1992; Wei Y et al., 1996; Brooks PC et al., 1994) thus leading to regulated cell adhesion and migration resulting in directed endothelial and tumor cell invasion. These functions can be readily accomplished due to the expression of different components of the PA system (uPA, uPAR, and PAI-1) by vascular endothelial cells (Grondahl-Hansen J et al.,1991; Bianchi E et al., 1994; Delbaldo C et al., 1995; Christensen L et al., 1996), which are regulated by the concerted actions of a variety of cytokines and growth factors with angiogenic activity including bFGF, VEGF, HGF, TNF, IL-1 and TGF-β (Salsela O et al., 1987; Pepper MS et al., 1991; Grant DS et al., 1993).

On the other hand, angiogenic factors (bFGF, VEGF, HGF,TGF-β) produced by tumor cells, stromal cells or endothelial cells may promote uPA/uPAR-mediated tumor invasion by up-regulating the expression of uPA, uPAR and PAI-1 (Pepper MS et al., 1991; Mandriota SJ et al., 1995; Mignatti P and Rifkin DB, 1996). uPA has been shown to provide signals for endothelial cell movement (Mignatti P and Rifkin DB, 1996; Busso N et al., 1994). Therefore, the induction of uPA increases plasmin formation, and co-induction of uPAR localizes the uPA activity to the cell surface and the migrating front. This leads to an increase in the efficiency of extracellular proteolysis on the cell surface. Co-induction of PAI-1 by angiogenic factors may play an important role during angiogenesis by preventing excessive and inappropriate matrix destruction. This ensures the presence of an intact matrix scaffold, into which appropriately stimulated endothelial cells can migrate to form capillary-like tubes.

1.6.4. Clinical Significance of the Plasminogen Activator System in Human Malignancies

1.6.4.a. Localization of the Components of the uPA System in Tumors

Cytological, immunohistochemical and in situ hybridization studies have demonstrated the presence and characteristic expression patterns of the multiple components of the uPA system in human tumor tissues (Skriver et al 1984; Dano K et al., 1985; Pollanen J et al., 1988; Andreasen PE et al., 1990). These studies strongly suggest an active involvement of the uPA system in tumor progression, invasion and metastasis. The presence of components of the uPA system in endothelial cells (Grondahl-Hansen J et al.,1991; Bianchi E et al., 1994; Delbaldo C et al., 1995; Christensen L et al., 1996) suggest a role in angiogenesis. Their presence on the surface of fibroblasts of the stroma (Grondahl-Hansen J. et al., 1991; Ohtani H et al., 1995; Christensen L et al., 1996) implicates a role in stromal remodelling. Their presence on various types of inflammatory cell (macrophages, neutrophils, eosinophils, granulocytes) (Pyke C et al., 1993b; Ohtani H et al., 1995; Bianchi E et al., 1994; Christensen L et al., 1996) is in agreement with findings in many non-neoplastic conditions (Vassalli JD, 1994).

Tumor cell expression of uPA, uPAR and PAI-I varies between tumors. uPA is mainly secreted by tumor cells in Lewis lung carcinoma (Skriver L et al., 1984), squamous cell carcinomas (Clayman G et al., 1993; Ornstein DL et al., 1991), astrocytoma (Yamamoto M et al., 1994) and renal cell carcinoma (Wagner SN et al., 1995). In colon cancer, the main source of uPA is from fibroblasts in the stroma (Grondahl-Hansen J et al., 1991; Pyke C et al., 1991; Delbaldo C et al., 1995), and in breast cancer (Jankun J et al., 1993; Christensen L et al., 1996), prostate cancer (Kirchheimer J et al., 1984; Van Veldhuizen PJ et al., 1996) and melanoma (De Vries TJ et al., 1994), uPA is produced by

both tumor cells and fibroblasts from the stromal environment.

With respect to uPAR, its expression is presented on the surface of various tumor cells of diverse origins (Pyke C et al., 1994; De Vries TJ et al., 1994; Ohtani H et al., 1995; Bianchi E et al., 1994; Wagner SN et al., 1995; Christensen L et al., 1996). The essential role of uPAR in mediating the actions of uPA is also supported by the observation that mere production of high levels of uPA, in the absence of a surface receptor, was inadequate in endowing a tumor cell with maximal invasive potential (Ossowski L et al., 1991). The pattern of localization of uPA and uPAR in tumor tissues suggest that uPAR localization is an essential event in the assembly of the machinery necessary for tumor invasion and metastasis. It also points to the diverse autocrine and paracrine interactions between tumor cells and between tumor and its surrounding stroma. Thus, uPA produced by one cell type can be utilized in a paracrine fashion to potentiate the invasive ability of another cell type which expresses uPAR.

Similar to the distribution of uPAR in tumor tissues, PAI-1 can be produced by tumor cells (Kristensen L et al., 1990; Reilly D et al., 1992; Jankun J et al., 1993; Bianchi E et al., 1995; Christensen L et al., 1996) in various human malignancies, by endothelial cells (Bianchi E et al., 1995), or by stromal cells (Bianchi E et al., 1995; Christensen L et al., 1996).

1.6.4.b. The Components of the uPA System as Prognostic Markers for Human Malignancies

Early studies have established that the level of uPA in malignant tumors is significantly higher than in the corresponding normal tissue or in benign tumors of the same tissue (Duffy MJ et al., 1988; Duffy MJ wt al., 1994; Duffy MJ, 1996a; Duffy MJ,

1996b). More importantly, the levels of uPA, uPAR, PAI-1 and PAI-2 have been found to vary considerably in malignant tumors and to be related to patient prognosis (Duffy MJ et al., 1996).

uPA was the first proteinase shown to be a strong and independent prognostic marker in human malignancy (Duffy MJ,1990; Janicke F et al., 1990; Schmitt M et al., 1992; Duffy MJ et al., 1992). Duffy et al. (1988; 1990) first reported that patients with breast tumors containing high levels of uPA had a significantly shorter disease-free interval and a shorter overall survival than patients with tumors containing low levels of uPA. Since then, similar results have been reported by many other research groups (Janicke F et al., 1990; Foekens JA et al., 1992; Grondahl-Hansen J et al., 1993, Ferno M et al., 1996). In particular, uPA has been shown to be a marker of disease outcome in axillary node-positive breast cancer patients (Janicke F et al.,1990; Duffy MJ et al., 1992; Duffy MJ et al., 1994; Ferno M et al., 1996). Apart from breast cancer, uPA has been shown to be a prognostic marker in other common human malignancies, including cancers of the prostate (Van Veldhuizen PJ et al., 1996), lung (Oka T et al., 1991), bladder (Hasui Y et al., 1992), stomach (Nekarda H et al., 1994), colorectum (Mulcahy HE et al., 1994), cervix (Kobayashi H et al., 1994b), ovary (Kuhn W et al., 1994), kidney (Hofmann R et al., 1996) and brain (Bindahl AK et al., 1994).

Accumulating evidence from recent clinical research has supported an emerging role for uPAR as a prognostic tumor marker in certain types of human cancers. High levels of uPAR have been shown to be correlated with poor prognosis, short disease-free survival and short overall survival in cancers of colorectum (Ganesh S et al., 1994b), breast (Duggan C et al., 1995; Grondal-Hansen J et al., 1995), prostate (Van Veldhuizen PJ et al., 1996), squamous carcinoma of the lung (Pedersen H et al., 1994a) and stomach (Heiss

MM et al., 1995). Similarly, another component of the PA system, PAI-I was first shown to be a prognostic marker in breast cancer by Janicke (Janicke F et al., 1993) with similar results reported in gastric cancer (Nakarda H et al., 1994), lung cancer (Pedersen H et al., 1994b) and advanced ovarian cancer (Kuhn W et al., 1994).

In summary, in human tumors, the correlation between uPA, uPAR and PAI-1 concentrations and poor prognosis is in agreement with the basic idea of uPAR-bound uPA at the surface of cancer cells being necessary for cancer cell invasion and metastasis. The results from clinical studies on the localization of the uPA system in tumor tissues, although not conclusive, provide compelling evidence of their multi-functional role in tumors, not only in cancer cell migration and invasion, but also in tissue remodelling events which occur in the tissue surrounding cancer cells. Some of the tissue remodelling events, e.g., angiogenesis, may be essential for the overall process of tumor metastasis.

1.6.5. The Role of uPA and uPAR in Prostate Cancer Progression

Prostatic carcinoma is one of the leading cancers affecting men with a high rate of mortality which is due to the presence of systemic metastatic disease (Chiarodo A, 1991; Shi XB et al., 1996). Prostate cancer arises from various genetic and epigenetic changes during malignant transformation. Once established, prostate cancer continues to progress under the influence of various growth factors and progression factors like proteases, which endow tumor cells with a more aggressive phenotype. A sustained increase in uPA production has been widely reported during the progression of various human malignancies including prostate cancer, where it is abundantly produced by tumor cells and their surrounding stroma (Kirchheimer J et al., 1984; Goltzman D et al., 1992; Peterson H et al., 1994; Van Veldhuizen J et al., 1996). Elevated levels of uPA have been reported in

the plasma of patients with prostate cancer as compared with those affected with benign prostatic hyperplasia. The highest levels of uPA were seen in patients with advanced disease associated with bone metastases (Van Veldhuizen J et al., 1996).

The production of uPA has been demonstrated in a panel of human (PC-3, DU-145) and rat (Dunning R3227 Mat Ly Lu) prostate cancer cell lines (Goltzman D et al., 1992), and it is particularly high in the highly aggressive human prostate cancer cell line PC-3 which was derived from tumor metastases to the lumbar vertebra (Van Veldhuizen J et al., 1996). Due to the lack of suitable *in vivo* models of prostate cancer and the species specificity of uPA actions, characterization of the role of uPA in prostate cancer progression has thus far remained an elusive goal. Various studies accomplished in our laboratory have contributed significantly towards our understanding of the molecular events underlying prostate cancer progression. A syngeneic model of uPA overexpression and under-expression by the rat prostate cancer cell line Mat Ly Lu was established by gene transfer techniques (Achbarou A et al., 1994). When tested *in vivo*, experimental animals receiving cells overexpressing uPA developed more extensive and larger metastases to various skeletal and non-skeletal sites. In contrast, animals inoculated with prostate cancer cells under-expressing uPA showed a delay in the emergence of bone metastasis and failed to develop metastases to non-skeletal sites. These results not only strongly demonstrated a direct role for uPA in prostate cancer progression and metastasis, but also showed the usefulness of this *in vivo* model which can serve to assess the efficacy of any uPA/uPAR targeted therapeutic strategy.

In a xenograft model of human prostate cancer, PC-3 cells, transfected with a catalytic mutant of uPA (Ser^{356}-Ala), failed to elicit the proteolytic effects of uPA. However, it retained its ability to bind to uPAR and was capable of blocking the interaction

between endogenous uPA and uPAR (Crowley CW et al., 1993). When tested *in vivo*, no significant difference in tumor growth was observed in the primary tumors of control and experimental animals. However, blockage of uPAR by mutant uPA resulted in a marked decrease in tumor metastases. These studies demonstrated the significance of the cell surface localization of uPA activity mediated via uPAR in prostate cancer progression and metastasis.

1.6.6. The Role of uPA/uPAR in Breast Cancer Progression

Adenocarcinoma of the breast is one of the leading malignancies affecting women causing a high rate of cancer-related death in its advanced stages (Russo J and Russo IH, et al., 1995). In this hormone dependent cancer, a direct correlation between elevated levels of uPA / uPAR and poor prognosis has been well established (Duffy MJ et al., 1988; Pyke C et al., 1993; Duffy MJ et al., 1994). Investigation of the mechanisms of tumor progression has demonstrated that uPAR expression within the tumor cell environment is a rate-limiting factor for uPA-mediated tumor cell invasion and metastasis (Ossowski L et al., 1991). Using *in vitro* quantitative radiography, tissue sections of breast carcinoma were found to contain 5 fold higher levels of uPAR and 19 times higher uPA levels by immunoenzymatic assays as compared to benign breast lesions (Del Vecchio S et al., 1993). Using monoclonal antibodies against different epitopes of uPAR, 85% of histologic sections of ductal breast carcinoma showed elevated levels of uPAR in the periductal tissue by immunohistochemistry (Pyke C et al., 1993). These and several other clinical studies provided compelling evidence for the role of uPAR as a prognostic marker, where higher uPAR levels in tumor extract and plasma of patients with breast cancer were associated with poor disease free and overall survival rates (Grondahl-Hansen J et al., 1993).

However, despite these convincing results from clinical studies, interspecies differences in the action of uPA/uPAR hindered the development of a suitable *in vivo* model of breast cancer to directly investigate the role of uPAR in breast cancer progression (Ramshaw IA and Badenoch-Jones P, 1985). Therefore, development of a syngeneic *in vivo* model where the role of uPA / uPAR in breast cancer progression can be readily assessed became the primary focus of my Ph. D. study.

1.6.7. uPA / uPAR Based Therapeutic Strategies

Elucidation of the role of uPA/uPAR in tumor progression over the last few years and their usefulness as molecular prognostic markers to follow disease progression have resulted in the establishment of uPA/uPAR as a potential target for anti-cancer therapy. These objectives can be effectively achieved by blocking the proteolytic effects of uPA using inhibitors of proteolytic actions of uPA, or via blocking its interaction with uPAR by designing uPAR antagonists (Fazioli F et al., 1994). These anti-tumor agents will specifically prove to be of benefit in those malignancies where increased expression of uPA/uPAR is associated with a higher invasive and metastatic potential.

Several recent studies have tried to evaluate the potential effectiveness of uPA / uPAR-based therapeutic approaches to prevent tumor growth and progression. Following implantation of tumor cells in chicken embryo chorioallantoic membrane (CAM), tumor cell metastases to the lungs of embryo have been effectively blocked by infusion of anti-uPA antibodies (Ossowiski L and Reich E, 1983a, b; Ossowiski L et al., 1994). Similarly, uPA anti-sense oligonucleotides inhibited human ovarian cancer cell growth following their intraperitoneal inoculation into nude mice (Wilhelm O et al., 1995). Despite the convincing evidence described above, these studies were limited in scope to allow

complete interactions between host environment, and uPA/uPAR produced by the inoculated tumor cells due to the species specificity of uPA actions. In order to overcome these limitations, our laboratory had used our syngeneic models of rat prostate cancer (Achbarou et al., 1994) to evaluate the usefulness of anti-uPA based therapeutic strategies. Amiloride, a potent potassium sparing diuretic was shown to be a specific inhibitor of uPA activity without effecting tPA or plasmin (Vassalli D and Belin D, 1987). By modifying the primary structure of amiloride, a highly selective inhibitor of the enzymatic activity of uPA, 4-iodo benzo [b] thiophene-2-carbon-amidine (B-428) was synthesized (Towle MJ et al., 1993). *In vitro*, B-428 treatment caused a dose-dependent inhibition of the invasiveness of human (PC-3) and rat (Mat Ly Lu) prostate cancer cells, as assessed by the Boyden chamber Matrigel invasion assay. When tested *in vivo*, infusion of B-428 to the tumor-bearing animals receiving cells overexpressing uPA effectively decreased tumor growth at the primary site. More significantly, the incidence and size of tumor metastases in the lungs and lymph nodes was markedly reduced following B-428 treatment, with complete prevention of macroscopic tumor metastases at kidneys and spleen (Rabbani SA et al., 1995b). A similar reduction in tumor growth by B-428 was reported in a murine mammary adenocarcinoma model *in vivo* (Alonso DF et al., 1996). Collectively, these results demonstrate that active site inhibitors of uPA, like B-428, can decrease tumor growth and block tumor metastasis without any noticeable side effects, thus further supporting the notion of uPA as a useful therapeutic target for cancer control.

In hormone dependent malignancies like breast and prostate cancer, due to the ability of sex steroids (estrogens and androgens) to promote cell proliferation, anti-estrogens like tamoxifen and anti-androgens like flutamide are currently included as a part of the standard therapeutic strategy for treating these patients (Lippman ME et al.,

1976; Jordan VC, 1995). However, due to the ultimate transition of breast and prostate cancer to the hormone insensitive variety, treatment of patients with anti-estrogens / anti-androgens becomes ineffective in the later stages of disease, resulting in the development of highly aggressive metastatic tumors (Jordan VC, 1995). Therefore, there is a great need for the development of new combinational therapeutic strategies by adding anti-proteolytic agents to the existing armamentarium of treatment regimens against cancer, which will block tumor invasion and metastasis by acting both at the stages of tumor initiation and progression. These approaches will not only result in increased disease free survival, but will also result in prolonged survival in patients where the uPA/uPAR system is involved.

1.7. DNA METHYLATION: SIGNIFICANCE IN CANCER BIOLOGY

Inherited genetic information is identical in all the different tissues of the body. However, the program that interprets the basic genetic information and enables its tissue-specific differential expression is distinguished by its diversity. While transcriptional factors play a critical role in orchestrating gene expression profiles of all organisms, other epigenetic mechanisms may contribute to the diversification of the genetic presentation. Epigenetic modifications caused by cytosine DNA methylation has been shown to play an important role in regulating mammalian gene expression. It is now well established that regulation of gene expression by DNA methylation is involved in allele-specific differential gene expression in X chromosome-inactivation (Zuccotti M and Monk M, 1995), parental imprinting (Sapienza C et al., 1987), as well as tissue-specific gene expression (Razin A and Cedar H, 1991).

1.7.1. Cytosine DNA Methylation: Formation of DNA Methylation Patterns

Cytosine DNA methylation is the reaction in which a methyl group from the methyl donor S-adenosylmethionine is transferred to the 5 position in cytosine of the CpG dinucleotide sequence. This reaction is catalyzed by DNA methyltransferase (DNA MeTase) [Figure 1.6]. The presence of 5-methylcytosine (5-mC) paired with guanine was first reported in 1948 (Hotchkiss RD, 1948). Approximately 4% of cytosine residues in DNA are methylated, predominantly at the dinucleotide CpG (Gruenbaum Y et al., 1981). DNA methylation has been recognized as one of the many processes that influence the function of genes by epigenetic modifications, without affecting the genetic code (Ramsahoye BH et al., 1996). Cytosine DNA methylation is characteristic of eucaryotic cells (Bestor T. 1990). Approximately 60% to 90% of the CpGs are methylated at the 5 position on the cytosine ring, and this accounts for most of the 5-mC in the vertebrate genome (Bird AP, 1986).

Methylation may be part of the process by which complex multicellular organisms compartmentalize their genomes into actively transcribed and non-transcribed parts, enabling the development of a varied tissue phenotype (Ramsahoye BH et al., 1996). The pattern of DNA methylation is established during development by sequential *de novo* methylation and demethylation events (Razin A and Cedar H. 1991; Brandeis M et al., 1993). DNA methylation patterns are tissue-specific and are maintained with high fidelity during cell division (Stein RY et al., 1982; Jones PA and Buckley JD, 1990). Immunofluorescence and immunocytochemical studies demonstrate that 5-mC is non-randomly distributed in chromosome. Housekeeping genes and tissue-specific genes differ in their distribution of CpG. The former contain clusters of CpG known as CpG islands in their promoter regions, whereas the latter characteristically lack CpG islands

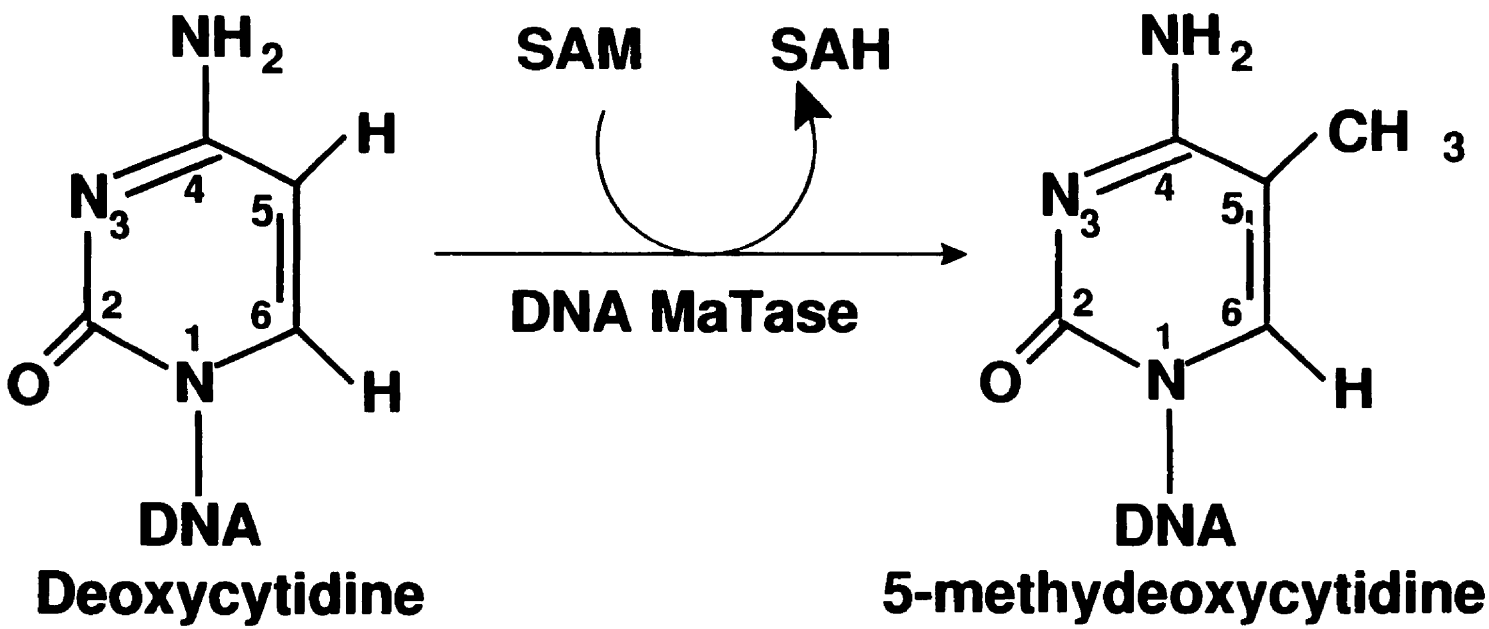


Figure 1.6: **The reaction of cytosine DNA methylation**

(Ramsahoye BH et al., 1996). However, it is not well understood how methylation patterns are formed and maintained. It has been suggested that the final methylation pattern is an equilibrium state between methylation and demethylation, probably determined by an interplay between methylation / demethylation events, and site-specific signals (Szyf M, 1994).

1.7.2. DNA Methylation and Cancer

An epigenetic mechanism, such as aberrant *de novo* methylation or generalized demethylation, may contribute to cancer pathogenesis or tumor-cell heterogeneity (Baylin SB et al., 1991). Several aspects of the biology of DNA methylation have been implicated in human malignancy:

1.7.2.a. Spontaneous Deamination of 5-mC

The most obvious mechanism implicating DNA methylation in cancer aetiology stems from the known propensity of methylcytosine to deaminate to thymine (Shen JC et al., 1994). Despite comprising only 1% of all nucleotides in the genome, 5-mC mutations are responsible for nearly one third of all germline mutations causing disease (Cooper DN and Krawczak M, 1990). The excessive number of mutations at CpG may be due to the relatively inefficient repair of G:T mismatches (Hatada I et al., 1995; Brown TC and Jiricny J, 1987).

1.7.2.b. Genome-wide Hypomethylation

The occurrence of genome-wide hypomethylation in most forms of cancer was first established in the early 1980s (Gama-Sosa MA et al., 1983). Since then, little progress

has been made in understanding the process leading to DNA hypomethylation and its significance. It might be envisaged that certain normally methylated genes capable of inducing malignant transformation might be activated by such a process. In this respect, DNA hypomethylation has been shown to increase the expression of a number of proto-oncogenes including *c-myc*, *c-fos* and *c-Ha-ras* (Christman JK et al., 1993; Feinberg AP et al., 1983).

1.7.2.c. CpG Island Hypermethylation

Despite evidence for genome-wide hypomethylation, many tumors demonstrate regional areas of hypermethylation. This is significant since genetic mutations on one allele of a tumor suppressor gene, combined with a methylation associated inactivation of the other allele, would satisfy Knudson's "two-hit" criteria for tumorigenesis (Knudson AG, 1983).

It is believed that CpG methylation is associated with a repressed state of gene expression (Yisraeli J and Szyf m, 1984; Razin A and Cedar H, 1991). The calcitonin gene was the first gene for which tumor-associated hypermethylation was demonstrated in primary material (Baylin SB et al., 1986). Since then, results from various studies have shown that many tumor suppressor genes (Rb, p16, p15, VHL) (Ohtani-Fujita N et al., 1993; Merlo A et al., 1995; Herman JG et al., 1996; Herman JG et al., 1994), as well as genes engaged in tumor progression, such as E-cadherin (Yoshiura K et al., 1995), ER (Ottaviano YL et al., 1994), endothelin receptor (Nelson et al., 1997), or inhibitors of angiogenic factors (Ahuja N et al., 1997) are hypermethylated and inactivated in various human malignancies. Accumulating evidence supports the notion that DNA methylation can mark certain genes for inactivation either directly, by interfering with the binding of

some transcriptional factors to regulatory sequences (Becker PB et al., 1987), or indirectly, by either attracting the binding of proteins that have high affinity to methylated DNA (Huang LH et al., 1984; Razin A and Cedar H, 1991; Nan X et al., 1997), or by precipitating an inactive chromatin structure (Kass SU et al., 1997).

1.7.2.d. Mutagenicity Induced by DNA Methyltransferase

There is some evidence suggesting that DNA MeTase itself may be a mutagen. Gene transfer experiments using constructs overexpressing the mammalian DNA MeTase, induce tumorigenicity in NIH 3T3 fibroblasts (Wu J et al., 1993). The uniqueness of DNA MeTase as a candidate key player in the transformation process is that it can act at different levels to coordinate the multiple changes required to shift a cell into a transformed state. A number of observations have shown increased production and activity of DNA MeTase in transformed cells compared to their normal counterparts in both cell lines and tumors (Kautiainen TL and Jones PA, 1986; el-Deiry WS et al., 1991), and high activity of DNA MeTase is required for cellular transformation (MacLeod AR and Szyf M, 1995; Laird PW et al., 1995). It was speculated that induction of DNA MeTase results in hypermethylation and simultaneous inactivation of a large number of genes that can either suppress tumorigenesis (Merlo A et al., 1995; Baylin SB et al., 1998; Graff JR et al., 1997), tumor invasion (Yoshiura K et al., 1995), angiogenesis (Ahuja N et al., 1997), or genes involved in maintenance of the cognate differentiated functions of a cell (Szyf M et al., 1989). Thus, overexpression of DNA MeTase might be a switch that turns off a cognate program of gene expression and induced a transformed gene expression profile (Szyf M, 1994). An alternative mechanism of DNA MeTase action could be that it is directly involved in controlling origins of DNA replication through protein-protein interactions at the replication

fork (Chuang L. S. H. et al., 1997; Rein T et al., 1997).

To summarize, the mechanisms responsible for the alterations in DNA methylation in tumorigenesis are not fully delineated. It could be simply that aberrant methylation in cancer cells is a consequence of random methylation errors which are selected and fixed during the tumorigenic process. Therefore, both hypomethylation or hypermethylation of specific loci may confer growth advantage upon cells. However, accumulating experimental evidence strongly supports the alternative hypothesis that changes in DNA methylation in cancer are critical components of carcinogenic programs (Szyf M, 1994; Szyf M, 1996). Several lines of evidence have associated the induction in DNA MeTase mRNA with known oncogenic programs. The regulatory regions of both mouse (Reuleau J et al., 1992) and human DNA MeTase bear a number of AP-1 recognition sites which are activated by the transcription factor *Jun* (Rouleau J et al., 1995), and the levels of DNA MeTase mRNA and DNA methylation are induced by expression of *Ras* or *Jun* (MacLeod AR et al., 1995). These results are consistent with the contention that the *Ras-Jun* oncogenic pathway regulates DNA MeTase and that increased DNA MeTase activity is a downstream effector of *Ras* (Szyf M, 1994; Yang J et al., 1997). Moreover, most recent studies reported the induction of a demethylase activity as a result of Ras overexpression (Szyf M et al., 1995). This is consistent with the widely observed generalized hypomethylation. The mechanisms responsible for coordinating the expression of DNA MeTase and DNA demethylase in cancer cells are not known. It is possible that both activities are regulated by the same oncogenic pathways as has been recently demonstrated for the Ras signalling pathway (Szyf M, 1994; Szyf M et al., 1995; MacLeod AR et al., 1995).

1.7.3. 5-Azacytidine: A DNA Methyltransferase Inhibitor

The nucleoside 5-azacytidine (5-AzaC) was first synthesized in 1964 (Piskala A and Sorm F, 1964). It differs from cytidine in having a nitrogen in place of carbon in the 5 position of the pyrimidine ring. 5-AzaC is incorporated into the DNA following its phosphorylation and forms an irreversible covalent bond with the DNA MeTase enzyme at carbon 6 in the cytosine moiety, resulting in trapping of the enzyme onto DNA and reduction of active DNA MeTase levels in the nucleus (Wu JC and Santi DV, 1985;). However, 5-AzaC is also a nucleoside analogue and a differentiating agent. Therefore, when interpreting the data involving 5-AzaC as the DNA MeTase inhibitor, it is essential to address the cytotoxic effect and alterations in the differentiation state of the cell.

1.8. OBJECTIVES OF THESIS

1. To elucidate the role of uPAR in breast cancer growth, invasion and metastasis *in vitro* and *in vivo*.

2. To evaluate the efficacy of a combinational therapeutic approach using anti-estrogen tamoxifen and a specific active site inhibitor of uPA in the prevention of breast cancer growth, invasion and metastasis *in vitro* and *in vivo*.

3. To study the molecular actions of androgen (DHT) on uPA gene expression and its resultant effect on prostate cancer growth, invasion and metastasis *in vitro* and *in vivo*.

4. To characterize uPA gene expression at different stages of breast and prostate cancer progression; and to investigate a potential role of cytosine DNA methylation in the differential regulation of uPA gene expression in hormone dependent malignancies like breast and prostate cancer.

CHAPTER 2

OVEREXPRESSION OF UROKINASE RECEPTOR IN BREAST CANCER CELLS RESULTS IN INCREASED TUMOR INVASION, GROWTH AND METASTASIS

2.1 SUMMARY

Results of studies on the role of uPAR overexpression in breast cancer invasion and metastasis *in vitro* and *in vivo* are presented in this chapter in the form of a published paper (*Int. J. Cancer. 67: 1-7. 1996*). I was responsible for all of the experimental work described in this chapter. Anti-uPAR antibody was kindly provided by American Diagnostic Inc. (Greenwich, CT).

These studies were carried out in a syngeneic model of rat breast cancer using female Fischer F344 rats and chemically induced rat mammary adenocarcinoma cell line Mat B III. Mat B III cells grew rapidly *in vitro* mainly as rounded cells in suspension and were found to be highly invasive in Boyden chamber Matrigel invasion assay. Subcutaneous inoculation of Mat B III cells into female Fischer rats resulted in the development of rapidly growing primary tumors and establishment of metastases to various distant organs including the liver, lung, spleen, as well as the regional and draining lymph nodes as described by Ramshaw and Badenoch-Jones (Ramshaw IA and Badenoch-Jones P, 1985). In our study, we chose the orthotopic route of tumor cell implantation into the mammary fat pad of host animals. This route of tumor implantation has been shown to best mimic the systemic nature of breast cancer dissemination seen in patients with metastatic breast disease (Price JE, 1990).

Results from our studies have, for the first time, provided compelling experimental evidence for the direct involvement of uPAR in the process of tumor growth, invasion and metastasis.

2.2. BOYDEN CHAMBER MATRIGEL INVASION ASSAY: AN *IN VITRO* MODEL OF INVASION

The aim of this study was to examine the effect of uPAR overexpression on tumor invasion and metastasis *in vitro* and *in vivo*. Boyden chamber Matrigel invasion assay was used to assess the invasiveness of Mat B III cell and Mat B III cells overexpressing uPAR (Mat B III-uPAR).

In order to better understand the underlying mechanisms involved in tumor cell invasion, various *in vitro* models have been developed which aim to isolate and mimic discrete steps within the process of tumor invasion and metastasis. Availability of these *in vitro* models has significantly contributed to our understanding of the multi-step process of tumor progression.

Among these models, the Boyden Chamber Matrigel Invasion Assay, which is a modification of the original two compartment chamber model proposed by Hart and Fidler (1978), is the most widely used conventional *in vitro* model used to study the behavior of tumor cells during the process of invasion. This assay, which measures the invasion of cells through a basement membrane-like barrier, was first described by Albini et al (1987). The system consists of a two compartment chamber separated by a polycarbonate membrane filter, precoated with a reconstituted basement membrane gel derived from the conditioned culture medium of the Engelbreth-Holm-Swarm mouse sarcoma line (Matrigel). The tumor cells are added to the upper chamber and allowed to penetrate the Matrigel. The invading cells can then transverse the 8 μM-pore filters and depending on the cells and assay conditions, can either be harvested from the lower chamber or may attach and spread on the lower surface of the filter where they can be stained and enumerated. The latter method has been used consistently throughout all my studies where invasion

assays were performed.

2.3. PUBLISHED PAPER

2.3.1. Abstract

We have examined the role of urokinase receptor (uPAR) in tumor invasion and metastasis by developing a homologous model of uPAR overexpression in a rat breast cancer cell line Mat B III using gene transfer techniques. Control (pRc-CMV) and experimental plasmid (pRc-uPAR-S) were transfected into Mat B III cells by using Lipofectin reagent. Levels of uPAR production were assessed by Northern blotting, immunofluorescence, by receptor binding and by ELISA. At least three experimental clones (pRc-uPAR-S), expressing 3-5 fold higher levels of uPAR than control (pRc-CMV), were selected for further analysis. Experimental cells overexpressing uPAR showed a 4-5 fold higher invasive capacity as compared to control cells in a Boyden chamber invasion assay. Both control and experimental cells (1×10^6 cells) were injected into the mammary fat pad of syngeneic female Fischer rats. Animals were sacrificed at timed intervals and evaluated for the development of tumor growth and metastasis. Animals receiving cells overexpressing uPAR had significantly larger tumor volumes and weights throughout this study. Furthermore, due to increased uPAR expression, experimental animals developed large metastatic lesions in the liver, spleen and lymph nodes. These studies therefore demonstrate the role of uPAR in tumor progression, due to its ability to localize uPA within the tumor cell milieu. Development of this syngeneic model of uPAR overexpression will allow us to evaluate various therapeutic strategies for blocking uPA activity in breast cancer invasion and metastasis, where the uPA / uPAR system plays a major role.

2.3.2. Introduction

A critical event in cancer invasion and metastasis is the ability of tumor cells to invade through the extracellular matrix (ECM). This is achieved through various proteolytic enzymes located at the plasma membrane, which localize their degradative effects on the invading cell surfaces (Stetler-Stevensen WG *et al.*, 1993). One such proteolytic enzyme, uPA, which belongs to the family of serine proteases has been implicated in the progression of many malignancies (Dano K *et al.*, 1994). Increased uPA production by tumor cells and their surrounding stroma is associated with higher invasive and metastatic potential in human malignancies (Hollas W *et al.*, 1991; Dano K *et al.*, 1994). In previous studies, we have reported that the amino terminal fragment (ATF) of uPA isolated from the human prostate cancer cell line PC-3, could act as a selective mitogen for osteoblastic cells (Rabbani SA *et al.*, 1990). Furthermore, we have demonstrated that uPA overexpression by the rat prostate cancer cells Mat LyLu results in increased tumor metastasis to both skeletal and non-skeletal sites (Achbarou A *et al.*, 1994).

The cell surface uPAR has recently been isolated and characterized from human, mouse and rat cell lines (Roldan AL *et al.*, 1990; Kristensen P *et al.*, 1991; Rabbani SA *et al.*, 1994). We and others have reported the presence of multiple uPAR isoforms, which arise from alternative splicing of uPAR mRNA (Kristensen P *et al.*, 1991; Rabbani SA *et al.*, 1994). uPAR is a 50-60 KD, highly glycosylated protein linked to the cell surface via a GPI anchor (Ploug M *et al.*, 1993). In malignant tumors, uPAR is believed to play a critical role in malignant tumors due to its ability to bind secreted uPA from tumor cells

and surrounding stromal cells (Dano K *et al.*, 1994). Binding of uPA to its cell surface receptor results in localization of the proteolytic effects of uPA within the tumor cell environment. In the process of tumor invasion and metastasis, increased uPAR expression by tumor cell could play a key role by localizing uPA within the tumor cell environment. The resulting uPA/uPAR complex leads to the activation of plasminogen, matrix metalloproteases (MMPs) and latent transforming growth factor ß to facilitate tumor cell invasion through the ECM (Stetler-Stevensen WG *et al.*, 1993; Grondahl-Hansen J *et al.*, 1991).

Adenocarcinoma of the breast is one of the leading malignancies affecting women, and is associated with high morbidity and mortality (Russo J and Russo IH, 1995). In this common malignancy, elevated levels of uPA have been correlated with higher relapse and poor prognosis (Jänicke F *et al.*, 1991). Accumulating evidence strongly supports the critical role of uPAR in breast cancer and invasive ductal carcinoma where high levels of uPAR are seen in tissue neutrophils and macrophage-like stromal cells located at the leading edge of invasive tumors (Pyke *C et al.*, 1993). In contrast to this, however, the enzymatic activity of uPA is located primarily in surrounding stromal cells.

Recent clinical studies strongly point towards the critical role of uPAR in breast carcinoma invasion and metastasis (Duggan C *et al.*, 1995). However, due to the reported interspecies blockage of the actions of uPA, development of an appropriate *in vivo* model to directly examine the role of uPAR in the process of tumor invasion and metastasis has thus far not been feasible (Duggan C *et al.*, 1995; Kariko *K et al.*, 1993). In the current study we have, for the first time, developed a syngeneic *in vivo* model of uPAR

overexpression by rat mammary adenocarcinoma cells Mat B III. This model of uPAR overexpression has the distinct advantage of allowing complete interaction between endogenous uPA and transfected uPAR of the same species located within the tumor cell environment. This *in vivo* model of advanced breast cancer may serve as a suitable system to further investigate the complex processes of breast cancer invasion and metastasis where uPA/uPAR plays a key role.

2.3.3. Materials and Methods

Plasmid Preparation

The rat (r) uPAR-1 cDNA previously isolated in our laboratory was digested with Hind III-Xba I and ligated in the sense orientation at the Hind III-Xba I cloning site of the plasmid pRc/CMV (Invitrogen) to prepare the expression vector pRc-uPAR-S (Rabbani SA *et al.*,1994). Control (pRc-CMV) and experimental (pRc-uPAR-S) plasmids were propagated in DH5 E. coli (BRL/GIBCO) to obtain sufficient amounts of plasmid DNA (Achbarou A *et al.*, 1994).

Cells and cell Culture

The control (pRc-CMV) and experimental (pRc-uPAR-S) plasmids were each transfected into the rat breast adenocarcinoma cells Mat B III by Lipofectin (BRL/GIBCO). Cells were selected for neomycin resistance gene with G418 (600 μg / ml) for 10 days and resistant colonies were isolated and allowed to grow as monoclonal cell populations. Clones expressing the highest amount of uPAR, as determined by Northern blot analysis,

immunoflourescence and receptor binding assays were selected for future studies.

Mat B III cells were obtained from American Type Culture Collection (Rockville, Maryland). Cells were maintained *in vitro* in McCoy's 5A modified medium supplemented with 2 mM L-glutamine (GIBCO), 10% fetal bovine serum (FBS), 100 units / ml of penicillin-streptomycin sulphate (GIBCO) and 0.2% gentamycin.

Animal Protocols

Inbred female Fischer 344 rats weighing 200-220 g were obtained from Charles River Inc. (St.Constant, Quebec). Before inoculation, control and experimental Mat B III tumor cells grown in serum-containing medium were washed with Hank's buffer and trypsinized for 5 min. Cells were then collected in Hank's buffer and centrifuged at 1500 rpm for 5 min. Cell pellets (1×10^6 cells) were resuspended in 0.2 mL saline and injected using one ml insulin syringes into the mammary fat pad of rats anesthetized with ethanol / Somnotal (MTC Pharmaceuticals, Cambridge, Ontario). All animals were numbered, kept separately and were examined for the development of tumors every third day for up to 21 days. The tumor mass of control and experimental animals was measured in two dimensions by callipers and the tumor volume calculated. Control and experimental animals were sacrificed at timed intervals and animals were examined and scored for the development of macroscopic metastases in various tissues. Tumor tissues were also removed from the site of inoculation and extracted for RNA analysis.

Northern Blot Analysis

Total cellular RNA was isolated from control and experimental MAT B III tumor cells and tumor tissues by acid guanidium thiocyanate-phenol-chloroform extraction. Twenty μg of total cellular RNA was electrophoresed on a 1.1% agarose-formaldehyde gel and transferred to a nylon membrane (Nytran) by capillary blotting. Filters containing RNA of Mat B III cells were hybridized with a ^{32}P-labelled rat uPAR cDNA or with an 800 bp *Bam* HI restriction fragment of rat cyclophilin cDNA as a control for the amount of RNA loaded (Achbarou A *et al.*, 1994). All filters were incubated at 42°C for 24 h and successively washed in 1 x SSC (10 x SSC is 1.5 M NaCl, 0.5 M sodium citrate, pH 7.0), 1% sodium dodecyl sulfate (SDS) for 15 min at room temperature, 0.5 x SSC, 0.5% SDS for 15 min at room temperature, 0.1 x SSC, 0.1% SDS twice for 15 min at room temperature, and then once for 30 min at 55°C. Autoradiography of filters was carried out at -70°C using XAR film (Eastern Kodak Co., Rochester, NY) with two intensifying screens. The level of uPAR expression was quantified by densitometric scanning.

Matrigel Invasion and Modified Receptor Binding Assay

The invasive capacity of Mat B III cells was tested by two-compartment Boyden chambers (Transwell, Costar, USA) and basement membrane Matrigel (Becton Dickinson Labware) as previously described (Liu DF *et al.*, 1995). In some experiments Mat B III cells (5 x 10^5 / mL) were cultured in the presence or absence of phosphotidyl inositol phospholipase C, PI-PLC (GIBCO), pre-immune rabbit IgG or with anti-rat uPAR IgG (100 μg/ml) (American Diagnostic Inc. Greenwich, CT). All receptor binding assays were

done by plating control and experimental cells in 100 mm plates and letting them grow to 70% confluence. Then plasma membrane isolated as previously described (Ploug M *et al.*, 1991).

Rat uPA (American Diagnostic Inc. Greenwich CT) was iodinated by the chloramine-T method (specific activity of 20 - 60 μCi / μg). Binding assays were performed on plasma membrane fractions isolated from control and experimental cells. 150 μg of membrane protein was pre-incubated with ^{125}I-ruPA (200,000 cpm) in a total volume of 0.5 ml containing 5% BSA, 0.5 mM Tris-Cl, 0.05 mM $CaCl_2$ pH 7.4 for 90 min at 25°C. At the end of incubation, the reaction mix was centrifuged at 4°C at 2000 rpm for 30 min and the pellet was washed in the same reaction buffer and centrifuged again to pellet. The pellet obtained was incubated with 2.0 ml PI-PLC (0.12 U) at 37°C for 30 min and centrifuged at 2000 RPM for 30 min, released ^{125}I-labelled uPA / uPAR complex was counted in a γ counter. In some experiments un-labelled rat uPA (0.1μM - 1.0μM) was used as a control of binding specificity.

PCR Amplification of Rat uPAR

Total cellular RNA was isolated from axillary, retroperitoneal and mesenteric lymph nodes of the rats receiving vector (pRc-CMV) transfected alone and pRc-uPAR-S transfected Mat B III cells. 2 μg of total RNA was used for reverse transcription. Rat uPAR cDNA was amplified using two PCR primers 1 and 2 corresponding to nucleotides 60-85 and 478-500 respectively of the reported nucleotide sequence of rat uPAR. All PCR reactions were analyzed on 1.1% DNA agrose gel containing ethidium bromide (Rabbani

SA *et al.*, 1994).

ELISA

The level of uPAR protein secreted into the cell conditioned culture medium was determined by indirect enzyme-linked immunosorbent assay (ELISA) as previously described (Liu and Rabbani, 1995). Anti rat uPAR IgG antibody diluted 1:500 and goat anti-rabbit IgG (American Diagnostica Inc., Greenwich, CT) conjugated to alkaline phosphatase (Sigma) diluted 1:1000 were used in the assay. ELISA plates were read by Microplate Reader (Model 3550, BIO-RAD). The concentration of uPAR in each sample was calculated by reading against a standard curve generated with rat uPA (American Diagnostica Inc. Greenwich, CT) and expressed in ng of uPAR/ml of conditioned culture medium.

Indirect Immunofluorescence

To examine the cell surface expression of uPAR by control (pRc-CMV) and experimental (pRc-uPAR-S) Mat B III cells, 5 x 10^4 cells were plated in Lab-Tek tissue culture chambers (Nunc Inc., Naperville, IL) and allowed to grow to 70-80% confluent. Cells were then incubated with 30% goat serum (Sigma) for 1 hour at room temperature and washed with PBS containing 1% BSA. Sequentially, cells were incubated with 100 ug/ml of rabbit anti-rat uPAR IgG (American Diagnostica Inc. Greenwich, CT) and with goat-anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) (Sigma). Photographs were taken at 250 X magnification using Zeiss MC-63 microscope (Liu DF

and Rabbani SA, 1995).

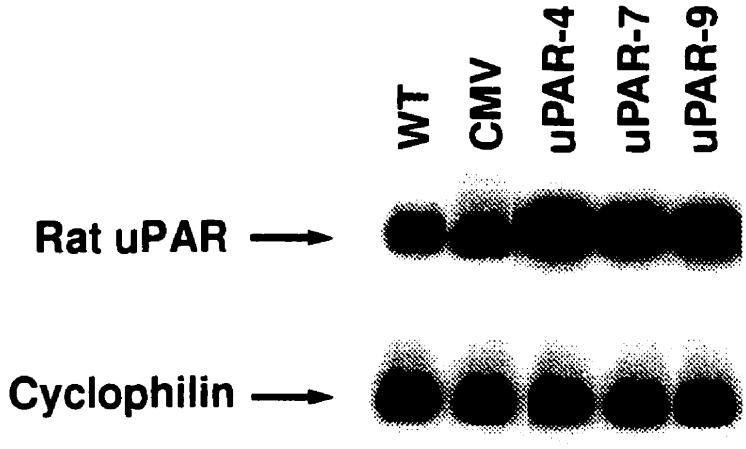
Statistical Analysis

Results are expressed as the mean ± SEM of at least triplicate determinations and statistical comparisons are based on the Student's t test or analysis of variance. A probability value of < 0.05 was considered to be significant.

2.3.4. Figures

Figure. 2.1: Northern blot analysis of Mat B III cell lines.

Total cellular RNA was extracted from wild type cells (WT), cells transfected with vector pRc-CMV (CMV) and from three cell lines (uPAR-S-4, uPAR-S-7 and uPAR-S-9) transfected with experimental plasmid pRc-uPAR-S (uPAR-S). 20 μg of total cellular RNA was electrophoresed on a 1.1% agrose/formaldehyde gel and blotted to a nylon membrane by capillary action. All blots were probed with a ^{32}P labelled rat uPAR cDNA or with a ^{32}P cyclophilin cDNA as described in "Materials and Methods." Blots were scanned by laser densimetric scanning and changes in uPAR mRNA expression determined by plotting the ratio of uPAR/ cyclophilin mRNA. Results are representative of at least three monoclonal cell lines in control groups and from a representative experimental cell line.


WT
CMV
uPAR-4
uPAR-7
uPAR-9
Rat uPAR
Cyclophilin

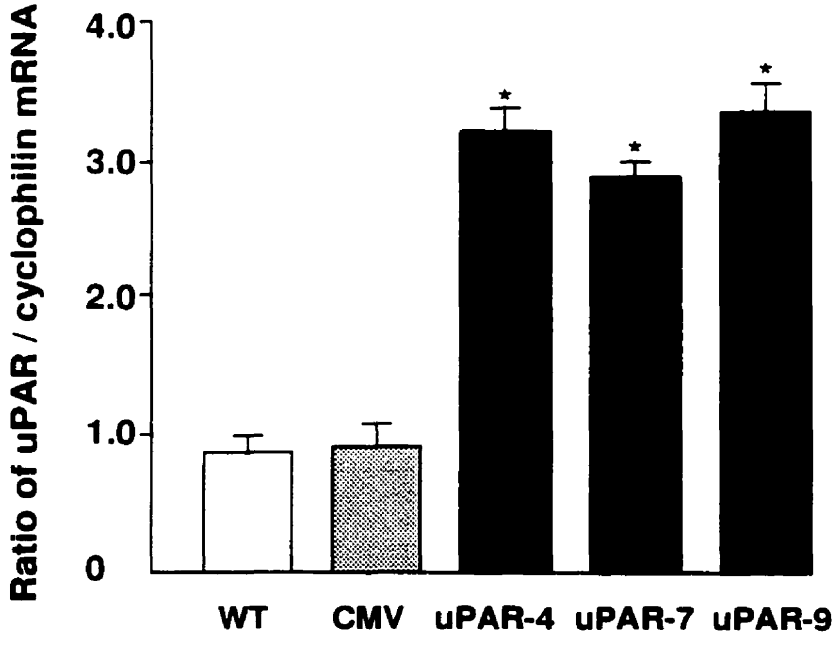
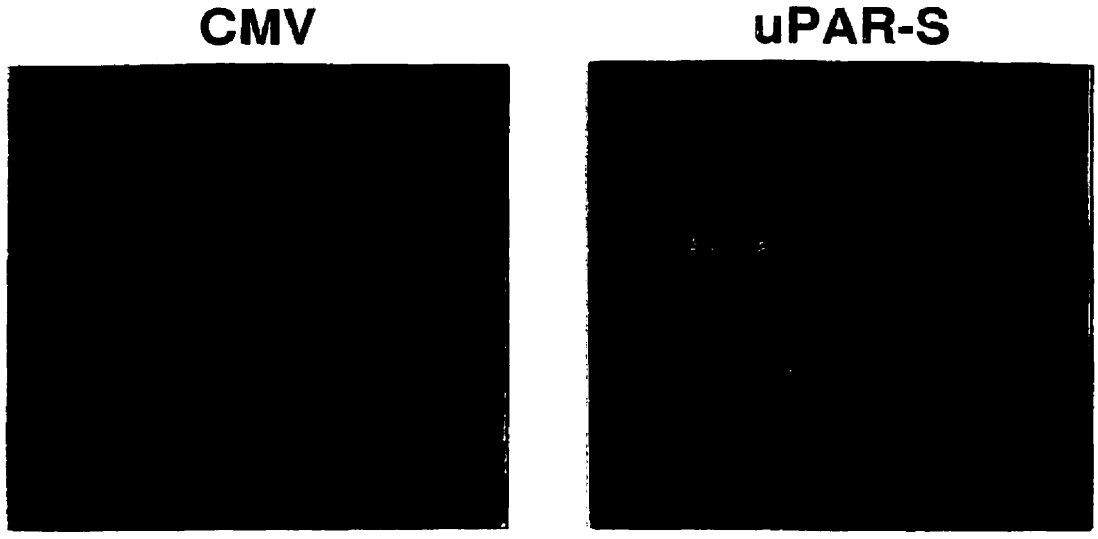

Ratio of uPAR / cyclophilin mRNA
4.0
3.0
2.0
1.0
0
*
*
*
WT
CMV
uPAR-4
uPAR-7
uPAR-9

Figure. 2.2: Determination of uPAR protein production

uPAR protein production was determined in Mat B III cells transfected with vector alone (CMV), and with the experimental plasmid pRc-uPAR-S (uPAR-S) by indirect immunofluoresence as described in "Materials and Methods" (Fig. 2A).

Release of uPAR into conditioned culture medium of wild type Mat B III cells (WT), cells transfected with vector alone (CMV), or with the experimental plasmid pRc-uPAR-S (uPAR-S) was also determined by ELISA as described in "Materials and Methods" (Fig.2B). Results are the mean ± SEM of three experimental cell lines and at least four independent determinations in each group. Significant difference from control is marked by asterisks* (*$P < 0.05$).

A:



B:

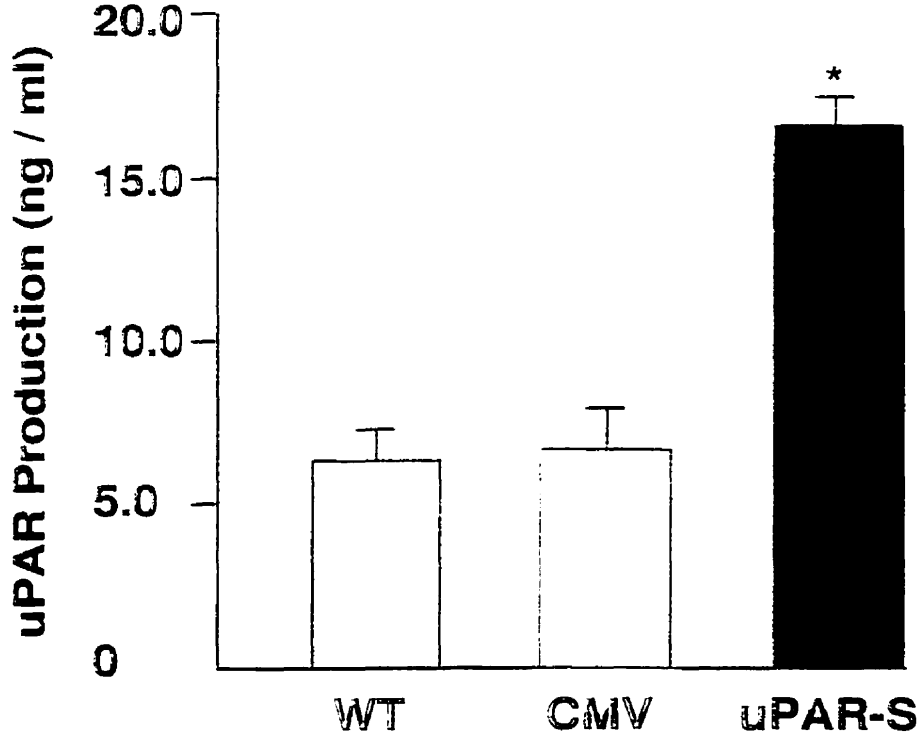


Figure. 2.3: Effect of uPAR overexpression on ^{125}I labelled uPA binding in Mat B III cell.

Plasma membranes from wild type (WT) vector transfected (CMV) and Mat B-III cells transfected with experimental plasmid (uPAR-S) were incubated with ^{125}I labelled rat uPA as described in "Materials and Methods.". In these plasma membranes, % of total binding of ^{125}I labelled rat uPA was determined. Results are the Mean ± SEM of ten different determinations of three experimental cell lines in four experiments in each group. Significant difference from control is marked by asterisks* (*$P < 0.05$).

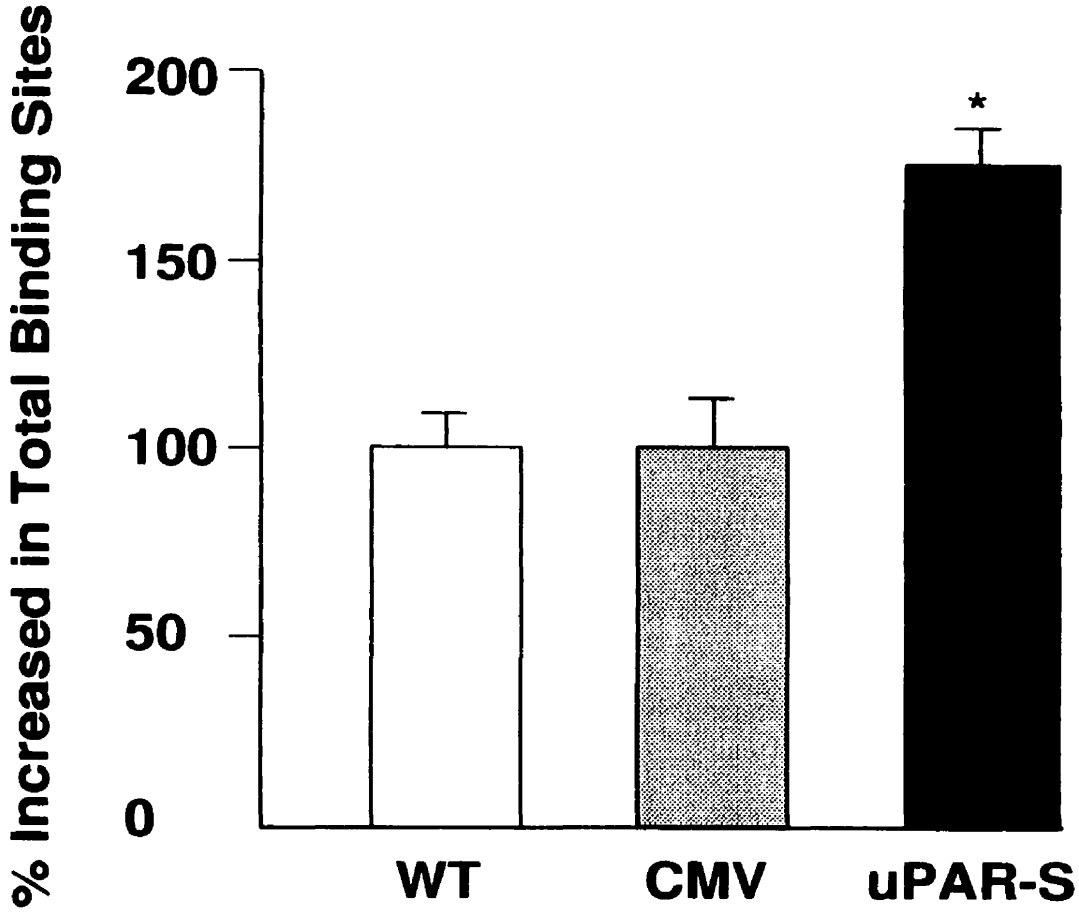

% Increased in Total Binding Sites
200
150
100
50
0
*
WT
CMV
uPAR-S

Figure. 2.4: Effect of uPAR overproduction on Mat B-III cell invasion.

Non-transfected wild type (WT), vector transfected (CMV) and experimental plasmid pRc-uPAR-S (uPAR-S) transfected Mat B III cells were grown in culture. Number of cells migrating to the lower aspect of the Boyden chamber filter were countered. Control vector transfected (CMV) and experimental cells (uPAR-S) were also incubated in the presence of PI-PLC (CMV + PI-PLC), (uPAR-S+PI-PLC), pre-immune rabbit IgG (uPAR-S+CTL) and with anti-rat uPAR IgG (uPAR-S+Ab) as described in "Materials and Methods". Results are the mean ± SEM of ten different determinations of three experimental cell lines in four experiments in each group. Significant difference from the control is marked by asterisks* (*$P < 0.05$).

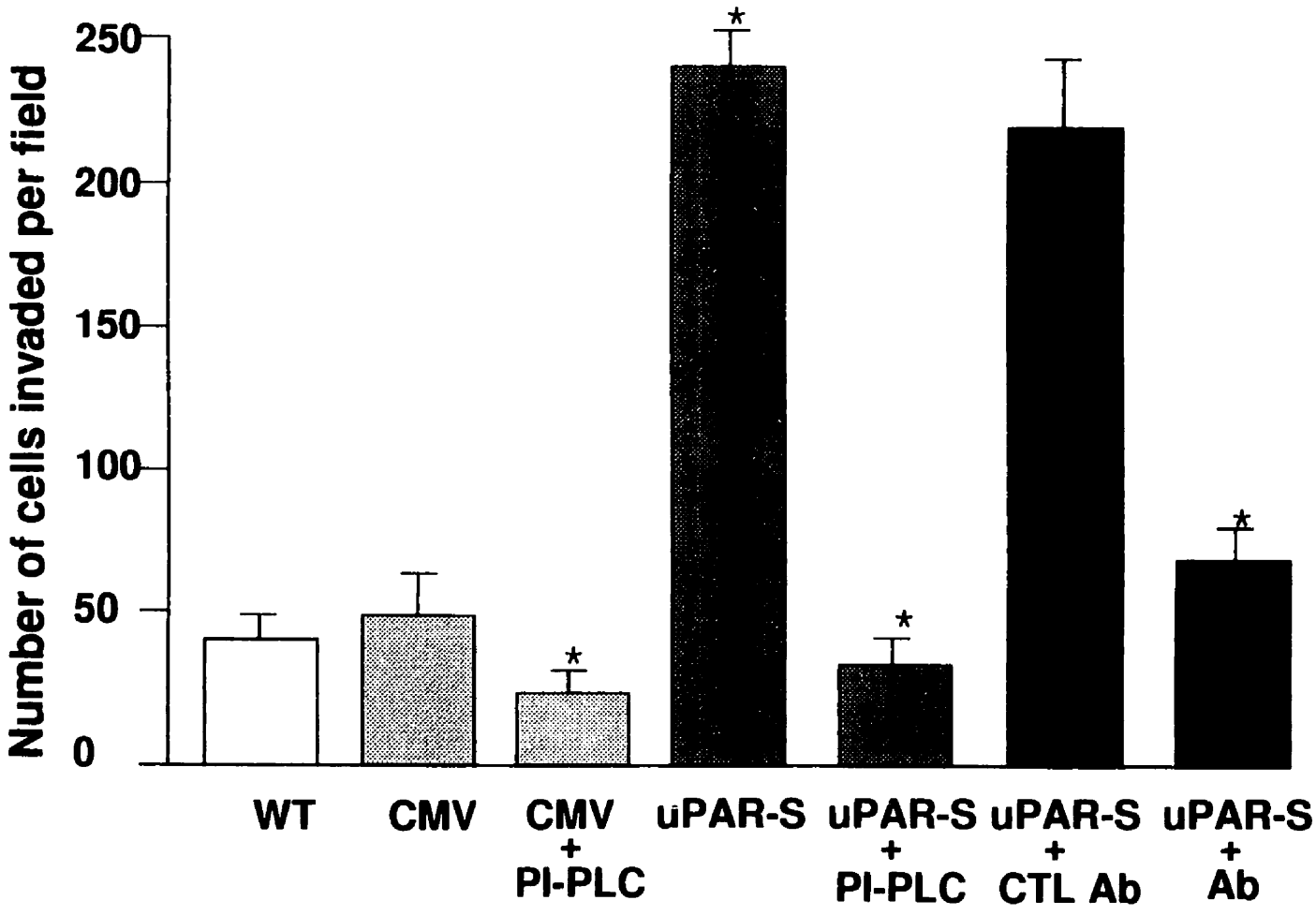

Number of cells invaded per field
250
200
150
100
50
0
*
*
*
*
WT
CMV
CMV + PI-PLC
uPAR-S
uPAR-S + PI-PLC
uPAR-S + CTL Ab
uPAR-S + Ab

Figure. 2.5: Effect of uPAR overexpression on tumor volume.

Tumor volume of control and experimental animals was determined at timed intervals as described in "Materials and Methods". Tumor volumes of animals receiving different cell lines overexpressing uPAR (uPAR-S-4, uPAR-S-7 and uPAR-S-9) was compared to control animals receiving cells transfected with vector alone (CMV). Results are the mean ± SEM of 10 starting animals in each group in three different experiments. Significant difference in tumor volume with control at each time point is marked by asterisks* (*$P < 0.05$).

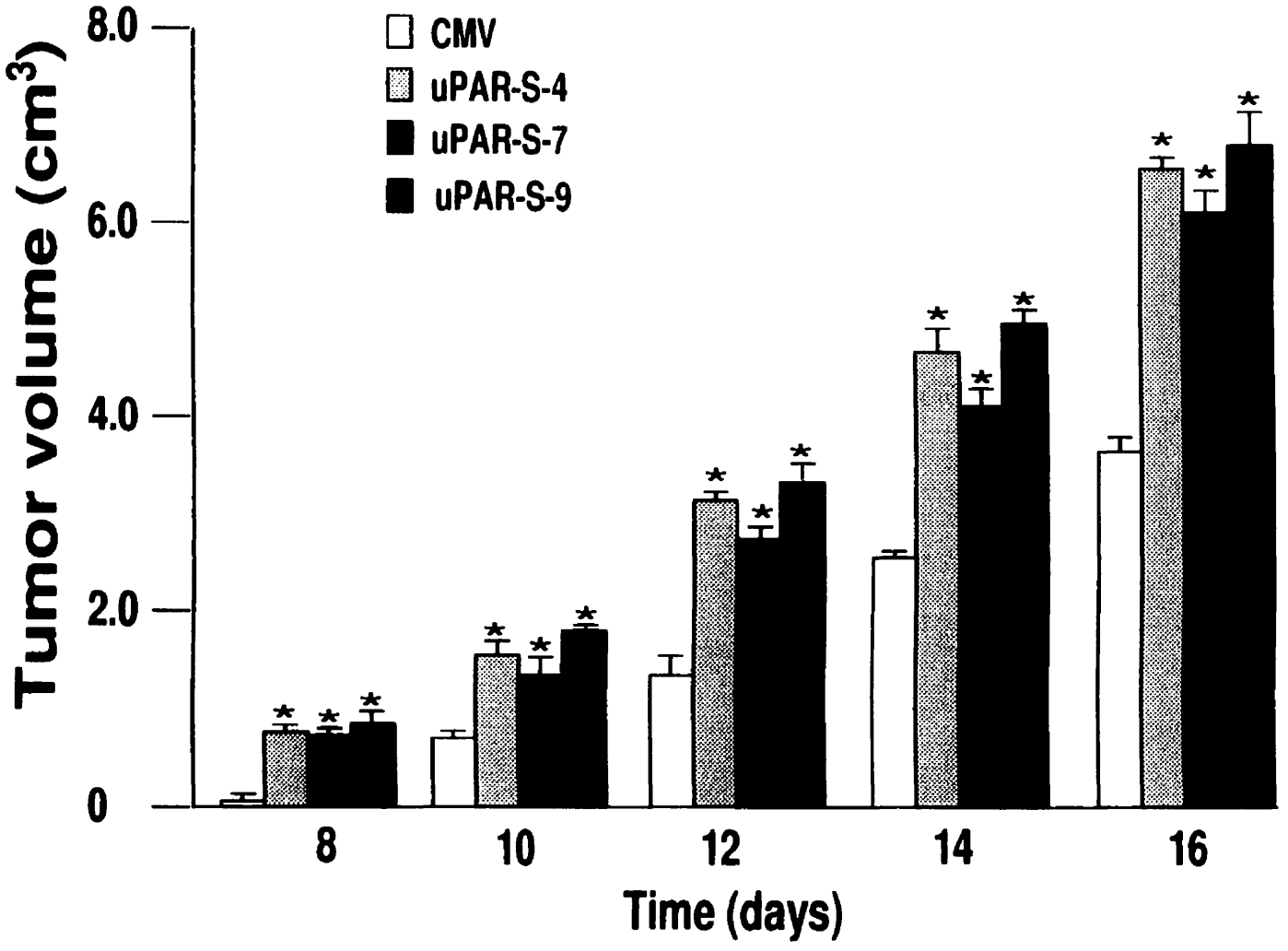

CMV
uPAR-S-4
uPAR-S-7
uPAR-S-9
Tumor volume (cm3)
8.0
6.0
4.0
2.0
0
8
10
12
14
16
Time (days)

Figure. 2.6: Amplification of uPAR from axillary lymph nodes.

2 μg of total cellular RNA from axillary lymph nodes of animals inoculated with Mat B III cells transfected with vector alone (CMV), and with experimental plasmid pRc-uPAR-S (uPAR-S) was reverse transcribed and uPAR DNA amplified with uPAR primers 1 and 2 as described in "Materials and Methods". Amplified products were analyzed on a 1.1% DNA agrose gel. The migration position of PCR amplified uPAR DNA is indicated. Results are representative of at least six animals in each group.

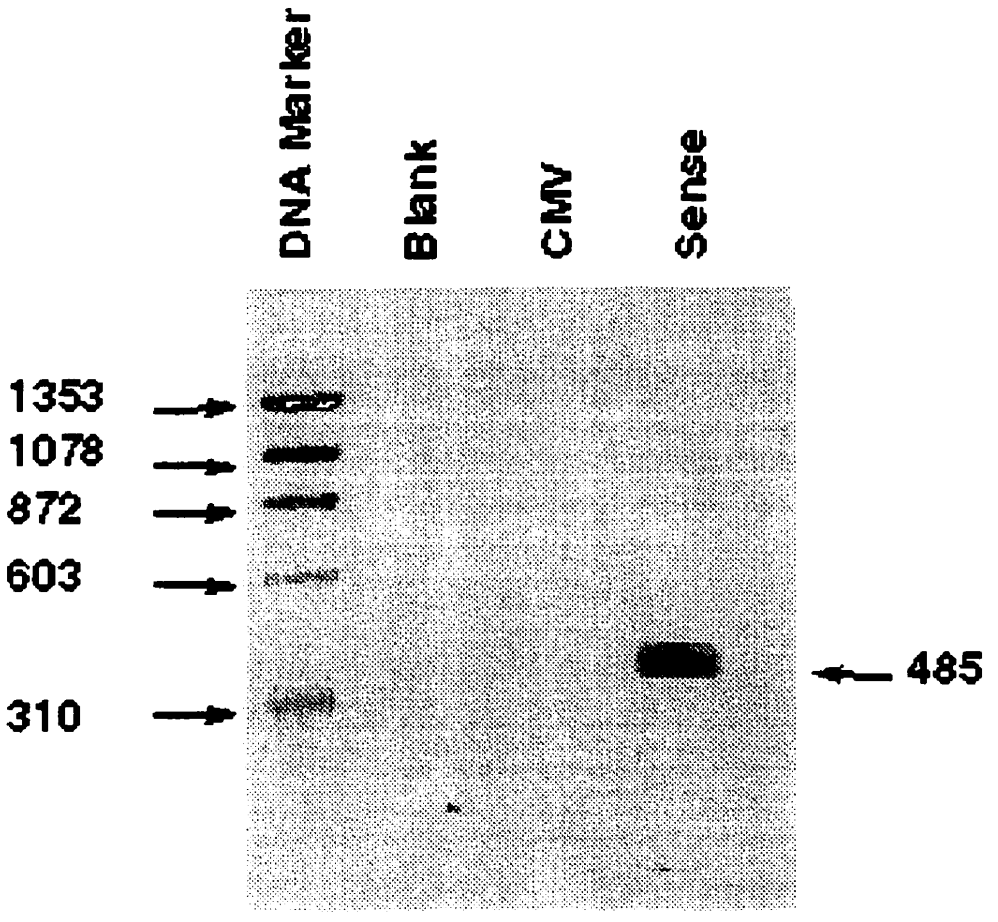
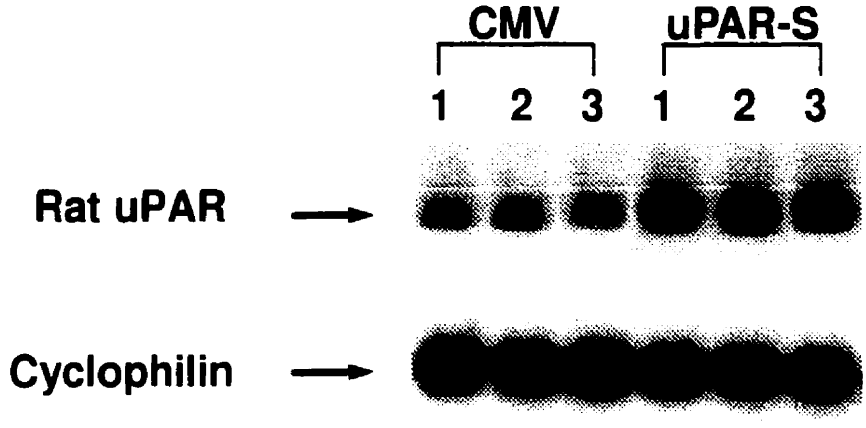

DNA Marker
Blank
CMV
Sense
1353
1078
872
603
310
485

Figure. 2. 7: Northern blot analysis of uPAR mRNA in Mat B III tumors.

Total tumoral RNA was extracted from tumors removed from three control animals inoculated with vector transfected Mat B III cells (CMV) and from three experimental animals inoculated with cells transfected with experimental plasmid pRc-uPAR-S (uPAR-S). 20 μg of total RNA was electrophoresed on 1.1% agrose / formaldehyde gel and blotted to a nylon membrane by capillary action. All blots were probed with a ^{32}P labelled rat uPAR cDNA or with a ^{32}P labelled cyclophilin cDNA as described in "Materials and Methods". Blots were scanned by laser densimetric scanning and change in uPAR mRNA expression determined by plotting the ratio of uPAR / cyclophilin mRNA. Results are representative of three different experiments.

Significant difference from the control is marked by asterisks* (* $P < 0.05$).


CMV
uPAR-S
1 2 3 1 2 3
Rat uPAR
Cyclophilin

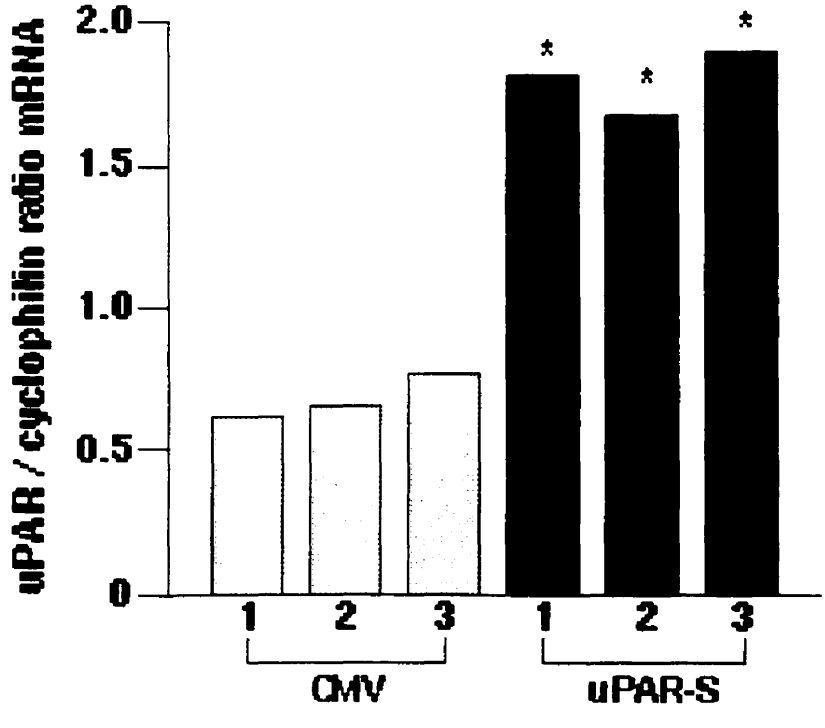

uPAR / cyclophilin ratio mRNA
2.0
1.5
1.0
0.5
0
*
*
*
1 2 3 1 2 3
CMV
uPAR-S

Table 2.1: Evaluation of tumor metastasis in control and experimental animals.

Control animals inoculated with Mat B III cells transfected with vector alone (CMV) and experimental animals receiving cells transfected with experimental plasmid pRc-uPAR-S (uPAR-S) were sacrificed at 16 days post tumor inoculation. Animals were examined for the presence of macroscopic tumor metastasis as described in "Materials and Methods". The total number of metastatic foci in liver and spleen is indicated. Results are representative of at least three animals in each group in three different experiments.

Sites	Effect of uPAR expression	
	uPAR / CMV	uPAR -S
Axillary LN	1±1	4±2
Retroperitoneal LN	2±2	6±3
Mesenteric LN	1±1	4±1
Liver	0	4±1
Spleen	0	3±1

2.3.5. Results

The rat breast adenocarcinoma cells Mat B III were transfected with either the vector alone (pRc-CMV) or with the experimental plasmid (pRc-uPAR-S). At least 15 control and experimental monoclonal cell lines were selected with G-418 for the presence of neomycin resistance gene. The level of uPAR mRNA expression in control and experimental cells lines were determined by Northern blot analysis. No significant difference in uPAR mRNA expression was seen between the wild type and control Mat B III cell lines transfected with vector alone. In contrast to this, transfection of experimental plasmid pRc-uPAR-S resulted in a significant increase (3-5 fold) in uPAR mRNA expression in at least three cell lines (uPAR - S - 4, 7 and 9), as compared to the non-transfected controls and in cells transfected with vector alone (Figure 2.1). These three monoclonal cell lines expressing 3-5 fold higher uPAR mRNA were selected for further analysis. The presence of stable vector transfection was also confirmed by probing all blots with the gene for neomycin resistance (data not shown). To assess if stable transfection of rat uPAR resulted in any change in uPA expression, control and experimental cell lines were also examined for uPA mRNA expression. Although these experimental cells expressed high levels of uPAR, no significant change in uPA mRNA expression was observed (data not shown).

To determine if this increase in uPAR mRNA expression led to a similar increase in uPAR protein production, both control and experimental cells were examined for uPAR production by immunoflurescence and ELISA. In addition, transfection of experimental plasmid (pRc-uPAR-S) resulted in a marked increase in uPAR protein production in these experimental cells, compared to control cells transfected with vector alone as determined by indirect immunofluorescence technique (Figure 2.2). An increase in uPAR protein was

also observed and quantitated by ELISA by determining the amount of cleaved uPAR released into cell conditioned culture medium from control and experimental cells (Figure 2.2). This increase in uPAR protein production paralleled the increase in uPAR mRNA expression, as determined previously by Northern blot analysis. The functional ability of transfected rat uPAR was assessed by a modified receptor binding assay. Total binding of ^{125}I labelled rat uPA was determined in plasma membranes of wild type, vector transfected cells and in cells transfected with the experimental plasmid. No significant difference in total binding of radio-labelled rat uPA was seen between plasma membranes of wild type and vector transfected Mat B III cells. In contrast to this, a significant increase in ^{125}I rat uPA binding was seen in plasma membranes isolated from Mat B III cells overexpressing uPAR. Unlabelled rat uPA (0.1μM - 1.0μM) was able to compete with ^{125}I rat uPA for binding to plasma membrane in a dose dependent manner. These results not only confirmed the increased production of uPAR by experimental Mat B III cells, but also demonstrated their enhanced functional ability to efficiently bind radio-labelled rat uPA *in vitro* (Figure 2.3).

The invasive capacity of the experimental cell lines overexpressing uPAR was determined by a Boyden Chamber Matrigel invasion assay. The total number of experimental cells invading through the Matrigel was significantly higher (4-5 fold) than both wild type and vector transfected Mat B III cells (Figure 2.4). This enhanced invasive capacity of experimental cells could be inhibited by pre-incubation of experimental cells with PI-PLC, which specifically cleaves GPI anchored proteins like uPAR, to release its cell surface associated receptor into cell conditioned culture medium. A similar decrease in the invasive capacity of experimental Mat B III cells overexpressing rat uPAR was seen after co-incubation of cells with anti-rat uPAR IgG, which demonstrated the specificity of

uPAR in mediating these effects (Figure 2.4). Treatment of vector transfected control cells with PI-PLC also caused a decrease in the invasive capacity of Mat B III cells. However, this decrease in cell invasion was far less than that seen in experimental cells overexpressing uPAR (Figure 2.4).

Following *in vitro* characterization of control and experimental cells, female Fischer rats were inoculated with both control and experimental cells, and animals were examined at timed intervals for the development of tumors. Control groups of animals inoculated with either wild type cells or with cells transfected with vector alone showed the presence of palpable tumors by day 10 at the site of tumor inoculation. These tumors continued to increase in volume throughout the course of this study. In contrast, experimental animals inoculated with cells overexpressing uPAR consistently developed tumors of significantly larger volume from day 8 (the day of appearance of palpable tumors) until day 16 (Figure 2.5). After day 16 post tumor inoculation, the differences in tumor volume between control and experimental animals were less pronounced, due to the onset of tumor necrosis (data not shown).

The effect of increased uPAR production on tumor associated metastasis was examined by sacrificing groups of control and experimental animals at day 16 post tumor implantation, a time when primary tumor volume achieved maximum levels. Examination of control animals revealed the presence of enlarged retroperitoneal lymph nodes and in some cases enlargement of axillary and mesenteric lymph nodes due to tumor metastasis. No macroscopic tumor metastases were seen in the liver and spleen of these control animals (Table 2.1). In contrast, experimental animals inoculated with cells overexpressing uPAR routinely showed the presence of metastatic enlargement of axillary, retroperitoneal and mesenteric lymph nodes. Furthermore, macroscopic tumor metastasis were also seen

in the liver and spleen of these experimental animals (Table 2.1). The presence of these macroscopic metastases was further confirmed by histologic examination of tumor tissue (data not shown). A single large axillary lymph node was found in some of the control animals. To assess whether this lymph node enlargement was benign lymphadenopathy, or due to the presence of metastatic Mat B III cells, these lymph nodes were examined microscopically and by RT-PCR for the presence of uPAR. Both techniques failed to show any evidence of metastatic Mat B III cells in these axillary lymph nodes from control animals. However a microscopic analysis of lymph nodes, from experimental animals confirmed the presence of metastatic Mat B III cells. Furthermore, RT-PCR failed to amplify the predicted uPAR DNA fragment from control lymph nodes, however, the predicted uPAR DNA fragment of 441 bp was readily amplified from enlarged lymph nodes of experimental animals inoculated with cells overexpressing rat uPAR (Figure 2.6). These experiments demonstrated that the occasional lymph node enlargement observed in control animals was due to benign lymphadenopathy, and not due to Mat B III cell metastasis to lymph nodes of these control animals.

We have also looked for the presence of uPAR mRNA in tumor tissues of both control and experimental animals. Examination of tumors removed on day 16 post tumor implantation revealed continued higher levels of uPAR mRNA expression in tumors of all experimental animals compared to low uPAR mRNA expression in tumors of control animals (Figure 2.7). These results confirmed that increased tumor volume and metastasis were due to a sustained overexpression of uPAR, and that our experimental plasmid was able to cause a stable increase in uPAR expression throughout the course of these studies. Histologic examination of tumor tissues from control and experimental animals also confirmed a continued increased level of uPAR production at the protein level (data not

shown) in tumors of experimental animals.

2.3.6. Discussion

Since the isolation and characterization of the cell surface receptor for uPA, intense efforts have been made to understand the role of uPAR in the process of tumor invasion and metastasis. Although these studies established a clear relationship between the level of uPAR expression and tumor progression, they were limited in scope due to interspecies barriers of uPA action (Kariko K *et al.*, 1993). In the present study, we have overcome these limitations by generating a syngeneic model of uPAR overexpression in the rat breast cancer cell line Mat B III transfected with rat uPAR, and injected orthotopically.

Up-regulation of the uPA system is associated with increased invasion in many malignancies including breast (Grondahl-Hansen J *et al.*, 1991). Using an expression vector with a strong cytomegalo virus (CMV) promoter, we obtained at least three monoclonoal cell lines expressing 4-5 fold higher levels of uPAR as compared to the parental Mat B III cells. The efficient translation of rat uPAR cDNA into mature receptor protein was confirmed by increased production of cell surface uPAR as detected by immunocytochemistry and by monitoring the release of cleaved uPAR in cells conditioned culture medium as assessed by ELISA. The functional ability of transfected uPAR was also demonstrated by increased binding of ^{125}I labelled rat uPA to plasma membranes isolated from different experimental cell lines. To quantitate receptor binding in control and experimental cells, we treated the cells with PI-PLC to remove ^{125}I uPA/uPAR complexes from the cell surface which demonstrated a higher total binding in experimental cells, thus confirming enhanced expression of uPAR. These cells also showed a higher invasive potential *in vitro*, which could be specifically inhibited by PI-PLC and anti-rat uPAR

antibodies. The ability of PI-PLC treatment to inhibit invasion in experimental cells, at levels greater than control, may be due to the ability of PI-PLC to cleave other GPI anchored proteins involved in tumor cell invasion.

Animals inoculated with control or experimental cells demonstrated a linear increase in their tumor volume up to day 16, with significant differences in tumor volumes between the two groups. However, past day 16, these differences in tumor volume were less pronounced due to the onset of tumor-associated necrosis. In addition, transfected cells were more invasive than control cells *in vitro*, and on inoculation into host animals spread to lymph nodes, thus closely mimicking the natural metastatic progression of breast cancer. This higher metastatic potential may well be due to larger tumor volumes in experimental animals, making available an increased number of cancer cells to metastasize to distant sites. No macroscopic skeletal metastasis was observed in this model, possibly due to the orthotopic route of injection and the short duration of this breast cancer model. However, we can not rule out the presence of any skeletal micro-metastasis, since we were only able to evaluate lesions observed macroscopically.

The higher invasive potential of tumor cells overexpressing uPAR in the absence of any change in uPA expression strongly supports the emerging role of uPAR in tumor invasion (Hollas W *et al.*, 1992). This higher invasive ability is directly related to enhanced uPAR expression on the tumor cell surface, which may potentiate the recruitment of uPA from the surrounding stroma, thereby localizing uPA activity to the tumor cell surface. This facilitates ECM degradation by activating plasminogen, thereby initiating a cascade of other proteases and release of growth factors (Stetler-Stevensen WG *et al.*, 1993). Our *in vitro* and *in vivo* results further underscore the importance of a syngeneic system. These results are particularly significant in breast carcinoma due to the reported higher levels of

uPAR expression in invading ductal breast carcinoma cells relative to benign adenomas and in normal breast tissue. Development of this *in vivo* model of breast cancer will permit us to test various therapeutic strategies aimed at blocking uPA activity, as well as antagonists which block binding of uPA to the cell surface (Crowley CW *et al.*, 1993; Rabbani SA *et al.*, 1995).

2.3.7. Acknowledgments

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CHAPTER 3

PREVENTION OF BREAST CANCER GROWTH, INVASION AND METASTASIS BY ANTI-ESTROGEN TAMOXIFEN ALONE OR IN COMBINATION WITH UROKINASE INHIBITOR B-428

3.1. SUMMARY

Results of studies aimed at evaluating the effectiveness of a combinational therapeutic approach using antiestrogen Tamoxifen (TAM) and a specific active site inhibitor of urokinase 4-iodo benzo[b]thiophene-2-carboxamidine (B-428) in preventing breast cancer invasion and metastasis *in vitro* and *in vivo* are presented in this chapter in the form of a published paper (*Cancer Res.. 57: 3585-3593, 1997*). Urokinase inhibitor B-428 was kindly provided by Dr. B. A. Littlefield of Eisai Research Institute, Andover, MA. I was responsible for all of the experimental work described in this chapter. I greatly appreciate the input of Drs. A. Mazar and J. Henkin from Abbott Laboratories (Abbott Park, Illinois) in the design of the experiments and preparation of the manuscript.

All of the studies were carried out with our syngeneic *in vivo* model of uPAR overexpression in a rat mammary adenocarcinoma cell line Mat B III as described in Chapter 2 of this thesis. Results from this study, for the first time, clearly demonstrated the advantage of adding an anti-proteolytic agent like B-428 to the standard hormone therapy in preventing breast tumor growth , invasion and metastasis. More significantly, in addition to its role as an active site inhibitor of uPA, B-428 was found to down-regulate the uPA system by decreasing the level of uPAR gene transcription.

3.2. REVIEW OF PUBLISHED DATA ON B-428

Urokinase inhibitor B-428 is a synthetic active site inhibitor of uPA. Characterization of this inhibitor by Towel MJ et al. (1993) demonstrated its high specificity in inhibiting the proteolytic activity of uPA of both human and murine origin.

Previous studies in our lab have shown the effectiveness of B-428 in preventing tumor growth and invasion in a dose dependent manner when tested in our syngeneic

model of prostate cancer overexpressing rat uPA (Rabbani SA et al., 1995). This inhibitor has also been shown to block tumor cell invasion *in vitro* and *in vivo* in a mouse mammary adenocarcinoma model (Alonso DF et al., 1996)

3.3. PUBLISHED ORIGINAL PAPER

3.3.1 Abstract

Urokinase (uPA) and its cell surface receptor (uPAR) play an important role in a variety of physiological and pathological processes requiring cell migration and tissue remodelling. Using our syngeneic model of uPAR overexpression by the rat breast cancer cell line Mat B III, we have examined the abiiity of the non-steroidal anti-estrogen, tamoxifen (TAM) and of a selective synthetic inhibitor of uPA, 4-iodo benzo[b]thiophene-2-carboxamidine (B-428) to inhibit the expression of uPA and uPAR as well as cell growth, invasion and metastasis of wild type Mat B III cells and of cells overexpressing uPAR (Mat B III-uPAR). Both TAM and B-428 inhibited uPAR gene transcription, mRNA expression, protein production and also decreased the proliferative and invasive capacity of Mat B III and Mat B III-uPAR. The effects of TAM and B-428 were more pronounced when these agents were tested in combination. Both control and experimental cells ($1x10^6$ cells) were inoculated orthotopically into the mammary fat pad of syngeneic female Fisher rats and animals were infused i.p. with either TAM and B-428 alone or in combination for two weeks. Control animals receiving vehicle alone developed large tumors and macroscopic metastases to the lungs, liver and lymph nodes. In contrast to this, experimental animals receiving TAM and B-428 showed a significant decrease in primary tumor volume and metastases. Combination therapy had especially marked effects on blocking progression of the primary tumor in experimental animals inoculated with

highly aggressive Mat B III-uPAR cells. These results underscore the utility of anti-proteolytic agents (B-428) in addition to standard hormone therapy (TAM) in advanced breast cancer patients where the uPA/uPAR system plays a key role in tumor progression.

3.3.2 Introduction

Adenocarcinoma of the breast is one of the leading malignancies affecting women resulting in a high incidence of morbidity and mortality (Russo J and Russo IH, 1995). In this hormone dependent cancer, the steroid hormone estrogen stimulates tumor cell proliferation resulting in the use of anti-estrogens as a standard therapeutic regimen for patients with estrogen receptor (ER) positive tumors (Lippman ME et al., 1976a). This treatment strategy has had limited success in controlling breast cancer progression, due to the transition of breast cancer cells from an estrogen sensitive to estrogen insensitive variety in later stages of disease when the tumor is more aggressive (Clarke R et al., 1993). In contrast to estrogens, the role of progestins in breast cancer remains poorly defined (Horwitz KB, 1992). The non-steroidal anti-estrogen, Tamoxifen (TAM) still remains the most common form of hormone therapy in patients suffering from hormone sensitive breast cancer (Jordan VC, 1995). Due to its ability to bind ER, TAM can trans-activate various estrogen responsive genes implicated in tumor cell proliferation and differentiation (Lippman ME et al., 1976b). Additionally, TAM can arrest cells in early G1 phase of the cell cycle by inhibiting cyclin-dependent kinase activity (Watts CKW et al., 1995). In addition to its anti-estrogenic effects, TAM can also modulate the expression of a variety of growth factors e.g. (TGFα and TGF ß) implicated in breast cancer progression (Dickson RB and Lippman ME, 1987).

A critical event in cancer cell invasion and metastasis is the ability of tumor cells to invade through the extracellular matrix (ECM) (Mullins DE and Rohrlich ST, 1983). This is achieved through various proteolytic enzymes located at the plasma membrane, which helps to focus these degradative events on the invading cell surfaces (Dano K et al., 1985; Vassali JD and Pepper MS, 1994). One such proteolytic enzyme, urokinase (uPA) , a serine protease, has been implicated in the progression of many malignancies (Dano K et al., 1994; Quax PHA et al., 1991). Increased uPA production by tumor cells and their surrounding stroma is associated with higher invasive and metastatic potential in human malignancies (Dano K et al., 1994; Hollas W et al., 1992). In our previous studies, we have demonstrated that overexpression of uPA by the rat prostate cancer cells Mat LyLu results in increased tumor metastasis to both skeletal and non-skeletal sites (Achbarou A et al., 1994). This higher invasive and metastatic potential is achieved at least partially via focalization of the proteolytic activity of uPA within the tumor cell environment by uPA binding to its receptor (uPAR) expressed on tumor cells (Janicke F et al., 1991; Grondahl-Hansen J et al., 1991). Several clinical studies have provided compelling evidence for the role of uPA/uPAR in breast cancer where higher plasma levels of uPAR were associated with lower overall survival (Duggan C et al., 1995). In order to directly examine the role of uPAR in tumor progression, we have developed a syngeneic model of uPAR overexpression by the rat breast cancer cell line Mat B III (Xing RH and Rabbani SA, 1996). Experimental cells overexpressing uPAR showed higher invasive capacity *in vitro*. Inoculation of these cells into the mammary fat pad of syngeneic female Fischer rats resulted in the development of large tumors and metastasis to several sites as compared to animals inoculated with control cells (Xing RH and Rabbani SA, 1996).

Since the uPA / uPAR system plays a key role in tumor invasion and metastasis, inhibition of cell surface uPA activity is an attractive therapeutic target for controlling cellular invasiveness in cancer (Fazioli F and Blasi F, 1994). The uPA active site specific inhibitor, 4-iodo benzo[b]thiophene-2-carboxamidine (B-428), was able to efficiently block tumor growth and invasion in a dose dependent manner when tested in our syngeneic model of prostate cancer overexpressing rat uPA (Rabbani SA et al., 1995). This inhibitor has also been shown to block tumor cell invasion *in vitro* and *in vivo* in a mouse mammary adenocarcinoma model (Alonso DF et al., 1996). Since breast cancer is a complex multistep process in which several growth factors and proteases play key roles, appropriate therapeutic strategies to specifically target these mechanisms are required.

In the current study we have utilized our syngeneic *in vivo* model of breast cancer to evaluate the ability of TAM alone and in combination with B-428 to block the invasion and metastasis of wild type Mat B III cells and cells overexpressing uPAR.

3.3.3 Materials and Methods

Urokinase Inhibitor B-428 and Anti-estrogen Tamoxifen

Urokinase inhibitor 4-iodo benzo[b]thiophene-2-carboxamidine was a kind gift from Dr. B. A. Littlefield of Eisai Research Institute, Andover, MA (Towle MJ et al., 1993). Tamoxifen citrate salt was purchased from Sigma (St. Louis, MO).

Cells and Cell Culture

Mat B III cells were obtained from American Type Culture Collection (Rockville, Maryland). The experimental cells overexpressing uPAR (Mat B III-uPAR) were generated by transfecting the expression vector pRc-uPAR containing the entire coding

region of rat uPAR cDNA as previously described (Xing RH and Rabbani SA, 1996). Cells were maintained in culture *in vitro* in McCoy's 5A modified medium supplemented with 2 mM L-glutamine (GIBCO), 10% fetal bovine serum (FBS), 100 units / ml of penicillin-streptomycin sulphate (GIBCO) and 0.2% gentamycin. For transfected Mat B III and Mat B III-uPAR cells, the medium was also supplemented with G418 (600 μg / ml).

Animal Protocols

Inbred female Fisher 344 rats weighing 200-220 g were obtained from Charles River Inc. (St. Constant, Canada). Before inoculation, control and experimental Mat B III cells were grown in serum-containing medium and washed with Hank's buffer and trypsinized for 5 min. Cells were then collected in Hank's buffer and centrifuged at 1500 rpm for 5 min. Cell pellets (1×10^6 cells) were resuspended in 0.2 mL saline and injected using one ml insulin syringes into the mammary fat pad of rats anaesthetized with ethanol / Somnotal (MTC Pharmaceuticals, Cambridge, Ontario).

To examine the effect of B-428 on tumor-bearing animals, Alzet osmotic minipumps (models 2ML4 and 2MN4, Alza, Palo Alto, CA) were implanted i.p. under light ether anesthesia. Each mini-pump contained B-428 dissolved at 2.5 mg/ml in 0.9% saline containing 5% DMSO and 5 mg/ml BSA, to deliver a continuous dose of the compound for up to 2 weeks at a delivery rate of 0.005 ml / h (Rabbani SA et al., 1995). Control animals received vehicle alone (0.9% saline, 5% DMSO, 5 mg / ml BSA) delivered in an identical manner. To examine the effect of TAM on tumor-bearing animals, TAM was given daily i.p. at a concentration of 3 mg / kg / day for 2 weeks. Combination treatment was given in an identical manner. These doses of B-428 and TAM are sufficient for decreasing tumor growth in rats without any noticeable side effects and represent the

pharmacological dose of TAM for humans (Rabbani SA et al., 1995; Huynh HT et al., 1993; Price JE, 1996; Love RR, 1989; Torczynski R et al., 1983).

All animals were numbered, kept separately and were examined for the development of tumors every second day for up to 17 days. The tumor mass at the site of tumor cell inoculation (mammary fat pad) of control and experimental animals was measured in two dimensions by callipers and the tumor volume calculated (Rabbani SA et al., 1995; Price JE, 1996). At timed intervals, control and experimental animals were sacrificed, examined and scored for the development of macroscopic metastases in various tissues by two experts blinded to therapy. Tumor tissues were also removed from the site of inoculation and extracted for RNA analysis.

Cell Proliferation Assay

Control and experimental cells were trypsinized, reseeded into 6-well tissue culture dishes (2 x 10^4 cells / well), and allowed to adhere overnight. The cells were treated the next day with either TAM (0.01 - 1.0 μM), B-428 (0.5-5.0 μM) or a combination of TAM and B-428 (1.0 μM TAM, 5.0 μM B-428). B-428 and TAM were dissolved in DMSO and ethanol respectively as 1000x stock solutions and were added directly into the fresh culture medium (2 ml / plate) 24 h after plating the cells. Cell culture medium was replenished every third day and the total number of control and experimental cells were counted using a Coulter Counter (model ZF; Coulter Electronics, Harpenden, Herts, U.K.) at 1,2, 3 and 4 days after the treatments.

Northern Blot Analysis

Total cellular RNA was isolated from tumor tissue, control and treated MAT B III cells by acid guanidium thiocyanate-phenol-chloroform extraction. Twenty μg of total cellular RNA was electrophoresed on a 1.1% agarose-formaldehyde gel and transferred to a nylon membrane (Nytran, Amersham, Oakville, Canada) by capillary blotting. Filters containing Mat B III cell RNA were hybridized with a ^{32}P-labelled rat uPAR and rat uPA cDNA (Xing RH and Rabbani SA, 1996). All blots were also hybridized with 18 s RNA probe labelled with [γ-^{32}P ATP] as a control for the amount of RNA loaded (Torczynski R et al., 1983). The filters were incubated at 42°C for 24 h, then successively washed in 1 x SSC (10 x SSC is 1.5 M NaCl, 0.5 M sodium citrate, pH 7.0), 1% sodium dodecyl sulfate (SDS) for 15 min at room temperature, 0.5 x SSC, 0.5% SDS for 15 min at room temperature, 0.1 x SSC, 0.1% SDS twice for 15 min at room temperature, and then once for 30 min at 55°C. Autoradiography of filters was carried out at -70°C using XAR film (Eastern Kodak Co., Rochester, NY) with two intensifying screens. The level of mRNA of various genes of interest was quantitated by densitometric scanning.

Matrigel Invasion Assay

The effect of TAM, B-428 and combination of TAM and B-428 treatment on the invasive capacity of control and experimental Mat B III cells was tested by two-compartment Boyden chambers (Transwell, Costar, USA) and basement membrane Matrigel (Becton Dikinson Labware) as previously described (Xing RH and Rabbani SA, 1996; Liu DF and Rabbani SA, 1995). In some experiments, control and experimental Mat B- III cells (5 x 10^5 / ml) were cultured in the presence of different concentrations of TAM

(0.01 μM -1.0 μM) , B-428 (0.5 μM -5.0 μM) or combination of TAM and B-428 (TAM 1.0μM, B-428 5.0 μM).

Nuclear Run-off Assay of Gene Transcription

Nuclear run-off assays were performed by harvesting cells after treatment with various agents, in cold PBS. Cells were collected and lysed in cold NP-40 lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40) for 5 min on ice. Cell nuclei were collected by centrifugation at 4 ^{0}C, and resuspended in storage buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 5 mM dithiothreitol). The nuclei were either used immediately or frozen in liquid nitrogen for later use. Nuclear run-off assays were carried out by adding 100 μl nuclear suspension (2-4 x 10^7 nuclei) to the 100 μl reaction buffer (50 mM Tris-HCl, pH 7.5, 0.3 M KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 0.5 mM each of ATP, CTP, and GTP, 50-100 μCi [^{32}P]a-uridine triphosphate, >600 Ci/mmol, ICN, Costa Mesa, CA) for 60 min at room temperature. After the incubation, DNase I (150 U per reaction) and proteinase K (0.2 mg/ml) were added sequentially and incubated for 30 min at 37 ^{0}C respectively (Liu B et al., 1993). Newly synthesized RNAs were isolated by spin column and ethanol precipitation, and pelleted by centrifugation. RNAs were hybridized with uPA, uPAR and 18S cDNAs and with Bluescript vector DNA (Stratagene, La Jolla, CA) previously immobilized on Nytran membranes using a slot blot manifold (Bio-Rad, Richmond, CA). These membranes were incubated in the hybridization solution (6 x SSC, pH 7.4, 50% formamide, 1% SDS, 0.1 mg/ml sonicated salmon sperm DNA) at 42 ^{0}C for 48 h. After hybridization, membranes were washed in a final wash solution of 0.1 x SSC, 0.1% SDS at 42 ^{0}C, and exposed to

Kodak XAR film with intensifying screens. The intensity of each band was quantitated using laser densitometry.

Indirect Immunofluorescence

To examine the effect of TAM and B-428 alone or in combination on uPAR expression on Mat B III cells, 5 x 10^4 cells were plated in Lab Tek tissue culture chambers (Nunc, Naperville, IL) and allowed to grow to 70% confluence. Cells were treated with vehicle, TAM (1.0μM), B-428 (5.0 μM) and in combination (TAM 1.0μM, B-428 5.0μM) for 6 days. Indirect immunofluorescence was performed as described previously (Xing RH and Rabbani SA, 1996) using anti-rat uPAR IgG (American Diagnostica Inc, Greenwich, CT).

Statistical Analysis

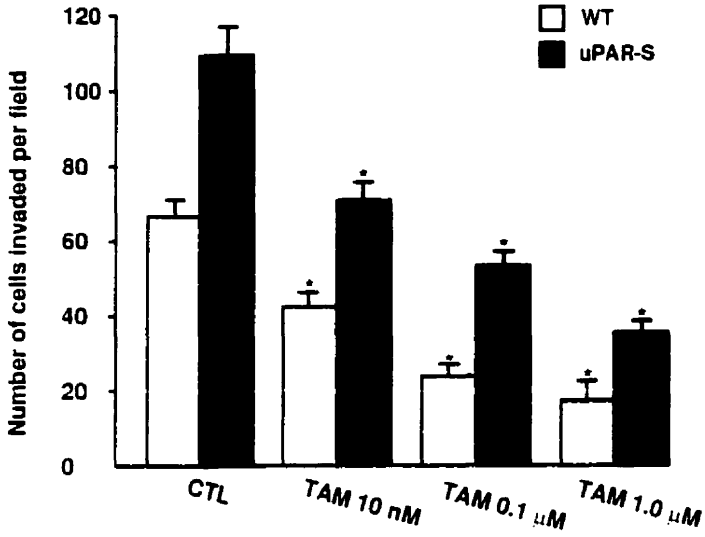
Results are expressed as the mean ± S.E. of at least triplicate determinations and statistical comparisons are based on the Student's *t* test or analysis of variance. A probability value of <0.05 was considered to be significant.

3.3.4 Figures

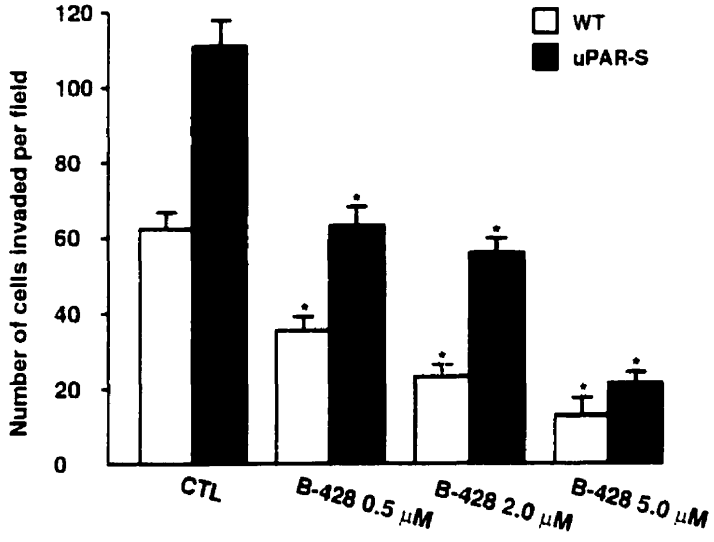
Figure. 3.1: Effect of TAM and B-428 alone or in combination on Mat B III cell invasion.

Non-transfected Mat B III (WT) and Mat B III cells overexpressing uPAR (uPAR) were grown in culture as described in "Materials and Methods". Number of cells migrating to the lower aspect of the Boyden chamber filter after treatment with vehicle alone or different concentrations of TAM (panel A) and B-428 (panel B) or TAM and B-428 in combination (panel C) were counted. Percentage inhibition of invasion was calculated by taking the number of cells invading in the presence of vehicle alone (CTL) as 100%. Results represent ± SEM of four different experiments. Significant difference in inhibition by each agent from control is represented by one and by combination treatment from either of these agents alone is represented by two asterisks ($P<0.05$).

A


WT
uPAR-S
Number of cells invaded per field
120
100
80
60
40
20
0
CTL
TAM 10 nM
TAM 0.1 μM
TAM 1.0 μM

B


WT
uPAR-S
Number of cells invaded per field
120
100
80
60
40
20
0
CTL
B-428 0.5 μM
B-428 2.0 μM
B-428 5.0 μM

C

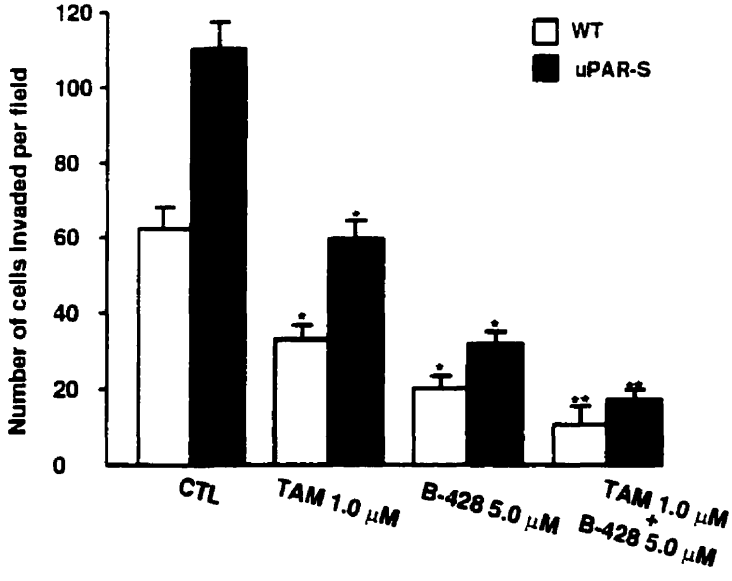
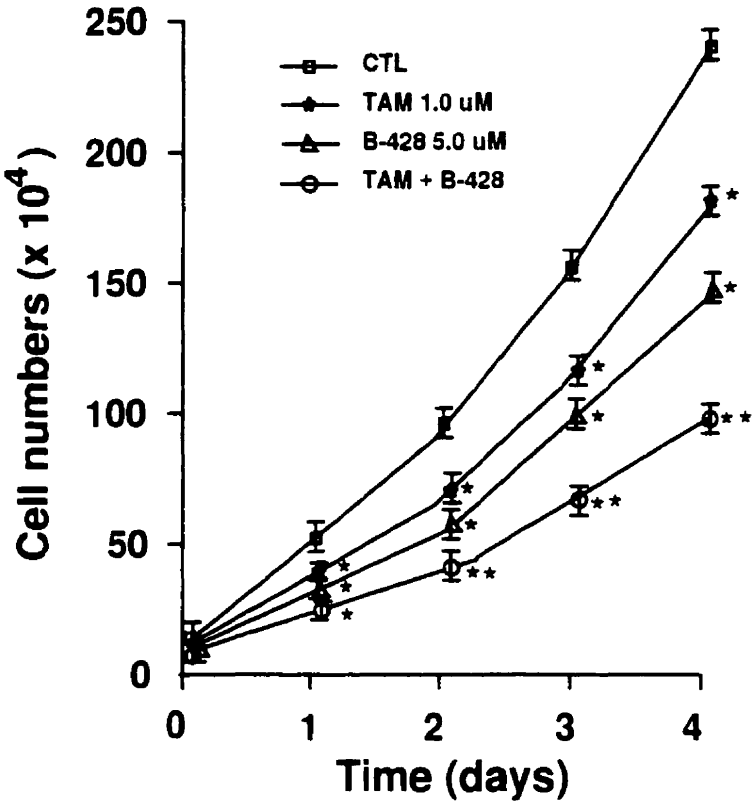

WT
uPAR-S
Number of cells invaded per field
120
100
80
60
40
20
0
CTL
TAM 1.0 μM
B-428 5.0 μM
TAM 1.0 μM
+
B-428 5.0 μM

Figure. 3.2: Effect of TAM and B-428 alone or in combinations on Mat B III cell growth.

Comparison of growth curves for nontransfected Mat B III (A) and Mat B III-uPAR cells overexpressing uPAR (B). Cell growth in each group was compared after incubating them in the presence of vehicle (CTL) or TAM and B-428 alone or a combination of TAM and B-428 for indicated periods of time. Cells from triplicate dishes were trypsinized and counted as described in "Materials and Methods". Each point represents ± SEM of four experiments. Significant difference from vehicle treated (CTL) cells, and cells treated with TAM and B-428 alone in each panel is represented by one asterisk, and by combination treatment from either of these agents alone by two asterisks ($P<0.05$).

A


CTL
TAM 1.0 uM
B-428 5.0 uM
TAM + B-428
Cell numbers (x 10^4)
250
200
150
100
50
0
0
1
2
3
4
Time (days)

B

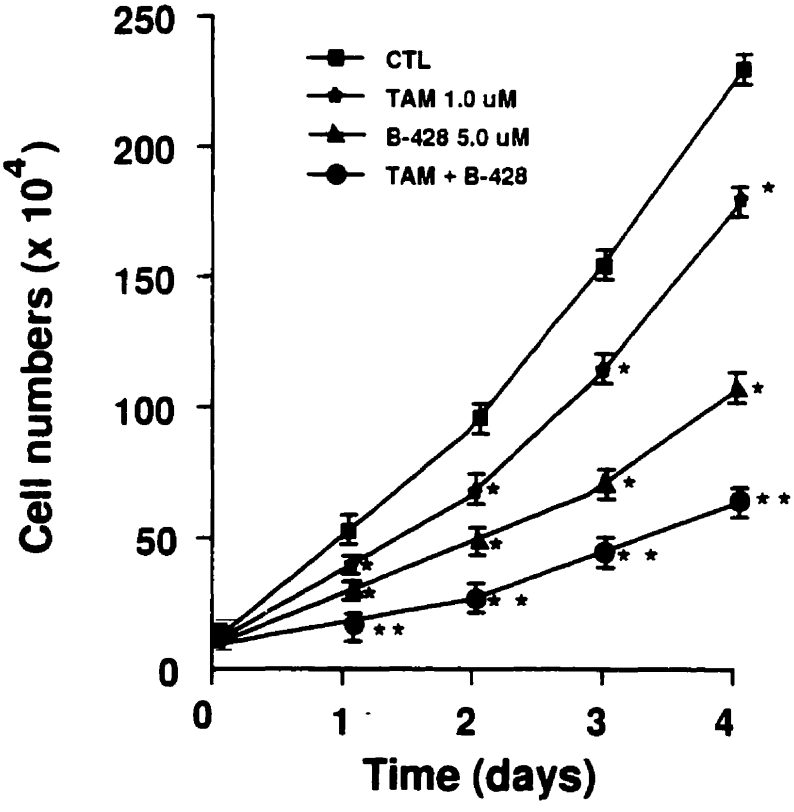

CTL
TAM 1.0 uM
B-428 5.0 uM
TAM + B-428
Cell numbers (x 10^4)
250
200
150
100
50
0
0
1
2
3
4
Time (days)

Figure. 3.3: Effect of TAM and B-428 on uPA and uPAR mRNA expression in wild type Mat B III cells.

Total cellular RNA was extracted from untreated (CTL) and cells treated with TAM (1.0 μM) and B-428 (5.0μM) alone or a combination of TAM and B-428. 20μg of total cellular RNA from each group were electrophoresed on a 1.1% agarose/formaldehyde gel and blotted to a nylon membrane by capillary action. All blots were hybridized with a ^{32}P labelled rat uPA cDNA, rat uPAR cDNA or with a ^{32}P labelled 18S cDNA as described in "Materials and Methods". Blots were scanned by laser densitometric scanning and changes in uPA (panel A) and uPAR (panel B) mRNA expression were determined by plotting the ratio of uPA or uPAR/18S mRNA and are shown in the lower panels. Results are representative of at least 4 different experiments. Significant difference from control cells is represented by asterisks ($P<0.05$).

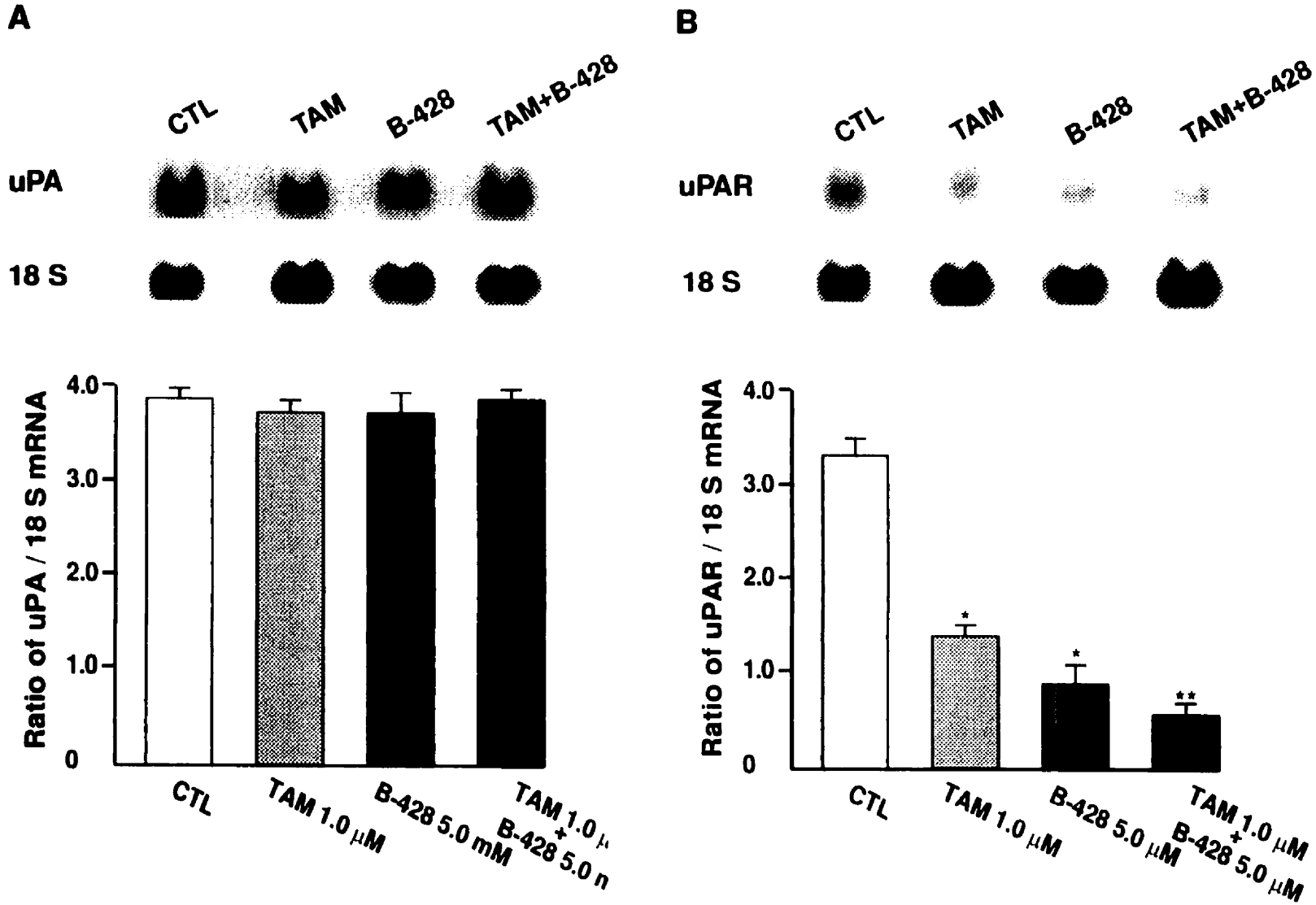

A
CTL
TAM
B-428
TAM+B-428
uPA
18 S
Ratio of uPA / 18 S mRNA
4.0
3.0
2.0
1.0
0
CTL
TAM 1.0 μM
B-428 5.0 mM
TAM 1.0 μ
+
B-428 5.0 n
B
CTL
TAM
B-428
TAM+B-428
uPAR
18 S
Ratio of uPAR / 18 S mRNA
4.0
3.0
2.0
1.0
0
*
*
**
CTL
TAM 1.0 μM
B-428 5.0 μM
TAM 1.0 μM
+
B-428 5.0 μM

Figure. 3.4: Effect of TAM and B-428 on uPA and uPAR mRNA expression in Mat B III-uPAR cells

Total cellular RNA was extracted from untreated (CTL) and Mat B III-uPAR cells treated with TAM (1.0 μM) and B-428 (5.0 μM) alone or in combination. 20μg of total cellular RNA from each group were electrophoresed on a 1.1% agarose/formaldehyde gel and blotted to a nylon membrane by capillary action. All blots were hybridized with a ^{32}P labelled rat uPA cDNA, rat uPAR cDNA or with a ^{32}P labelled 18S cDNA as described in "Materials and Methods". Blots were scanned by laser densitometric scanning and changes in uPA (panel A) and uPAR (panel B) mRNA expression were determined by plotting the ratio of uPA or uPAR/18S mRNA in the lower panels. Results are representative of at least 4 different experiments. Significant difference from control cells is represented by asterisk ($P<0.05$).

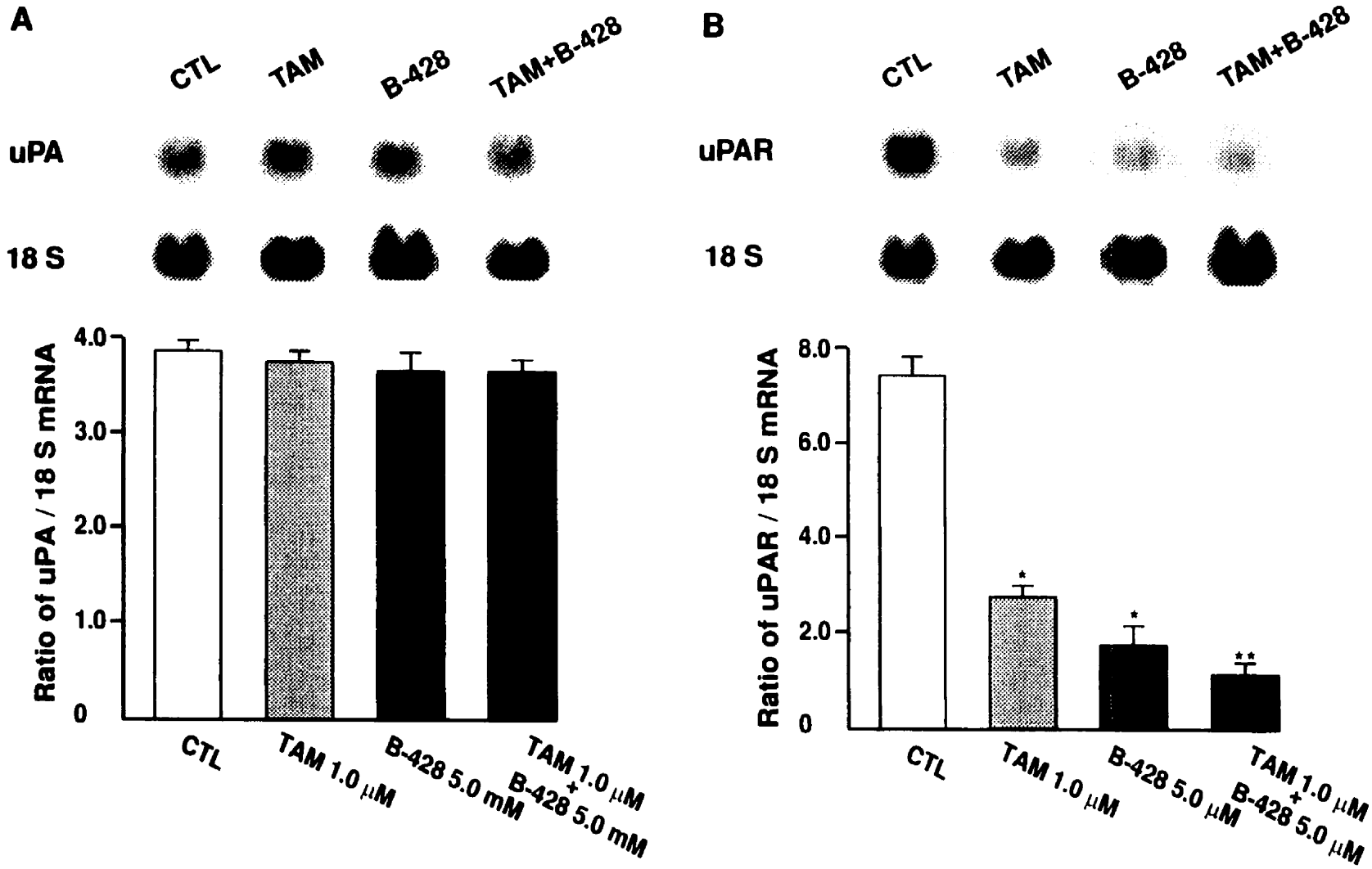

A
CTL
TAM
B-428
TAM+B-428
uPA
18 S
Ratio of uPA / 18 S mRNA
4.0
3.0
2.0
1.0
0
CTL
TAM 1.0 μM
B-428 5.0 mM
TAM 1.0 μM + B-428 5.0 mM
B
CTL
TAM
B-428
TAM+B-428
uPAR
18 S
Ratio of uPAR / 18 S mRNA
8.0
6.0
4.0
2.0
0
*
*
**
CTL
TAM 1.0 μM
B-428 5.0 μM
TAM 1.0 μM + B-428 5.0 μM

Figure. 3.5: Effect of TAM and B-428 alone and in combination on uPA and uPAR gene transcription in Mat B III cells.

Nuclear run-off assays were performed as described in "Materials and Methods". ^{32}P labelled run-off transcripts were prepared from Mat B III cell nuclei following treatment with TAM, B-428 alone or in combination. Probes used were uPA, uPAR and 18S cDNAs. All blots were scanned by laser densitometry. The fold stimulation of the rate of gene transcription of uPA and uPAR relative to 18S was determined.

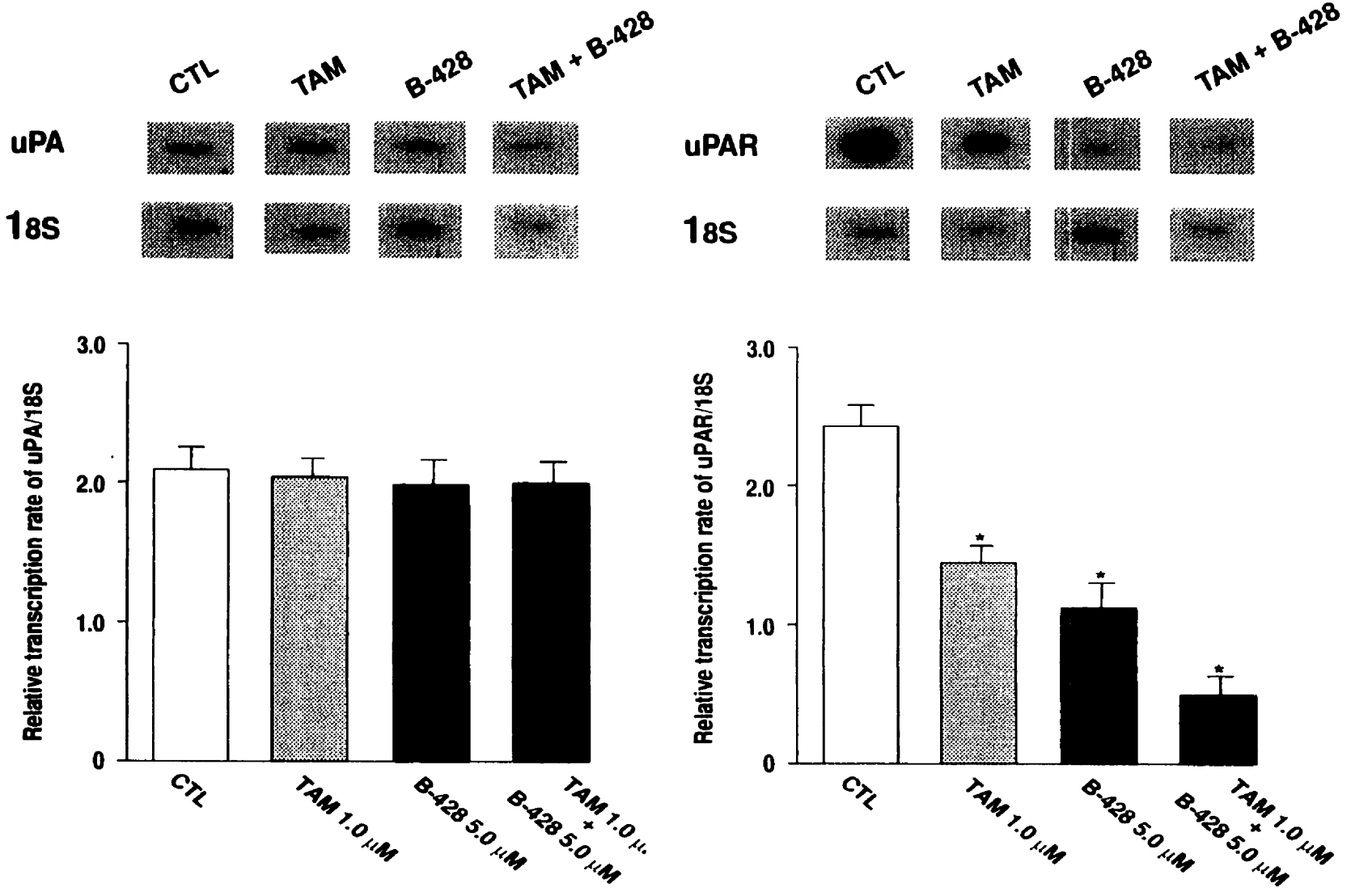

CTL
TAM
B-428
TAM + B-428
uPA
18S
uPAR
18S
Relative transcription rate of uPA/18S
3.0
2.0
1.0
0
CTL
TAM 1.0 μM
B-428 5.0 μM
TAM 1.0 μM + B-428 5.0 μM
Relative transcription rate of uPAR/18S
3.0
2.0
1.0
0
CTL
TAM 1.0 μM
B-428 5.0 μM
TAM 1.0 μM + B-428 5.0 μM

Figure. 3.6: Effect of TAM and B-428 alone and in combination on uPAR protein production in Mat B III cells.

Expression of uPAR on Mat B III cell surface following treatment with vehicle alone (CTL), TAM, B-428 and a combination of TAM and B-428 was examined by indirect immunofluorescence as described in "Materials and Methods". Results are representative of 4 different experiments.

CTL

TAM

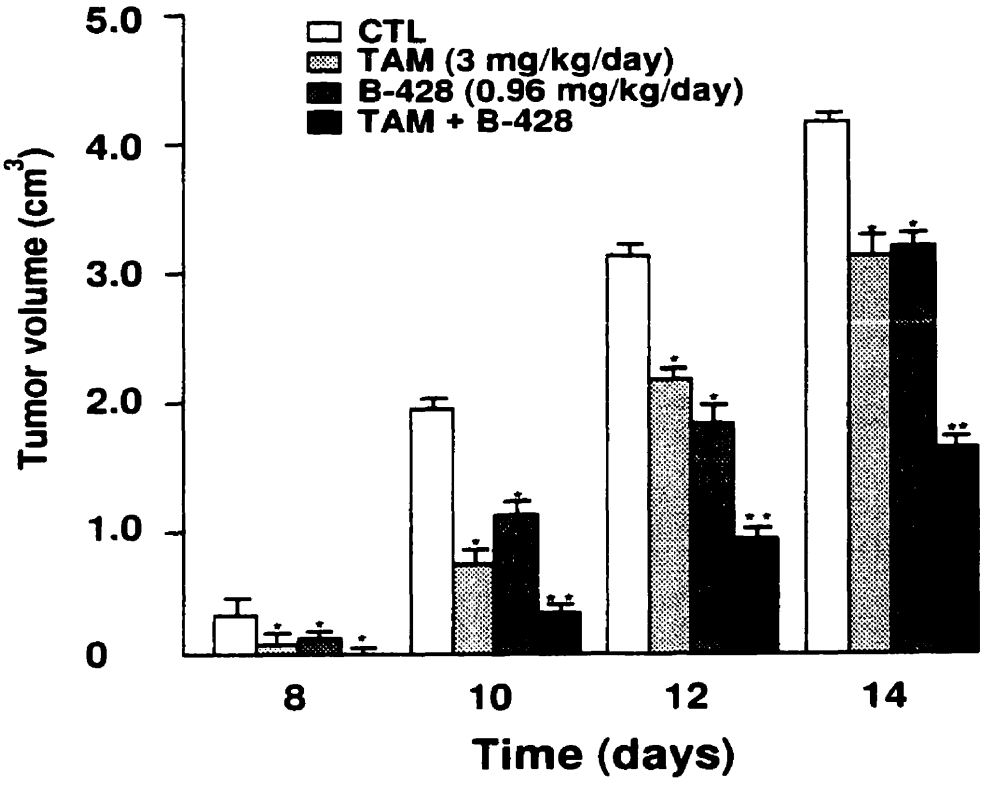
B-428

TAM+B-428

Figure. 3.7: Effect of TAM and B-428 alone or in combination on tumor volume.

Tumor volume in control (Mat B III) and experimental (Mat B III-uPAR) animals was determined at timed intervals as described in "Materials and Methods." Tumor volume of animals inoculated with wild type Mat B III cells (panel A) and Mat B III-uPAR cells overexpressing uPAR (panel B) was determined after treatment with TAM (1.0μM), B-428 (5.0μM) alone or in combination and was compared with animals receiving vehicle alone (CTL). Results represent the mean ± SEM of 6 starting animals in each group in 4 different experiments. Significant difference from control tumor bearing animals after treatment with TAM and B-428 alone is marked by one, and by combination treatment from either of these agents alone by two asterisks ($P<0.05$).

A


CTL
TAM (3 mg/kg/day)
B-428 (0.96 mg/kg/day)
TAM + B-428
Tumor volume (cm3)
5.0
4.0
3.0
2.0
1.0
0
8
10
12
14
Time (days)

B

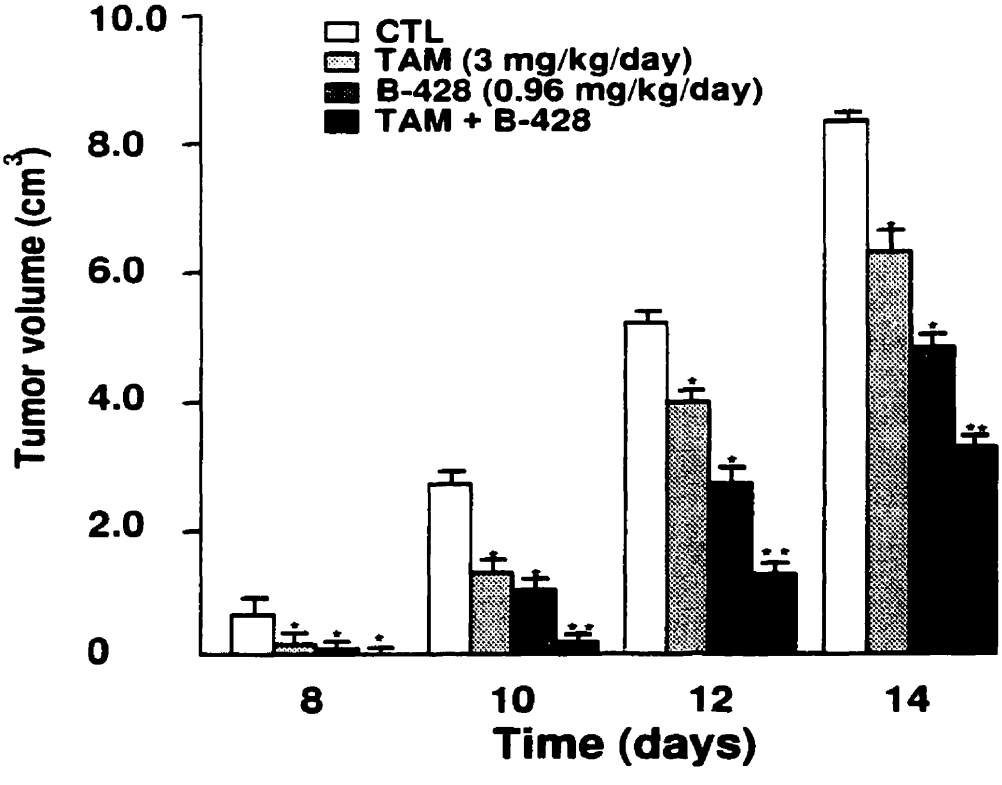

CTL
TAM (3 mg/kg/day)
B-428 (0.96 mg/kg/day)
TAM + B-428
Tumor volume (cm3)
10.0
8.0
6.0
4.0
2.0
0
8
10
12
14
Time (days)

Figure. 3.8: Evaluation of tumor metastasis in control and experimental animals.

Female Fischer rats were inoculated with wild type (WT) Mat B III and Mat B III-uPAR (uPAR) cells and infused with either vehicle (CTL), TAM (1.0μM), B-428 (5.0μM), alone or in combination as described in "Materials and Methods". All control and experimental animals were sacrificed at day 16 post tumor inoculation and examined for the presence of macroscopic tumor metastasis. The total number of macroscopic metastatic foci were counted and compared. Results are representative of at least 4 animals in each group in 4 different experiments.

	SITE	CTL	TAM	B-428	TAM+B-428
WT	Lung	3 ± 2	2 ± 1	1 ± 0	1 ± 0
	Liver	2 ± 1	2 ± 1	1 ± 0	1 ± 0
	Ax. Lymph nodes	3 ± 1	3 ± 1	1 ± 1	1 ± 1
uPAR	Lung	6 ± 2	5 ± 2	3 ± 1	3 ± 2
	Liver	5 ± 2	5 ± 2	2 ± 1	2 ± 1
	Ax. Lymph nodes	5 ± 3	4 ± 2	2 ± 2	2 ± 1

3.3.5 Results

Effect of TAM and B-428 on Mat B III Cell Invasion *In Vitro*

The effects of TAM (0.01 - 1.0 μM) and B-428 (0.5 - 5.0 μM) on the invasive capacity of Mat B III and Mat B III-uPAR cells were examined by Boyden Chamber invasion assays. Both TAM and B-428 were able to cause a significant decrease in the ability of control and experimental Mat B III cells to invade through the Matrigel in a dose-dependent manner. The number of cells invading through Matrigel following treatment with these reagents was counted and compared with cells treated with vehicle alone. Although an increased number of experimental cell invaded through the Matrigel as compared to control cells as previously reported (Xing RH and Rabbani SA, 1996), TAM (0.01-1.0 μM) was equally effective (25-52%) in decreasing the invasive capacity of both control and experimental cells (Figure 3.1A). In contrast to this, B-428 (0.5-5.0 μM) was more effective (36-67%) in inhibiting the invasive capacity of Mat B III-uPAR as compared to control Mat B III cells (27-52%) [Figure 3.1B]. Furthermore, an additive inhibitory effect on cell invasion was observed with both control and experimental cells receiving the combination treatment of TAM (1.0 μM) and B-428 (5.0 μM) [Figure 3.1C].

Effect of TAM and B-428 on Mat B III Cell Growth *In Vitro*

Wild type nontransfected Mat B III cells (control) and Mat B-III-uPAR cells overexpressing uPAR (experimental) were maintained in culture in the absence or presence of TAM (1.0 μM) and B-428 (5.0 μM) alone and in combination for 1-4 days, and the effect of these treatments on cell proliferation *in vitro* were examined. Both TAM and B-428 inhibited the growth of control Mat B III cells for up to 4 days. TAM inhibited Mat B-III cell growth by 30% whereas a 40% decrease in cell proliferation was seen

following treatment with B-428 (Figure 2.2A). Treatment of these control cell with TAM and B-428 in combination resulted in a significantly greater (60%) inhibition in cell growth as compared to either of these agents alone (Figure 2.2A). Under similar experimental conditions, treatment of Mat B III-uPAR cells with TAM caused a 29% decrease in cell proliferation whereas a significantly more marked decrease (55%) in cell growth was seen following treatment of these cells with B-428 (Figure 2.2B). Furthermore, combination of TAM and B-428 had a more pronounced (75 %) effect on the growth of these experimental cells overexpressing uPAR as compared to either of these agents when tested alone (Figure 2.2B). Overall, TAM was equipotent in blocking the growth of Mat B III and Mat B III-uPAR cells, however, B-428 alone or in combination with TAM was found to be a more effective inhibitor of Mat B-III-uPAR cell growth. The viability of both groups of cells after treatment with these agents was determined by Trypan blue assay at each time point. No evidence of cytotoxicity was observed. Furthermore, these concentrations of TAM and B-428 did not alter cell morphology (data not shown).

Effect of TAM and B-428 on uPA and uPAR mRNA Expression, Gene Transcription and on uPAR Protein Production *In Vitro*

Both control (Mat B III) and experimental (Mat B III-uPAR) cells were treated with either TAM (0.1 - 1.0 μM), B-428 (0.5 - 5.0 μM), or a combination of TAM and B-428 (1.0 μM TAM, 5.0 μM B-428) for 6 days. Incubation of control and experimental Mat B III cells with both TAM and B-428 for 6 days had no cytotoxic effects. In our previous study, we have shown that Mat B III-uPAR cells express 4 fold higher levels of uPAR mRNA as compared to control nontransfected cells without altering the level of uPA mRNA expression(Xing RH and Rabbani SA, 1996). Treatment of Mat B III cells with

TAM, B-428, or a combination of the two failed to show any significant change in uPA mRNA expression as determined by Northern blot analysis (Fig.3A). In contrast, TAM and B-428, alone or in combination, caused a significant decrease in the rate of uPAR mRNA expression in the control Mat B-III cells (Figure 3.3B). Similarly, no statistically significant change in uPA mRNA expression was seen in Mat B III-uPAR cells (Figure 3.4A), whereas a marked inhibition of uPAR mRNA levels was observed following treatment with TAM and B-428 alone or in combination (Figure 3.4B). The effects of B-428 on uPAR mRNA expression were especially more profound in Mat B-III-uPAR.

In order to explore the molecular mechanisms of the actions of TAM and B-428, the effect of these agents alone and in combination on uPA/uPAR gene transcription was examined by nuclear run-off assay. Following treatment with TAM (1.0 μM), B-428 (5.0 μM) or a combination, cellular nuclei were isolated from control Mat B III cells and the rate of uPA/uPAR gene transcription was determined. TAM and B-428 alone or in combination had no significant effect on uPA gene transcription (Figure 3.5A). However, treatment of these cells with either TAM or B-428 caused a marked decrease in uPAR gene transcription and the effects were additive when these reagents were tested in combination (Figure 3.5B). A similar effect on uPA/uPAR gene transcription was observed following treatment of experimental Mat B III-uPAR cells with TAM and B-428 (data not shown). This decrease in uPAR mRNA expression and gene transcription resulted in a similar lower level of uPAR expression on the Mat B III cell surface as determined by indirect immunofluorescence and receptor binding assays (data not shown).

To determine if a decrease in uPAR gene transcription and mRNA expression by TAM and B-428 results in a similar lower level of uPAR protein production, Mat B III cells were treated with either vehicle TAM (1.0μM), B-428 (5.0 μM) alone or a

combination of TAM and B-428. Treatment of these cells with both TAM and B-428 resulted in a marked decrease in uPAR expression on the Mat B III cell surface, as assessed by immunofluorescence (Figure3.6). These effects were additive when TAM and B-428 were given in combination and showed decreased uPAR expression at similar levels as seen by Northern blot analysis. A similar decrease in total binding of ^{125}I labelled rat uPA was seen in Mat B III cells following treatment with TAM and B-428 alone or in combination (data not shown).

Effect of TAM and B-428 on Tumor Growth

Female Fisher rats were inoculated with Mat B III (control) and Mat B III-uPAR (experimental) cells. Animals were divided into four experimental groups receiving vehicle alone, TAM (3 mg/kg/day), B-428 (0.96 mg/kg/day) alone, or TAM and B-428 in combination. Animals were monitored for tumor growth and were sacrificed at day 15 post tumor inoculation for evaluation of tumor metastasis. Animals receiving Mat B III (Figure 3.7A) and Mat B-III-uPAR (Figure 3.7B) treated with either TAM or B-428 alone showed a marked decrease in tumor volume as compared with the control group of animals receiving vehicle alone. Consistent with our *in vitro* observations of the effect of TAM on tumor cell growth, TAM was equipotent in inhibiting tumor growth in animals receiving Mat B III (control) and Mat B III-uPAR (experimental) cells. In these studies, B-428 was equally effective as TAM in blocking tumor growth of animals inoculated with control cells (Figures 7.7A and 7.7B). However, infusion of B-428 into animals inoculated with experimental Mat B III-uPAR cells resulted in a significantly greater decrease in tumor volume as compared with TAM alone (Figure 3.7B). Most significantly, although combination therapy with TAM and B-428 showed an additive effect on tumor

reduction in animals inoculated with either control (Figure 3.7A) or experimental cells (Figure 3.7B), these effects were more pronounced in animals receiving experimental Mat B-III uPAR cells. Furthermore, infusion of TAM and B-428 alone or in combination was well tolerated by experimental animals without any noticeable side effects.

Effect of TAM and B-428 on Tumor Metastasis

Comparison of tumor metastases in animals inoculated with wild-type control Mat B III cells and experimental cells overexpressing uPAR confirmed our previous observations that animals receiving cells overexpressing uPAR developed more extensive macroscopic tumor metastases to the lungs, livers and auxiliary lymph nodes as compared to animals receiving wild-type Mat B-III cells (Xing RH and Rabbani SA, 1996). On day 15 days post tumor inoculation, animals inoculated with Mat B III and Mat B III-uPAR cells receiving either vehicle, TAM, B-428, or TAM and B-428 in combination which showed marked effects of these agents in decreasing tumor volume were sacrificed and evaluated for the presence of macroscopic tumor metastasis. TAM treatment had no significant effect on the development of tumor metastasis in animals inoculated with Mat B III or Mat B III-uPAR cells. In contrast, infusion of B-428 into animals receiving Mat B III or Mat B III - uPAR cells resulted in a significant reduction in tumor metastasis, with an even greater reduction in those animals receiving Mat B III-uPAR cells (Figure 3.8). Treatment of these groups of animals with a combination of TAM and B-428 failed to show any additive effect in preventing tumor metastasis in either group as compared to infusion of B-428 alone (Figure 3.8).

3.3.6. Discussion

The efficacy of currently available therapies for breast cancer is restricted by the disseminated nature of the disease which is characterized by the progression of the majority of tumors to a phenotype which is resistant to both cytotoxic and hormonal therapies, and the inability of these therapies to effectively control tumor spread to various metastatic sites (Clarke R et al., 1993). Therefore, the development of a complementary approach which involves modifying the tumor microenvironment and reducing the propensity for tumor cell invasion, neovascularization and metastasis is required. The role of cell associated uPA and its cell surface receptor (uPAR) in extracellular matrix (ECM) degradation and cellular invasiveness and tumor progression is well documented in clinical and experimental studies (Dano K et al., 1994; Quax PHA et al., 1991; Hollas W et al., 1992; Achbarou A et al., 1994; Janicke F et al., 1991; Grondahl-Hansen J et al., 1991; Duggan C et al., 1995; Xing RH and Rabbani SA, 1996; Pyke C et al., 1993; Kobayashi H et al., 1994). Inhibition of uPA activity and interruption of uPA/ uPAR interaction is therefore an attractive target for blocking cellular invasiveness in cancer (Xing RH and Rabbani SA, 1996; Rabbani SA et al., 1995). Due to the species specificity of uPA and uPAR interaction, a syngeneic model of breast cancer is best suited for evaluation of certain therapeutic strategies aimed at the plasminogen activator system (Achbarou A et al., 1994; Xing RH and Rabbani SA, 1996). Using this model, in which uPA/uPAR plays a key role, we have evaluated the anti-invasive and anti-metastatic abilities of the recently developed synthetic uPA active site inhibitor B-428 alone and in combination with TAM.

We have previously reported the ability of B-428 to block tumor progression in our syngeneic model overexpressing uPA (Rabbani SA et al., 1995). The availability of control and experimental Mat B III cells overexpressing uPAR in the current study

allowed us to evaluate the usefulness of not only the anti-estrogen agent TAM, but also the efficacy of any adjuvant anti-proteolytic therapy against uPA. In breast cancer (and certain other cancers), uPA may not be expressed directly by the tumor cells but rather recruited from the surrounding stroma (Nielsen BS et al., 1996). Furthermore, overexpression of uPAR by the tumor cells themselves creates a system of localization of uPA to the cell surface. Therefore, even though the tumor cells themselves do not express high levels of uPA, an anti-uPA antagonist would nevertheless be potentially useful due to the localization of uPA in the tumor milieu. This model may also prove to be of benefit in assessing the usefulness of inhibitors of other proteases (e.g. MMP, cathepsin, cadherins) and growth factors (e.g.EGF) implicated in tumor progression (Dano K et al., 1994; Elliott E and Sloane BF, 1996; Mbalaviele G et al., 1996; Pirinen R et al., 1997).

In the current study, both TAM and B-428 decreased the invasive capacity of control and experimental Mat B III cells in a dose dependent manner. Combination of TAM and B-428 treatment demonstrated an additive anti-invasive effect which was more marked in experimental cells. Although B-428 has previously been shown to be an anti-invasive agent in prostate cancer cells, this is the first report demonstrating the anti-proliferative effects of this protease inhibitor (Rabbani Sa et al., 1995). TAM and B-428 were also anti-proliferative in the absence of cytoxicity suggesting a role for these inhibitors in tumor cell signal transduction . The molecular mechanism of these effects was further examined by Northern blot analysis and nuclear run-off assays for uPA/uPAR (Wang Y et al., 1994). Although neither TAM or B-428 had any effect on uPA mRNA expression, these agents caused a significant decrease in uPAR mRNA expression and protein production in control and experimental cells. These results suggest several potential mechanisms of the action for B-428: direct inhibition of the uPA active site can down regulate the uPA dependent

cell-surface proteolytic cascade which can ultimately activate latent growth factors via the action of plasmin or metalloproteases (Dano K et al., 1985). Blocking the start of this cascade leads to lower growth factor activity in the tumor milieu resulting in decreased proliferation and invasion of the tumor. Decreased growth factor activity may act in *trans* on uPAR expression, resulting in the down-regulation of its expression as well. Alternatively, the observed effects could be a direct result of uPAR down-regulation. The increased proliferative response of tumors formed *in vivo* by the Mat B III-uPAR could reflect the direct signal transducing role of uPAR which is mediated at least partially by the binding of uPA. uPA/ uPAR is endocytosed in some cells by virtue of the cell-surface uPA complexing with PAI-1. It is not clear what the down-stream consequences of this internalization are. In the presence of B-428, it is possible that PAI-1 may not complex to uPA, thereby creating a long-lived cell surface uPA/uPAR complex, which could impact signal transduction both in *cis* and *trans*. It is interesting that the magnitude of the effect observed in the presence of B-428 is greater in the transfected Mat B-III-uPAR. This observation suggests that these effects are due to down-regulation of the proteolytic cascade resulting in decreased growth factor effects. Finally, a novel protein has been recently identified which regulates uPAR expression at a post-transcriptional level by modulating the half-life of uPAR mRNA (Shetty S et al., 1997). This uPAR mRNA binding protein binds a 51 nucleotide segment within the coding region and it is possible that B-428 has an effect on the expression of this protein which could also explain the ability of B-428 to down-regulate uPAR levels to a similar degree in control (Mat B III) and transfected (Mat B III-uPAR) cells.

The cytotoxic effects of TAM have been reported to result from the ability of TAM to inhibit cyclin-dependent kinases resulting in cell cycle arrest at the G_0 / G_1 checkpoint

(Dickson RB and Lippman ME, 1987). In addition to these ER mediated effects, TAM has also been found to act on several other targets implicated in breast cancer progression including TGF- ß, TGF- α, insulin -like growth factor (IGF-I) and to reduce cell-matrix adhesion (Kalkhoven E et al., 1996; Pratt S and Pollak MN, 1993). The current study however is the first report describing the effects of TAM on the plasminogen activator system and its ability to decrease invasiveness by down-regulating uPAR expression.

Using previously established doses, TAM and B-428 caused a significant decrease in tumor growth of animals inoculated with control and experimental Mat B III cells (Rabbani SA et al., 1995). Tumor progression and metastasis *in vivo* requires neovascularization of the tumor. Previous studies have implicated uPA/uPAR expression on endothelial cells with angiogenesis (Min HY et al., 1996). Therefore, *in vivo* administration of B-428 could have an effect not only on the invasiveness of tumor cells but also on endothelial cell dependent capillary tube morphogenesis. In addition, breast cancer is known to be highly vascularized and numerous recent studies have attempted to correlate the degree of neovascualrization with prognosis (Gasparini G, 1995). Therefore, one potential mechanism of action for TAM and B-428 could be the inhibition of angiogenesis as a result of uPAR down-regulation (as well as a direct effect on the uPA proteolytic activity). The fact that metastasis is decreased by B-428 in addition to proliferation is consistent with blocking angiogenesis. In our model system, TAM which only decreases uPAR expression, acts as an anti-proliferative and anti-invasive agent resulting in a marked decrease in primary tumor growth and local invasion. In contrast to this, the addition of B-428, which has dual actions of decreasing uPAR expression and anti-catalytic activity of uPA leads to both anti-invasive and anti-metastatic characteristics. Overall, these studies point to the potential use of combination therapies to inhibit tumor angiogenesis, primary

tumor growth, local invasion and metastases to the secondary sites (Avery RL et al., 1990). Further studies are in progress to elucidate the mechanism of B-428 in regulating uPAR expression and the role of this expression in angiogenesis.

In summary, these results not only provide support for the notion that inhibiting plasminogen activator-mediated cellular invasiveness is an effective therapeutic intervention, but also demonstrate the effectiveness of a combination regimen aimed at targeting different steps of breast cancer progression. With recent advances demonstrating the need for combination therapy for malignancies and viral diseases, a strategy that adds B-428 and other anti-uPA/uPAR compounds will lead to the development of novel therapeutic regimens that approach the ultimate goal of total suppression of mammary carcinogenesis and progression.

3.3.7. Acknowledgements

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We thank Ms. Julienne Gladu and Penelope Harakidas for their assistance during these studies.

CHAPTER 4

REGULATION OF UROKINASE PRODUCTION BY ANDROGENS IN HUMAN PROSTATE CANCER CELLS: EFFECT ON TUMOR GROWTH AND METASTASES *IN VIVO*.

4.1. SUMMARY

While uPA and uPAR, as discussed in Chapters 2 and 3, play important roles in promoting the progression of hormone dependent malignancies, the mechanisms which lead to their overexpression in these malignancies are poorly understood. In spite of the well documented regulation of uPA by growth factors and cytokines, the role of sex steroids in the regulation of uPA gene expression during the progression of hormone dependent malignancies like breast and prostate cancer is not well characterized.

Results of studies aimed at investigating the regulation of uPA production by androgens in human prostate cancer cells and the resultant effect on tumor growth and metastases *in vitro* and *in vivo* are presented in this chapter in the form of a manuscript submitted to Journal of Clinical Investigation for publication. I was responsible for all of the experimental work described in this chapter. PC-3T cell line was a kind gift from Dr. A. Keating (Toronto, Canada).

4.2. ORIGINAL PAPER

4.2.1. Abstract

During the complex multi-step process of tumor progression, prostate cancer is initiated as an androgen-sensitive, non-metastatic cancer, followed by a gradual transition into a highly metastatic and androgen-insensitive variety which lacks the expression of functional androgen receptors. Urokinase (uPA), a member of the serine protease family has been implicated in progression of various human malignancies including prostate cancer. Elevated levels of uPA are associated with a poor prognosis and short overall disease-free survival. Although uPA production is regulated by various growth factors and cytokines, the role of sex steroids (androgens) in regulating uPA gene expression in

prostate cancer is poorly understood. In the current study, we have examined the regulation of uPA production by androgens in the androgen insensitive PC-3 cells transfected with the full length human androgen receptor cDNA (PC-3T). The effect of androgens on uPA production was monitored, and tumor cell invasiveness was evaluated *in vitro* and *in vivo*. Restoration of androgen responsiveness in PC-3T cells caused a marked decrease in cell doubling time. Treatment of PC-3T cells with dihydroxytestosterone (DHT) caused a dose-dependent decrease in uPA mRNA and protein production. Nuclear run-off assays revealed that these effects were due to the ability of DHT to inhibit uPA gene transcription. Down regulation of uPA production by androgen decreased the ability of PC-3T cells to invade through the Matrigel. Androgen receptor antagonist flutamide (Flu) reversed the effect of DHT on proliferation and invasion of PC-3T cells. Both control (PC-3) and experimental (PC-3T) cells were injected into the right flank of male BALB/c nu/nu mice. Control animals developed palpable tumors and microscopic tumor metastases at lymph nodes, lungs and liver at 6 week post tumor cell inoculation. In contrast to this, due to androgen sensitivity of PC-3T cells, palpable tumors were observed only at week 12, with occasional tumor metastases in lungs. Furthermore, inoculation of PC-3T cells into surgically castrated host animals resulted in the development of tumors at a much earlier time (week 10) and a high incidence of metastases as compared to regular animals receiving PC-3T cells. Collectively, these results demonstrate the ability of androgen to regulate uPA production which may directly effect prostate cancer growth, invasion and metastasis *in vitro* and *in vivo*.

4.2.2. Introduction

Adenocarcinoma of the prostate is a common hormone dependent malignancy resulting in a high incidence of cancer related morbidity and mortality (Wilson JMG, 1987; Chiarodo A, 1991). Sex steroid androgens play key roles in the growth and differentiation of normal prostatic tissues to promote the initiation of malignant transformation and the progression of prostate cancer (Sandberg AA, 1980; Griffiths K, 1987). These effects of androgens are mediated via androgen receptors (AR), expressed in both the stromal and epithelial compartments of the prostate (Masai M et al., 1990; Sadi MV and Barrack ER, 1993; Ruizeveld-de-Winter JA et al., 1994). During the complex multistep process of tumor progression, prostate cancer is initiated as a low virulent and androgen sensitive variety, which gradually transforms into a highly metastatic and hormone insensitive variety due to the outgrowth of androgen receptor negative cells resulting in the establishment of hormone resistance (Scott WW et al., 1980; Chiarodo A, 1991). This hormone insensitivity is closely associated with a lack or mutation of the androgen receptor (Veldsholte J et al., 1990; Veldsholte J et al., 1992; Kaighn ME et al., 1979). Due to the close relationship between hormonal status and prostate cancer progression, treatment of early stage prostate cancer consists of strategies aimed at eliminating the sources of circulating androgens via medical or surgical castration, or administration of anti-androgens (Trachtenberg J, 1987). However, continued use of these therapeutic strategies in treating late stage prostate cancer results in limited beneficial effects which may be attributed to the loss of functional androgen receptors in affected tumor cells. Additionally, various growth factors, hormones and proteases are also implicated in prostate cancer progression.

The role of growth factors, steroids and proteases in the acquisition of hormonal independence and the underlying molecular mechanism involved in this process remain

poorly understood. The androgen insensitive human prostate cancer cell line PC-3 which lacks a functional androgen receptor has been used extensively as a model for androgen independent prostate cancer (Kaighn ME et al., 1979).

Urokinase (uPA), a member of the serine protease family, is strongly implicated to promote tumor progression in several malignancies including breast and prostate cancer (Janicke F et al., 1991; Achbarou A et al., 1994; Rabbani et al, 1995). These effects are due to the ability of uPA to breakdown various components of the extracellular matrix (ECM) including laminin, fibronectin and collagen (Dano K et al., 1985; Vassali JD and Pepper MS, 1994). Although uPA is produced by normal and benign hyperplastic prostatic tissue, elevated levels of uPA are observed in patients with prostate cancer (Kirchheimer J et al., 1997). In previous studies, we have demonstrated that overexpression of uPA by the rat prostate cancer cell line Dunning R3227 Mat Ly Lu results in increased tumor invasion and metastases in both skeletal and non skeletal sites (Achbarou A et al., 1994). These effects of uPA could be blocked by treating tumor bearing animals with an active site inhibitor of uPA which resulted in decreases in both tumor volume and tumor metastases (Rabbani SA et al., 1995b). Although the expression of human uPA gene has been shown to be under the regulation of various growth factors and cytokines (Riccio A et al., 1985; Blasi F, 1988; Roghani M et al., 1996; Ossowski L et al., 1979; Mira-Lopez R et al., 1983; Liu DF and Rabbani SA, 1995), the role of sex steroids (estrogens and androgens) in regulating uPA production in hormone dependent malignancies like breast and prostate cancer is poorly understood.

In the current study, we have examined the regulation of uPA production by androgens in the androgen insensitive human prostate cancer cell line PC-3, which lacks a functional androgen receptor, and in androgen sensitive PC-3 cells, transfected with the full

length human androgen receptor cDNA (PC-3T). The effect of androgens on uPA production, tumor cell growth, invasion and metastasis was evaluated both *in vitro* and *in vivo*.

4.2.3. Materials and Methods

Cell Lines, and Reagents

The human prostate cancer cell line PC-3 (Kaighn ME et al., 1979) was obtained from American Type Culture Collection (Rockeville, MD). PC-3 cells transfected with a functional full length human androgen receptor cDNA (PC-3T) was kindly provided by Dr. T. J. Brown (The Toronto Hospital Research Institute, Toronto). PC-3 cells were maintained in F-12 (Gibco BRL, Gaitherburg, MD), and the transfected PC-3T cells were maintained in RPMI 1640 (Gibco BRL, Gaitherburg, MD) supplemented with 100 μg / ml hygromycin B (Sigma Chemicals, St. Louis MO). All culture media were supplemented with 10% fetal bovine serum (Gibco BRL), 25 mM 4-(2-hydroxyethy)-1piperazineethanesulfonic acid, 26 mM sodium bicarbonate, 5000 units/ml penicillin G (Gibco BRL) and 5000 mg/ml streptomycin (Gibco BRL). Cells were incubated at 37°C in 5% CO_2. Stripped fetal bovine serum (Gibco BRL), which is depleted of steroids, was used during androgen treatment. PC-3T cells were treated with different concentrations of androgen DHT and the androgen receptor antagonist flutamide (Sigma Chemicals, St. Louise, MO).

Stable Transfection of PC-3 and PC-3T Cells with Green Fluorescent Protein (GFP)

The expression vector containing the codon optimized hGFP-S65T gene was obtained from Clontech Laboratories, Inc. (Palo Alto, CA). PC-3 and PC-3T cells were transfected with hGFP-S65T using lipofectin reagent (Gibco BRL). Cells with stably integrated plasmids were selected for neomycin resistant gene with G418 (Xing, RH and Rabbani, SA, 1996).

Cell Proliferation

Cell growth was determined by cell proliferation assays. 10,000 PC-3 and PC-3T cells were plated in 2 ml of medium in 6-well tissue culture plates. Where indicated, PC-3T cells were treated with either 10 nM of DHT alone or with 10 nM DHT and 10 nM flutamide. Cell culture medium was replenished every third day. At 4 hr and at the indicated time point, cells were trypsinized and counted using a Coulter Counter (model ZF, Coulter Electronics, Harpenden, herts, U. K.). After 4 hr of incubation, the number of cells in each well was determined to establish that equal number of cells were present in all wells.

Northern Blot Analysis

Total cellular RNA was isolated from control and experimental PC-3 and PC-3T cells as previously described (Xing, RH and Rabbani, SA, 1996). Briefly, 20 μg of total cellular RNA was electrophoresed on a 1.1% agarose-formaldehyde gel and transferred to a nylon membrane (Nytran) by capillary blotting. Filters containing RNA of Mat B III cells were hybridized with a ^{32}P-labelled human uPA cDNA or with 18S cDNA as a control for

the amount of RNA loaded (Achbarou A et al., 1994; Xing RH and Rabbani SA, 1996). All filters were incubated at 42°C for 24 h and successively washed in 1 x SSC (10 x SSC is 1.5 M NaCl, 0.5 M sodium citrate, pH 7.0), 1% sodium dodecyl sulfate (SDS) for 15 min at room temperature, 0.5 x SSC, 0.5% SDS for 15 min at room temperature, 0.1 x SSC, 0.1% SDS twice for 15 min at room temperature, and then once for 30 min at 55°C. Autoradiography of filters was carried out at -70°C using XAR film (Eastern Kodak Co., Rochester, NY) with two intensifying screens. The level of uPA mRNA expression was quantified by densitometric scanning.

Indirect Immunofluorescence

To examine the cell surface expression of uPA by PC-3 and control and experimental PC-3T cells, 5 x 10^4 cells were plated in Lab-Tek tissue culture chambers (Nunc Inc., Naperville, IL) and allowed to grow to 70-80% confluent. Cells were then incubated with 30% goat serum (Sigma) for 1 hour at room temperature and washed with PBS containing 1% BSA. Cells were subsequently incubated with 100 μg/ml of rabbit anti-rat uPA IgG (American Diagnostica Inc. Greenwich, CT) and with goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) (Sigma). Photographs were taken at 60 X magnification using a Zeiss MC-63 microscope (Liu DF and Rabbani SA, 1995).

Nuclear Run-off Assay of Gene Transcription

Nuclear run-off assays were performed by harvesting cells after treatment with various agents in cold PBS. Cells were collected and lysed in cold NP-40 lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40) for 5 min on ice. Cell nuclei were collected by centrifugation at 4°C, and re-suspended in storage buffer (50 mM

TrisHCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 5 mM dithiothreitol). The nuclei were either used immediately or frozen in liquid nitrogen for later use. Nuclear run-off assays were carried out by adding 100 µl nuclear suspension (2-4 x 10^7 nuclei) to 100 µl reaction buffer (50 mM Tris-HCl, pH 7.5, 0.3 M KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 0.5 mM each of ATP, CTP, and GTP, 50-100 µCi [^{32}P]α-uridine triphosphate, >600 Ci/mmol, ICN, Costa Mesa, CA) for 60 min at room temperature. After the incubation, DNase I (150 U per reaction) and proteinase K (0.2 mg/ml) were added and incubated for 30 min at 37°C respectively (Liu B et al., 1993). Newly synthesized RNAs were isolated by spin column and ethanol precipitation, and pelleted by centrifugation. RNAs were hybridized with uPA and 18S cDNAs and with Bluescript vector DNA (Stratagene, La Jolla, CA) previously immobilized on Nytran membranes using a slot blot manifold (Bio-Rad, Richmond, CA). These membranes were incubated in the hybridization solution (6 x SSC, pH 7.4, 50% formamide, 1% SDS, 0.1 mg/ml sonicated salmon sperm DNA) at 42 °C for 48 h. After hybridization, membranes were washed in a final wash solution of 0.1 x SSC, 0.1% SDS at 42°C, and exposed to Kodak XAR film with intensifying screens. The intensity of each band was quantitated using laser densitometry.

Boyden Chamber Matrigel Invasion Assay

The invasive capacities of PC-3 and PC-3T cells were determined by two compartment Boyden chambers (Transwell, Costar, USA) and basement membrane Matrigel (Becton Dikinson Labware) invasion assay as previously described (Xing RH and Rabbani SA, 1996; Liu DF and Rabbani SA, 1995). In some experiments, PC-3T cells were cultured in stripped FBS in the presence or absence of DHT (10 nM), or with DHT

and androgen receptor antagonist flutamide (10 nM), or with rat anti-uPA antibody (100 μg/ml).

Animal Protocols

6-week-old BALB/c nu/nu male normal and castrated mice were obtained from Charles River, Inc. (St.Constant, Quebec). Before inoculation, GPF labelled PC-3 and PC-3T tumor cells (PC-3-GFP and PC-3T-GFP) grown in serum containing medium were washed with Hank's buffer and trypsinized for 5 min. Cells were then collected in Hank's buffer and centrifuged at 1500 rpm for 5 min. Cell pellets (3×10^6 cells) were re-suspended in 200 μl of a matrigel and saline mixture (20% matrigel) and injected subcutaneously into the flank region of the hind leg of the mice. All animals were numbered, kept separately and examined for the development of tumors weekly. The tumor mass was measured in two dimensions by callipers and the tumor volume calculated. Animals were sacrificed at timed intervals and were examined and scored for the development of macroscopic metastases in various tissues.

Statistical Analysis

Results are expressed as the mean ± SEM of at least triplicate determinations and statistical comparisons are based on the Student's t test or analysis of variance. A probability value of < 0.05 was considered to be significant.

4.2.4. Figures

Figure. 4.1: Effect of androgen on PC-3 cell growth

Growth curves were compared between the wild type PC-3 and PC-3 cells transfected with the full length human androgen receptor (PC-3T). The rate of PC-3T cell doubling time was also compared following treatment of PC-3T with DHT (PC-3T+DHT) alone or in combination with androgen receptor antagonist flutamide (PC-3T+DHT+Flu). Cells from triplicate dishes were tripsinized at each time point and counted as described in "Materials and Methods". Each point represents ± SEM of three experiments. Significant difference in the growth of PC-3T cells from the control is represented by asterisks * ($P<0.05$).

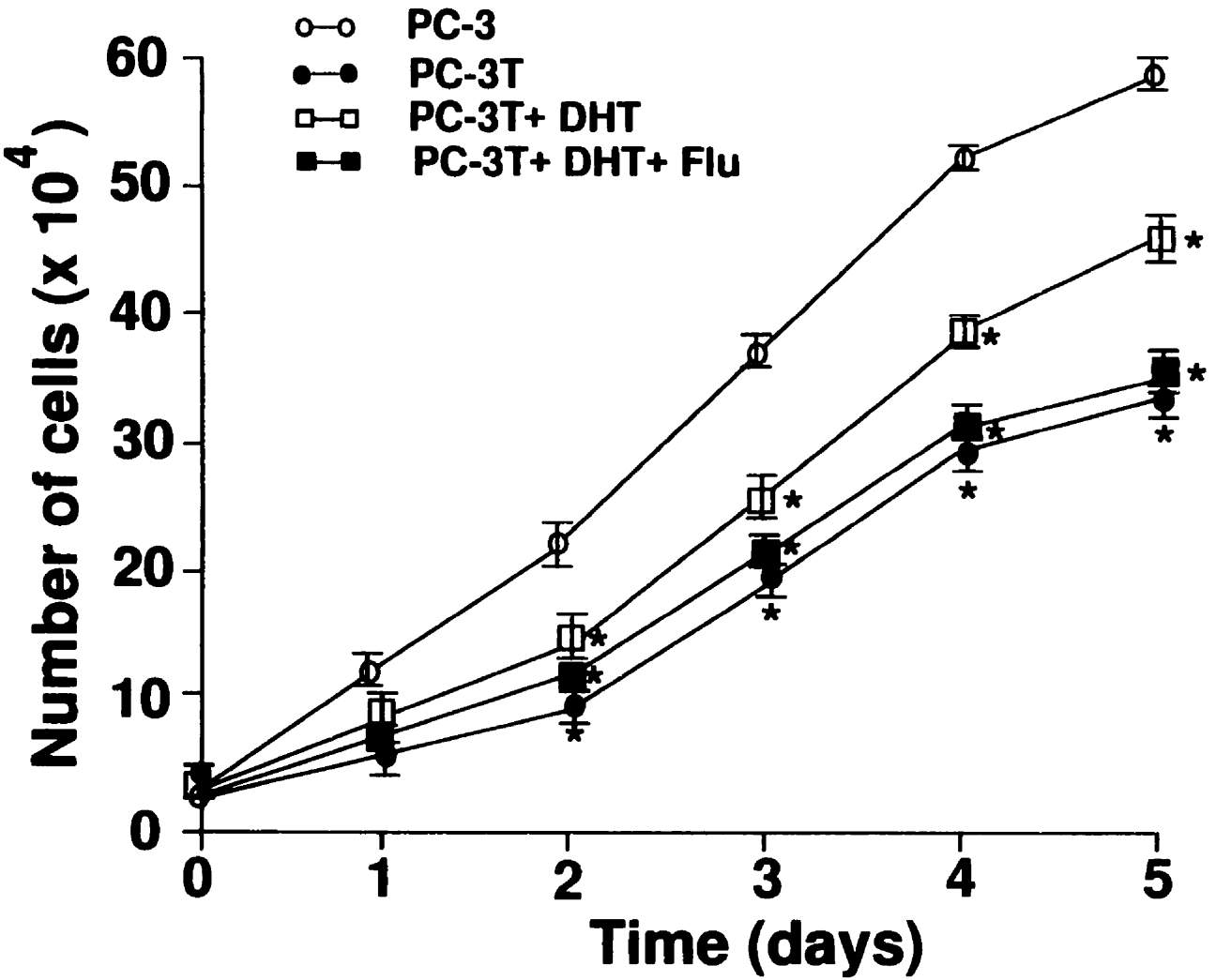
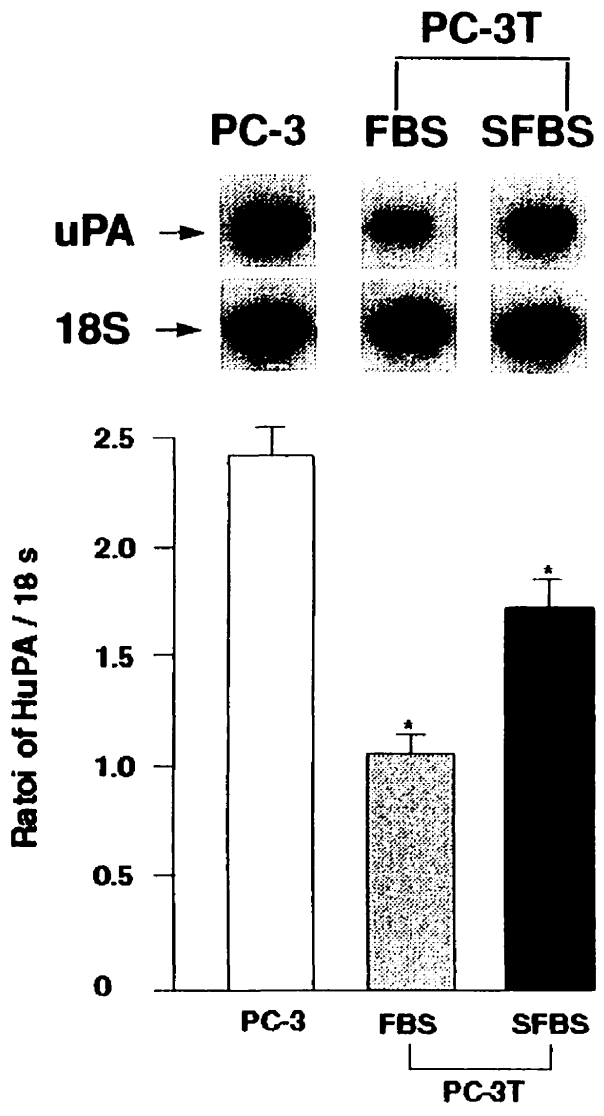

PC-3
PC-3T
PC-3T+ DHT
PC-3T+ DHT+ Flu
Number of cells (x 10^4)
60
50
40
30
20
10
0
0
1
2
3
4
5
Time (days)
*

Figure. 4.2: Effect of restoration of hormone responsiveness on uPA production

Panel A: Total cellular RNA was extracted from PC-3 and PC-3T cells cultured in medium containing 10% FBS (FBS) and in culture medium supplemented with charcol stripped FBS (SFBS). 20μg of total cellular RNA from each group of cells were electrophoresed on a 1.1% agarose/formaldehyde gel and blotted to a nylon membrane by capillary action. All blots were hybridized with a ^{32}P-labelled human uPA cDNA, or with a ^{32}P-labelled 18S cDNA as described in "Materials and Methods". Blots were scanned by laser densitometeric scanning and change in uPA mRNA expression was determined by plotting the ratio of uPA and 18S mRNA. Results are representative of at least 4 different experiments. Significant difference from control cells is represented by asterisks * ($P<0.05$).

Panel B: uPA protein production was determined in PC-3 cells and PC-3T cells maintained in culture medium containing FBS (PC-3T-FBS) and in charcol stripped FBS (PC-3T-SFBS) by indirect immunofluoresence under 60X magnification as described in "Materials and Methods".

A


PC-3T
PC-3
FBS
SFBS
uPA
18S
Ratoi of HuPA / 18 s
2.5
2.0
1.5
1.0
0.5
0
PC-3
FBS
SFBS
PC-3T

B

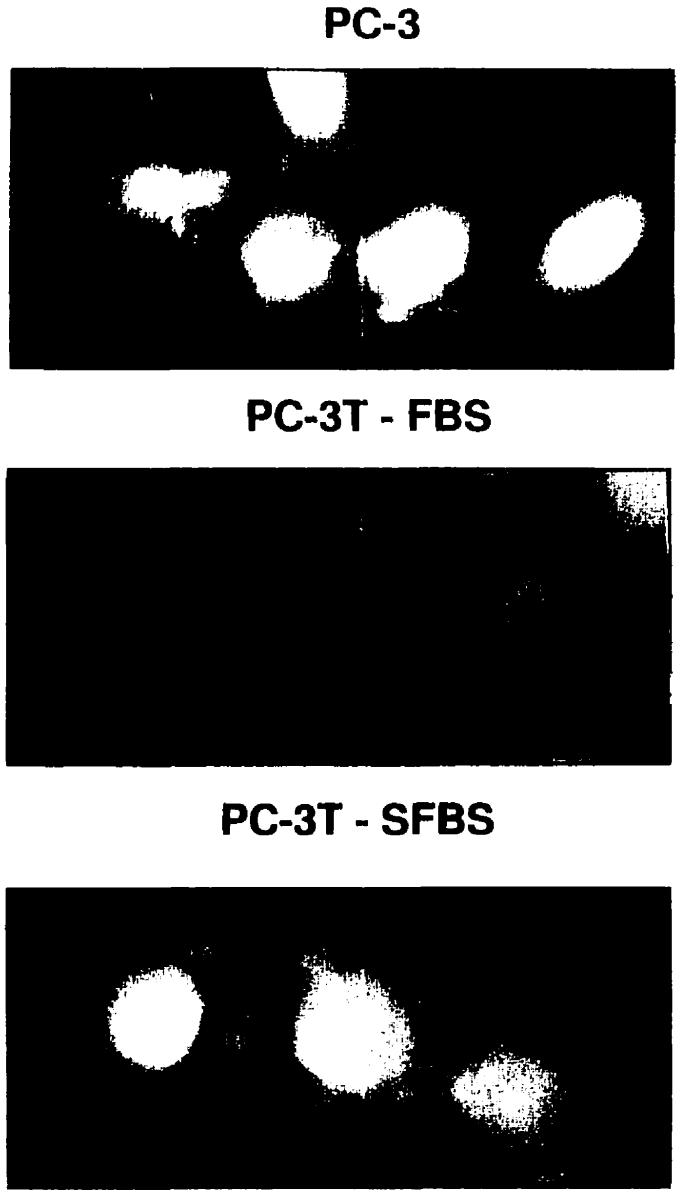

PC-3
PC-3T - FBS
PC-3T - SFBS

Figure. 4.3: Effect of androgen on uPA production in PC-3T cells

Panel A: Total cellular RNA was extracted from vehicle-treated PC-3T cells (CTL) and PC-3T cells treated with different concentrations of DHT (DHT). 20μg of total cellular RNA from each group were electrophoresed on a 1.1% agarose/formaldehyde gel and blotted to a nylon membrane by capillary action. All blots were hybridized with a ^{32}P-labelled human uPA cDNA or with a ^{32}P-labelled 18S cDNA as described in "Materials and Methods". Blots were scanned by laser densitometric scanning and changes in uPA mRNA expression were determined by plotting the ratio of uPA and 18S mRNA. Results are representative of at least 4 different experiments. Significant difference from control cells is represented by asterisks * ($P<0.05$).

Panel B: uPA protein production was determined in vehicle-treated PC-3T cells (CTL) and PC-3T cells receiving androgen treatment (DHT) by indirect immunofluoresence under 60X magnification as described in "Materials and Methods".

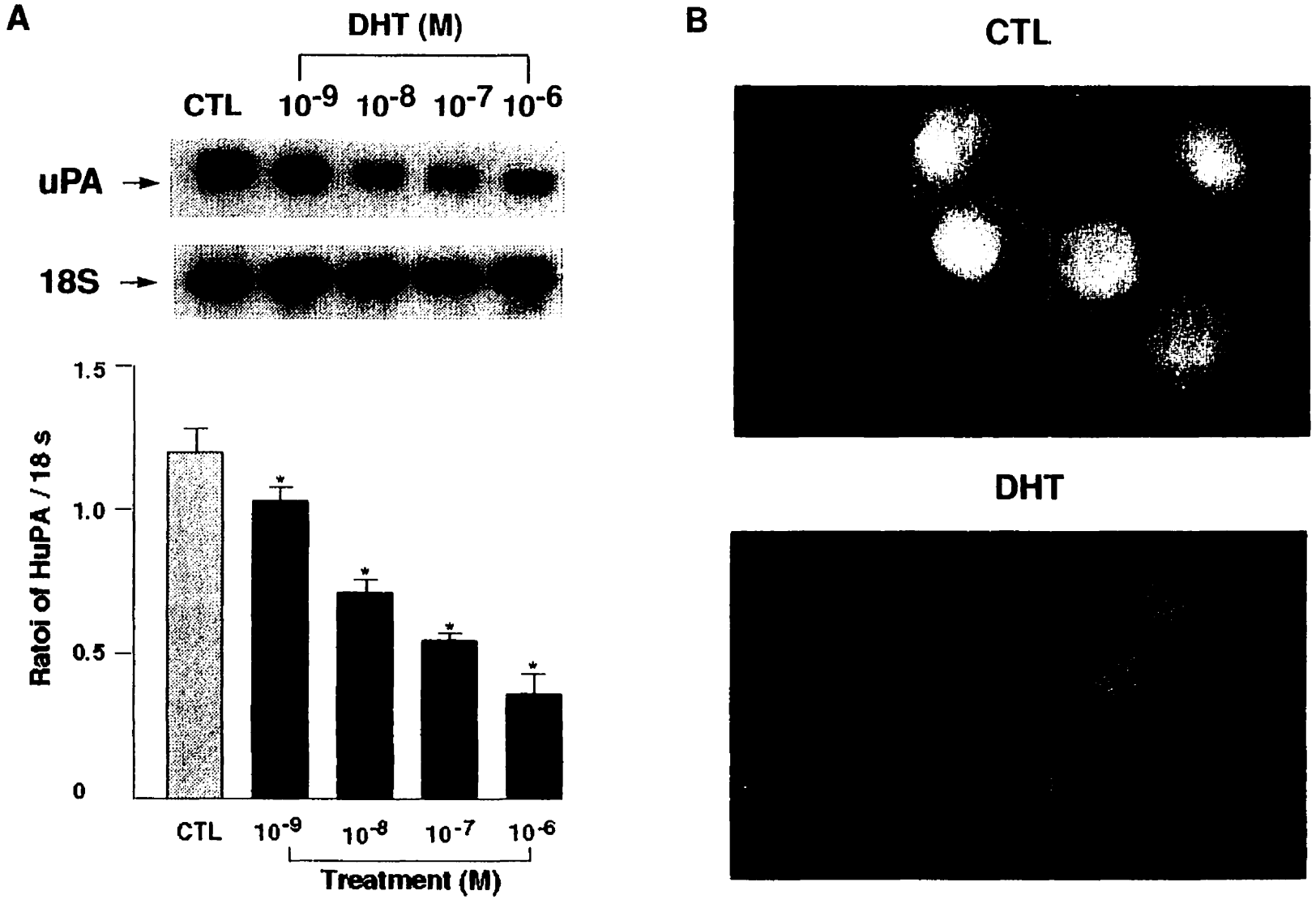
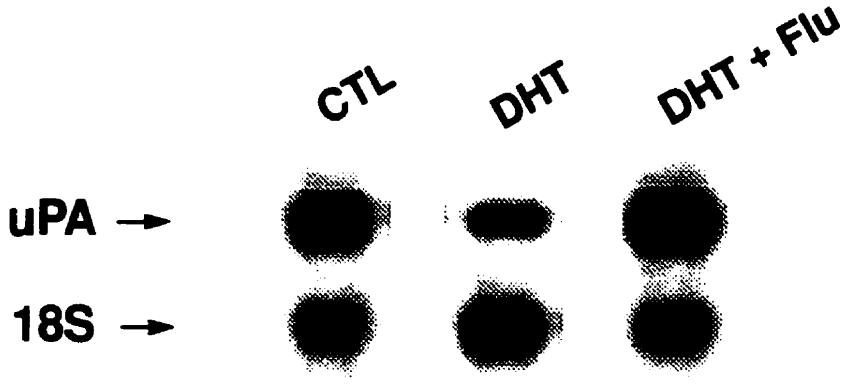

A
DHT (M)
CTL
10-9
10-8
10-7
10-6
uPA
18S
Ratoi of HuPA / 18 s
1.5
1.0
0.5
0
Treatment (M)
B
CTL
DHT

Figure. 4.4: Role of androgen receptor in mediating the inhibitory effect of androgen on uPA mRNA expression

The role of the androgen receptor in mediating the inhibitory effect of androgen on uPA mRNA expression was determined by Northern blot analysis. Total cellular RNA was extracted from vehicle-treated PC-3T cells (CTL), PC-3T cells treated with 10 nM of DHT (DHT), and PC-3T cells treated with both 10^{-8} M DHT and 10^{-8} M flutamide (DHT+Flu). 20μg of total cellular RNA from each group were electrophoresed on a 1.1% agarose/formaldehyde gel and blotted to a nylon membrane by capillary action. All blots were hybridized with a ^{32}P-labelled human uPA cDNA or with a ^{32}P-labelled 18S cDNA as described in "Materials and Methods". Blots were scanned by laser densitometric scanning and the changes in uPA mRNA expression was determined by plotting the ratio of uPA and 18S mRNA. Results are representative of at least 4 different experiments. Significant difference from control cells is represented by asterisks * ($P<0.05$).


CTL
DHT
DHT + Flu
uPA
18S

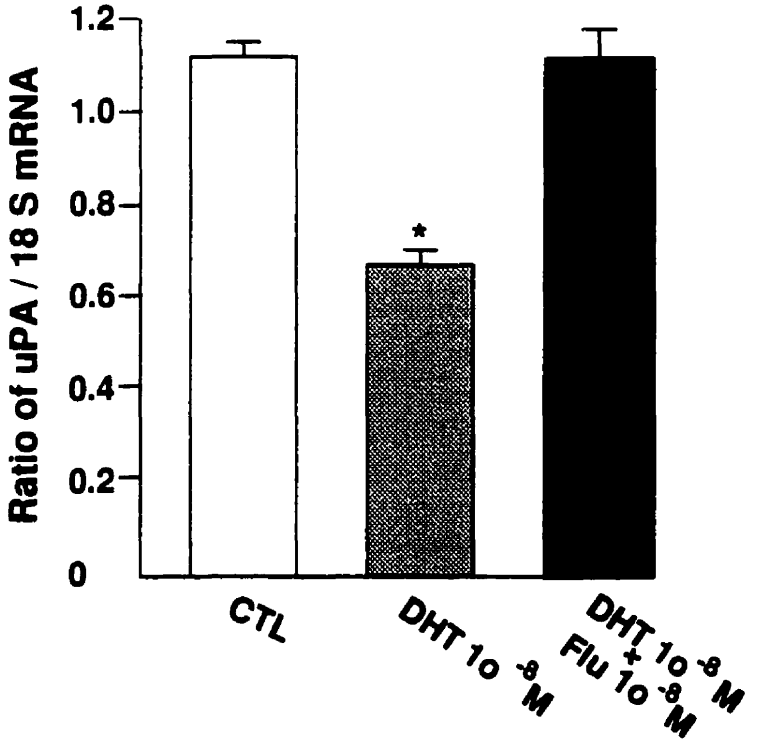
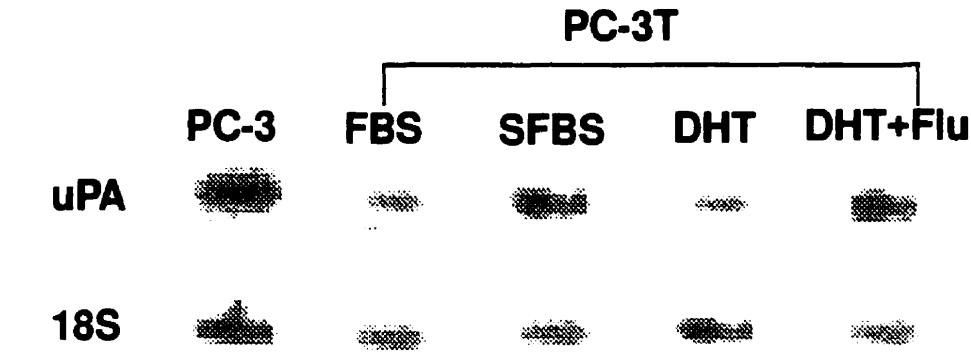

Ratio of uPA / 18 S mRNA
1.2
1.0
0.8
0.6
0.4
0.2
0
*
CTL
DHT 10 -8 M
DHT 10 -8 M + Flu 10 -8 M

Figure. 4.5: Effect of androgen on uPA gene transcription

Nuclear run-off assays were performed as described in "Materials and Methods". 32P-labelled run-off transcripts were prepared from PC-3 cells and PC-3T cells following treatment with 10% FBS (FBS), stripped FBS (SFBS), 10 nM DHT alone (DHT) or 10 nM DHT and 10 nM flutamide (DHT+Flu). All blots were probed with human uPA and 18S cDNA, scanned by laser densitometric scanning and fold stimulation of uPA gene transcription relative to that of 18S was determined. Results are representative of three different experiments. Significant difference from control cells is represented by asterisks * ($P<0.05$).


PC-3T
PC-3
FBS
SFBS
DHT
DHT+Flu
uPA
18S

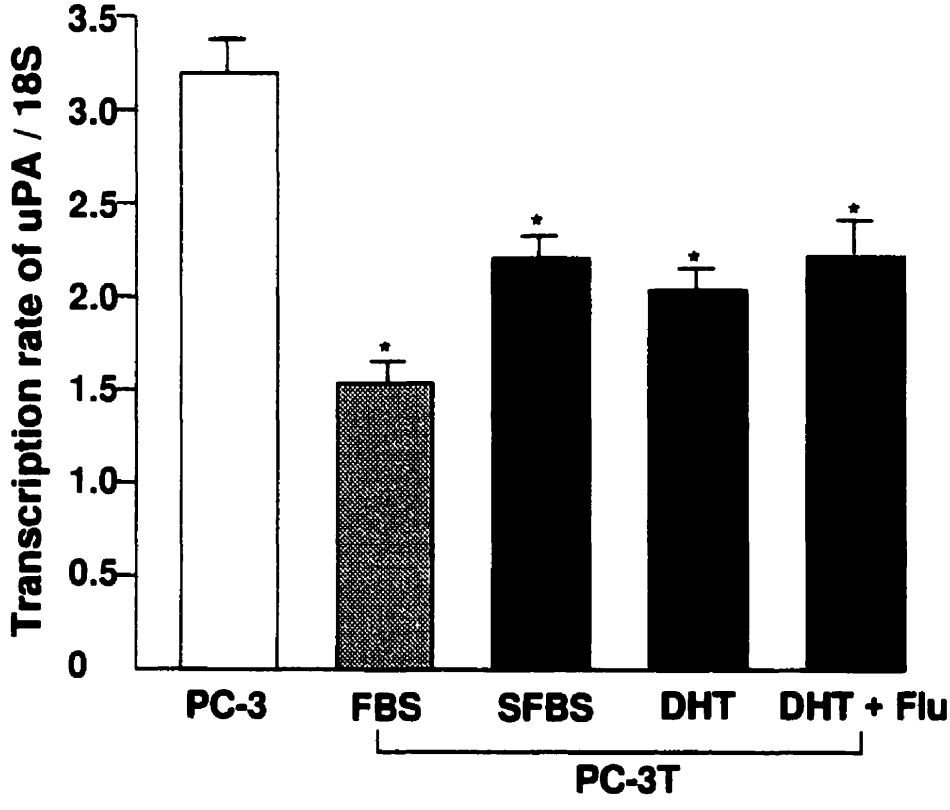

Transcription rate of uPA / 18S
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
PC-3
FBS
SFBS
DHT
DHT + Flu
PC-3T

Figure. 4.6: Effect of androgen on PC-3 and PC-3T cell invasion

Both PC-3 and PC-3T cells were grown in culture as described in "Materials and Methods". Number of PC-3T cells migrating to the lower aspect of the Boyden chamber filter after treatment with stripped FBS (SFBS), or with 10 nM of DHT (DHT), or with 10 nM DHT, 10 nM flutamide (DHT+Flu) and 50 μg/ml of anti-uPA antibody (SFBS+uPA IgG) were counted and compared with vehicle-treated controls (FBS). Results represent ± SEM of four different experiments. Significant difference in cell invasion from the controls is represented by asterisks * ($P<0.05$).

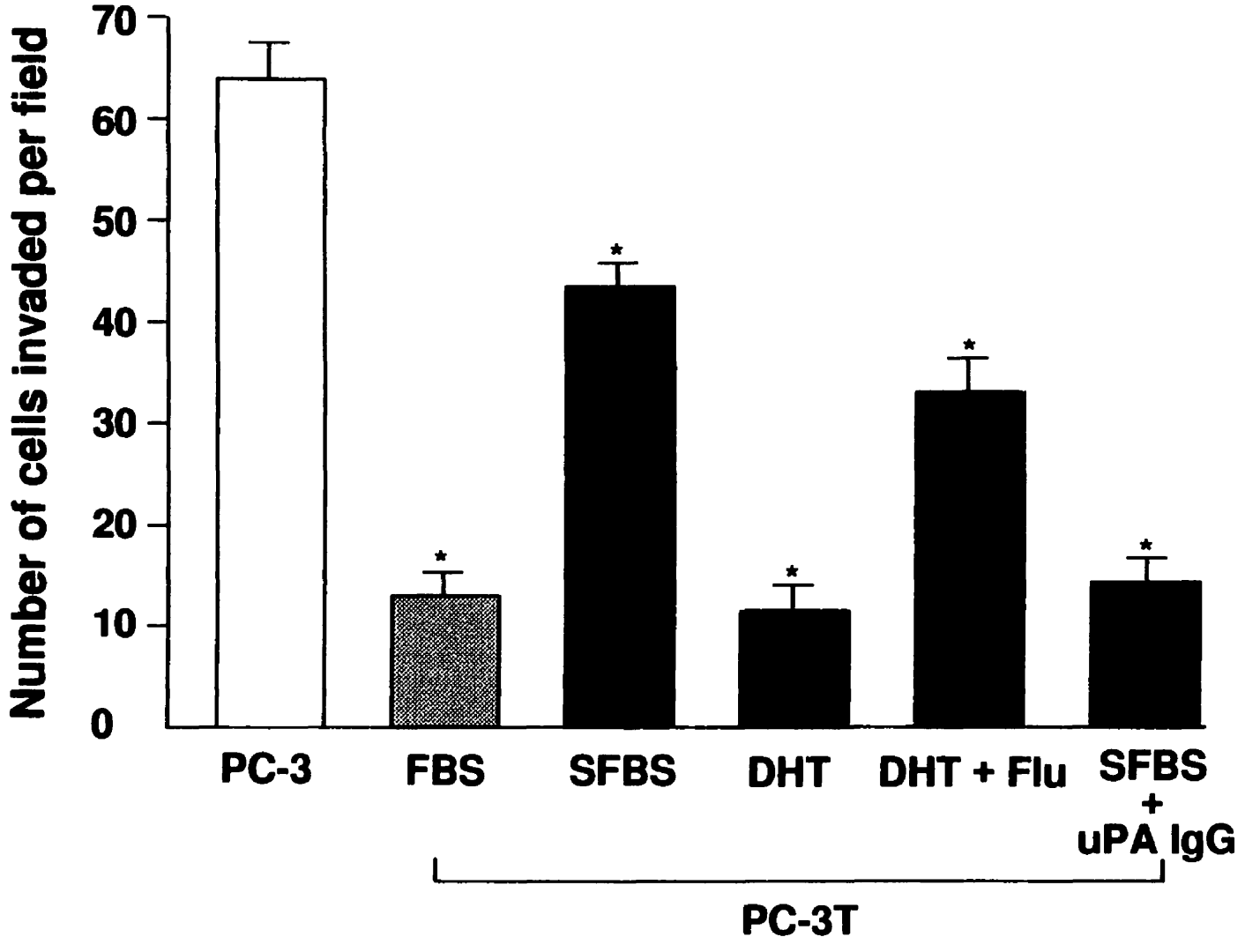

Number of cells invaded per field
70
60
50
40
30
20
10
0
PC-3
FBS
SFBS
DHT
DHT + Flu
SFBS + uPA IgG
PC-3T

Figure. 4.7: Effect of restoration of androgen sensitivity on PC-3 tumor growth

Tumor volumes of non-castrated BALB/c nu/nu male nude mice receiving PC-3 or PC-3T cells, and of castrated animals inoculated with PC-3T cells were determined at timed intervals as described in "Materials and Methods." Results represent the mean ± SEM of 6 starting animals in each group in 3 different experiments. Significant difference from control PC-3 tumor bearing animals is marked by asterisks * ($P<0.05$).

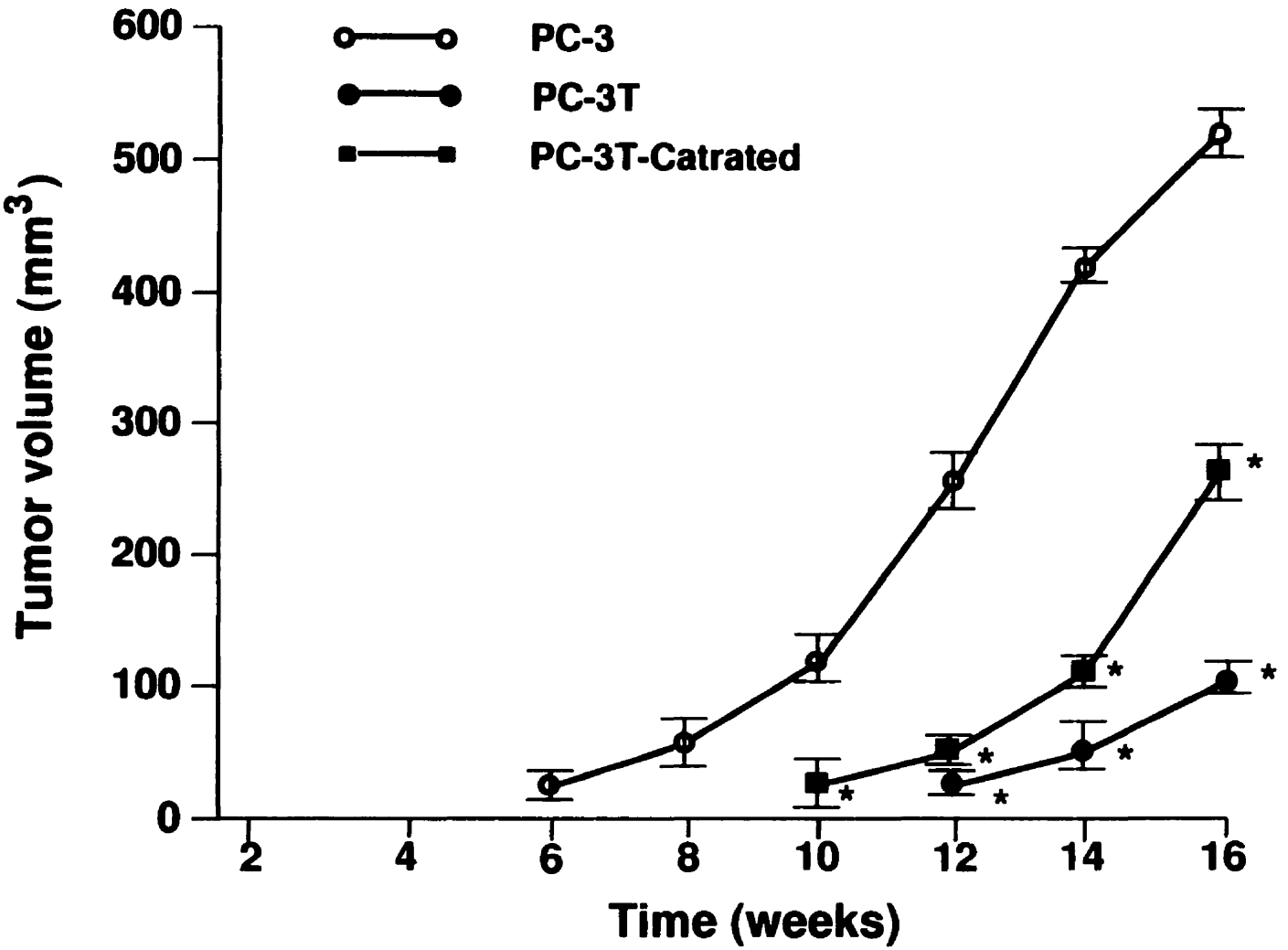

PC-3
PC-3T
PC-3T-Catrated
Tumor volume (mm3)
600
500
400
300
200
100
0
2
4
6
8
10
12
14
16
Time (weeks)
*

Table. 4. 1: Effect of restoration of androgen sensitivity on PC-3 tumor metastases

Male BALB/c nu/nu nude mice were inoculated with either hormone insensitive PC-3-GFP cells or with hormone sensitive PC-3T-GFP cells as described in "Materials and Methods". In order to evaluate the effect of androgen on PC-3T tumor progression and metastasis, castration procedure was performed on a group of animals before receiving PC-3T cells. At the time of development of palpable tumors (week 6, 10, 12), animals were sacrificed and examined for the presence of tumor metastases by counting the number of green colonies in the lungs, livers and axillary lymph nodes under the microscope as described in "Materials and Methods". Results represent the mean ± SEM of 6 starting animals in each group in 3 different experiments.

EFFECT OF RESTORATION OF HORMONE SENSITIVITY AND ANDROGEN ON PC-3 TUMOR METASTASES

	PC-3	PC-3T	PC-3T-Castrated
Presence of palpable tumor	Week 6	Week 12	Week 10
Auxiliary LN	3±1	–	–
Liver	4±2	–	2±1
Lungs	7±2	2±1	3±1

4.2.5. Results

Effect of Androgen on Prostate Cancer Cell Growth *In Vitro*

The effect of restoration of androgen responsiveness was examined by a cell proliferation assay in PC-3T cells and comparison was made with cell doubling time of wild type PC-3 cells over five days. PC-3T cells exhibited a significantly reduced (40-50%) cell doubling time throughout the course of this study (Figure.1). Following culture of PC-3T cells in a culture medium containing charcoal stripped FBS to remove androgens, treatment of PC-3T cells with 10 nM dihydroxytestosterone (DHT) showed a marked increase in PC-3T cell growth. The specificity of androgen receptor (AR) in mediating this growth stimulating effect of androgen was confirmed by coincubation of PC-3T cells with DHT and 10 nM of AR antagonist flutamide (Flu), which completely abolished the increase in PC-3T cell growth seen after DHT treatment (Figure.1).

Effect of Androgen on uPA Production

In order to examine the effect of restoration of androgen responsiveness of PC-3 cells on uPA mRNA expression, Northern blot analysis was performed on PC-3 and PC-3T cells cultured in the presence of 10% FBS or charcoal stripped FBS to remove androgens. Restoration of androgen responsiveness resulted in a marked decrease in uPA mRNA expression in PC-3T cells as compared with wide type PC-3 cells. This inhibition in uPA mRNA expression could be reversed in PC-3T cells following removal of androgens from FBS with charcoal treatment (Figure.2A). A similar change in uPA protein production was seen in PC-3T cells as determined by immunohistochemistry (Figure.2B). These effects of induction of uPA expression in PC-3T cells by stripped

serum treatment were time dependent, with a maximal induction of uPA following 24h of androgen removal (data not shown).

The specificity of the effect of DHT on uPA production was further evaluated by treating PC-3T cells with different concentrations of DHT following 24h of androgen withdrawal. DHT inhibited uPA mRNA expression in a dose dependent manner (1nM-1μM DHT) [Figure.3A]. A similar decrease in uPA protein production was seen in DHT treated PC-3T cells (Figure.3B). In order to determine the role of AR in mediating these effects, PC-3T cells were treated with either DHT alone, or in combination with the androgen receptor antagonist flutamide. Due to the ability of flutamide to compete with androgens for AR binding, coincubation of PC-3T cells with DHT and flutamide resulted in a complete restoration of uPA mRNA expression as determined by Northern blot analysis (Figure.4).

Effect of Androgen on uPA Gene Transcription

In order to explore the underlying molecular mechanisms involved in the regulation of uPA gene expression by androgen in PC-3T cells, the effect of DHT on uPA gene transcription was examined by nuclear run-off assays. Comparison of uPA gene transcription in wild type PC-3 and PC-3T cells showed a marked reduction in uPA gene transcription in PC-3T cells. This reduction in uPA gene transcription could be restored following stripped FBS (SFBS) treatment (Figure.5). Treatment of PC-3T cells grown in SFBS with 10 nM DHT showed a marked decrease in uPA gene transcription, and such a reduction was effectively prevented by coincubation of PC-3T cells with DHT (10 nM) and androgen receptor antagonist flutamide (10nM) [Figure.5]. Collectively, these results

demonstrate that DHT can decrease uPA gene transcription, and these actions of DHT are mediated via the AR.

Effect of Androgen on PC-3T Cell Invasion

The effect of restoration of hormone sensitivity on the invasive capacity of PC-3 cells was determined by Boyden Chamber Matrigel invasion assays. Androgen sensitive PC-3T cells exhibited a significantly lower invasive capacity through Matrigel as compared with wild type PC-3 cells which are hormone insensitive (Figure.6). However, the number of PC-3T invading through the Matrigel was high when these cells were incubated in the presence of SFBS. In contrast to this, following DHT treatment, a marked decrease in PC-3T cell invasion was observed. The inhibitory effect of androgen on PC-3T cell invasion could be blocked by addition of androgen receptor antagonist flutamide or anti human uPA IgG (Figure.6). Collectively these results demonstrated that the inhibitory effect of androgen on PC-3T cell invasion was due to its ability to inhibit uPA production, and these actions of androgen are mediated by the androgen receptor.

Results from these *in vitro* studies clearly demonstrated that restoration of androgen responsiveness rendered PC-3 cells in a less virulent phenotype, which was characterized by lower rate for proliferation, decreased uPA production, and the resultant decreased capability of tumor cells to invade through the Matrigel.

Effect of Androgen on Tumor Growth, Invasion, and Metastasis *In Vivo*

Following *in vitro* characterization of the effect of restoration of androgen responsiveness of PC-3T cells on cell growth, invasion and uPA production, we compared the growth and metastatic characteristics of PC-3 and PC-3T cells *in vivo*. Subcutaneous

inoculation of the wild type PC-3 cells into the right flank of 6-week-old male BALB/c nu/nu mice resulted in the development of palpable tumors by 6 week post tumor cell inoculation (Figure.7). In contrast to this, development of primary tumors was significantly delayed when the animals were inoculated with PC-3T cells, where tumors could be palpated only at 12 weeks following tumor cell inoculation. Once developed, both PC-3 and PC-3T tumors showed a linear growth rate up to week 16 (Figure.7). In order to evaluate the effect of androgen ablation on PC-3T tumor development, PC-3T cell were inoculated into castrated animals. Castrated nude mice developed palpable tumors by 10 week post tumor cell inoculation which was at least two weeks earlier as compared to non-castrated PC-3T tumor bearing animals. Although the tumor volume of castrated animals was significantly smaller compared with PC-3 tumors, it was markedly larger than that of non-castrated animals (Figure.7).

In order to assess the effect of restoration of androgen sensitivity on PC-3 and PC-3T tumor metastases, we used tumor cells which had previously been transfected with the plasmid containing GFP protein. These green fluorescent cells showed similar growth characteristics *in vitro* and *in vivo* as compared to the wild type PC-3 and PC-3T cells (data not shown). However, the presence of green cells allowed us to easily visualize and characterize metastatic tumor foci in the distant organs of host animals. At the time of development of palpable PC-3 tumors (week 6), tumor metastases were seen in the lymph nodes, livers and lungs. In contrast to this, at the time of PC-3T tumor development (week 12), no evident tumor metastases were detected in the lymph nodes and livers, only a few small metastatic foci were seen in the lungs of these animals. However, when PC-3T cells were inoculated into castrated hosts, tumor metastases were observed in the livers and lungs by week 10 (Table 1). Removal of the primary tumors and lung metastases followed

by Northern blot analysis showed significantly lower levels of uPA mRNA expression in animals inoculated with PC-3T cells compared with animals bearing PC-3 tumors. Moreover, tumoral level of uPA mRNA was significantly higher in castrated PC-3T tumor-bearing animals compared with that of non-castrated controls (data not shown).

These results collectively, for the first time, demonstrated that restoration of hormone responsiveness in PC-3 cells resulted in a decrease in tumor growth, invasion and metastases *in vivo*. These results also demonstrated that this change in growth and metastasis of tumor cells following induction of their androgen responsiveness is directly due to uPA production, which is required for tumor growth and metastasis.

4.2.6. Discussion

Patients with prostate cancer initially respond to hormone therapies including androgen ablation and anti-androgen treatment. However, due to a slow transition into androgen independent tumor, currently available hormonal and chemotherapeutic strategies have limited success in controlling prostate cancer progression (Crawford ED et al., 1989; Yagoda A and Petrylak D, 1993). In the present study, we have investigated the mechanisms of high invasiveness and poor responsiveness to anti-cancer therapies of hormone insensitive prostate cancer cells. For these studies, we have utilized androgen insensitive human prostate cancer cell line PC-3 and compared its growth, invasion and metastatic behavior to PC-3 cells transfected with a functional androgen receptor (PC-3T) [Yan S et al., 1993]. Restoration of PC-3T cells into a hormone sensitive state significantly reduced the cell doubling time and their ability to invade through the Matrigel. This change in the phenotype of PC-3T cells was due to regulation of a key protease uPA involved in

the invasion and metastasis of several human malignancies including prostate cancer. Treatment of PC-3T cells with DHT decreased uPA mRNA and protein production.

Since higher levels of uPA production are observed in prostate cancer (Achbarou A et al., 1994; Rabbani SA et al., 1995; Kircheimer J et al., 1997), the inhibitory effect of androgens on uPA production may explain the non-invasive and low virulent phenotypes of tumor cells at the initial hormone sensitive state of prostate cancer. This may also provide a mechanism for a role of androgens in the acquisition of a more malignant phenotype during hormone treatment to promote the growth of hormone insensitive tumor cells. Decreased availability of androgens to tumor cells in the presence of competing anti-androgens may also promote a subset of hormone responsive prostate cancer cells to produce higher levels of uPA, which in turn endows tumor cells with a higher invasive and metastatic potential. On the other hand, increased uPA production may have a direct effect on promoting tumor progression via its stimulating effect on angiogenesis, cell adhesion and migration (Liotta LA et al., 1991; Stahl A and Mueller BM, 1994). It is not yet known whether the effect of androgen on uPA gene expression is direct, however, the presence of a putative androgen receptor responsive element (ARE) within uPA promoter region (Blasi F, 1988) strongly suggests that AR can directly down regulate the expression of uPA mRNA in hormone sensitive PC-3T cells.

When tested *in vivo*, athymic nude mice inoculated with hormone responsive PC-3T cells developed significantly smaller tumors and exhibited a later onset of tumor development as compared with animals receiving wild type hormone insensitive PC-3 cells. Moreover, PC-3T-tumor bearing animals developed very limited micro-metastases only at their livers as compared with the development of extensive macroscopic and microscopic tumor metastases in the livers, lungs and auxiliary lymph nodes of animals

inoculated with PC-3 cells. Detection of micro-metastases was made possible by using GFP-labelled PC-3 and PC-3T cells (Morin J and Hastings J, 1972). Previous *in vivo* studies by Chishima et al. (Chishima T et al., 1997) have demonstrated the effectiveness, simplicity and sensitivity of the GFP gene as a marker to visualize micro-metastases in fresh viable target organs such as the livers, lungs and draining and regional lymph nodes at the single cell level. In order to examine the effect of uPA induction by androgen ablation on tumor growth, invasion and metastases, PC-3T cells were implanted into the castrated nude mice. The early onset of PC-3T tumor development in castrated host further underscored the role of androgens in tumor development. Despite castration, tumor development was still delayed as compared to wild type animals receiving PC-3T cells. Decreased production of uPA in primary tumors and metastatic sites strongly points to the role of uPA in altering the growth and metastatic ability of tumor cells *in vivo*. Recent clinical studies have shown that in castrated human males, intra-prostatic DHT concentrations range as high as 20-50% of that measured before castration (Labrie F et al., 1993), illustrating the significant contribution of extragonadal sources of androgen and implicating a similar source of androgens in castrated animals. Results from our studies demonstrate that the extragonadal sources of androgen are sufficient to provide the required amount of androgen for the initiation and progression of prostate cancer, a finding with significant clinical implications. It points to the importance of complete deprivation of any androgenic source to prevent prostate tumor growth. This can be achieved by performing both surgical and medical castrations. Medical castration can be achieved by drug induced suppression of the hypothalamic-pituitary-testicular axis by administrating of GnRH analogs. If complete androgen ablation is not satisfied, the residual amount of androgens could not only stimulate tumor growth, but also promote a subset of hormone sensitive

tumor cells to acquire a more malignant phenotype via increased production of tumor progression factors like uPA.

4.2.7. Acknoledgements

This work was supported by Medical Research Council of Canada grants MT 12609 and MT 10603 to SAR.

CHAPTER 5

TRANSCRIPTIONAL REGULATION OF UROKINASE (uPA) GENE EXPRESSION IN HORMONE-DEPENDENT MALIGNANCIES: ROLE OF DNA METHYLATION.

5.1. SUMMARY

The study presented in Chapter 4 provided important experimental evidence for an inhibitory role of sex steroids (estrogens and androgens) in the regulation of uPA gene expression in human breast and prostate cancer. It also demonstrated a strong correlation between hormone responsiveness, uPA expression and cell invasiveness in hormone dependent malignancies prostate cancer. However, the mechanisms conferring the hormonal regulation of uPA production are poorly understood.

Results of studies aimed at exploring the molecular mechanisms underlying hormonal regulation of uPA gene expression in breast and prostate cancer, and evaluating the potential role of DNA methylation in regulating uPA production at different stages of breast and prostate cancer are presented in this chapter in the form of an original paper submitted to *Cancer Research* for publication. I was responsible for all of the experimental work described in this chapter.

This study clearly demonstrated for the first time that the uPA gene is differentially expressed in HR and HI breast and prostate cancer cells and that the production of uPA is correlated with hormone-responsiveness of these tumor cells. More significantly, it provided compelling evidence for DNA methylation as an unique molecular mechanism for the transcriptional regulation of uPA gene expression to allow this critical protease to be turned-on and turned-off at different stages of these common hormone-dependent malignancies.

5.2. 5-AZACYTIDINE: A DNA METHYLTRANSFERASE INHIBITOR

The nucleoside 5-azacytidine (5-AzaC) was first synthesized in 1964 (Piskala A and Sorm F, 1964) as a potent demethylating agent. It differs from cytidine in having a

nitrogen in place of carbon in the 5 position of the pyrimidine ring. 5-AzaC is incorporated into the DNA following its phosphorylation and forms an irreversible covalent bond with the DNA methyltransferase (DNA MeTase) enzyme and carbon 6 in the cytosine moiety, resulting in trapping of the enzyme onto DNA and reduction of active DNA MeTase levels in the nucleus (Wu JC and Santi DV, 1985;). However, 5-AzaC is also a nucleoside analogue and a differentiating agent. Therefore, when interpreting the data involving 5-AzaC as the DNA MeTase inhibitor, it is essential to address the cytotoxic effect and alterations in the differentiation state of the cell.

5.3. ORIGINAL PAPER

5.3.1. Abstract

Carcinoma of the breast and prostate are the two leading hormone dependent malignancies, resulting in a high rate of morbidity and mortality. During the complex multi-step process of tumor progression, these malignancies are initiated as a hormone-responsive (HR) non-metastatic cancer, followed by a gradual transition into a highly metastatic hormone-insensitive (HI) variety which lacks the functional estrogen and androgen receptors. The transition of these cancers into an estrogen / androgen insensitive variety causes them to become refractory to hormonal treatments.

Urokinase (uPA), a member of the serine protease family has been implicated in progression of various human malignancies including breast and prostate cancer. In the current study, we have examined the correlation between hormone sensitivity and uPA expression in several (HR) and (HI) breast and prostate cancer cell lines. uPA mRNA expression was seen only in the highly invasive, HI breast (MDA-231) and prostate (PC-3) cell lines. Failure of uPA expression in the minimally invasive, HR breast (MCF-7) and

prostate (Ln-CAP) cells was due to transcriptional suppression of uPA gene as determined by nuclear run-off assays. Since alteration of DNA methylation status of CpG islands in the 5' sequence of oncogenes and tumor suppressor genes can change their expression, we examined DNA methylation as a potential molecular mechanism regulating uPA gene transcription in these cancer cells. Southern blot analysis using methylation sensitive enzymes revealed that CpG islands of uPA gene are hypermethylated in HR, MCF-7 and Ln-CAP cells whereas they are hypomethylated in HI, MDA-231 and PC-3 cells. Treatment of MCF-7 cells with cytosine DNA methyltransferase inhibitor 5' azacytidine caused a dose-dependent induction of uPA mRNA due to demethylation of the CpG islands of uPA gene which led to increased invasive ability of these HR cancer cells. These results demonstrate that DNA methylation can regulate the transcription of uPA gene to alter the invasive behaviour of these HR breast and prostate cancer cells.

5.3.2. Introduction

Adenocarcinoma of the breast and prostate are two common hormone dependent malignancies where female and male hormones estrogen and androgen play a critical role in cancer initiation and progression (Hennderson BE et al., 1988). This hormonal interaction is crucial in tumor development since these malignancies are initiated as a hormone sensitive, low virulence type which in their later stages become hormone resistant due to the lack of functional estrogen and androgen receptors (Russo J and Russo HI, 1995; Chiarodo A, 1991). This gradual transition is closely related to these malignancies becoming refractory to any hormonal treatment in their late stages. In cases of breast cancer, estrogen receptor positive HR tumors grow slowly, respond to hormone treatment with anti-estrogen and result in prolongation of survival (McGuire WL, 1978). However,

these patients often eventually become unresponsive to hormone therapy due to the development of hormone resistance. This HI stage is generally associated with a mutation in either the estrogen receptor (ER) or interference in the ER signalling pathway (Zhang QX et al., 1993; Kitzenellenbogen BS, 1991). The role of growth factors, steroids and proteases in this complex transition and the underlying molecular mechanism remain poorly understood. In previous studies, we and others have shown that increased uPA expression is closely related to tumor invasion and metastasis of several malignancies including breast and prostate cancer (Achbarou A et al., 1994; Janicke F et al., 1991). These characteristics are due to the ability of uPA to break down various components of the extracellular matrix (ECM) including laminin, fibronectin and collagen which allow tumor cells to extravasate and form tumor metastases at distant sites (Dano K et al., 1985; Vassali JD and Pepper MS, 1994). The human uPA gene, located on chromosome 10, spans 7.25 Kb to encode a 2.5 Kb mRNA whose expression is regulated by growth factors and steroids (Riccio A et al., 1985; Blasi F, 1988; Roghani M et al., 1996; Ossowski L et al., 1979; Mira-Lopez R et al., 1983). uPA mRNA is expressed abundantly in breast and prostate cancers as compared to normal mammary tissues, benign breast adenomas and benign prostatic hyperplasia (Xing RH et al., 1997; Duffy MJ et al., 1996; Rabbani SA et al., 1995b; Xing RH and Rabbani SA, 1996). However, the underlying molecular mechanism controlling uPA gene expression in these hormone dependent malignancies is poorly understood. One possible mechanism is DNA methylation of the effected gene to cause epigenetic changes in the chromatin structure without altering the DNA sequence (Bird AP, 1986). This DNA methylation takes place at CpG islands located in the 5' region of the DNA sequence. Recent data has shown that many tumor suppressor genes (Rb, p16, p15, VHL) (Ohtani-Fujita N et al., 1993; Merlo A et al., 1995; Herman JG et al.,

1996; Herman JG et al., 1994), as well as genes engaged in tumor progression such as E-cadherin (Yoshiura K et al., 1995), ER (Ottaviano YL et al., 1994), endothelin receptor (Nelson et al., 1997), or inhibitors of angiogenic factors (Ahuja N et al., 1997) are hypermethylated and inactivated in various human malignancies including breast and prostate cancer. In contrast, hypomethylation of CpG islands is responsible for overexpression of various oncogenes including *ras* and *myc* to promote tumor progression (Feinberg AP et al., 1983a; Christman JK et al., 1993; AP and Vogelstein B, 1983).

In the current study, we have examined uPA gene expression in human breast and prostate cancer cell lines maintained in culture to establish a correlation between uPA expression and hormone responsiveness in these cancer cell lines. For these studies, we have used HR breast (MCF-7) and prostate (Ln-CAP), and HI breast (MDA-231) and prostate (PC-3) cell lines. These HR cell lines also represent early stages of malignancy, while HI cell lines which have a higher invasive potential represent a later and highly malignant tumor phenotype. By functional analysis, we have also evaluated the methylation of CpG islands of uPA gene as the underlying molecular mechanism controlling uPA production at various stages of these common cancers.

5.3.3. Materials & Methods

Cell Lines and Reagents

All cell lines were obtained from American Type Culture Collection (Rockville, MD). Cells were maintained in MEM (MCF-7), F-15 (MDA-231), RPMI-1620 (Ln-CAP) and F-12 (PC-3) culture mediums (Gibco BRL, Gaitherburg, MD). All culture media were supplemented with 10% fetal bovine serum (Gibco BRL), 25 mM 4-(2-hydroxyethy)-1-piperazineethanesulfonic acid, 26 mM sodium bicarbonate, 5000 units/ml

penicillin G (Gibco BRL) and 5000 mg/ml streptomycin (Gibco BRL). Cells were incubated at 37°C in 5% CO_2. MCF-7 and Ln-CAP cells were treated with 5'-azacytidine (Aza-C) (Sigma Chemicals, St. Louis MO) at different concentrations (10-25 μM) for 10 days, followed by extraction of genomic DNA or cellular RNA of treated and untreated cells (Herman JG et al., 1996; Ottaviano YL et al., 1994).

Northern Blot Analysis and Nuclear run-off Assay

Total cellular RNA was isolated from different breast and prostate cancer cell lines by acid guanidium thiocyanate-phenol-chloroform extraction. Filters containing different cellular RNA were hybridized with a ^{32}P-labelled human uPA or an 18S cDNA as a control for the amount of RNA loaded at 42°C for 24 h (Xing RH and Rabbani SA, 1996). The level of mRNA of various genes of interest was quantitated by densitometric scanning.

Nuclear run-off assays were performed as previously described (Xing RH et al., 1997). Briefly, the nuclei were extracted from different cell lines and nuclear run-off assays were carried out by adding 100 μl nuclear suspension (2-4 x 10^7 nuclei) to 100 μl reaction buffer containing 50-100 μCi [^{32}P]a-uridine triphosphate for 60 min at room temperature. Newly synthesized RNAs were isolated by spin column and ethanol precipitation. RNAs were hybridized with uPA and 18S cDNAs and with Bluescript vector DNA (Stratagene, La Jolla, CA) previously immobilized on Nytran membranes using a slot blot manifold (Bio-Rad, Richmond, CA). These membranes were incubated in the hybridization solution (6 x SSC, pH 7.4, 50% formamide, 1% SDS, 0.1 mg/ml sonicated salmon sperm DNA) at 42 °C for 48 h. The rate of uPA gene transcription was quantitated by densitometric scanning (Xing RH et al., 1997).

Matrigel Invasion Assay

The capacity of cell invasiveness was tested by two-compartment Boyden chambers (Transwell, Costar, USA) and basement membrane Matrigel invasion assay (Becton Dikinson Labware, San Jose, CA) (Xing RH and Rabbani SA, 1996). The 8 μm pore polycarbonate filters were coated with basement membrane Matrigel (50 μg/filter) and dried under a tissue culture hood. Matrigel was then reconstructed by adding 0.1 ml serum-free culture medium to the upper chamber and incubated for 90 min. After removal of medium, cells (5×10^4) in 0.1 ml of medium were added to the upper chamber and placed in a lower chamber pre-filled with 1.0 ml of serum-free medium supplemented with 25 μg/ml fibronectin (Sigma), and incubated at 37°C for 24 hr. At the end of incubation, medium was removed, filters were fixed in 2% paraformaldehyde, 0.5% glutaraldehyde in 0.1 M phosphate buffer, pH7.4 at room temperature for 30 min. After washing with PBS, all filters were stained with 1% toluidine blue and cells on the surface of Matrigel were removed with a Q-tip. Filters were mounted onto glass slides and cells were examined under a light microscope. Ten fields under 400x magnification were randomly selected and the mean cell number was calculated. In some experiments, MCF-7 cells were treated with 5'Aza-C (25μm) alone or in combination with human uPA IgG (American Diagnostica Inc., Greenwich, CT., USA).

Southern Blot Analysis

Genomic DNA was isolated using TRISOL method. Briefly, genomic DNA was precipitated from the interphase and organic phase with 100% ethanol, washed in 10% ethanol containing 0.1 M sodium citrate and subsequently washed in 75% ethanol. Cellular DNA was dissolved in 8 mM NaOH to get a final concentration between 0.2-0.3 mg/ml.

The isolated genomic DNA was analyzed for uPA gene expression and methylation status of CpG islands of uPA gene by Southern blotting. Ten μg of genomic DNA isolated from different cells were digested with *EcoR I* or *Hind III* or *Pst I*. *Pst I/Hpa II* or *PstI/HhaI* (8 units/μg of DNA) for 18h at 37°C and were electrophoresed on a 0.8% agrose gel and transferred to a nylon membrane (Nytran, Amersham, Oakville, Canada) by capillary blotting. Filters were then hybridized with a ^{32}P-labelled human uPA cDNA or a 0.79 Kb *SmaI-AvrII* promoter probe (gift from Dr. F. Blasi, Milan, Italy). All filters were incubated at 42°C for 24 h, then successively washed in 1 x SSC (10 x SSC is 1.5 M NaCl, 0.5 M sodium citrate, pH 7.0), 1% sodium dodecyl sulfate (SDS) for 15 min at room temperature, 0.5 x SSC, 0.5% SDS for 15 min at room temperature, 0.1 x SSC, 0.1% SDS twice for 15 min at room temperature, and then once for 30 min at 55°C. Autoradiography of filters was carried out at -70°C using XAR film (Eastern Kodak Co., Rochester, NY) with two intensifying screens.

5.3.4 Figures

Figure. 5.1: Northern blot analysis of breast and prostate cancer cell lines.

Panel A: Twenty micrograms of total cellular RNA was isolated from MCF-7, MDA-231, Ln-CAP and PC-3 cells and electrophoresed on 1.1% agrose-formaldehyde gel and blotted to a nylon membrane. All blots were probed with a ^{32}P-labelled human uPA, estrogen receptor (ER), androgen receptor (AR), and with 18S cDNA, as described in "Materials and Methods" (upper panel).

Panel B: The invasive capacity of these cancer cells was assessed by Boyden Chamber invasion assay as described in "Materials and Methods". + signs denote ten cells per field of examination. Results are representative of at least 3 different experiments.

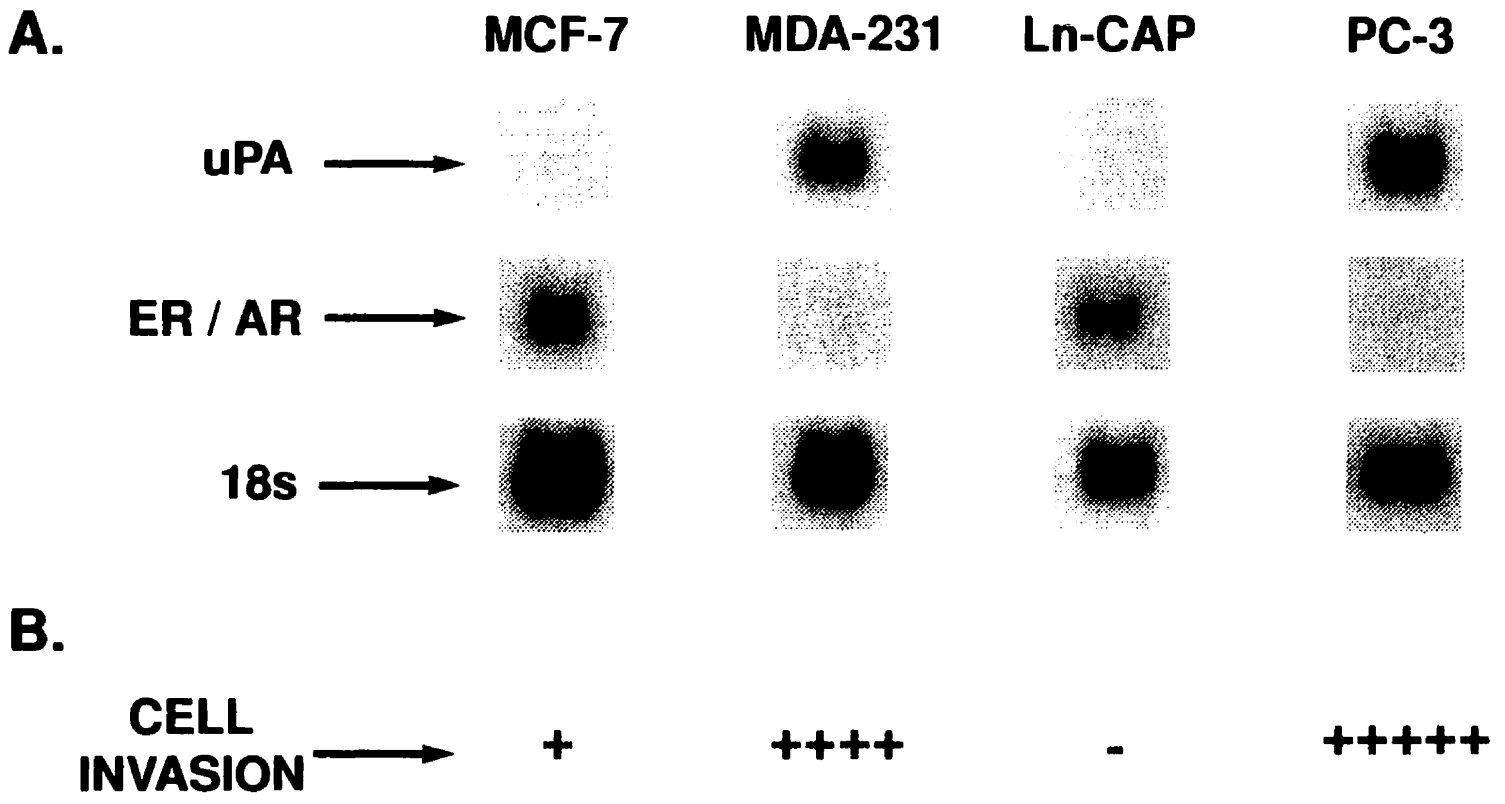
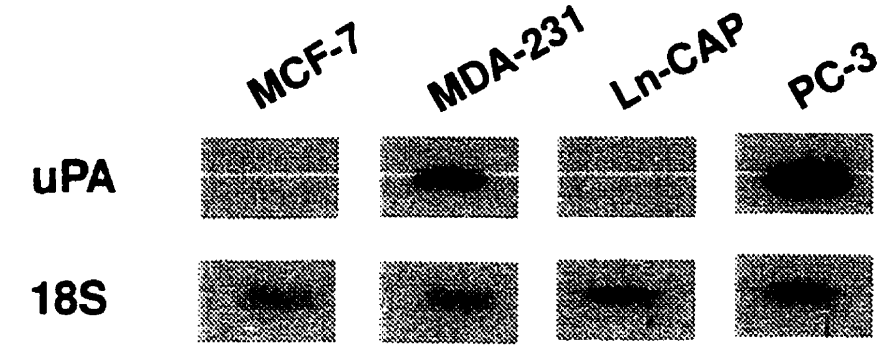

A.
MCF-7
MDA-231
Ln-CAP
PC-3
uPA
ER / AR
18s
B.
CELL INVASION
+
++++
-
+++++

Figure. 5.2: Evaluation of uPA gene transcription.

Nuclear run-off assay was carried out as described in "Materials and Methods". ^{32}P labelled run-off transcripts were prepared from various breast (MCF-7, MDA-231) and prostate (Ln-CAP, PC-3) cancer cells and probed with human uPA and 18S cDNA. All blots were scanned by laser densitometric scanning and fold stimulation of uPA gene transcription relative to that of 18S was determined. Results are representative of three different experiments.


MCF-7
MDA-231
Ln-CAP
PC-3
uPA
18S

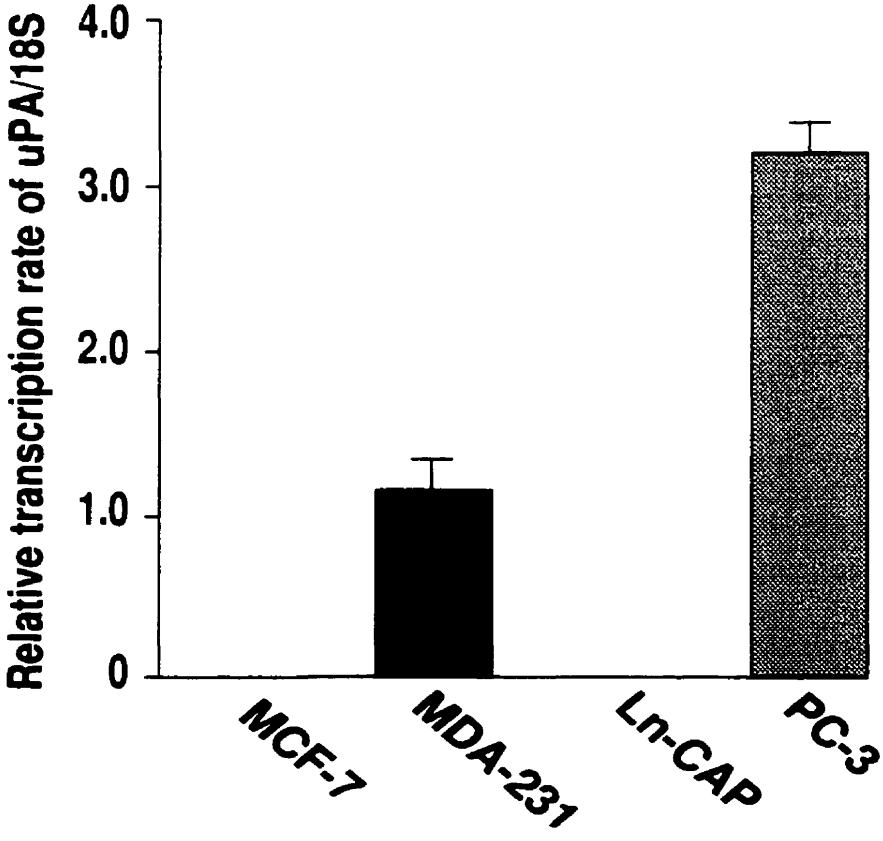

Relative transcription rate of uPA/18S
4.0
3.0
2.0
1.0
0
MCF-7
MDA-231
Ln-CAP
PC-3

Figure. 5.3: Southern blot analysis of genomic DNA.

Genomic DNA was isolated from breast (MCF-7, MDA-231) and prostate (Ln-CAP, PC-3) cells and digested with *EcoRI* or *Hind III*. Restriction products were electrophoresed in 1.1% DNA agrose gel, blotted to nylon membrane and probed with ^{32}P labelled uPA cDNA as described in "Materials and Methods". Results are representative of three different experiments when genomic DNA was digested with *EcoRI*.

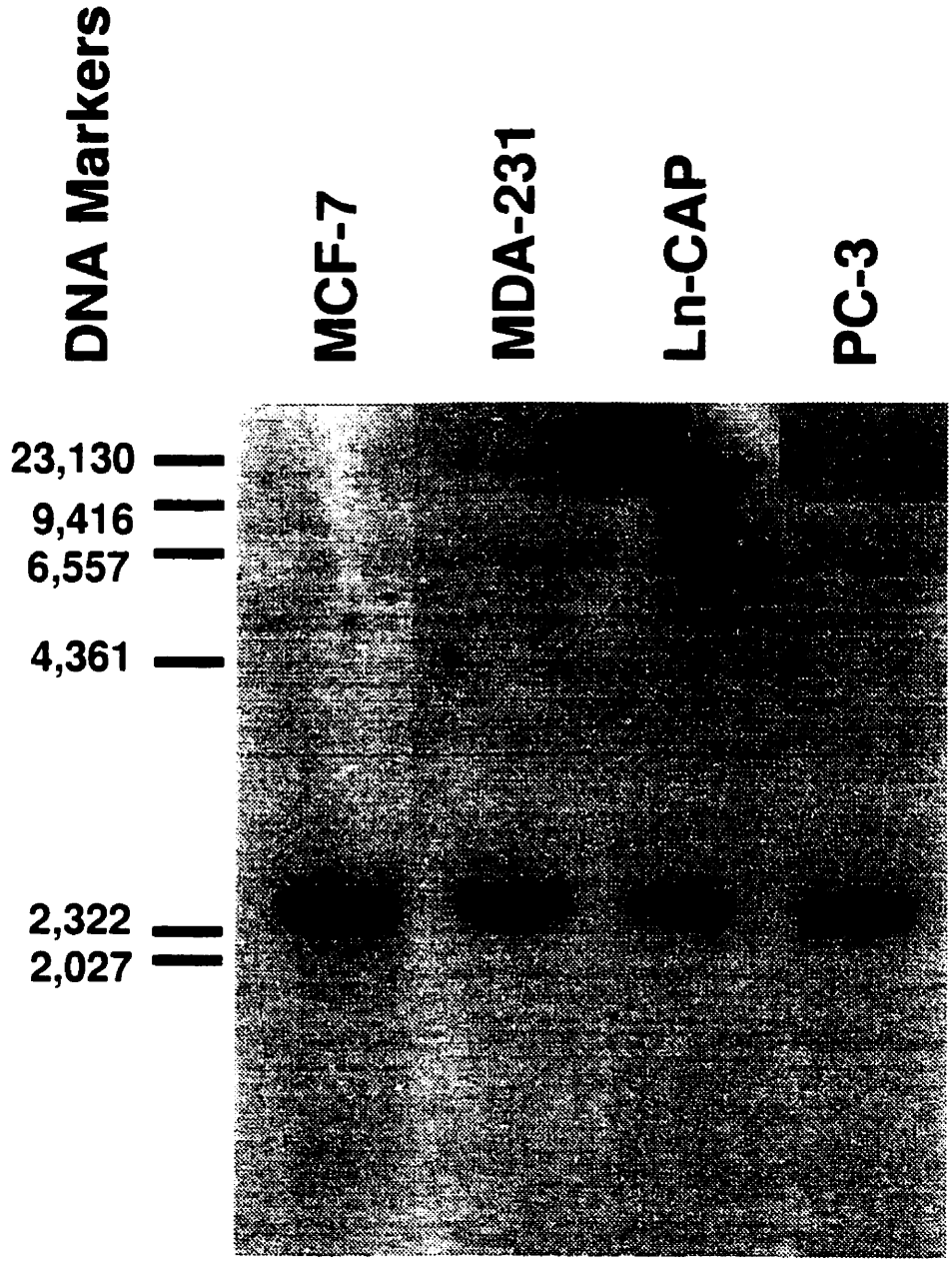
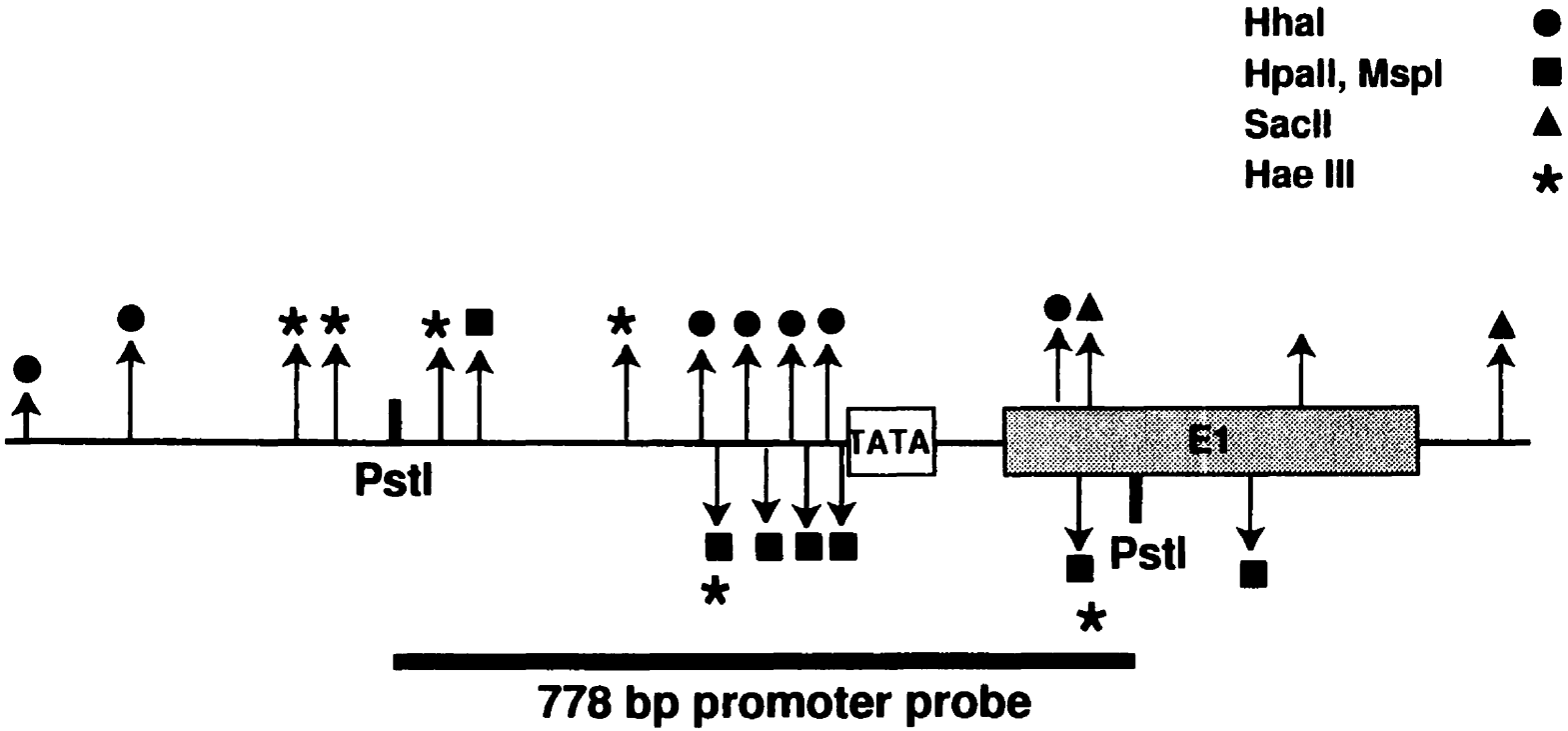

DNA Markers
MCF-7
MDA-231
Ln-CAP
PC-3
23,130
9,416
6,557
4,361
2,322
2,027

Figure. 5.4: Restriction map of the 5' region of human uPA gene.

Exon 1 (E1) and 5' region outlining various promoters (CAAT,TATA), and potential restriction sites for various enzymes involved in DNA methylation is indicated.

METHYLATION SENSITIVE SITES ON THE uPA GENE



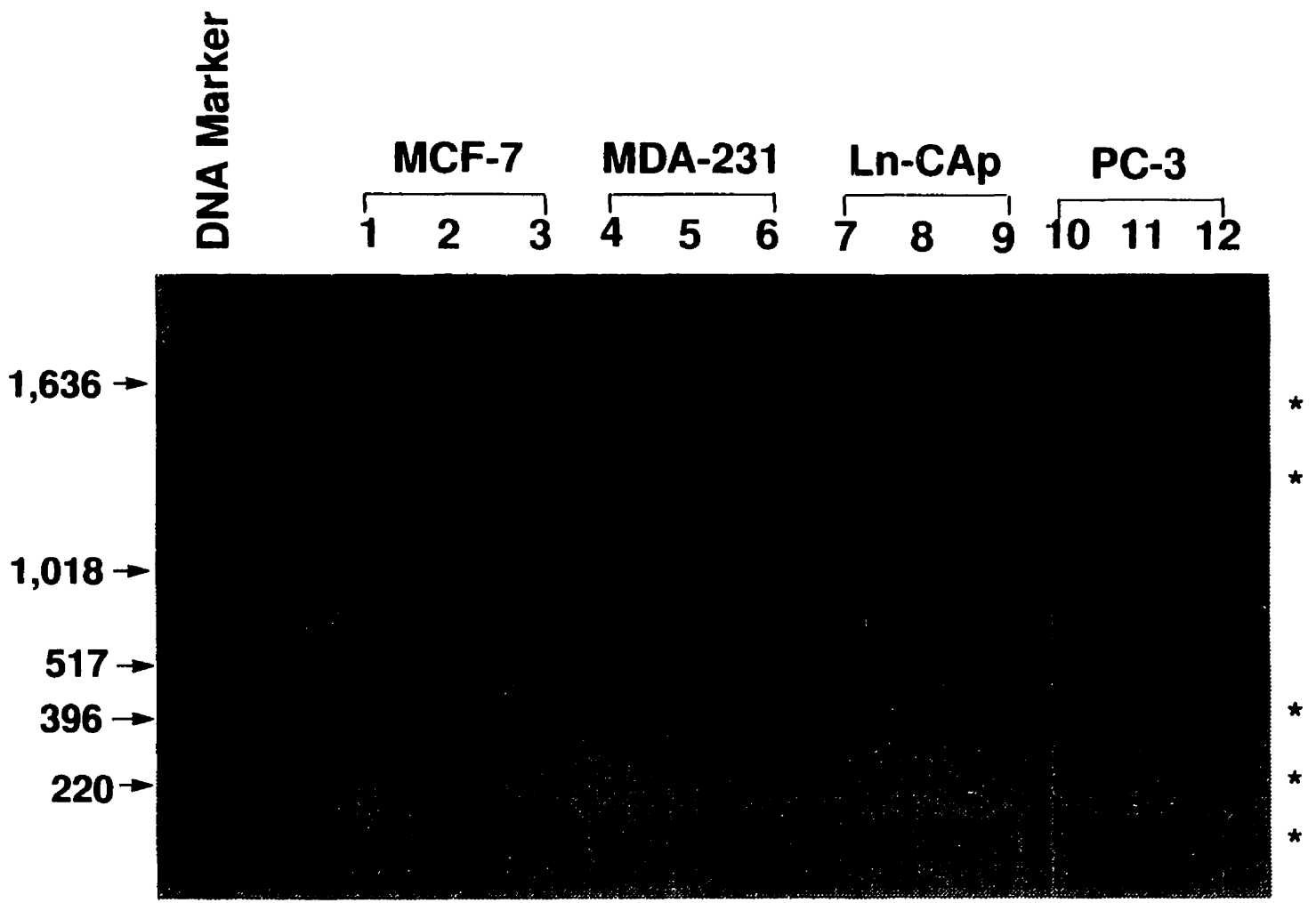
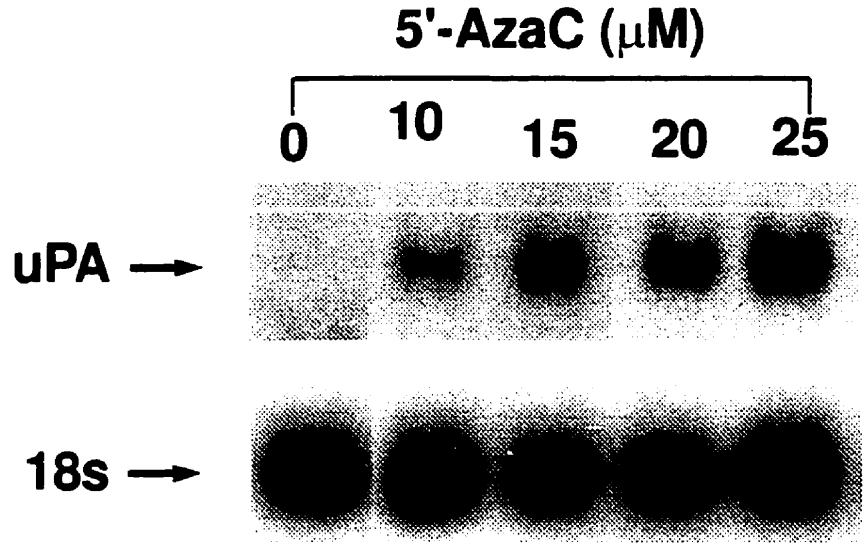

DNA Marker
MCF-7
MDA-231
Ln-CAp
PC-3
1 2 3
4 5 6
7 8 9
10 11 12
1,636
1,018
517
396
220
*
*
*
*
*

Figure. 5.5: Methylation status of representative sites in CpG island of human uPA gene.

10 μg of genomic DNA isolated from HR (MCF-7, Ln-CAP) and H1 (MDA-231, PC-3) breast and prostate cancer cells were digested with non-methylation sensitive Pst1 alone (lanes 1,4,7,10) or with Pst1 and methylation sensitive enzymes Hpa1 1 (lanes 2,5,8,11) or Hha1 (lanes 3,6,9,12) as described in "Materials and Methods". Digests were run on 0.8% agrose gel and Southern blot analysis was performed. All blots were probed with a 778 bp *SmaI* and *AvrII* promoter fragment of human uPA gene. Results are representative of three different experiments.

Figure. 5.6: Effect of DNA demethylation on uPA gene expression in MCF-7 cells.

HR human breast cancer cells MCF-7 maintained in culture were treated with different concentrations of DNA methylation inhibitor 5'-azacytidine (5'-azaC) (10 - 25 μm) for 10 days. Northern blot analysis was performed on total cellular RNAs isolated from the un-treated control and 5'-azaC-treated MCF-7 cells to monitor the induction of uPA mRNA. All blots were probed with ^{32}P-labelled human uPA and 18S cDNA (upper panel). Blots were scanned by laser densitometry and level of uPA mRNA expression determined by plotting the ratio of uPA/18S (lower panel). Results are representative of three individual experiments. Significant difference from control cells is represented by asterisks ($p<0.05$).


5'-AzaC (μM)
0
10
15
20
25
uPA
18s

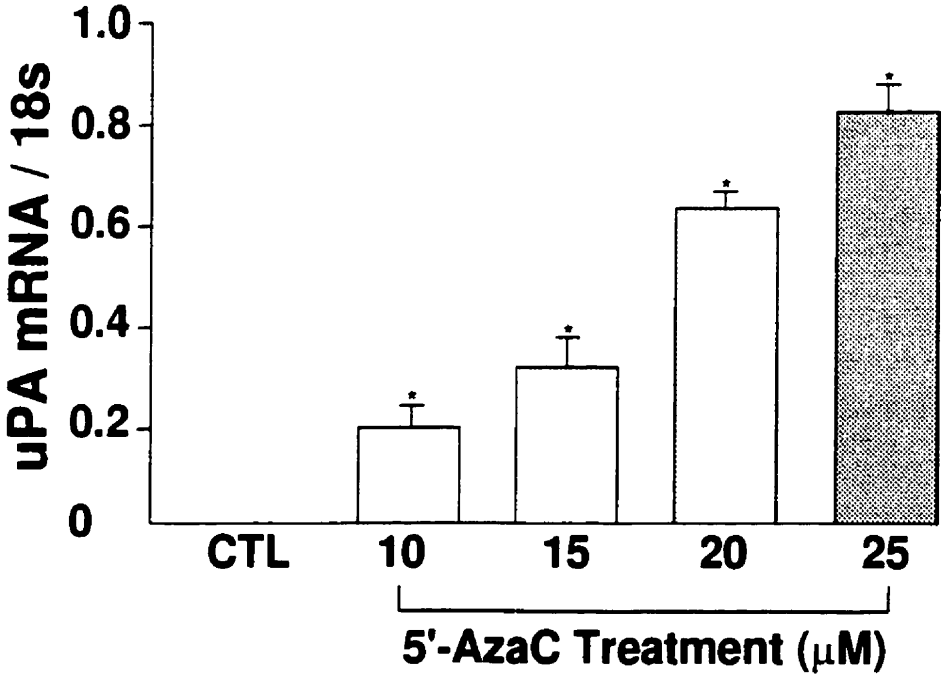

uPA mRNA / 18s
1.0
0.8
0.6
0.4
0.2
0
CTL
10
15
20
25
5'-AzaC Treatment (μM)

Figure. 5.7: Methylation status of representative sites of CpG islands of human uPA gene after treatment with DNA methylation inhibitor 5-azaC.

10μg of genomic DNA isolated from MCF-7. MCF-7 cells treated with 5'-azaC for 10 days and MDA-231 cells were digested with Pst1 alone (lanes 1,4,7) or with Pst1 and methylation sensitive enzymes Hpa11 (lanes 2,5,8) or Hha1 (lanes 3,6,9). DNA digests were resolved on 0.8% agrose gel and Southern blot analysis was performed. All blots were probed with a 778 bp *SmaI* and *AvrII* promoter fragment of human uPA gene. Results are representative of three different experiments.

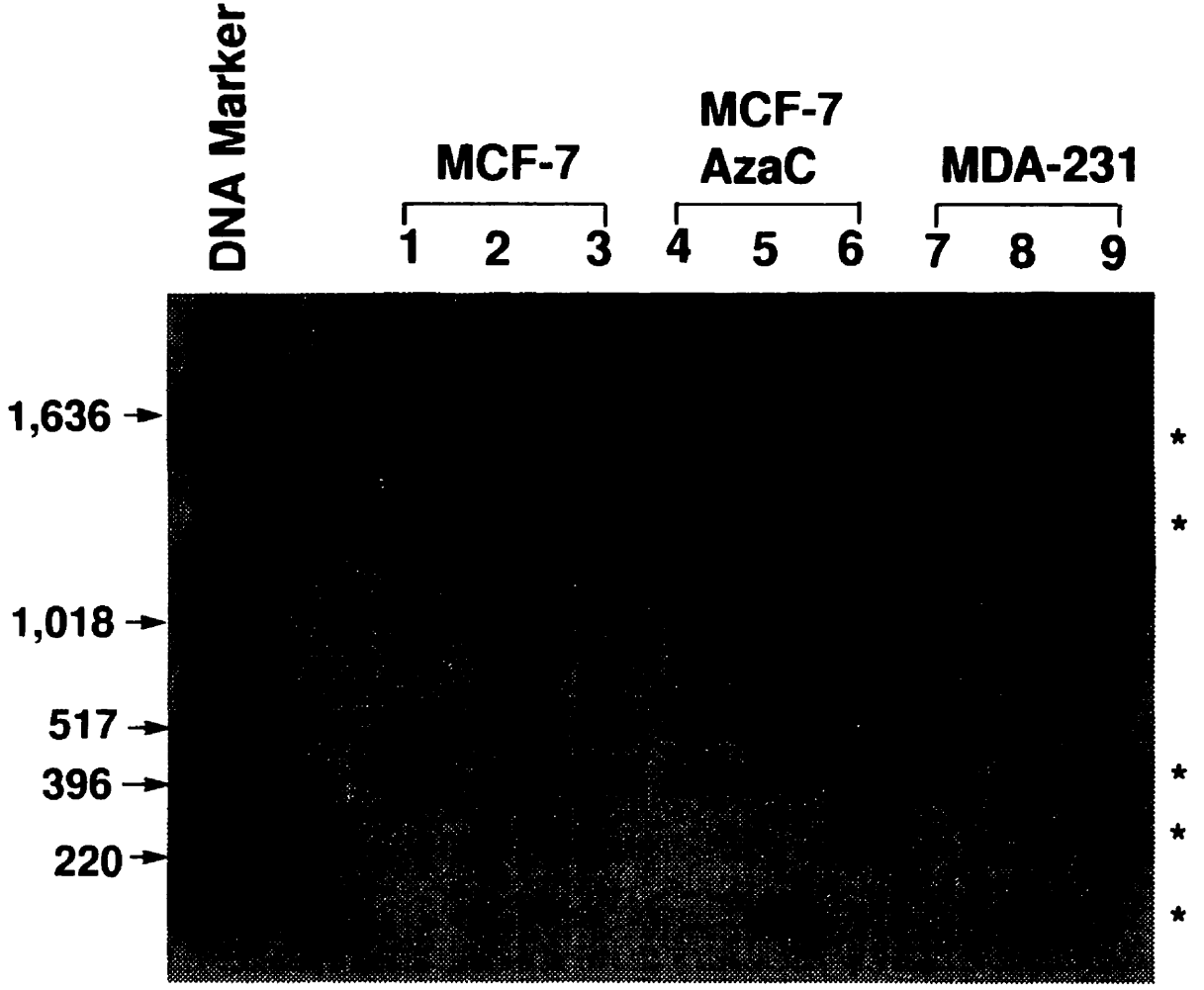
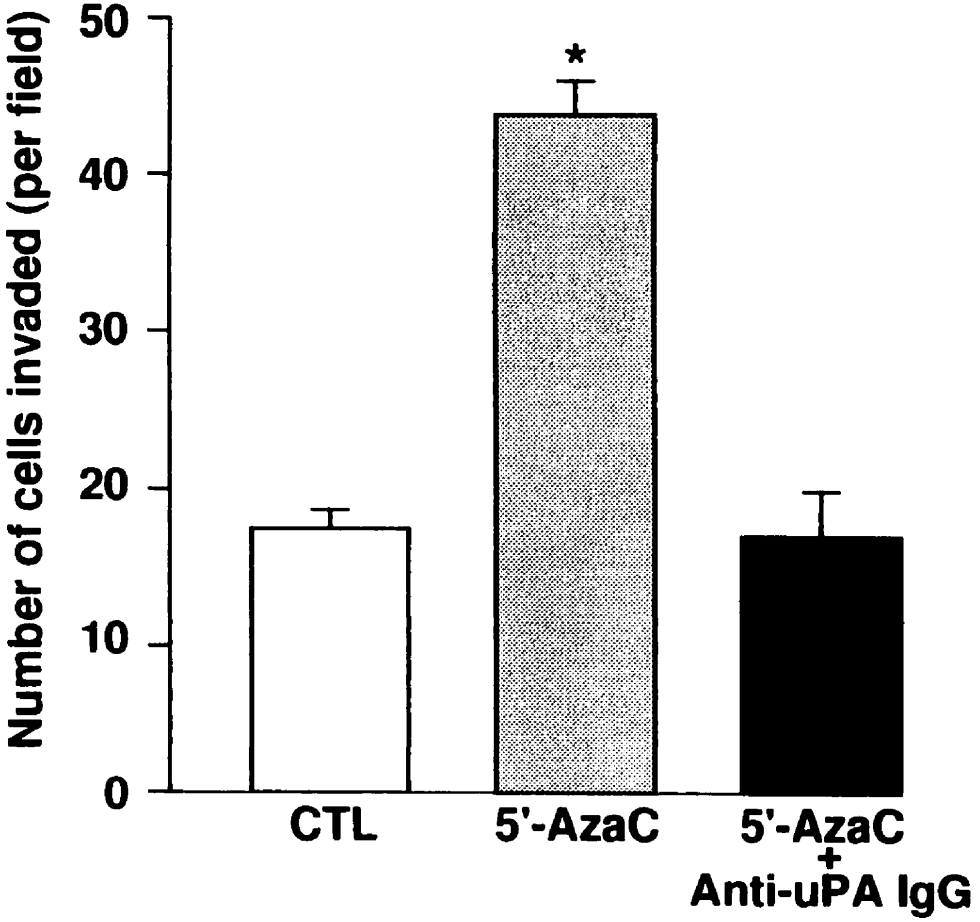

DNA Marker
MCF-7
MCF-7 AzaC
MDA-231
1 2 3
4 5 6
7 8 9
1,636
1,018
517
396
220
*
*
*
*
*

Figure. 5.8: DNA demethylation in MCF-7 cell invasion.

Human breast cancer cells MCF-7 maintained in culture were treated with 20 μm of DNA methylation inhibitor 5'-azacytidine (5'-azaC) for 10 days. The invasive capacity of control and 5'-azaC-treated MCF-7 cells were assessed *in vitro* by Boyden Chamber Matrigel invasion assay. The specificity of uPA in mediating these effects was confirmed by co-incubation of 5'-azaC treated MCF-7 cells with anti-human uPA IgG. Results are representative of three different experiments. Significant difference from control cells is represented by asterisks ($p<0.05$).


Number of cells invaded (per field)
50
40
30
20
10
0
*
CTL
5'-AzaC
5'-AzaC
+
Anti-uPA IgG

5.3.5 Results

uPA Expression and its Correlation with Cellular Invasion

In order to determine the presence of any relationship between uPA expression and estrogen/androgen responsiveness of human breast and prostate cancer cells, total cellular RNA was isolated from HR breast (MCF-7) and prostate (Ln-CAP) cancer cells expressing a functional estrogen/androgen receptor. Comparison of uPA mRNA expression was also made between HI breast (MDA-231) and prostate (PC-3) cells by Northern blot analysis. The level of uPA mRNA was determined by probing these blots with a human uPA cDNA and with a control 18S cDNA probe. Presence of uPA mRNA expression was only seen in HI (MDA-231 and PC-3) cancer cells (Figure 5.1A). Exposure of these blots for extended periods (7 days) and monitoring the presence of very low levels of uPA mRNA by RT-PCR also failed to show any uPA mRNA in HR MCF-7 and Ln-CAP cells (data not shown).

Both HR and HI cell lines were evaluated for their ability to invade through Matrigel in Boyden Chamber invasion assay. Only HI cells (MDA-231, PC-3) were found to be highly invasive whereas HR (MCF-7, Ln-CAP) either failed to invade or did so poorly in this assay (Figure 5.1B). This tumor cell invasion was directly associated with the level of uPA mRNA expression in these tumor cell lines.

uPA Gene Transcription

In order to determine the mechanism of lack of uPA mRNA expression in MCF-7 and Ln-CAP cells, uPA gene transcription was examined in these breast and prostate cancer cells by nuclear run-off assays. Following the isolation of nuclei from these cells, RNA synthesis *in vitro* was allowed to proceed in the presence of γ^{32}P-UTP, followed by

isolation and slot blot analysis of their RNA. Presence of uPA gene transcription was seen only in MDA-231 and PC-3 cells, both of which are hormone insensitive and lack a functional estrogen/androgen receptor (Figure5.2). Reproducible transcription of 18S RNA observed in all cell lines demonstrated the authenticity and efficacy of this technique. These results show that lack of uPA mRNA expression in HR (MCF-7, Ln-CAP) cancer cells is due to a block of uPA gene transcription.

Presence of uPA Gene

A simple explanation for the lack of uPA mRNA expression and failure of uPA gene transcription in these HR cells could be deletion of both alleles of uPA gene in MCF-7 and Ln-CAP cells. To examine this possibility, total genomic DNA was isolated from HR and HI breast and cancer cells. Following digestion of genomic DNA with EcoRI or Hind III restriction enzymes, digestion products were analyzed by Southern blotting using uPA cDNA as a hybridization probe. Identical DNA restriction patterns were seen in HR (MCF-7, Ln-CAP) and HI (MDA-231, PC-3) cancer cells when genomic DNAs were digested with EcoRI (Figure 5.3).

Analysis of uPA Gene Sequence

The human uPA gene located on chromosome 10, consists of 11 exons and spans 7.25 Kb to encode a 2.5 Kb mRNA. In order to assess the potential role of DNA methylation in regulation of uPA gene transcription in HR and HI breast and prostate cancer cells, restriction analysis of the uPA promoter and first exon regions was performed. Restriction digest with appropriate enzymes (*Pst I, HpaII, HhaI*) revealed that these regions of uPA genes are densely populated with CpG islands which can serve as

potential sites for DNA methylation (Figure5.4), a molecular event which causes epigenetic changes without changing the nucleotide sequence.

Analysis of DNA Methylation Status of the uPA Gene

In order to test the role of DNA methylation in uPA gene transcription, genomic DNA was isolated from HR and HI breast and prostate cancer cells. Total cellular DNA was digested with non-methylation-sensitive endonuclease Pst 1 alone (Figure 5.5; lanes 1,4,7,10) or with Pst 1 and methylation-sensitive endonucleases Hpa1 1 (lanes 2,5,8,11) or Hha1 (lanes 3,6,9,12). The methylation status of *Hpa1 1* and *Hha1* sites were analyzed by Southern blotting. All blots were probed with a 778 bp *Sma I* and *Avr II* promoter fragment of uPA gene. Hypermethylation of the CpG islands of uPA gene in HR MCF-7 and Ln-CAP-cells prevented *Hpa1 1* and *Hha1* from DNA digestion, yielding restriction patterns identical to *Pst 1* digestion alone as indicated by the asterisks (Figure 5.5; lanes 1,2,3,7,8,9). In contrast to this, CpG islands of uPA gene in HI MDA-231 and PC-3 cells were non-methylated or hypomethylated, resulting in the complete digestion of *Hpa1 1* and *Hha1* to generate multiple lower molecular weight bands as compared to *Pst1* digestion alone (Figure 5.5; lanes 4,5,6,10,11,12). These results indicate that the inhibition of uPA gene transcription in HR, MCF-7 and Ln-CAP is due to hypermethylation of CpG islands of uPA gene.

Effect of 5-azaC on uPA Gene Expression

5'-azacytidine, a nucleoside analog has been shown to be a potent demethylating agent. Treatment of hypermethylated CpG islands of some inactivated tumor suppressor genes results in the re-activation of their gene expression. In order to examine the capacity

of 5'-azaC in demethylating the CpG islands of uPA gene in MCF-7 cells, cells were treated with different concentrations of 5'-azaC (10-25 μm) for 10 days. During this period, cells were examined for their viability following treatment with different doses of 5'-azaC. Treatment of MCF-7 cells with 5'-azaC failed to show any sign of cytotoxicity (data not shown). uPA mRNA expression following 5'-azaC treatment was determined by Northern blot analysis. MCF-7 cells treated with vehicle alone failed to show any uPA mRNA expression. However, treatment of MCF-7 cells with 5'-azaC caused a dose-dependent induction of uPA mRNA expression (Figure 5.6). These results suggest that demethylating agents like 5'-azaC are able to induce the re-expression of uPA in HR MCF-7 and Ln-CAP cells. Genomic DNA isolated from 5'-azaC treated MCF-7 cells was analyzed for the methylation status of CpG islands of uPA gene using methylation sensitive endonucleases Hpa1 1 and Hha1. CpG islands of untreated MCF-7 cells were hypermethylated which were resistant to Hpa1 1 and Hha1 digestion and yielded identical restriction digestion patterns as compared to Pst1 digestion alone (Figure 5.7; lanes 1,2,3). In contrast to this, 5'-azaC treatment of MCF-7 cells caused a complete demethylation of previously hypermethylated Hpa1 1 and Hha1 sites yielding multiple lower molecular weight bands as compared to Pst1 digestion alone (Figure 5.7; lanes 5,6) which were identical to those seen in MDA-231 cells (lanes 8,9) whose CpG islands of uPA gene are hypomethylated. Following this treatment, a significant level of uPA protein production was seen in the conditioned culture medium of MCF-7 cells by Western blotting (data not shown). These results demonstrate that the induction of uPA mRNA expression in MCF-7 cells after 5'-azaC treatment is due to the demethylation of CpG islands of the uPA gene, resulting in the abrogation of transcriptional inhibition on uPA gene. The hormone-responsiveness in MCF-7 cells after 5'-azaC treatment was assessed by measuring the

mRNA level of ER by Northern blotting analysis. There was a significant decrease in ER mRNA with the induction of uPA in 5'-azaC-treated MCF-7 cells (data not shown).

In order to examine the functional significance of induction of uPA gene expression by MCF-7 cells after 5'-azaC treatment, the invasive capacity of control and treated cells was assessed *in vitro* by Boyden Chamber Matrigel invasion assay. As a result of induction of uPA mRNA expression by 5'-azaC, MCF-7 cells showed a significantly higher invasive capacity as compared to the control un-treated cells (Figure 5.8). In order to confirm the specificity of uPA in mediating these effects, 5'-azaC-treated MCF-7 cells were co-incubated with anti-human uPA IgG which completely abolished the increase in MCF-7 cell invasiveness after 5'-azaC treatment.

This study clearly demonstrated for the first time that uPA gene is differentially expressed in HR and HI breast and prostate cancer cells and that the production of uPA is correlated with hormone-responsiveness of the tumor cells. More significantly, DNA methylation plays an important role in transcriptional regulation of uPA gene expression in hormone-dependent malignancies like breast and prostate cancer.

5.3.6 Discussion

In the current study, we have demonstrated a positive correlation between tumor cell invasiveness and estrogen/androgen responsiveness in human breast and prostate cancer cell lines. Since recent studies have shown that uPA plays a key role in the progression of several malignancies including breast and prostate cancer (Achbarou A et. al., 1994; Janicke F et al., 1991; Rabbani SA et al., 1995b; Xing RH et al., 1997), we evaluated the relationship between uPA expression, hormone responsiveness and tumor cell invasion. Only HI cancer cell lines (MDA-231, PC-3) expressing abundant amounts

of uPA mRNA exhibited high invasive capacity through the Matrigel, whereas HR (MCF-7, Ln-CAP) cell lines expressing functional estrogen and androgen receptor exhibited poor invasive ability. Nuclear run-off assays performed on nuclei isolated from various cancer cells lines confirmed the results seen by Northern blot analysis and showed that the lack of uPA expression in these cells was due to blockage of uPA gene transcription. Failure of uPA gene transcription in HR MCF-7 and Ln-CAP cells may be due to the deletion of both alleles of uPA gene. However, this possibility was excluded by Southern blot analysis which showed the presence of intact uPA gene in all tumor cell lines examined. Collectively, these results indicated that the presence of transcriptional inhibition of uPA gene in HR cells allowed them to maintain a low virulence phenotype as characterized by their poor invasive capacity. These results clearly point towards the differences in transcription control mechanisms which allow these tumor cells to turn off and turn on uPA production at different stages of tumor progression.

The differences in uPA gene transcription in HR and HI breast and prostate cancer cells are of particular significance, since no differential changes in uPA mRNA expression have been reported at different stages of tumor progression in non-hormone-dependent malignancies (Sordat I et al., 1997; Bolon I et al., 1997). This blockage in uPA gene transcription could be due to differences in trans-acting transcription factors or due to changes in uPA gene promoter sequences to cause cis-acting alterations resulting in inactivation of the uPA promoter in HR breast and prostate cancer cells (Imagawa M, 1996; Tollervey D, 1996). There is accumulating evidence supporting an important role of DNA cytosine methylation resulting in epigenetic modifications to alter genomic functions and regulation of gene transcription in human malignancies (Baylin SB et al., 1991; Szyf M, 1996). Disregulation of DNA methylation resulting in either hypomethylation or

hypermethylation of CpG islands in effected genes has been reported in human malignancies (Feinberg AP and Vogelstein B, 1983b; Ramsahoye BH et al., 1996). Hypermethylation of DNA has been attributed to the inactivation of a number of tumor suppressor genes including Rb (Ohtani-Fujita N et al., 1993), E-cadherin (Herman JG et al., 1996), endothelin receptor (Nelson JB et al., 1997), estrogen receptor (Ottaviano YL et al., 1994) and P53 (Denissenko MF et al., 1997). In contrast, hypomethylation of CpG islands is believed to be responsible for overexpression of various oncogenes including *ras* and *myc* to promote tumor progression (Feinberg AP and Vogelstein B 1983a). Since DNA methylation causes epigenetic changes to regulate promoter activity of the target gene without changing its nucleotide sequences, it clearly represents a potential control mechanism for the differential regulation of uPA gene expression in HR and HI breast and prostate cancer cells.

In the present study, a detailed restriction analysis, using methylation-sensitive restriction enzymes revealed that the promoter and exon 1 regions of uPA gene are densely populated with these CpG islands which are potential sites for DNA methylation. These observations led us to further explore DNA methylation as a potential molecular mechanism controlling uPA gene transcription in HR and HI breast and prostate cancer cells. In order to investigate these possibilities, Southern blot analysis using methylation sensitive enzymes Hpa I I and Hha I and a sequence-specific promoter probe spanning the areas of uPA promoter regions containing multiple Hpa I I and Hha I sites was performed. In HI MDA-231 and PC-3 cells Hpa I I and Hha I sites were found to be completely un-methylated or hypomethylated, whereas the same sites were fully methylated in HR MCF-7 and Ln-CAP cells. These results demonstrated that inhibition of uPA gene transcription in HR MCF-7 and Ln-CAP cells is due to hypermethylation of CpG sites of uPA gene.

Induction of uPA gene expression in MCF-7 cells following treatment with 5'-aza-C, a potent inhibitor of DNA methylation, further confirmed the role of DNA methylation on uPA gene transcription. Evaluation of the previously hypermethylated *Hpa II* and *Hha I* sites in MCF-7 cells after 5'-aza-C treatment demonstrated that the release of transcriptional inhibition of the uPA gene is due to demethylation of CpG sites. Induction of uPA production was also seen at the protein level which resulted in a significant increases in the invasive capacity of 5'-azaC treated MCF-7 cells. The specificity of uPA in mediating this increase in invasion was also confirmed by co-incubation of 5'-azaC treated MCF-7 cells with anti-human uPA antibody which completely reversed the observed increase in tumor cell invasion. The current study provides compelling evidence for DNA methylation as a unique molecular mechanism involved in the transcriptional regulation of uPA gene expression to allow this critical protease to be turned-on and turned-off at different stages of these common hormone-dependent malignancies.

The results obtained from the current study has opened new avenues for further investigation of the mechanism of regulation of uPA gene expression and the role of DNA methylation in these events. Due to the strong correlation between hormonal status, uPA gene expression and demethylation of CpG sites of uPA gene in MCF-7 and Ln-CAP cells, it is highly like that these events are also regulated by hormones. DNA methyltransferase, the key enzyme catalyzing the DNA methylation reaction (Szyf M, 1994) has recently been shown to be under the regulation of *ras* oncogene which in turn is strongly associated with various hormones and growth factors (Szyf M et al., 1991; Rouleau J et al., 1995; Macleod AR et al., 1995). Several recent studies have also demonstrated the ability of *ras* oncogene to regulate an endogenous demethylase activity in tumor cells (Szyf M et al., 1995), pointing towards the role of additional intracellular

signal transduction pathways involved in regulating DNA methylation. Studies are currently underway to investigate the role of different hormones regulating key enzymes associated with DNA methylation and to investigate various signalling pathways involved in these complex processes. A better understanding of the molecular basis of malignant progression in these hormone-dependent malignancies will not only enhance our understanding of the biological basis of these cancers but will also lead towards the development of new therapeutic approaches for controlling these common malignancies.

5.3.7 Acknowledgements

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CHAPTER 6

GENERAL DISCUSSION

6.1. ORIGINAL CONTRIBUTIONS OF THE CURRENT STUDIES

The original contributions of the studies described in Chapters 2-5 are:

1. The development of a syngeneic *in vivo* model of breast cancer in which the direct involvement of uPAR in breast cancer invasion and metastasis can be readily assessed. Results from these studies provide convincing experimental evidence for an essential role of uPAR overexpression in breast cancer progression due to its capacity to promote tumor growth, invasion and metastasis.

2. The establishment of the validity and effectiveness of a new combination therapeutic strategy in the treatment of metastatic breast cancer by adding anti-invasive agents (uPA inhibitors) to the standard hormone therapy (anti-estrogens); and demonstration of these anti-tumor agents to block tumor progression by inhibiting uPAR gene transcription.

3. Sex steroids like androgens play important roles in the initiation and progression of hormone-dependent malignancies such as prostate cancer. These effects are due to transcriptional regulation of uPA gene by androgens in hormone responsive prostate cancer cells to prevent the development of hormone independent state of tumor growth. These studies demonstrate that changing the level of bioavailable androgens can alter the growth characteristics of prostate cancer *in vivo*.

4. Results from these studies demonstrate for the first time that the uPA gene is differentially expressed in hormone responsive and hormone insensitive breast and prostate cancer cells, and that the production of uPA is correlated with hormone responsiveness of tumor cells.

5. These studies also demonstrate for the first time a role of cytosine DNA methylation in the differential transcriptional regulation of uPA gene expression in hormone dependent malignancies like breast and prostate cancer, which allows the transcription machinery of uPA to be turned on and turned off at different stages of these malignancies.

6.2. uPAR IN TUMOR INVASION AND METASTASIS

The process of tumor metastasis is a cascade of linked sequential events (Liotta IA, 1986; Fidler IJ,1990). A critical step in cancer invasion and metastasis is the breaching of the extracellular matrix by tumor cells (Bernstein, LR and Liotta, LA, 1994). This event is regulated by various proteolytic enzymes such as uPA (Blasi F, 1993; Liotta LA et al., 1980; Mignatti P et al., 1986; Rochefort H et al., 1990; and Sato H et al., 1994). We and others have demonstrated that overexpression of uPA results in increased tumor growth and metastasis (Mignatti, P et al., 1986; Crowley CW et al., 1993; Achbarou A et al., 1994; Kobayashi H et al., 1994; Rabbani SA et al, 1995b; Xing RH et al., 1997). Up-regulation of the uPA system is associated with increased invasion in various common human malignancies including hormone-dependent malignancies like breast and prostate cancer (Duffy MJ et al., 1988; Janicke F et al., 1990; Foekens JA et al., 1992; Grondahl-Hansen J et al., 1993; Duffy MJ et al., 1994; Duffy MJ, 1996a; Duffy MJ, 1996b; Ferno M et al., 1996; Van Veldhuizen PJ et al., 1996). uPA is now recognized as a strong independent prognostic marker for breast and prostate cancer where increased uPA production is associated with poor prognosis, short rate of overall survival and high rate of relapse

(Duffy MJ et al., 1990; Duffy MJ, 1996a; Duffy MJ, 1996b; Ferno M et al., 1996; Van Veldhuizen PJ et al., 1996).

The proteolytic activity of uPA is localized on the cell surface by membrane-bound, GPI-anchored uPAR (Andreasen PA et al., 1997; Bernhard EJ et al., 1994; Dano K et al., 1994; Ploug M et al., 1991). Accumulating evidence from clinical studies strongly support a critical role of uPAR in breast and prostate cancer invasion and metastasis (Del Vecchio S et al., 1993; Pyke C et al., 1993; Ganesh S et al., 1994b; Pedersen H et al., 1994a; Duggan C et al., 1995; Grondal-Hansen J et al., 1995; Heiss MM et al., 1995; Van Veldhuizen PJ et al.). However, due to the reported interspecies specificity of the interactions between uPA and uPAR (Ramshaw IA and Badenoch-Jones P, 1985), there was no appropriate *in vivo* model available to us to directly examine the role of uPAR in the process of tumor invasion and metastasis. In addition, the localization of uPAR in turmor tissues had not been established. Therefore, it was hypothesized that the contribution of uPAR to tumor invasion was merely due to its ability to localize the proteolytic activity of uPA in the tumor cell environment. The molecular mechanism underlying some of the uPA-independent functions of uPAR was not well defined. In the current study (Chapter 2: Xing RH and Rabbani SA, 1996), the development of a syngeneic *in vivo* model of uPAR overexpression by the rat mammary adenocarcinoma cell line Mat B-III is of significant value. This syngeneic model allows complete interaction between the endogenous uPA / uPAR and that expressed by the inoculated tumor cells to demonstrate the role of uPAR in breast cancer progression. The higher invasive potential of tumor cells overexpressing uPAR in the absence of any changes in uPA level strongly supports the role for uPAR in tumor progression. These results are of particular

significance in consolidating the role of uPAR in breast cancer progression since high levels of uPAR are observed only in invasive breast cancer cells compared to the benign and normal breast tissues (Del Vecchio S et al., 1993). Furthermore, development of this syngeneic *in vivo* model will permit us to evaluate various therapeutic strategies aimed at blocking uPA activity, or preventing uPA/uPAR interactions.

Although uPAR lacks cytoplasmic domains, there is overwehelming evidence suggesting that uPAR may promote tumor progression by directly engaging in the activation of intracellular signal transduction pathways (Rabhani SA et al., 1992; He CJ et al., 1991; Dumler I et al., 1993; Anichini E et al., 1994; Busso N et al., 1994; Cao D et al., 1995; Clark EA and Brugge JS, 1995; Li C et al., 1995; Resnati M et al.,1996; Rabbani SA et al., 1997). The close association of uPAR with protein tyrosine kinases (Dumler I et al., 1993; Busso N et al., 1994; Resnati M et al.,1996), vitronectin (Wei Y et al., 1994; Nip J et al., 1995; Deng G et al., 1996; Kanse SM et al., 1996) and β1 -and β2-integrins (Xue W et al., 1994; Bohuslav J et al., 1995; Sitrin RG et al., 1996; Wei Y et al., 1996) strongly supports the emerging role of uPAR in mediating some of the cellular functions that are independent of the proteolytic activities of uPA, such as cell proliferation, adhesion and migration. However, the molecular mechanisms underlying these events are not known, and their contribution to the process of tumor progression has not been well defined *in vivo*.

It is now known that the processes of tumor angiogenesis, invasion and metastasis are intimate interplays between proteolytic enzymes and angiogenic factors (Dvorak HF et al., 1988 ; Liotta LA et al., 1991). Angiogenic factors could promote the process of tumor metastasis by (1) up-regulation of the production of various proteolytic enzymes such as

uPA / uPAR and metalloproteases to enance ECM degradation, or (2) by increasing extravasation of tumor cells through the newly formly leaky blood vessles. On the other hand, uPAR could increase tumor angiogenesis by plasmin-dependent morbilization and activation of angiogenic factors (TGF-β, bFGF). Furthermore, due to its ability to modulate integrin functions, uPAR expressed by vascular endothelial cells could directly promote tumor angiogenesis by a paracrine / autocrine effect on endothelial cell migration, invasion and adhesion. Using our homologous model of uPAR overexpression, the effect of uPAR over-production by breast cancer cells on endothelial cell adhesion and migration can be readily assessed by a co-culture system *in vitro* (Janvier R et al., 1997; Zhau HE et al.. 1997). The contribution of uPAR in tumor angiogenesis could be determined *in vivo* by immunocytohistochemical measurement of the micovessel density in tumor tissues. Expansion of uPAR function to tumor angiogenesis could help to explain our *in vivo* data on increased primary tumor growth as a result of uPAR overexpression.

Studies on tumor growth and metastasis in uPAR-/- mice remain to be reported and will help to further elucidate the significance of uPAR in this process. However, the lack of severe phenotypic abnormalities in uPAR deficient mice implicates that redundancy of functions may occur in cancer progression.

The significance of another class of protease-metalloproteases in tumor progression has not been discussed in this thesis. However, this does not undermine their important contribution to the process of tumor progression. For references on the role of MMPs in tumor invasion and metastasis, refer to a few recent reviews for more details (DeClerck YA and Laung WE, 1996; Hewitt R and Dano K, 1996; Mignatti P and Rifkin DB, 1996; Durko M and Brodt P, 1996; Rabbani SA, 1998). Elucidation of the relative importance of

uPA/Plasmin system and MMPs in tumor progression will not only help to achieve a better understanding of the actions of different classes of proteases in this critical event, it will also help to develop new diagonostic and therapeutic strategies to control tumor invasion and metastasis more effectively and efficiently. This issue could be studied in our syngeneic *in vivo* models of breast and prostate cancer by using inhibitors of uPA and MMPs alone or in combination. If plasmin-independent MMP activation exists *in vivo* and contributes to tumor progression, MMP inhibitors will further potentiate the inhibitory effect of anti-uPA agents like B-428 on tumor invasion and metastasis. Studies in uPA and MMP knock out mice on tumor progression will also provide valuble information on the role of these proteases in human malignancies.

6.3. COMBINATION THERAPEUTIC APPROACH TO THE TREATMENT OF HORMONE DEPENDENT MALIGNANCIES

Hormone dependent malignancies like breast and prostate cancer are metastatic by nature (Lee YTN, 1983; Koscielny S et al., 1984) and are associated with a high incidence of morbidity and mortality (Franks LM, 1973; Cannon-Albright LA and Skolnick MH, 1996) The dependence on sex steroids for initiation and early stage tumor growth results in the utilization of hormonal therapy aimed at eliminating the sources of these hormones and/or interferring with the interactions between hormones and their receptors (Hsieh WS and Simons JW, 1993; Vogel CL, 1996). Anti-estrogens and anti-androgens are included as part of the standard therapeutic regimen for patients with hormone receptor positive tumors (Jordan VC, 1994; Vogelzang NJ and Kennealey GT, 1992). However, anti-hormone therapy has had limited success in controlling breast and prostate cancer

progression, due to the heterogeneous nature of the tumor cells present in the primary tumor, due to the progression of the majority of tumors to a phenotype that is resistant to hormonal manipulations, and due to the inability of these therapies to effectively control tumor metastasis.

The marked heterogeneity of breast and prostate tumors is a major restriction of the curative potential of single modality treatments (Van Netten JP et al., 1988; Clarke R et al., 1992). Even in tumors expressing high levels of hormone receptors, there are areas within the tumor that do not possess these receptors (Van Netten JP et al., 1988). The selective pressure applied by endocrine manipulation would remove the hormone sensitive populations and could facilitate the emergence of tumors comprised of predominantly endocrine-resistant cells. Therefore, the development of a complementary approach that involves modifying the tumor microenvironment and reducing the propensity for tumor cell invasion, neovascularization and metastasis is essential for a better control of these metastatic diseases. Although combinational therapy begins to emerge as an effective approach to treat human cancers, studies using experimental animals are needed to establish its advantege over tranditional therapeutic strategies.

Elucidation of the role of uPA/uPAR in tumor progression over the last few years (Achbarou A et al., 1994; Kobayashi H et al., 1994; Rabbani SA et al, 1995b; Xing RH and Rabbani SA, 1996; Xing RH et al., 1997) and their usefulness as molecular prognostic markers to follow disease progression (Duffy MJ, 1990; Grondahl-Hansen J et al., 1993, Ferno M et al., 1996 ; Van Veldhuizen PJ et al., 1996) have resulted in the emergence of uPA/uPAR as potential targets for anti-cancer therapy. Inhibition of the proteolytic activity of uPA and interruption of the association between uPA and uPAR represent attractive

therapeutic targets for blocking cellular invasiveness in cancer (Fazioli F and Blasi F, 1994). Several studies have attempted to achieve this goal via interfering with interactions between between uPA and uPAR by soluble uPAR and uPAR antagonists, by inhibiting the proteolytic activity of uPA by active site inhibitors of uPA, or by anti-sense technology to inhibit uPA and uPAR gene expression (Kook YH et al., 1994; Wang Y et al., 1994; Billstrom A et al., 1995; Quattrone A et al., 1995; Alonso DE et al., 1996; Min HY et al., 1996). In the current study (Chapter 3: Xing RH et al., 1997), using our syngeneic *in vivo* model of uPAR overexpression, in which uPA and uPAR play key roles in breast cancer progression, we have evaluated the anti-invasive and anti-metastatic abilities of the recently developed synthetic uPA active site inhibitor B-428 alone, and in combination with anti-estrogen tamoxifen. We hypothesized that a combination of anti-invasive and hormonal treatment could result in the inhibition of growth of the hormone insensitive, invasive and metastatic subpopulations by anti-invasive drugs, and the elimination of the hormone-dependent cells by hormonal agents.

Although we have previously shown that B-428 is an effective anti-invasive and anti-metastatic agent in preventing prostate cancer progression (Rabbani SA et al., 1995b), the present study is the first report demonstrating the anti-proliferative effects of this protease inhibitor. Results from these studies have suggested several potential mechanisms of the actions of B-428 in preventing breast cancer progression. First, it acts as an active site inhibitor of uPA to directly inhibit uPA-mediated proteolysis. Second, decreased proteolytic activity of uPA further leads to decreased activation of latent growth factors like HGF and TGF-β by plasmin and metalloproteases which may account for the observed decrease in cell proliferation. Third, B-428 has been shown for the first time, to inhibit the

functions of the uPA system by down-regulating uPAR gene transcription in addition to its role as the active site inhibitor of uPA. This decreased uPAR expression could be a direct action of B-428 through a yet undefined mechanism, or could be an indirect effect resulting from decreased growth factor activation by B-428. Since B-428 is a derivative of amiloride, which has been shown to alter gene expression via a mechanism involving modulation of the activity of Na^+/H^+ antiporter, B-428 could inhibit uPAR gene transcription via a similar mechanism. The observed decrease in uPAR expression may not only prevent tumor invasion and metastasis by inhibiting uPA-mediated proteolysis, but also inhibit tumor progression via its signalling functions to modulate cell migratory events. Anti-estrogen tamoxifen used in combination therapy has been shown to be an anti-proliferative agent due to its ability to interfere with the actions of estrogen (Sutherland RL and Jordan VC, 1981; Jordan VC, 1986), as well as its inhibitory effects on the actions of several growth factors (TGF-α, IGF-1) [Pollak MN et al., 1990 ; Huynh HT et al., 1993; Grainger DJ and Metcalfe JC, 1996] implicated in breast cancer progression. The cytostatic effect of tamoxifen is attributed to its ability to cause cell cycle arrest at the G_0-G_1 checkpoint (Jordan VC, 1988; Osborne CK et al., 1983; Sutherland RN et al., 1983). The present study, however, is the first report to describe an inhibitory effect of tamoxifen on cell invasion by down-regulating uPAR gene transcription. The mechanisms underlying the inhibitory action of TAM on uPAR gene expression can not be clearly pointed out at this moment. TAM is capable of regulating gene expression directly by interacting with ER-signaling pathway, or indirectly by modulating the production of growth factors or cytokines. Since TAM selectively decreases uPAR gene transcription without affecting the expression of uPA, it is most likely that the effect of TAM on uPAR gene expression is

mediated via a ER-independent mechanism. Combination teatments have clearly demonstrated advantages in preventing breast cancer growth, invasion and metastasis over either therapeutic approach alone.

In summary, results from the current study not only support the notion that inhibiting plasminogen activator-mediated cellular invasiveness is an effective therapeutic intervention, but also demonstrate the effectiveness of a combination regimen aimed at targeting different steps of tumor progression. These findings have significant clinical implications. Better understanding of the molecular nature of tumor progression in hormone dependent malignancies has allowed the characterization of additional prognostic markers for these cancers. Here, we propose a prognostic marker-based adjuvant therapy for breast cancer treatment as illustrated in Figure 6.1. In addition to assessing the lymph node status and hormone receptor status, node positive patients who are at a higher risk of developing metastatic lesions at distant sites will also be evaluated for expression of well established molecular tumor markers, such as EGF/EGFR, cyclin D and proteases like uPA / uPAR, cathepsin D and metalloproteases. Based on this information, in addition to the standard hormone therapy or / chemotherapy, a cocktail containing agents against the detected tumor markers will also be included in the regimen as anti-invasive and anti-metastatic therapy. Using this strategy, specific treatment plans can be designed based on the profile of molecular risk factors, thus avoiding unnecessary side effects associated with cytotoxic chemotherapy which has minimal effectiveness in preventing tumor growth and progression where these growth factors and proteases are involved. The promising results from combination treatment will encourage the development of novel therapeutic

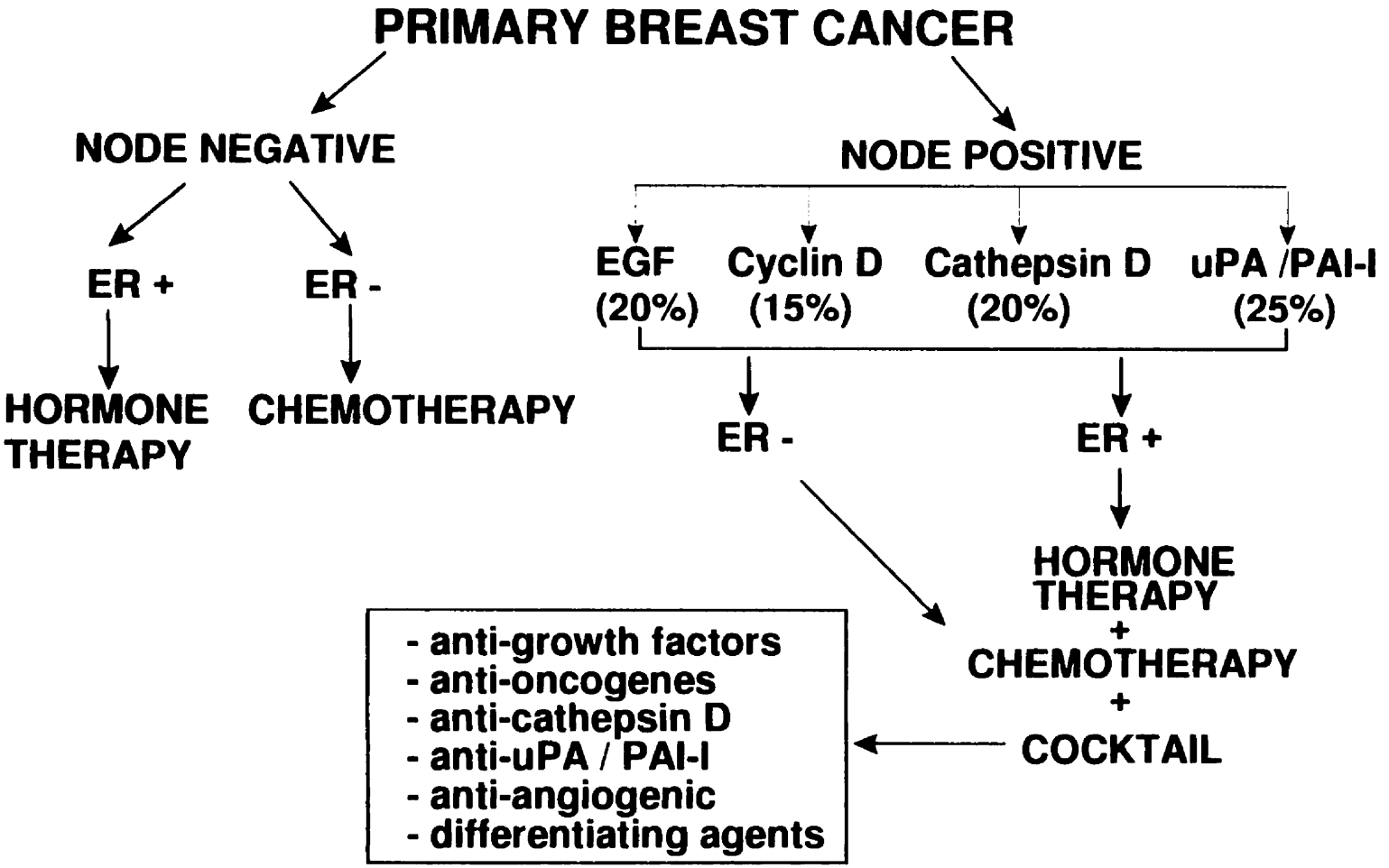

Figure 6.1: **Prognostic marker-based systemic adjuvant therapy in breast cancer**

strategies which will help to eventually achieve the goal of total control of tumor progression. Future studies employing combination treatment could be expanded to include in the treatment regimens a cocktail containing anti-estrogens / antiandrogens, cytostatic agenets, nucleoside analogous, anti-invasive / anti-metastatic agents, and anti-angiogennic agents.

6.4. HORMONAL REGULATION OF uPA GENE EXPRESSION IN HORMONE DEPENDENT MALIGNANCIES

While uPA and uPAR, as described above, have been implicated in the progression of hormone dependent malignancies, the mechanisms which lead to their overexpression in these malignancies are poorly understood. Stromal influences (i.e. increased production of uPA and uPAR by stromal cells, or paracrine stimulation of the tumor cell production of uPA and uPAR by stromal-derived growth factor or cytokines) have been shown to play important roles in the regulation of uPA and uPAR production by tumor cells (Hewitt R and Dano K, 1996; Jonca F et al., 1997; Sordat I et al., 1997). However, the control mechanisms governing uPA and uPAR expression in tumor cells are largely unknown. Mechanisms contributing to the control of a particular gene expression include gene amplification, regulation of the stability of mRNA transcripts and transcriptional regulation. Information from various sources demonstrate that among these mechanisms, uPA and uPAR expression by tumor cells is mainly regulated at the level of gene transcription (Rorth P et al., 1990; Reifel-Miller AE et al., 1996; Lengyel E et al., 1996), although stabilization of the uPA and uPAR transcripts has also been shown to lead to higher level of the protein production (Gaido KW and Maness SC, 1995; Nanbu R et al., 1995).

The expression of the uPA gene is regulated by the 2.3 Kb 5' flanking sequence which contains binding sites for various transcription factors such as PEA3, AP-1, NF-kB, SP1 and cAMP response elements (Verde P et al., 1988). Transcription of the gene yields a 2.5 Kb message which is translated into a single chain glycosylated proenzyme with a MW of 55 kDa (Wun TC et al., 1982; Rabbani SA, 1995). uPA gene expression is regulated by a wide variety of agents including growth factors, angiogenic factors and cytokines (Laiho M and Keski-Oja J, 1989; Pepper MS et al., 1992; Desruisseau S et al., 1996; Guerra F et al., 1996; Koolwijk P et al., 1996; Liu DF and Rabbani SA, 1996; Panozzo MP et al., 1996; Roghani M et al., 1996; Aguirre Ghiso JA et al., 1997). The induction of uPA gene expression requires intact binding sites for AP-1, PEA3 and NF-kB (Rorth P et al., 1990; Reifel-Miller AE et al., 1996; Lengyel E et al., 1996). There is ample evidence demonstrating that the activity or / and synthesis of these transcriptional factors is controlled by the interplay of multiple signal transduction pathways which are known to mediate the actions of growth factors and cytokines (Basheeruddin K et al., 1995; Newton R et al., 1996; Urban RJ et al., 1996; Yan GZ and Ziff EB, 1997). Therefore, it is very possible that growth factors and/or cytokines derived from the tumor cells or the surrounding stromal cells, bind to their receptors leading to the sequential activation of *ras*, *raf*, MAPKKs and ERKs which modulate the activity and/or synthesis of transcription factors.

In addition to transcriptional mechanisms regulating uPA gene expression, an interplay between the different cell types via paracrine interactions may be an essential requirement for the function of the uPA system in tumor progression. The expression pattern of the components of the uPA system probably depends on the complex signalling

network mediated by growth factors and cytokines, through which tumor cells communicate with stromal cells and vice versa (Ossowski L et al., 1991). The uPA system itself may be involved in this process by activating latent growth factors which may in turn regulate the expression of uPA and uPAR (Salsela O and Rifkin DB, 1990; Odekon LE et al., 1994; Baillie CT et al., 1995; Andreasen PA et al., 1997).

In spite of the well documented regulation of uPA by growth factors and cytokines, the role of sex steroids in the regulation of uPA gene expression during the progression of hormone dependent malignancies like breast and prostate cancer is not well understood. In addition, although growth factors, hormones and proteases have been implicated in breast and prostate cancer progression, the underlying molecular events and mechanisms involved in the transition to and establishment of the hormone insensitive state has not been well characterized. In the literature, a number of studies have attempted to address the issue of hormonal regulation of uPA gene expression in breast cancer *in vitro* by monitoring the activity and production of uPA after adding exogenous estrogen to the culture medium, or after removal of the estrogenic agents from the culture medium (Ossowski L et al., 1979; Casslen B et a., 1995; Fujimoto J et al., 1996; Long BJ and Rose DP, 1996). Due to the difficulty of controlling assay conditions, these studies resulted in conflicting results. Furthermore, there was no appropriate *in vivo* model of breast or prostate cancer to test the effect of hormones on tumor progression and metastasis. In the current study (Chapter 4), generation of a hormone sensitive PC-3 cell line (PC-3T) by insertion of a functional human androgen receptor has provided a useful model for delineating the relationship between androgen and uPA gene expression in hormone sensitive prostate cancer cells. Results from this study indicate that maintenance of hormone sensitivity helps to keep a

non-invasive and low virulent phenotype at the initial hormone sensitive state of prostate cancer.

Prostate cancer is characterized by its progression to a hormone independent state which is the major cause of the failure of the current therapeutic strategies (Jordan VC, 1995). At present, the mechanisms underlying the arise of such hormone insensitivity in prostate cancer is not known. Results from the current study may also implicate a potential mechanism of androgen action in mediating the acquisition of a more malignant phenotype during hormonal treatment. Clinical studies have shown that in pre-menopausal women, anti-estrogen therapy at the initial hormone responsive stage of breast cancer results in increased tumor growth, invasion and metastasis (Grainger DJ and Metcalfe JC, 1996). This strategy decreases the availability of androgens to tumor cells in the presence of competing anti-estrogen, thus promoting a subset of hormone-responsive cells to produce more uPA, which in turn endows tumor cells with a higher invasive and metastatic potential. On the other hand, increased uPA production could promote tumor progression via its stimulatory effect on angiogenesis, cell adhesion and migration, and cell invasion and metastasis. It is not yet known whether the effect of androgen on uPA gene expression is a direct effect. However, the presence of a putative androgen responsive element (ARE) in the published sequence of uPA promoter (Blasi F, 1988) opens the possibility that AR could directly down-regulate the expression of uPA mRNA in hormone sensitive PC-3T cells. Future studies can be carried out to address this issue by assessing the effect of androgen on uPA gene expression after mutating the androgen responsive elements of the uPA gene.

In Chapter 3, we reported the lack of effect of the estrogen receptor antagonist TAM on uPA gene expression in a rat breast cancer model MAT B-III, implicating a lack of regulation of uPA by hormones. In contrast to this, results from Chapter 4 on regulation of uPA production in androgen-responsive human prostate cancer cells PC-3T clearly demonstrated a positive correlation between hormone responsiveness and uPA gene expression, implicating a regulatory role of androgens. However, the potent capacity of flutamide, the androgen receptor antagonist, in reversing the inhibition of uPA by androgens clearly indicates that actions of androgens are most likely mediated by AR. The lack of effect of TAM on uPA production in Mat B-III cells could be due to a ER-independent mechanism of the action of TAM. The discrapencies between the two observations could also be due to species-specific differences in the transcriptional regulation of uPA gene expression by hormones in hormone dependent malignancies.

With the help of this model, we have clearly demonstrated an inhibitory role of androgen on uPA gene expression; and for the first time, the effect of hormone responsiveness on tumor growth, invasion and metastasis has been assessed *in vivo*. Athymic nude mice inoculated with hormone responsive PC-3T cells developed significantly smaller tumors and exhibited a later onset compared with animals receiving non-transfected hormone insensitive PC-3 cell. The more rapid progression (higher rate of primary tumor growth and increased metastases in distant organs) of prostate cancer in castrated mice compared to non-castrated controls has significant clinical implications. It points to the importance of complete deprivation of any androgenic source to prevent prostatic tumor growth. If complete androgen ablation is not achieved, residual amounts of androgens could not only stimulate tumor growth, but also promote a subset of hormone

sensitive tumor cells to acquire a more malignant phenotype via increased production of tumor progression factors like uPA.

Detection of micrometastase was made possible by using GFP-labelled PC-3 and PC-3T cells *in vivo*. GFP gene was cloned from the bioluminecent jellyfish (Morin J and Hastings J, 1972). GFP-labelling of cancer cells provides an effective, simple and sensitive way to visualize micrometastases in fresh viable target organs such as the livers, lungs and draining and regional lymph nodes at the single-cell level. It will also be useful to detect the presence of tumor cells in the circulation which is essential to address questions related to the process of tumor progression, such as the onset of tumor invasion and metastasis and the relationship between primary tumor burden and the number of tumor cells shedding into the circulation. Differences in distant metastases at various organs among different animal group are consistent with our *in vitro* finding that hormone sensitivity is necessary for the maintanence of a low virulent malignant phenotype by inhibiting the expression of tumor progression factors such as uPA. In contrast, decreased availability of androgens to androgen-sensitive prostate cancer cells can promote these cells to acquire a more invasive and metastatic phenotype by increasing the production of agents like uPA. Results of the current study have significant clinical implications in the management and treatment of prostate cancer.

6.5. ROLE OF DNA METHYLATION IN THE DIFFERENTIAL TRANSCRIPTIONAL REGULATION OF GENE TRANSCRIPTION IN HORMONE DEPENDENT MALIGNANCIES

As discussed above, the progression of hormone dependent malignancies like breast and prostate cancer is characterized by the transition of an early hormone responsive, low virulent phenotype to the hormone insensitive variety accompanied by increased invasive and metastatic potential which becomes refractory to hormonal therapy (Russo J and Russo IH, 1995; Chiarodo A, 1991). Acquisition of a more malignant phenotype during tumor progression is associated with the loss of functional hormone receptors due to mutations in the receptor or interference in hormone receptor signalling pathways (Kitzenellenbogen BS, 1991; Zhang QX et al., 1993). However, the molecular mechanisms regulating this complex transition remain poorly understood. The fact that increased uPA production is associated with higher invasive and metastatic potential, and uPA mRNA is expressed abundantly in breast and prostate cancers as compared to normal mammary tissues, benign breast adenomas and benign prostatic hyperplasia strongly implicates a link between the level of uPA production and the progression of hormone dependent malignancies like breast and prostate cancer.

In the current study (Chapter 5), we have characterized uPA gene expression in several human breast and prostate cancer cell lines which represent hormone responsive and hormone insensitive states of tumor growth. Results from this study have clearly demonstrated, for the first time, a differential transcriptional regulation of uPA gene expression in hormone dependent malignancies and a correlation between hormone responsiveness and cell invasiveness. Demonstration of a transcriptional inhibition of uPA gene expression in hormone responsive human breast and prostate cancer cells has provided a mechanism for these hormone responsive cells toturn off uPA gene expression under normal physiological state and in early hormone responsive stage of breast and

prostate cancer. Furthermore, the absence of uPA gene expression by health and non-invasive mammary epithelial cells indicates that uPA expression is not only a feature of the malignant state, but more importantly, it is characteristic of the invasive and metastatic phenotype. The differences in uPA gene transcription in hormone responsive and hormone insensitive cells are of particular significance, since no differential changes in uPA mRNA expression have been reported at different stages of tumor progression in non-hormone dependent malignancies such as cancers of lung, colon and leukemia (Bolon I et al., 1997; Sordat I et al., 1997).

Recently, cytosine DNA methylation, which regulates gene transcription by epigenetic mechanisms, has been the focus of cancer research. Epigenetic modifications mediated by DNA methylation have been shown to play important roles in the regulation of mammalian gene expression and genetic presentation (Sapienza C et al., 1987; Razin A and Cedar H, 1991; Zuccotti M and Monk M, 1995). Deregulation of cytosine DNA methylation, either by hypermethylation or hypomethylation, has been reported in various human malignancies including breast and prostate cancer (Feinberg AP et al., 1983; Yisraeli J and Szyf m, 1984; Baylin SB et al., 1991; Razin A and Cedar H, 1991; Christman JK et al., 1993). Hypermethylation is responsible for the inactivation of various tumor suppressor genes (Ohtani-Fujita N et al., 1993; Herman JG et al., 1994; Ottaviano YL et al., 1994; Merlo A et al., 1995; Yoshiura K et al., 1995; Herman JG et al., 1996; Ahuja N et al., 1997; Nelson et al., 1997). Accumulating evidence suggests that DNA methylation can mark certain genes for inactivation either directly, by interfereing with the binding of some transcriptional factors to regulatory sequences (Becker PB et al., 1987), or indirectly, by either attracting the binding of proteins that have high affinity to methylaed

DNA (Huang LH et al., 1984; Razin A and Cedar H, 1991; Nan X et al., 1997), or by precipitating an inactive chromatin structure (Kass SU et al., 1997). DNA hypomethylation, on the other hand, is associated with the activation of oncogenes and tumor promoting genes to promote tumor progression (Feinberg AP et al., 1983; Christman JK et al., 1993). The most significant finding of the current study is that it provides compelling evidence for DNA methylation as a unique molecular mechanism involved in the transcriptional regulation of uPA gene expression to allow the production of this critical protease to be turned-on and turned-off at different stages of these hormone dependent malignancies.

A question that remains unanswered is whether the uPA gene becomes unmethylated / demethylated during breast cancer progression, as a consequence of loss of hormone responsiveness. Due to the strong correlation between hormonal status, uPA gene expression and the methylation status of the CpG islands of the uPA gene in these hormone sensitive and hormone insensitive breast and prostate cancer cells, it is highly likely that these events are hormonally regulated. Although aberrant DNA methylation is associated with malignant transformation and progression, the mechanisms regulating the event of DNA methylation are not fully delineated. The present study has opened new avenues to investigate the regulation of DNA methylation in hormone dependent malignancies. Cytosine DNA methylation is catalyzed by a specific enzyme known as DNA methyltransferase (Szyf M, 1994) which has recently been shown to be under the regulation of *ras* oncogenes (Szyf M et al., 1991; Macleod AR et al., 1995; Szyf M et al., 1995). Therefore, changes in DNA methylation in cancer could be critical components of the oncogenic programs. Moreover, recent studies have reported the presence and induction

of a demethylase activity as a result of *ras* overexpression (Syzf M et al., 1995). These studies collectively suggest that the methylation status of a given gene is most likely determined by the methylase and demethylase activity. Therefore, it is quite possible that DNA methyltransferase and the uncharacterized demethylase are ER-responsive genes, thus during the transition of breast and prostate cancer into a hormone insensitive variety, the balance of methylation / demethylation becomes in favor of demethylation which leads to a genome wide hypomethylation. This will lead to the activation of oncogenes and tumor promoting genes like uPA to promote tumor growth, invasion and metastasis. However, the mechanisms responsible for coordinating the expression of DNA methyltransferase and demethylase in cancer cells are not known. It is quite likely that both activities are regulated by the same oncogenic pathway. In hormone dependent malignancies like breast and prostate cancer, sex steroids estrogen and androgen may play a key role in the differential regulation of DNA methylation events to control the transcription of a specific gene.

A better understanding of the molecular basis of malignant progression in these hormone dependent malignancies will not only enhance our knowledge of the biological basis of these cancers, but will also lead to the development of new therapeutic approaches for controlling these common malignancies. Since inactivation of tumor suppressor genes is an early event in breast and prostate cancer, and overexpression of tumor promoting factors like uPA is a relatively late event, new therapeutic strategies aimed at altering the methylation statuscould be developed to activate tumor suppressor gene expression in patients diagnosed with hormone responsive and locally confined early stage breast cancer to reverse or stabilize the malignant state; or to inactivate oncogenes and tumor promoting

genes in patients diagnosed with HI and invasive late stage breast and prostate cancer to prevent / decrease the rate of tumor progression.

6.6. Suggestions for Future Research

The following aspects require further investigation:

1. Elucidation of the effects of increased uPA and uPAR production on tumor angiogenesis and its role in mediating primary tumor growth and establishment of distant metastasis.

2. Evaluation of the functional significance of the GPI anchor in mediating the signal transduction function of uPAR.

3. Analysis of the potential interactions between uPA-plasmin system and metalloproteinases (MMPs) in the progression of breast and prostate cancer and the relative importance of the two during tumor progression.

4. Development and evaluation of new uPA / uPAR-based therapeutic stretegies in the prevention of tumor growth and tumor progression.

5. Elucidation of the molecular mechanisms underlying the differential regulation of uPA gene transcription in breast and prostate cancer by cytosine DNA methylation: (i) the role of DNA methyltransferase; (ii) regulation of DNA methyltransferase gene expression and activity by growth factor, oncogene and hormone-mediated signalling pathways.

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