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Urokinase plasminogen activator and metalloproteinase two during urogenital sinus development in rodents

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UROKINASE PLASMINOGEN ACTIVATOR AND METALLOPROTEINASE
TWO DURING UROGENITAL SINUS DEVELOPMENT IN RODENTS

by

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DISSERTATION

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UROKINASE PLASMINOGEN ACTIVATOR AND METALLOPROTEINASE TWO DURING UROGENITAL SINUS DEVELOPMENT IN RODENTS

FRED ELFMAN

ABSTRACT

Cellular proliferation, migration, and an increase in the turnover of the extracellular matrix (ECM) are features that are common to the tissue remodeling that occurs during both prostatic organogenesis and carcinogenesis. Urokinase plasminogen activator (uPA) and metalloproteinase two (MMP-2) are proteolytic enzymes that are suggested to mediate the remodeling of the ECM. Rat VPs express uPA and MMP-2 during development. The current study examined the tissue expression of uPA, uPA receptor (uPAR), and MMP-2 during development of the rat VP; the activation of MMP-2 during development of the mouse BUG; as well as the function of uPA and uPAR during the development of rat VPs. We report that *In situ* hybridization analysis of developing rat VPs demonstrated that uPA and MMP-2 mRNA were expressed exclusively by the mesenchyme. uPA and MMP-2 mRNA were also expressed in the adult rat VP exclusively in the stroma. Zymographic analysis demonstrated that uPA and MMP-2 protein were localized in both epithelium and mesenchyme of newborn rat VP. Zymographic analysis of detergent extracts suggested that levels of activated MMP-2 decrease progressively with age in the mouse bulbourethral gland (BUG), as has been reported previously in rat VPs. Histochemical staining for activated uPA in frozen tissue sections of adult rat VP demonstrated that activated uPA was localized in smooth muscle and epithelium of adult rat VPs. The role of uPA ligand and uPAR during VP development was assessed by perturbing the action of uPA and uPAR with specific chemical and peptide inhibitors added to the culture medium of newborn rat VPs cultured for one week. uPA antagonists severely disrupted ductal branching morphogenesis of

newborn rat VPs cultured for one week under serum-free conditions as demonstrated by significantly reduced numbers of terminal ductal tips, as well as retarded histodifferentiation of the epithelium and mesenchyme. Newborn rat VPs treated with uPAR antagonists also demonstrated reduced growth and ductal branching morphogenesis as measured by reduced numbers of terminal ductal tips. In addition, retarded histodifferentiation was observed in both the epithelium and mesenchyme. In the current study, we demonstrated that uPA and MMP-2 are important components of epithelial-smooth muscle interactions that regulate development of the rat VP.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER ONE

INTRODUCTION

INTRODUCTION

1.1 General Introduction

Carcinoma of the prostate is a serious health problem. Recently, an hypothesis that unifies prostatic development and carcinogenesis has been proposed (Cunha, 1994; Hayward et al., 1996b). The foundation of this hypothesis is a reciprocal interaction of epithelium and mesenchyme during prostatic development followed thereafter by a reciprocal homeostatic interaction of epithelium and smooth muscle in adulthood. In the normal adult prostate, the growth quiescent epithelium is organized into ducts lined with tall, secretory, columnar epithelial cells. Layers of smooth muscle cells surround the epithelial ducts. During prostatic carcinogenesis, dynamic tissue modeling disrupts smooth muscle-epithelial interactions, resulting in adverse consequences for both tissues. The epithelium dedifferentiates, proliferates, invades extracellular matrix (ECM), and often metastasizes to pelvic bones. The smooth muscle tissue dedifferentiates into fibroblastic cells (fig. 1.1). The paracrine factors that mediate these processes are poorly understood.

The predominant cell type surrounding epithelial ducts in the adult prostate is the smooth muscle cell (Ichihara and Pelliniemi, 1975; Ichihara et al., 1978), which in the rodent prostate is organized into thin peri-ductal bundles, while in the human prostate is organized into thick bundles (fig. 1.2) (Shapiro et al., 1992). However, in both human and rodent prostates smooth muscle cells are each in intimate contact with the adjacent basement membranes (BM's) of epithelial ducts. Therefore, in humans and rodents, the androgen receptor (AR)-positive smooth muscle cells are ideally positioned for paracrine interactions with prostatic epithelium, and in adulthood the continued interaction of prostatic epithelium with its immediate cellular micro-environment is in fact an

Figure 1.1

Smooth muscle in the adult human prostate

Immunohistochemical staining for smooth muscle alpha actin in and around a human prostate tumor. The left of the field shows benign ducts surrounded by well-differentiated smooth muscle. To the right of this area is the margin of the tumor with progressively less differentiated smooth muscle at the tumor margins, towards the right side of the field. Higher magnifications of normal and cancerous tissue can be viewed in the lower left and right panels, respectively. In the cancerous tissue, smooth muscle alpha actin is limited to thin loosely organized strands of tissue between the abortive ductal structures. Some stromal cells that are alpha actin-negative can be seen.

Figure 1.1

Smooth Muscle in Adult Human Prostate

Immunohistochemical Staining for Smooth Muscle α–Actin in Normal Adult Prostate and in Adenocarcinoma of the Prostate

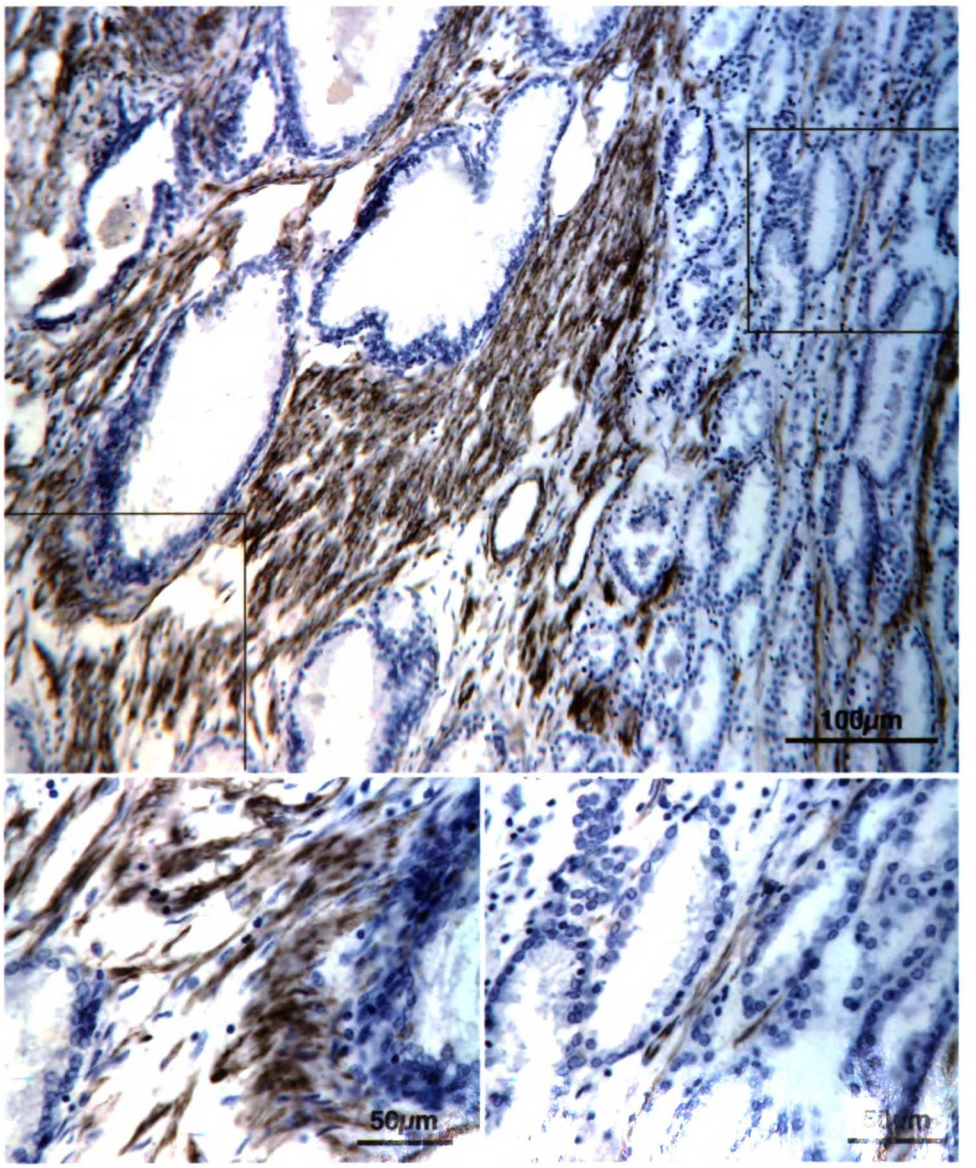


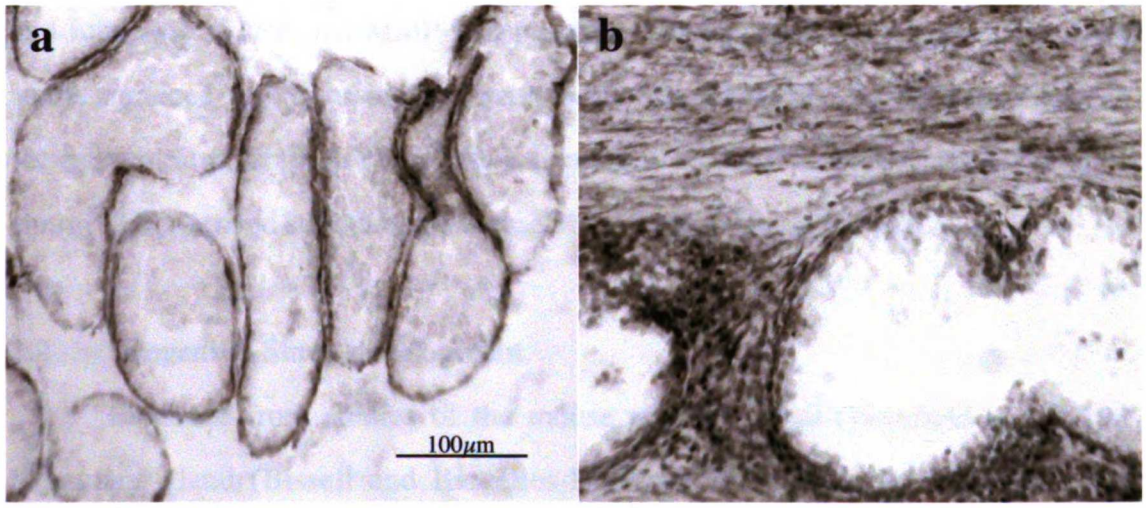
Figure 1.2

Rat and human adult prostates

Immunohistochemical staining for smooth muscle alpha actin in adult rat ventral prostate and adult human prostate. In the rat prostate (a), smooth muscle is organized into thin sheathes as 3-5 cells thick around epithelial ducts. In contrast, in the human prostate (b), smooth muscle is widely distributed throughout the stromal component of the gland.

Figure 1.2

a) adult rat ventral prostate
b) adult human prostate
(immunohistochemical staining for smooth muscle alpha actin)



epithelial-smooth muscle interaction. Although the human prostate contains many more layers of smooth muscle than the rodent, the rat prostate offers a useful model system for studying epithelial-smooth muscle interactions.

The ECM-degrading proteinases urokinase plasminogen activator (uPA) and metalloproteinase-2 (MMP-2) are among the postulated mediators of tissue remodeling during organogenesis and are therefore likely to be involved in prostatic epithelial-smooth muscle interactions. The expression and activation of uPA and MMP-2 are generally increased during organogenesis (Alexander, 1991). Moreover, the substrates of uPA, plasmin, and MMP-2 are at the "molecular crossroads" of many processes considered to be vital to tissue remodeling: ECM molecules, most importantly the basement membrane (BM), cell-ECM linking receptors, cell-cell linking receptors, proteinases, and cytokines (Alexander, 1991). However, the precise tissue expression and functions of uPA and MMP-2 in prostatic organogenesis are unclear. The current study examined the expression and function of uPA and MMP-2 during organogenesis of the rat ventral prostate (VP) and the activation of MMP-2 in another rodent urogenital sinus (UGS)-derived structure, the mouse bulbourethral gland (BUG).

1.2 Urogenital Sinus Development

Evidence from studies of the mouse salivary gland (Bernfield et al., 1984), mammary gland (Bissell and Barcellos-Hoff, 1987; Kratochwil, 1987; Daniel and Robinson, 1992; Imagawa et al., 1990), and the prostate (Cunha et al., 1983; Cunha et al., 1987) suggest that hormones, cytokines, and the ECM provide biochemical information required for ductal branching morphogenesis and histodifferentiation. In all of these glands, ductal branching morphogenesis entails invasive growth of epithelial ducts into the mesenchyme. During this process, BM and mesenchymal interstitial ECM must be constantly remodeled to accommodate the expanding epithelial mass. In some organs, organogenic processes are absolutely dependent upon steroid and/or peptide hormones,

e.g., the mammary gland (Daniel and Silberstein, 1987; Kratochwil, 1987; Sakakura, 1987; Cunha and Hom, 1996), as well as the androgen-dependent seminal vesicle (Higgins et al., 1989b; Higgins et al., 1989a), prostate (Cunha et al., 1987), and BUG (Cooke et al., 1987a; Cooke et al., 1987b).

Mesenchymal-epithelial interactions play crucial roles in prostatic and BUG development. The term mesenchymal-epithelial interaction refers to a cell-cell interaction initiated during embryonic periods in which mesenchyme induces epithelial development. For the developing prostate, urogenital sinus mesenchyme (UGM) induces prostatic bud formation, ductal branching morphogenesis, epithelial proliferation, as well as histodifferentiation (expression of epithelial androgen receptors and prostatic lobe-specific secretory proteins) (Cunha, et al., 1987; Chung and Cunha, 1983; Sugimura, et al., 1986a; Sugimura, et al., 1986c). While it is evident that UGM induces prostatic epithelial differentiation, the reciprocal is also true, that is, prostatic epithelium induces the adjacent mesenchyme to differentiate into smooth muscle (Cunha and Donjacour, 1989; Cunha, et al., 1992).

In the embryo, the Mullerian and Wolffian ducts are present within the urogenital ridges and terminate caudally by emptying into the sexually indifferent urogenital sinus (UGS). In males, the testis differentiates forming seminiferous cords. Leydig cells differentiate in the mesenchyme between the seminiferous cords and produce testosterone (T). T prevents the normal programmed cell death of the mesonephric tubules and the Wolffian duct, as well as stimulates the mesonephric tubules to differentiate into the efferent ducts. Further androgenic stimulation elicits regional development of the Wolffian duct into the epididymis, ductus deferens, and seminal vesicles, whose epithelia are derived from the mesodermal germ layer. Androgens also induce the UGS to form the prostate and BUG, as well as to evoke masculinization of the urethra. The epithelia of the prostate, BUG, and urethra are derived from the endodermal germ layer. Masculinization of the UGS and external genitalia is triggered by the more potent

androgen, dihydrotestosterone (DHT), produced by the enzyme 5-alpha-reductase which converts T to DHT in the developing UGS and external genitalia. DHT and T bind to nuclear androgen receptors. Ligand-associated androgen receptors regulate gene expression as trans-acting enhancer elements (Evans, 1988).

Epithelial buds of the prostate and BUG appear as cord-like protrusions from the urethra on embryonic day 17.5 in the mouse and day 19 in the rat. Unlike the human prostate, the rodent prostate is divided into anterior, dorsolateral, and ventral lobes. The nascent epithelial buds elongate and undergo branching through the formation of clefts. Newly formed buds at branch points are stabilized and elongate during the process of ductal branching morphogenesis (fig. 1.3). Ductal branching morphogenesis continues throughout neonatal as well as pubertal periods to form a highly arborized ductal network that is organized into ductal-acinar structures. Epithelial cytodifferentiation occurs when the solid "ducts" (fig. 1.4) undergo canalization to form a lumen, while the mesenchyme differentiates into smooth muscle immediately around ducts beginning proximally at the urethra and extending distally (Hayward et al., 1996a). In the adult prostate and BUG, the epithelium is composed of mixed populations of tall columnar secretory cells and dendritic basal epithelial cells that are organized into ductal-acinar structures (fig. 1.4d). In post-pubertal rodents, prostatic and BUG epithelium synthesizes, stores, and releases components into the seminal fluid that facilitate, but are not necessary for fertility (Cunha et al., 1987). The fibromuscular stroma of the adult prostate and BUG (both derived from UGM) contains fibroblasts, smooth muscle cells, nerves, and vasculature.

The VP and BUG of the rat and mouse undergo virtually all of their ductal branching morphogenesis neonatally. The newborn rat VP can be isolated and cultured as an explant under serum-free conditions (Sugimura et al., 1986b). The development of rat VPs in organ culture mimics morphogenesis and differentiation of this gland *in vivo*. At birth, rat VPs consists of 4 main ducts that each contain one to three terminal tips.

Figure 1.3

Ductal Branching morphogenesis of the rat ventral prostate

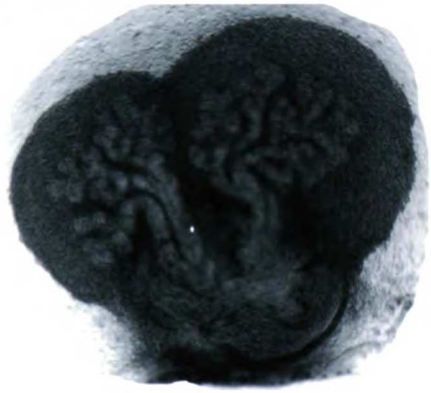
Whole mount Fisher 344 strain rat ventral prostates. a) newborn; b) cultured for 3 days; c) cultured for 6 days.

Figure 1.3

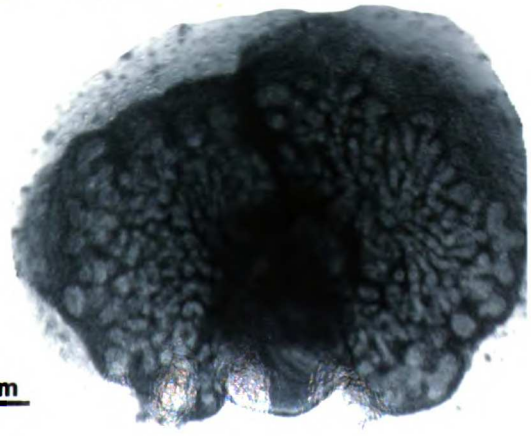
Ductal branching morphogenesis of the rat ventral prostate

a) newborn

b) cultured for 3 days



c) cultured for 6 days



0.5mm

Figure 1.4

Histodifferentiation of the rat ventral prostate

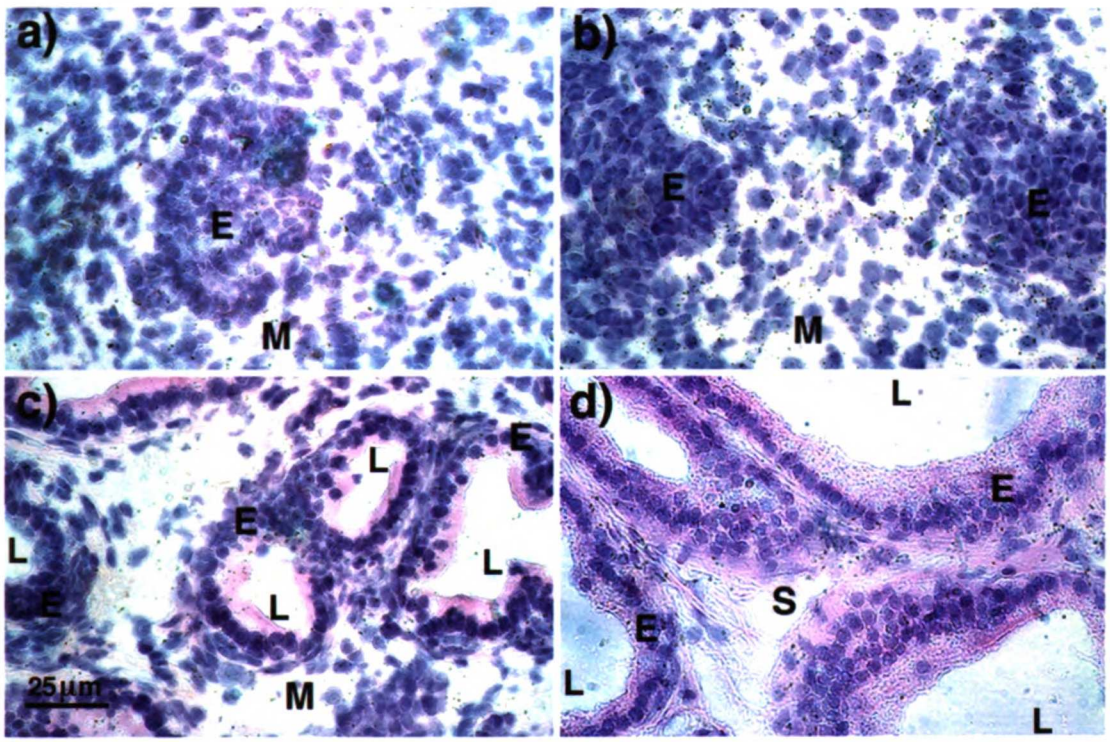
Hematoxylin and eosin stained frozen sections of Fisher 344 rat ventral prostates from: a) newborn, b) 3 day, c) 14 day, d) adult. The epithelial-mesenchymal/stromal border contains the basement membrane, although it is *not* visible in this preparation. Bar, 25 mm.

Figure 1.4

Histodifferentiation of rat ventral prostates

Hematoxylin/eosin staining of frozen tissue sections from developing and adult Fisher 344 rat ventral prostates

a) newborn, b) 3 day, c) 14 day, d) adult
E: epithelium; M: mesenchyme; S: stroma; L: lumen



The epithelial ducts are solid and uncanalized. The mesenchyme is composed of vimentin-positive fibroblasts and a few alpha actin-positive smooth muscle cells in intimate association with the epithelium (Hayward et al., 1996a).

During one week of culture in the presence of 10^{-8}M T, extensive ductal branching morphogenesis occurs with the formation of approximately 50 terminal ductal tips. Ductal branching morphogenesis induced by T is associated with ductal canalization and the differentiation of cytokeratin 8- and 18-positive columnar luminal cells and cytokeratin 5- and 14-positive basal cells from the solid "ducts" that originally were composed of epithelial cells co-expressing all of these cytokeratins. Prostatic epithelial cells induce the differentiation and patterning of the UGM into smooth muscle immediately adjacent to the ducts. As smooth muscle differentiates, vimentin expression is reduced in the peri-ductal mesenchyme as sleeves of smooth muscle form. Following one week of culture in the absence of androgens, rat VPs exhibited reduced proliferation as well as ductal branching morphogenesis and generated only about 20 terminal ductal tips that remain uncanalized. Compared to rat VPs cultured with T, peri-ductal mesenchymal fibroblasts expressed less smooth muscle alpha actin, while vimentin expression was maintained throughout the mesenchyme, reminiscent of newborn rat (Hayward et al., 1996a).

Thus, organ culture of newborn rat VPs provides a good model system for studying the tissue regulation and function of uPA and MMP-2 during ductal branching morphogenesis. Insights gathered in these studies may hopefully benefit the understanding of the tissue regulation of these proteinases in prostate cancer.

1.3 Proteinases

a) Families and Background

At the turn of the century, *in vitro* cell culture utilized plasma clots for culture media. From the onset of these studies, two observations became apparent. First, that

embryonic and tumor cells tended to proliferate and migrate more rapidly than normal adult cells. Second, that tumor cells also degraded the surrounding clot more readily than normal cells. Recently, tumor cells have been shown to express higher levels of ECM-degrading proteinases than their normal, less-proliferative counterparts. Proteinases were viewed as effectors of ECM degradation. Their roles in regulating cellular proliferation and migration were postulated to be indirect and limited to mediating the degradation of structural as well as biochemical substrates in the ECM. This classical (and intuitive) model of proteinase function during tissue remodeling has persisted. However, recent data suggest that ECM-degrading enzymes also can act as ligands, directly mediating cellular proliferation and migration.

Proteinases are endopeptidases that can hydrolyze peptide bonds in the primary amino acid sequence of a polypeptide. After their synthesis by either cytoplasmic or endoplasmic reticulum-associated ribosomes, proteinases can be: a) retained in intracellular vesicles, eg., in lysozomes and other vesicles, b) associated with the plasma membrane, or c) secreted extracellularly (Alberts et al., 1989). Intracellular proteinases are thought to regulate the trafficking of free and vesicle-associated proteins. In addition, evidence suggests that a calcium-dependent family of calpain-type proteinases regulates the cytoskeleton, cell shape, and in turn cell-ECM adhesion (Turner and Burridge, 1991). Plasma membrane-associated and secreted extracellular proteinases are active in the extracellular environment as well as within exo-and endocytitic vesicles. Many plasma membrane and secreted proteinases are active at neutral pH. These proteinases have a variety of substrates and can degrade soluble and ECM-associated molecules such as glycosaminoglycans, glycoproteins, other proteinases, proteinase inhibitors, and cytokines (Flaumenhaft and Rifkin, 1991). In addition, proteinases can degrade plasma membrane-associated glycosaminoglycans and glycoproteins (Alexander, 1991).

Proteinases are classified according to the presence of an amino acid residue or metal atom in the enzyme's active site. The five major classes are: threonine, aspartic,

cysteine, serine, and metallo-proteinases (Alexander, 1991). The latter four include members that can be secreted extracellularly. Serine and metalloproteinases are suggested to mediate tissue remodeling. Proteinases that remodel tissue are often referred to as ECM-degrading proteinases although other functions have been suggested. ECM-degrading proteinases hydrolyze peptide bonds in substrates that include: a) cell surface molecules, including cell surface receptors, b) ECM molecules; c) cytokines that directly affect cell growth (Naldini et al., 1992); and d) cytokine binding proteins (Alexander, 1991; Bonner and Brody, 1991. ECM-degrading proteinase function is controlled at several levels including: a) transcription and translation, b) activation by other proteinases, c) inactivation by proteinase inhibitors, and d) cell-surface localization. ECM-degrading proteinases are suggested to play a central role in tissue remodeling during processes that include: inflammation (Alexander and Werb, 1989), wound repair (Clark, 1989), neoplastic invasion and metastasis (Liotta et al., 1986; Alexander, 1991), as well as implantation, early embryonic development and ductal branching morphogenesis (Bode and Dziadek, 1979; Brenner et al., 1989).

Plasminogen activator cascade

Serine proteinases are grouped into 32 families. Many families are thought to have arisen from convergent evolution. Each contains the amino acid serine as the catalytic residue. The chymotrypsin family of serine proteinases has a linear order of three catalytically active amino acid residues: His, Asp, Ser, and shares this tertiary structural characteristic with several other related families to form a serine proteinase Clan (Alexander, 1991). The chymotrypsin family includes trypsin, thrombin, clotting factor X, some complement components, and enzymes of the plasminogen activator cascade. The plasminogen activator cascade is a cascade of two or more chymotrypsin type enzymes that include a) enzymes: plasminogen, urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA), b) receptors: the uPA receptor (uPAR)

and plasminogen receptor, and c) inhibitors (fig 1.5a). uPA and tPA are the physiological activators of plasminogen. tPA is believed to mediate blood and perivascular fibrinolysis (Kwaan, 1992) as well as to participate in disease and embryonic development (Dano, 1985). uPAR-associated latent uPA is suggested to cleave plasminogen into plasmin, as the furthest upstream physiological activator of the plasminogen activator cascade. Activation of uPA is the presumed rate limiting step in the plasminogen activator cascade because once activated, uPA rapidly converts the latent zymogen plasminogen into plasmin. Plasmin is the terminal effector of the plasminogen activator cascade which rapidly degrades a broad range of substrates that appear to be important in tissue remodeling (Dano, 1985). Plasmin has a broad specificity including uPA, tPA, plasminogen and other serine proteinases, several metalloproteinases, various ECM components, and several cytokines (Alexander, 1991). Rodent and human uPA, uPAR, and plasminogen are approximately 90% homologous and are thought to function similarly, although not with each other. For example, rodent uPA will not bind to human uPAR and vice versa (Blasi, 1993).

Plasminogen is a 92kD glycoprotein synthesized by the liver and is among the most abundant proteins in blood and pericellular fluids at a concentration of 1-2 μM (Plow, 1986; Kwaan, 1992). The concentration of plasminogen is not considered to be rate-limiting in any reactions in the plasminogen activator cascade due to its many-fold higher concentration compared to other components. However, the existence and the role of locally produced plasminogen has not been examined. Plasminogen has a biological half life of 2.2 days. Structurally, plasminogen contains 24 disulfides and 5 kringle domains that are suggested to mediate formation of complexes with other serine proteinases, inhibitors and the ECM. The kringle domains contain binding sites for fibrin and uPA (Kwaan, 1992). Soluble plasminogen is expressed at a high concentration in the blood and pericellular fluids and is readily localized to cell surfaces by binding to a low-affinity receptor. Proteolytic activation of plasminogen into plasmin occurs by cleavage

of the Arg_{560}-Val_{561} peptide bond. Plasmin has broad specificity among substrates considered to be important in tissue remodeling including fibrin, fibronectin, vitronectin, laminin, and type IV collagen (Alexander, 1991). In addition, plasmin can hydrolyze plasminogen, uPA, thrombin, MMP-3, and MMP-1 (Dano, 1985; Quigley, 1990). Therefore, plasmin is suggested to be a target and an effector molecule of the plasminogen activator cascade in fibrinolysis, development, and disease (Dano, 1985; Kwaan, 1992).

uPA is secreted in rodents as an inactive single chain protein (scuPA) with a molecular weight of 48 kD, although rodent uPA migrates at a range of 40-48kD in substrate zymography (fig. 1.5a) (Dano, 1985). uPA is cleaved by plasmin at Arg_{156}-Phe_{157} or Lys_{158}-Ile_{159} (fig. 1.5b, arrows) into a disulfide linked, enzymically active two-chain molecule (tcuPA). Structurally, the 20kD A chain (Ser_1-Lys_{158}) of uPA contains the amino-terminal residue and an epidermal growth factor-like(EGF)/transforming growth factor alpha-like domain which mediate binding to the uPA receptor (uPAR) (fig. 1.5b, blue), followed by a kringle domain (fig. 1.5b, yellow). The A chain is connected to the B chain by a single cysteine-linked disulfide bridge. The 34 kD B chain contains the serine residue and the rest of the catalytically active domain (fig. 1.5b, red) (Kwaan, 1992).

In rodents, uPA expression begins with activation of embryonic genome at the two blastomere stage (Zhang, 1994) and is elevated in embryonic development throughout the mouse (Bode and Dziadek, 1979), in the avian heart (McGuire, 1992), and urogenital sinus (Timme et al., 1994) in comparison to normal adult tissues. Under normal adult conditions, uPA mRNA and protein are found in vasculature (Dano, 1985), leukocytes (Ellis et al., 1992), kidney (Kanalas, 1995), epididymis, testis, and prostate (Wilson et al., 1988; Wilson, 1995). Expression of uPA is increased during

Figure 1.5a

Urokinase plasminogen activator (uPA) outline

The enzymes, receptors, and inhibitors that are suggested to interact with uPA in the plasminogen activator cascade. Also, the location and regulation of uPA expression, and proposed functions of uPA.

Figure 1.5b

uPA amino acid sequence and domains

Primary amino acid sequence of uPA including: domain 1, amino terminal through amino acid residue 48 which includes the epidermal growth factor (EGF)/(transforming growth factor alpha (TGF-a)-like domain that mediates uPA binding to uPAR (**blue**); domain 2, amino acid residues 49-138, includes the kringle domain that is suggested to mediate the docking of uPA to plasminogen, plasmin, PAI-1, and PAI-2 (**yellow**). Unlike the kringle domain found in plasminogen, the kringle domain in uPA lacks two lysine residues that mediate localization to fibrin; domain 3, amino acid residues 204-353 which contain the catalytic residue, Ser-235, as well as the His and Asp residues that contribute to the active proteolytic domain (**red**), from (Kwaan92).

Figure 1.5a

Urokinase plasminogen activator (uPA) outline

Cofactors:
Plasminogen
Plasminogen receptor
uPA receptor
Plasminogen activator inhibitors 1,2

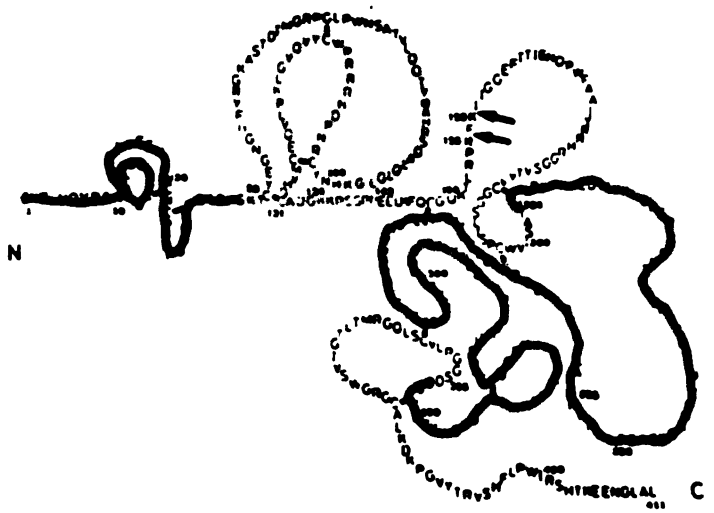
Means of expression:
AP-1, PEA3
ER-Golgi

Observed pattern of expression:
During extracellular matrix degradation and cellular migration in development; normal kidney, gut, vasculature, and urogenital function; inflammation; wound healing; cancer initiation and dissemination

Proposed functions:
extracellular matrix degradation
cellular migration
cellular proliferation
cytokine activation

Figure 1.5b

uPA amino acid sequence and domains



tumorigenesis and metastasis of prostate cancer (Wilson, 1995), as well as the migration and invasion of prostate cancer cells (Hoosein et al., 1991).

The function of uPA was initially believed to be restricted to the activation of plasminogen into plasmin. More recently, uPA has been shown to degrade certain substrates independent of plasmin, such as plasminogen, hepatocyte growth factor/scatter factor (HGF/SF) (Naldini et al., 1992), uPAR (Hoyer-Hansen et al., 1992), fibronectin (Quigley et al., 1987), and MMP-2 (Keski-Oja et al, 1992). uPA can also serve as a mitogenic ligand for endothelial cells and prostate cancer cells (Hoosein et al., 1991). Active uPA has a limited half life in solution, as do many other active serine proteinases (Dano, 1985). In addition, uPA is rendered inactive through the binding of both specific and broad spectrum inhibitors (Blasi et al., 1987). By contrast, uPAR-bound uPA is resistant to inactivation by inhibitors. Thus, uPAR regulates uPA activation, stability, and localizes its activity, thereby mediating cellular migration, mitogenesis and internalization of cell surface uPA (Blasi, 1993).

The *in vivo* expression and effects of the plasminogen activator cascade suggest an important role in development and disease. uPA and uPAR expression in development and disease has been widely studied and found to temporally and spatially correlate with tissue remodeling (Blasi, 1993). In carcinogenesis, uPA ligand and uPAR are frequently over-expressed in comparison to normal tissue, and are localized to either the neoplastic stroma (Pyke et al., 1993; Ohtani et al., 1995) or both stroma and epithelium (Bianchi et al., 1994; Romer et al., 1994). However, at the present time a physiological role for the plasminogen activator cascade has not yet been established since transgenic knockout mice develop normally even following targeted disruption of the genes encoding plasminogen (Bugge et al., 1995a), uPA (Carmeliet et al., 1994), or uPAR (Bugge et al., 1995b).

Matrix Metalloproteinases

In addition to the plasminogen activator cascade, other families of degradative enzymes can mediate ECM degradation during development. The first described vertebrate collagenase was identified in regressing amphibian tails as interstitial collagenase, metalloproteinase one (MMP-1). Metalloproteinases contain a zinc ion in the active site (Alexander, 1991). There are 25 families of metalloproteinases including the matrixins, which contain at least 14 members in 4 subclasses: a) the interstitial/fibrillar collagenases, b) the stromelysins, c) the membrane-tethered metalloproteinases (MT-MMPs), and d) the gelatinases. Matrixins are in the same Clan as the astacins (including BMP-1), the reprolysins (including rattle snake venom toxins), and the serralysins.

The interstitial/fibrillar collagenases include MMP-1 which is expressed in fibroblasts, macrophages and neoplastic epithelia; neutrophil collagenase (MMP-5) expressed in macrophages and neutrophils; and an interstitial collagenase (MMP-13) expressed in breast cancer epithelium, developing bone, and in the ovary (Pendas et al., 1997). These enzymes cleave alpha chains of collagen types I, II, and III at a single site producing peptide fragments that are substrates for other classes of metalloproteinases (Alexander, 1991).

The stromelysin family includes at least four members: Stromelysin-1 (MMP-3), stromelysin-2 (MMP-10), matrilysin (MMP-7), and stromelysin-3 (MMP-11). All are expressed by mesenchymal cells or fibroblasts (Matrisian, 1992). Expression of MMP-11 in the stroma of breast cancer is induced by association with breast cancer epithelial cells (Basset et al., 1990). The substrates for the stromelysin family include proteoglycans and glycoproteins.

The membrane tethered matrix metalloproteinases (MT-MMPs) have at least four members and are expressed in embryonic and neoplastic epithelium (Kinoh et al., 1996).

MT-MMP-1 has been implicated in the activation of MMP-2 during development and carcinogenesis (Basbaum and Werb, 1996).

Other matrixins, the putative type IV collagenases, 72 kD Gelatinase-A (MMP-2) and 92kD Gelatinase-B (MMP-9) are suggested to mediate the remodeling of basement membranes during normal development carcinogenesis, and metastasis (Matrisian, 1992; Stetler-Stevenson, 1990). MMP-2 and MMP-9 degrade denatured collagens such as gelatin (Fisher and Werb, 1991). In addition, MMP-2 and MMP-9 are suggested to degrade type IV collagen and therefore mediate the turnover of the basement membrane (Stetler-Stevenson, 1990; Bosman, 1993). Therefore, based on substrate specificity MMP-2 is postulated to regulate ECM degradation during tissue remodeling. In the newborn mouse, MMP-2 is expressed in the mesenchyme of the salivary and tracheal glands, as well as in the epithelium of both of these organs (Rennie, 1984; Lim, 1995). However, MMP-2 mRNA is found only in the mesenchyme of the branchial arches, eye, heart, kidney, lung (Reponin, 1992). Transcripts for MMP-2 are expressed by fibroblasts adjacent to several types of tumors (Stetler-Stevenson, 1990). As measured with immunohistochemistry, MMP-2 protein is localized near the basement membrane in the rat mammary gland (Talhouk et al., 1991). By zymography, MMP-2 is expressed by the ovary (Mann et al., 1991), and prostate (Wilson, 1992). MMP-9 is required for human cytotrophoblast invasion (McMaster et al., 1994).

The bioactivity of uPA and MMP-2 are regulated at four levels: 1) transcriptional and translational regulation of the amount of zymogen (or pro-enzyme); 2) the rate of conversion from latent to active enzyme; 3) the rate of inactivation by inhibitors (and clearance); 4) regulation and focalization of activity by receptors such as uPAR or MT-MMP 1. Clearly, many proteinases are important in tissue remodeling. However, uPA and MMP-2 are postulated to affect basement membrane turnover, and therefore are suggested to mediate tissue remodeling during organogenesis. Therefore, the tissue

expression and function of uPA, uPAR, and MMP-2 during prostatic development will be the focus of the current study.

b) Regulation of expression of uPA and MMP-2

Transcription of the gene encoding uPA is regulated by many factors. Peptide and steroid hormones, cytokines, cell shape change, and extracellular shear forces have been shown to affect transcription of uPA mRNA (Saksela et al., 1987). The human uPA gene has a constitutive and an inducible 5' enhancer (-2000-1870) that contains a PEA3/AP-1 element that can bind the nuclear oncoproteins cfos, cjun, h-Ets-1, c-Ets-1, c-Ets-2. In addition, the uPA enhancer element can bind the cellular oncoproteins Ha-ras, v-src, v-mos, v-raf, as well as the serum response factor (De Cesare et al., 1995; Lengyel et al., 1995a; Lengyel et al., 1996). Expression of uPA in squamous carcinoma cells is driven by a constitutively active c-raf dependent signaling pathway (Lengyel et al., 1995b). Members of the steroid receptor super family have been shown to bind to response elements near uPA and other proteinase genes (Alexander, 1991). In castrated males, the prostate regresses in parallel with an increase in the tissue levels of uPA mRNA and protein (Andreasen, 1990; Rennie, 1984), suggesting that uPA mediates the tissue remodeling associated with prostatic regression. In addition, uPA is developmentally regulated in the prostate (Wilson et al., 1988).

Expression of uPA is also regulated by cytokines in a cell type-specific pattern. Epidermal growth factor (EGF) increases the expression of uPA transcripts. In epithelial cells, transforming growth factor-beta (TGF-β_1) decreases expression of uPA and increases the expression of plasminogen activator inhibitor-1 (Massague, 1990). Mesenchymal cells transfected with a gene encoding latent TGF-β_1 express more uPA than cells transfected with an empty vector (Arrick, 1992). HGF/SF increases the production of uPA and uPAR in an epithelial cell line (Pepper, 1992). EGF, platelet-

derived growth factor (PDGF), and basic fibroblast growth factor (bFGF), each have been shown to increase production of uPA by epithelial cells.

Expression of MMP-2 is regulated by cytokines and cell-cell contact. EGF and TGF-alpha increase expression of MMP-2 in cultured rat lung cells (Ganser, 1991). Production of MMP-2 in human keratinocytes is also increased by TGF-β_1 (Salo et al., 1991). The adhesion molecule V-CAM can also regulate the expression of MMP-2. V-CAM expressed on murine endothelial cells increases the expression and activation of MMP-2 in T cells and increases T cell invasiveness of endothelial monolayers (Romanic et al., 1997).

There are many examples of increased production of uPA, uPAR, and MMP-2 by neoplastic epithelium and/or associated stroma (Blasi, 1993; Matrisian, 1992). Compared to normal adult human tissue, uPA and uPAR expression and activation are increased in tumors of the mammary gland (Holst-Hansen et al., 1996), colon (Pyke et al., 1994) and prostate (Wilson, 1995). In some cases the increase in expression levels for uPA ligand and receptor are increased in tumor stroma. In other cases uPA is expressed in both the tumor stroma and epithelium (Blasi, 1993). Also, cell lines derived from secondary metastasis of a thyroid carcinoma, produce more activated MMP-2 than their normal counterparts or cell lines derived from non-invasive primary tumor cells (Demeure et al., 1992).

c) Activation and Inactivation of uPA and MMP-2

An important level of regulation of uPA and MMP-2 function is the proteolytic conversion of the latent zymogens into the active enzymes. The latent serine proteinase plasminogen, is converted into the catalytically active plasmin by the action of uPA. Plasmin, in turn, has been shown to convert zymogen forms of a variety of serine and metalloproteinases into active conformations (Alexander, 1991) and is suggested to regulate plasma and tissue proteolytic cascades. uPAR is suggested to play an important

role in uPA activation and proteolytic activity by localizing the proteinases to the cell surface, as well as by protecting active uPA from inactivation (Dano, 1985; Blasi, 1993). According to the prevailing model, uPAR is the immediate upstream regulator of uPA activity, plasmin activity and subsequent ECM degradation (Dano, 1985). The metalloproteinases, pro-collagenase and pro-stromelysin, are converted into their active proteinases by plasmin (Alexander, 1991). uPA can also directly activate MMP-2 (Keski-Oja, 1992). Therefore, elements of the plasminogen activator cascade and other chymotrypsins such as thrombin are considered upstream of certain MMPs. MMP-2 activates latent MMP-9 (Fridman et al., 1995). Thus, MMP-2 may directly degrade the ECM, activate a matrixin cascade, and be regulated upstream by a serine proteinase cascade.

uPA and MMP-2 activity in tissues is regulated by proteinase inhibitors. Inhibitors bind the active forms of uPA and MMP-2 in solution and render them proteolytically inactive. Although this was the initially postulated function for inhibitors, more recently inhibitors have been proposed to have roles as mitogenic ligands and metalloproteinase activation cofactors. Specific inhibitors of uPA include plasminogen activator inhibitors-1 and 2 (PAI-1 and PAI-2), in addition to protease nexin-1. PAI-1 is a mitogen for some cancer cell lines. uPA is also inhibited by broad-spectrum serine proteinase inhibitors such as alpha 2-macroglobulin (α_2M) and alpha 1-antitrypsin (Alexander, 1991). α_2M acts by binding to the active uPA catalytic domain with a substrate-like baiting domain (Bonner and Brody, 1991).

MMP-2 and MMP-9 are inhibited by a different class of inhibitors. Tissue inhibitor of metalloproteinase-2 (TIMP-2) binds to latent and active MMP-2, while TIMP-1 binds to MMP-9. TIMP-binding inactivates active gelatinases. TIMP-2 levels in stroma adjacent to mammary carcinoma cells are decreased compared to normal tissue, whereas MMP-2 and MMP-9 levels are increased (Visscher et al., 1994). In the mammary gland, tissue ratios of proteinases (stromelysin) and inhibitors (TIMP-1) are

hormonally regulated and proteinase-inhibitor ratios appear to modulate the integrity of the basement membrane (Talhouk et al., 1991). Targeted over-expression of MMP-3 to the mammary gland of transgenic mice has been shown to enhance degradation of basement membranes, as measured with immunohistochemical staining of tissue sections for collagen type IV (Sympson et al., 1994). This further suggests the importance of matrix metalloproteinase-inhibitor ratios on BM morphology, and in turn, differentiated epithelial function such as casein production (Streuli et al., 1991). TIMP 1 and 2 were originally suggested to serve as inhibitors of soluble MMP-9 and MMP-2, respectively. However, recently TIMP-2 has also been implicated as a mitogenic ligand (Imren et al., 1996) and as an activator of MMP-2. TIMP-2 appears to serve as bridge between activated MT-MMP1 and the binding and activation of MMP-2 (Basbaum and Werb, 1996).

The types of proteinases that activate MT-MMPs remains unknown, but recent evidence suggests that transmembrane serine proteinases of the subtilisin family termed furin/PACE/kex 2-like proteinases may be upstream activators of MT-MMPs and other MMPs, perhaps mid-exocytosis. The furthest upstream regulation of furin may be phosphorylation-induced conformational changes, perhaps by a cell cycle-regulated kinase such as cyclin (Basbaum and Werb, 1996). It is intriguing to speculate how uPA and the PAC may participate in the activation of serine proteinases such as furins that may be upstream of MMP activation.

d) Receptors

The uPAR regulates uPA activation, stability, and focalization, thereby mediating cell migration, mitogenesis and internalization of cell surface uPA. uPAR is a heavily glycosylated 55kd cell surface protein. It has been found in calveolae of the M24met human melanoma cells (Stahl, 1995). uPAR is linked to the plasma membrane by a glycosyl-phosphatidylinositol moiety and can be released by phosphatidylinositol-

specific phospholipase C (Del Rosso et al., 1992) and D (Metz et al., 1994). uPAR binds both single and two chain uPA at nanomolar avidity, but not plasminogen or tPA (Blasi et al., 1987). In the normal adult rodent, uPAR is expressed in fibroblasts, leukocytes, migrating epithelial cells, vascular endothelium, and kidneys (Blasi, 1993). uPAR expression is increased in the stromal cells of metastatic human colon adenocarcinomas (Pyke et al., 1993) and in the stroma of metastatic human mammary adenocarcinomas (Bianchi et al., 1994). The expression of human uPAR is regulated at the transcriptional level by a 140 bp region that is 400 bp upstream of the 5' end of the transcription site. The promoter lacks canonical TATA and CAAT boxes and contains G-C rich stretches (Soravia et al., 1995). In the RKO human colon cancer cells, uPAR gene expression requires c-jun binding and is regulated in part by an extracellular signal-regulated kinase 1 (ERK 1)-dependent signaling pathway (Lengyel et al., 1995a; Lengyel et al., 1996). uPAR mRNA expression is regulated by cytokines such as EGF, PDGF, IL-6, TNF-alpha, and TGF-β_1 (Lund et al., 1995). Thrombin, but not the structurally similar clotting factor X, increases uPAR expression in DU-145 human prostate cancer cell line (Yoshida et al., 1994).

uPAR functions in tissue remodeling in a variety of ways: scuPA bound to uPAR is 20 times more active in converting plasminogen to plasmin than soluble scuPA (Dano, 1985). Following binding of scuPA to uPAR, conversion to tcuPA by plasmin is enhanced approximately 50-fold (Blasi et al., 1987). Moreover, uPAR enhances scuPA enzymatic activity 100 times in human monocytes (Manchanda, 1991), and uPAR-associated tcuPA converts plasminogen to plasmin at a greater rate than free uPA (Blasi, 1993). Therefore, uPAR increases the efficiency of uPA and plasmin activation. uPAR also affects tcuPA inactivation by inhibitors. The inhibition of uPA by PAI-1 and PAI-2 are reduced about 40% when uPA is bound to uPAR. Also, uPAR mediates internalization of uPA-PAI-1 complexes in association with low density lipoprotein receptor (LDLR)-related protein, mediating the removal of the complex from the cell

surface (Olson et al., 1992). Therefore, uPAR mediates the localization, inactivation, internalization of uPA.

uPAR also binds to ECM glycoproteins such as vitronectin (Kanse et al., 1996) and fibronectin (Moser et al., 1995) as well as glycosaminoglycans, such as heparin (Kwaan, 1992). uPAR mediates the migration of bovine endothelial cells (Pepper et al., 1993) and endocardial-derived mesenchyme cells in quail (McGuire, 1993). uPAR induces migration of gut epithelial cells (Kristensen et al., 1991), as well as migration and invasion by prostatic carcinoma cells (Hoosein, 1991). uPA bound to uPAR is localized to focal contacts, which are specialized areas of the plasma membrane where integrins link to the cytoskeleton via their β_1 subunits (Pollaen, 1988). Therefore, uPAR mediates the binding of uPA and several other ECM ligands that are utilized for cellular migration as well as localizing these adhesive contacts to specialized regions of the cytoskeleton. In addition, uPAR can signal without ligand (Resnati et al., 1996). Recently, uPAR has been shown to modulate integrin avidity and adhesion in a non-proteolytic manner (Wei et al., 1996). uPAR can signal when associated with c-src and another yet uncharacterized protein.

uPA amino acids 1-138 (uPA/ATF) can mediate migration and the induction of c-fos expression in PC3 cells as well as serving as a morphogen for cultured human endothelial cells (Schnaper et al., 1995). In contrast, the entire proteolytically active uPA protein is necessary for uPAR signaling and mitogenesis in human dermal fibroblasts (Anichini, 1994). Therefore, it is unclear which domains of uPA are necessary to mediate uPAR mediated functions. uPAR directly mediates signaling, migration, and mitogenesis in association with other plasma membrane proteins and indirectly by localizing the activation of HGF/SF by uPA alone (Naldini et al., 1992), or of TGF-β_1, in association with plasmin (Odekon et al., 1994).

The plasminogen activator cascade is regulated by additional receptors that function in cooperation with uPAR. Plasminogen and plasmin bind to low affinity

receptors in the plasma membranes of GM1380 fibroblasts (Plow, 1986). Compared to soluble plasminogen, plasminogen bound to the cell surface is more readily activated by uPA and is more resistant to inactivation by soluble inactivators (Kwaan, 1992).

MMP-2 is localized to the surface of mesenchymal and osteogenic tissues in fetal mice by an MT-MMP-1-TIMP-2 complex (Kinoh et al., 1996). There may be additional receptors for MMP-2 and other MMPs. For example, MMP-2 is localized to the invaidapodia of RSV-transformed embryonic chicken fibroblasts (Monsky and Chen, 1993). Recently, 80 kd and 200kD trans-membrane metalloproteinases have been observed and suggested to mediate invasiveness (Chen et al., 1994).

Therefore, both uPA and MMP-2 are regulated in part by plasma membrane receptors. In the case of uPA, the uPAR activates uPA and localizes its proteolytic activity at focal contacts, affecting the degradation of ECM components and the activation of cytokines. In addition, uPAR mediates adhesion, migration, signaling and mitogenesis. Also, MMP-2 is localized to the cell surface by TIMP-2 and MT-MMP 1 during embryogenesis and cancer (Nomura, 1995), which may regulate ECM degradation and mitogenesis. The precise functions of uPA and MMP-2 receptors during prostatic organogenesis and carcinogenesis are unclear.

e) Substrates

There are many important substrates for activated proteolytic enzymes including other enzymes of proteolytic cascades, ECM and cell adhesion molecule receptors on the cell surface, as well as ECM-associated adhesion ligands and cytokines (Alexander, 1991). The ECM is defined as the glycoprotein and glycosaminoglycan-rich intercellular material. Originally the ECM was considered to be merely an inert structural scaffold, devoid of regulatory activities. More recently, however, the ECM is viewed as a dynamic regulatory network (Bissell and Aggeler, 1988). ECM contains cytokines, structural and

adhesive molecules, proteinases, as well as proteinase inhibitors, each with influential roles in processes that include development, disease, and wound repair.

Several studies suggest that cytokines such as TGF-β_1 (Flaumenhaft et al., 1992; Massague, 1990, and HGF/SF (Naldini et al., 1992) are substrates for plasmin, while HGF/SF is a substrate of uPA (Naldini et al., 1992). bFGF complexed with heparan sulfate proteoglycan, is released from the ECM in an active form by plasmin through the action of uPA produced by endothelial cells (Saksela and Rifkin, 1990). Plasmin, uPA, tPA, thrombin, and several serine proteinase inhibitors (thrombin inhibitor-1, protease nexin-1, and PAI-1) are ECM-tethered as well as soluble (Kwaan, 1992). The proteolytic activation of ECM-tethered cytokines is a highly speculative, but intriguing aspect of the regulation of tissue remodeling by proteinases.

Glycoproteins, glycosaminoglycans, and cytokines in the ECM have been studied in tissue remodeling in several model systems including the rat mammary and salivary glands (Silberstein et al., 1992; Kratochwil, 1987; Bernfield and Banerjee, 1982). There are two main types of ECM's in organs with an epithelial parenchyma: the interstitial fibrous ECM and the epithelial basement membrane.

Synthesized primarily by fibroblasts, interstitial fibrous matrices contain collagens I and III, fibronectins, the B chains of laminin, and entactin. Glycosaminoglycans present in the interstitial ECM are hyaluronic acid and heparan sulfates, often in proteoglycans, chondroitin sulfates, dermatan sulfates, as well as the more complex syndecans and tenascin. In the developing salivary gland, epithelial form is regulated by the fibrillar collagen-type glycoproteins and the heparan sulfate proteoglycan-type glycosaminoglycans of the epithelial basement membrane (Bernfield et al., 1984). Turnover of epithelial BM glycosaminoglycans is regulated by a mesenchymally derived enzyme. A role for type III collagen has been suggested in cleft formation during ductal branching morphogenesis of the embryonic salivary gland by three observations: First, purified collagenase inhibits branching morphogenesis (Fukuda et al., 1988); second, a

collagenase inhibitor stimulates branching morphogenesis of the salivary gland (Nakanishi et al., 1986); third, collagen type III is localized in forming clefts (Nakanishi et al., 1988).

The BM is localized on the basal aspect of the epithelium facing the mesenchyme, and contains primarily collagens IV and V in addition to laminin, entactin, hyaluronic acid, heparan sulfate, and syndecan. The BM is highly negatively charged and is the primary physical barrier separating epithelium and stroma. BM morphology is regulated by the coordinated synthesis and degradation of its glycoprotein and glycosaminoglycan components (Bernfield et al., 1984). Although classically suggested to be synthesized by the epithelium, it is evident that during the development of the gut, molecular components of the epithelial basement membrane are synthesized by both the epithelium and mesenchyme (Haffen et al., 1987). Laminin and type IV collagen are also required for morphogenesis of the salivary gland (Cutler, 1990) and kidney (Ekblom, 1984). BM morphology regulates epithelial differentiation and casein synthesis in cultured mammary epithelium (Streuli et al., 1991). Several human carcinoma cells grafted into nude mice have basement membranes containing molecules derived from both the carcinoma cells and stromal cells of the host (Bosman, 1993). BM integrity, a function of coordinated synthesis and degradation, is suggested to mediate neoplastic differentiation, proliferation and containment. The BM is important in normal and diseased tissue because it contains chemical information that regulates cell behavior (Bosman, 1994).

Plasmin substrates such as fibronectin and laminin, as well as the MMP-2 substrate collagen type IV, are localized to the BM. In addition, co-localization of uPA and MMP-2 proteins in the BM region suggest that these enzymes would probably participate in the remodeling of the BM. The substrates of uPA include plasminogen, fibronectin, HGF/SF, TGF-β_1, uPAR, and MMP-2. Therefore, uPA and MMP-2 are likely to mediate the remodeling of the BM, and therefore regulate ductal branching morphogenesis in a variety of organs including the prostate.

1.4 Summary

uPA is a mitogen, a motogen, and a mediator of ECM turnover and cytokine activation. MMP-2 is a mediator of ECM turnover. Both are suggested to mediate tissue remodeling during development and involution of the rat ventral prostate. Precisely where these enzymes are synthesized, localized and activated, as well as the specific roles of uPA and MMP-2 in prostatic development are unclear. Defining the roles of uPA and MMP-2 in prostatic development are important because tissue remodeling during normal prostatic development and prostatic carcinogenesis are considered similar. Both include cellular proliferation, cellular migration, increased proteinase production, and ECM turnover. However, during normal prostatic development, cellular proliferation as well as tissue remodeling is orderly and leads to epithelial and mesenchymal histodifferentiation. Conversely, during prostatic carcinogenesis tissue remodeling is chaotic and leads to cellular dedifferentiation. Recently, a hypothesis has been proposed that smooth muscle-epithelial interactions regulate human and rodent prostatic morphology during development, adulthood, and carcinogenesis. The factors that may mediate this paracrine cross-talk include cytokines such as keratinocyte growth factor (KGF), HGF/SF, and TGF-β's. The latter two cytokines require proteolytic activation by a plasmin-like enzyme and are suggested to be paracrine mesenchymal to epithelial factors during prostatic development (K. Dano, F.Blasi, and L. Ossowski, personal communications).

uPA, uPAR, and MMP-2 expression is increased in prostate and other cancers (Dano, 1985; Kwaan, 1992; Matrisian, 1992; Stearns, 1993; Blasi, 1993; Wilson, 1995). Also, uPA and uPAR mediate the migration and invasion of prostate cancer cells in vitro. In the current study, we postulate that uPA and MMP-2 are among the mediators of smooth muscle-epithelial interactions during prostatic development. We report that by *in situ* hybridization, both uPA and MMP-2 mRNA are expressed exclusively by mesenchymal cells in the developing rat VP and exclusively by stromal cells in the adult rat VP. By zymography, uPA and MMP-2 protein are localized to both the epithelium

and the mesenchyme of newborn rat VP. Levels of activated MMP-2 decline with age in the Fisher 344 strain rat ventral prostate and bladder, as well as in the mouse BUG. In addition, perturbation of uPA and uPAR function by inhibitory peptides resulted in severely disrupted ductal branching morphogenesis and histodifferentiation in both epithelium and smooth muscle of cultured newborn rat VP. Thus, our findings support the hypothesis that uPA and MMP-2 mediate prostatic development as factors in paracrine smooth-muscle-epithelial interactions.

CHAPTER TWO

UROKINASE PLASMINOGEN ACTIVATOR IS NECESSARY FOR DEVELOPMENT OF THE RAT VENTRAL PROSTATE, BUT PLASMINOGEN IS NOT

2.1 Abstract

Urokinase plasminogen activator (uPA) is postulated to be involved in development, inflammation, wound healing, and cancer. uPA expression has been reported in rat ventral prostates (VPs) during neonatal development and prostatic involution in androgen-deprived adult rats. The current study has examined the expression and function of uPA during growth and branching morphogenesis of neonatal rat VPs. We report that by zymography, newborn rat VPs expressed uPA protein in both the mesenchyme and epithelium. *In situ* hybridization of VPs from newborn, 3 day, 14 day, and adult rats demonstrated that uPA transcripts are expressed in mesenchymal cells during prostatic development and in stromal fibroblasts as well as smooth muscle in the adult prostate. Biotinylated ecotin M84R/M85R binds to catalytically active uPA and was localized to both epithelium and peri-epithelial smooth muscle in tissue sections of adult rat VP. In order to study the role of uPA in growth and ductal branching morphogenesis, newborn rat VPs were cultured for one week under serum-free conditions with a specific high affinity uPA inhibitor, ecotin M84R/M85R. Such treatment significantly disrupted testosterone-induced ductal branching morphogenesis and histodifferentiation of both epithelial and mesenchymal tissues. The effect was dose-dependent and reversible. Also, addition of ecotin M84R/M85R after three days of culture with testosterone alone, arrested and reversed the ductal branching morphogenesis of newborn rat VP. Amiloride, a chemical inhibitor of uPA, also significantly perturbed ductal branching morphogenesis of newborn rat VPs. These data suggest an important role for uPA or a uPA-like enzyme in the development of the rat VP. Further, plasminogen mRNA was not detected in newborn rat VPs by RT-PCR, and ductal branching morphogenesis of newborn rat VPs was not inhibited by aprotinin, a plasmin inhibitor. Thus, development of newborn rat VPs does not require plasminogen, suggesting that the uPA may act independently of plasminogen.

2.2 Introduction

The postulated role of urokinase plasminogen activator (uPA) is to activate plasminogen into the potent, broad-spectrum endopeptidase, plasmin. Plasmin degrades extracellular matrix (ECM) and activates cytokines. In addition, uPA has been shown to directly degrade substrates such as: Hepatocyte growth factor/scatter factor (HGF/SF) (Naldini et al., 1992), MMP-2 (Keski-Oja, 1992), activating each, fibronectin (Quigley, 1987), and the uPA receptor (uPAR) (Hoyer-Hansen et al., 1992). Further, uPA, bound to uPAR, is a motogenic ligand for vascular endothelium (Pepper et al., 1993), endocardial mesenchymal cells (McGuire, 1993), and prostate cancer cells (Hoosein, 1991), as well as a mitogenic ligand for vascular smooth muscle cells, dermal fibroblasts (Anichini, 1994) and prostate cancer cells (Hoosein, 1991). uPA is suggested to regulate tissue remodeling during inflammation, wound healing, cancer, and development (Dano, 1985; Blasi et al., 1987). However, development was normal in mice with targeted disruption of the genes encoding uPA (Carmeliet et al., 1994), uPA receptor (uPAR) (Bugge, 1995b), or plasminogen (Bugge, 1995a). Thus, the role of the plasminogen activator system in development is unclear.

In rodents, uPA expression begins with activation of embryonic genome at the two blastomere stage (Zhang, 1994) and continues during embryonic mouse development (Bode and Dziadek, 1979), and mouse urogenital sinus (Timme et al., 1994). The rodent prostate is composed of three lobes: the anterior prostate, the dorsolateral prostate, and the ventral prostate (VP). uPA protein is expressed in the developing neonatal rat prostatic complex, which is composed of the prostatic urethra, peri-urethral fat, ventral, dorsolateral, and anterior prostatic lobes. In pubertal rats, uPA protein is expressed in all of these prostatic lobes (Wilson et al., 1988; Wilson, 1995). In adult rats, only the ventral prostatic lobe expresses uPA (Wilson et al., 1988; Wilson, 1995). Castration of adult rats

elicits prostatic involution in all lobes, but the expression of uPA is only increased in VPs (Andreasen, 1990; Rennie, 1984; Wilson et al., 1988).

The prostate originates from solid epithelial outgrowths (prostatic buds) that emerge from the embryonic urogenital sinus (UGS) immediately below the developing bladder (Cunha et al., 1987). The prostatic buds grow, elongate, and invade into urogenital sinus mesenchyme (UGM) and arborize to form a complex ductal network (Sugimura et al., 1986c). Androgens are required for the development of the prostate. Androgen receptor-positive UGM induces ductal branching morphogenesis, epithelial cytodifferentiation, expression of epithelial AR, and specifies synthesis of lobe-specific secretory proteins (Chung et al., 1981; Cunha et al., 1983). Mesenchyme is also required for epithelial growth and differentiation and specifies the patterning of epithelial ductal branching in each prostatic lobe (Cunha et al., 1987). In a reciprocal manner, the developing prostatic epithelium also induces the differentiation of smooth muscle in the mesenchyme (Cunha et al., 1996). In the adult rat VP reciprocal interactions between smooth muscle and epithelium maintain normal tissue phenotypes.

In order to study the role of uPA during ductal branching morphogenesis, uPA's activity was perturbed by specific peptide or chemical antagonists in a serum-free system that allows for the culture of newborn rat VPs for one week (Sugimura et al., 1986b). At birth, the VP contains 4 main epithelial ducts each having one or two branch points. The epithelial ducts are solid and uncanalized with the mesenchyme appearing densely cellular with fibroblasts. Smooth muscle cells are just beginning to differentiate directly adjacent to the epithelium but are sparse at birth (Hayward et al., 1996a). Following one week of culture in the presence of 10^{-8}M testosterone (T), newborn rat VPs form approximately 50 terminal ductal tips in a highly arborized pattern that extends to the outer edge of the mesenchyme. This ductal branching morphogenesis is associated with ductal canalization as well as epithelial differentiation into luminal cells and dendritic basal cells. Associated with these events in the epithelium, the mesenchyme

differentiates into smooth muscle tissue that condenses into peri-ductal sheaths. Newborn rat VPs cultured in the absence of added androgens or with cytokine perturbing antibodies, exhibited retarded ductal branching morphogenesis and impaired epithelial and mesenchymal histodifferentiation (Sugimura et al., 1986c).

Small peptide inhibitors have been used to inhibit proteinases in many systems. Macromolecular proteinase inhibitors more closely and efficiently mimic the action of biological inhibitors. Further, macromolecular inhibitors offer many advantages regarding drug delivery systems. Among such macromolecular proteinase inhibitors is wild-type ecotin, a serine proteinase inhibitor found in E. coli periplasm. Wild-type ecotin has nanomolar avidity for several chymotrypsin family serine proteinases, but only micromolar avidity for uPA. Ecotin M84R/M85R is a derivative of wild-type ecotin that has nanomolar avidity for uPA (Wang et al., 1995). Co-injection of ecotin M84R/M85R with prostate cancer cells results in reduced tumorigenicity and metastasis to the lungs as well as liver as measured by expression and localization of luciferase reporter protein (Melnyk et. al., manuscript in preparation).

In the current study, *in situ* hybridization was used to demonstrate that uPA mRNA was expressed by mesenchymal cells during development and by interstitial stromal fibroblasts as well as by smooth muscle in the adult rat VP. In addition, zymography of detergent tissue extracts of newborn VPs detected uPA protein in both mesenchyme and epithelium. Further, the requirement for uPA during development of newborn rat VPs was demonstrated by inhibiting its activity with a specific peptide inhibitor, ecotin M84R/M85R that was added to the culture media of newborn rat VPs grown for one week *in vitro*. We report that ecotin M84R/M85R or the uPA chemical inhibitor, amiloride, perturb the *in vitro* growth of rat VPs as demonstrated by impaired ductal branching morphogenesis and retarded epithelial and mesenchymal histodifferentiation. Therefore, uPA is necessary for the development of newborn rat VPs. Since plasminogen was not added to the culture system, nor synthesized in situ, it

was not surprising that the plasmin inhibitor, aprotinin, failed to impair development of newborn rat VPs. Thus, independent of plasminogen, uPA is necessary for development of newborn rat VPs.

2.3 Materials and Methods

Inbred Fisher 344 rats (Simenson Inc., Gilroy, CA.) were sacrificed by barbiturate overdose and bilateral thoracotomy in accordance with NIH guidelines. Specimens for *in situ* hybridization or histology were embedded in OCT compound (Sakura Inc., Torrence, CA.) and frozen on dry ice. Frozen sections were cut on a Jung Frigocut 2800N cryostat and thaw mounted onto presialized Fisher-plus microscope slides (Fisher Inc., Springfield, NJ.)

In situ hybridization was performed on uPA in developing rat VPs using frozen tissue sections as described previously (Lim, 1995). Frozen sections were hybridized with ^{35}S-labeled anti-sense and sense riboprobes for uPA mRNA according to standard protocols (Robinson et al., 1991). Following hybridization, the slides were washed at high stringency and digested with RNAase as described previously (Lim, 1995). The positive hybridization signal was detected autoradiographically, and the sections counterstained. Nonspecific hybridization was assessed by using an ^{35}S-labeled sense riboprobe. RT-PCR of newborn rat VP was performed as described previously using a 1st strand cDNA synthesis kit for RT-PCR (AMV), Boehringer Mannheim (Mannheim, Germany). mRNA was isolated from newborn rat VPs using RNA-STAT 60 brand reagent according to the manufacturer's instruction (Tel-Test, Inc., Friendswood, TX.). Primers for plasminogen were designed with Amplify 1.0 software (Olson, University of Wisconsin-Madison-Genetics) and were constructed commercially (Berringer Mannhiem-UCSF Cell Culture Facility custom synthesis) based on sequence data obtained from Genebank. For rat plasminogen, sense (5'-GGAGTACTGTGAGATTCCGTCCTGC-3') and anti-sense (5'-TACGTCAGACAGAGTCTCCACGGAC-3') primers were targeted to the nucleotide sequence at positions 30 to 446 with a predicted product of 416 bp. No product was observed from genomic DNA with these primers when RT was omitted in the PCR reaction.

A modification of the methods of Wilson (Wilson et al., 1988) was used for zymographic analysis of tissue extracts and conditioned media. For zymography, *in situ* hybridization, or organ culture, newborn VPs were harvested within 24 hours of birth. The epithelium and mesenchyme were surgically separated with fine gauge needles. All visible traces of mesenchyme were removed from the epithelium and collected. The separated epithelium and mesenchyme were homogenized with a glass mortar/pestle in the presence of 0.25% Triton X-100 (Sigma Inc., St. Louis, MO.) at 4^0 C for 30 minutes with ~100 strokes, centrifuged in Eppendorf tubes at 12,000g for 2 minutes, with the supernatants frozen on dry ice. Conditioned media (CM) of cultured rat VPs were concentrated with Centriprep-10 concentrators (Amicon Inc., Bedford MA.). Protein content of extracts and CM's was determined by the BCA method (Pierce Inc., Rockford, IL.). Tissue extracts or CM's containing equal amounts of protein were mixed with 4X Lameli electrophoresis sample buffer and loaded onto SDS-PAGE gels containing 10% polyacrylamide (acrylamide, ammonium persulfate, and SDS, Bio-Rad Inc., Richmond, CA.) mixed with 1 mg/ml casein (Sigma Inc., St. Louis, MO.) and 50µg human plasminogen (American Diagnostica Inc., Greenwich, CT.) and electrophoresed on a mini-gel apparatus (Hoefer Inc., San Francisco CA.). Following constant current electrophoresis with prestained molecular weight markers, the slabs were washed in 2.5% Triton X-100 (in Tris-HCL buffer) in order to remove SDS. Next, the Triton X-100 was washed out and the slabs were incubated in a Tris-HCL buffer at pH 7.5-8.0 for 24 hours at 37^0 C. Gels were stained with coomassie blue (Bio-Rad Inc., Richmond, CA.), destained, fixed, rubbed with glycerol (Sigma, St. Louis, MO.), mounted, and dried in Biodesign gel wrap (Biodesign Inc., Carmel, NY.).

Newborn rat VPs were isolated by dissection and transferred with micro-forceps to 5mm by 5mm square floating Millipore-CM membrane rafts (Millipore Inc., Bedford MA.) in 0.5ml of growth medium contained in four well plates (Nunc. Inc., Roskilde, Denmark). Culture medium was a DME H-16 /F-12 (1/1) mix with 50 µg/ml gentamycin

(UCSF Cell Culture Facility) supplemented with insulin (10μg/ml), tranferrin (50 μg/ml) plus and minus 10^{-8} M testosterone (T) (Sigma Inc., St Louis MO.). This culture system allows free exchange of nutrients and additives such as antibodies or peptides through the filter (Sugimura et al., 1986a). Organs were cultured for one week with media changes every two days. At the termination of the culture, unfixed glands were imaged as whole mounts on a Zeiss microscope using a Lumina scanner system (Leaf Systems Inc., Southborough, MA.). Images were collected on an Apple Power Macintosh 8200 (Apple Computer, Cupertino, CA.) using Adobe Photoshop 3.0 and 4.0 software (Adobe Systems, Mountain View, CA.). Statistical analysis was performed with Statview software (Abacus Concepts Inc., Berkeley, CA.).

Amiloride and aprotinin were purchased from Sigma Inc. (St Louis, MO.). Ecotin M84R/M85R and wild-type ecotin were prepared as previously described (Wang et al., 1995): uPA-binding ecotin-expressing phage clones were generated by random mutagenesis. Mutant ecotin M84R/M85R, a double amino acid-substitution variant of ecotin with nanomolar avidity for uPA, was isolated by ligand-binding panning and cloned into an E. Coli expression system. Ecotin M84R/M85R was purified by chromatography and stock solutions of 10mg/ml were diluted in culture medium to final concentrations of 1.25 to 50 μg/ml. Results shown were obtained using three separate preparations of ecotin M84R/M85R. Biotinylation of ecotin M84R/M85R was prepared as follows: ecotin M84R/M85R (1 mg) was dissolved in 1 ml of 50 mM $NaHCO_3$, pH 8.5. To this solution was added 0.4 ml of 1mg/ml NHS-LC-Biotin (Pierce) dissolved in water. The resulting solution was placed upon ice for two hours. The reaction mixture was dialyzed into 10 mM sodium phosphate, 150 mM NaCl, 0.1% sodium azide, pH 7.2. The HABA method for the determination of biotin incorporation (Pierce Inc. Rockford IL.) showed that 0.75 moles of biotin were incorporated for every 1 mol of ecotin M84R/M85R. Biotinylation of ecotin M84R/M85R did not affect uPA inhibition when assayed as described. Biotinylated ecotin was incubated with thawed frozen sections for

2.3 Results

1) Expression of uPA

The expression of uPA protein by epithelial and mesenchymal tissues in newborn rat ventral prostates

Newborn rat VPs were isolated with epithelial and mesenchymal tissues separated by microdissection, extracted with detergents, and electrophoresed on plasminogen/casein substrate zymography gels. Isolated epithelium and mesenchyme expressed equivalent amounts of a plasminogen activator of approximately 44 kD (fig. 2.1, lanes 4, 5). Zymographic degradation of casein at 44kD was not observed in the absence of plasminogen, nor in the presence of the uPA inhibitor amiloride, suggesting that uPA is the identity of the 44kD enzyme described (not shown).

uPA protein in conditioned media from newborn rat ventral prostates cultured with and without testosterone

VPs grown *in vitro* with testosterone (T) are significantly more developed than those grown without T as reported previously (not shown) (Sugimura et al., 1986b). Newborn VPs were cultured for one week in the presence or absence of 10^{-8}M T. Conditioned media (CM) were collected, concentrated, normalized for protein concentration, and electrophoresed on plasminogen/casein substrate zymography gels. Despite drastic differences in the ductal branching morphogenesis and histodifferentiation of androgen-deprived VPs compared to VPs cultured with androgens, zymographic

Figure 2.1

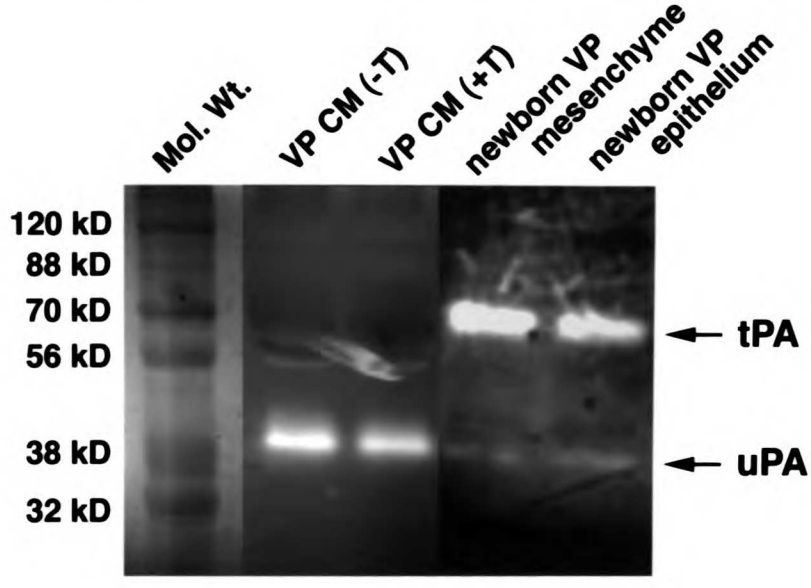
Zymography of plasminogen activators in newborn rat ventral prostates

Plasminogen/casein substrate gel zymography for plasminogen activators performed on concentrated conditioned media from newborn rat ventral prostates cultured for one week in the presence or absence of exogenous testosterone (T) as well as detergent tissue extracts from separated mesenchyme and epithelium of newborn rat ventral prostates. Organ culture, conditioned media processing, tissue detergent extraction, and plasminogen/casein zymography were performed as described in Materials and Methods. Lane 1, molecular weight standards; lane 2, conditioned media of newborn rat ventral prostates cultured serum-free for one week in the absence of androgens; lane 3, conditioned media of newborn rat ventral prostates cultured serum-free for one week in the presence of 10^{-8}M T; lane 4, detergent extract of newborn rat ventral prostate mesenchyme; lane 5, detergent extract of newborn rat ventral prostate epithelium.

Figure 2.1

Zymography of plasminogen activators in newborn rat ventral prostates

Plasminogen/casein substrate gel of newborn rat ventral prostates: tissue extracts and conditioned media from organs cultured for one week



analysis revealed equal amounts uPA were released into the CM in the presence or absence of added T (fig. 2.1 lanes 2, 3). Thus, removal of T in the newborn rat VP does not induce an increase in the production of uPA, as reported previously in adult rat prostates following castration.

uPA mRNA expression in developing and adult rat ventral prostates

In VPs from newborn rats the epithelial "ducts" are mostly unbranched, solid, and uncanalized (fig. 2.2c). The mesenchyme is dense with fibroblasts and contains a few peri-ductal smooth muscle cells (fig. 2.2c). Frozen sections of rat VPs of all ages examined exhibited a diffuse, weak background signal when hybridized with ^{35}S-labled sense riboprobes for uPA mRNA (fig. 2.2b). By contrast, frozen sections gave a strong signal for uPA mRNA that was localized homogeneously throughout the prostatic mesenchyme when hybridized with an ^{35}S-labled anti-sense riboprobe. The hybridization signal did not vary in relationship with distance from the epithelium (fig. 2.2d).

In VPs from 3 day old rats, the solid, uncanalized epithelial ducts are beginning to branch (fig. 2.3a). Ducts are circumferentially surrounded by mesenchymal fibroblasts with a few peri-ductal smooth muscle cells (fig. 2.3a). Frozen sections gave a signal for uPA mRNA that was localized to fibroblasts throughout the mesenchyme when hybridized with ^{35}S-labled anti-sense riboprobes (fig. 2.3b).

In VPs from 14 day old rats, the canalized epithelial ducts are highly branched and contain polarized tall columnar luminal epithelial cells and basal cells (fig. 2.3c). The stroma contains well differentiated smooth muscle sleeves circumferentially surrounding each epithelial duct and interstitial fibroblasts between ducts (fig. 2.3c).

Figure 2.2

***In situ* hybridization for uPA mRNA in newborn rat ventral prostates**

Distribution of uPA mRNA in newborn rat ventral prostates as detected by *in situ* hybridization. The 7 μm thick frozen tissue sections of newborn rat VPs were hybridized with sense uPA mRNA ^{35}S-labled-riboprobes (a, b), or anti-sense uPA mRNA ^{35}S-labled riboprobes (c, d). Sense and anti-sense probes were prepared, and hybridizations were performed as described in Materials and Methods. Tissue sections were viewed by bright field (a, c) and dark field (b, d). "E" and "M" in (c) indicate epithelial and mesenchymal tissue, respectively. Compared to the weak diffuse signal obtained with the sense probe (b), hybridization with the anti-sense probe gave a strong signal for in uPA mRNA in the mesenchyme (d). Sections were counterstained with hematoxylin/eosin. Bar, 25 μm.

Figure 2.2

In situ hybridization for uPA mRNA in newborn rat ventral prostates

a) bright field, sense probe; b) dark field, sense probe
c) bright field, anti-sense probe; d) dark field, anti-sense probe
M: mesenchyme E: epithelium

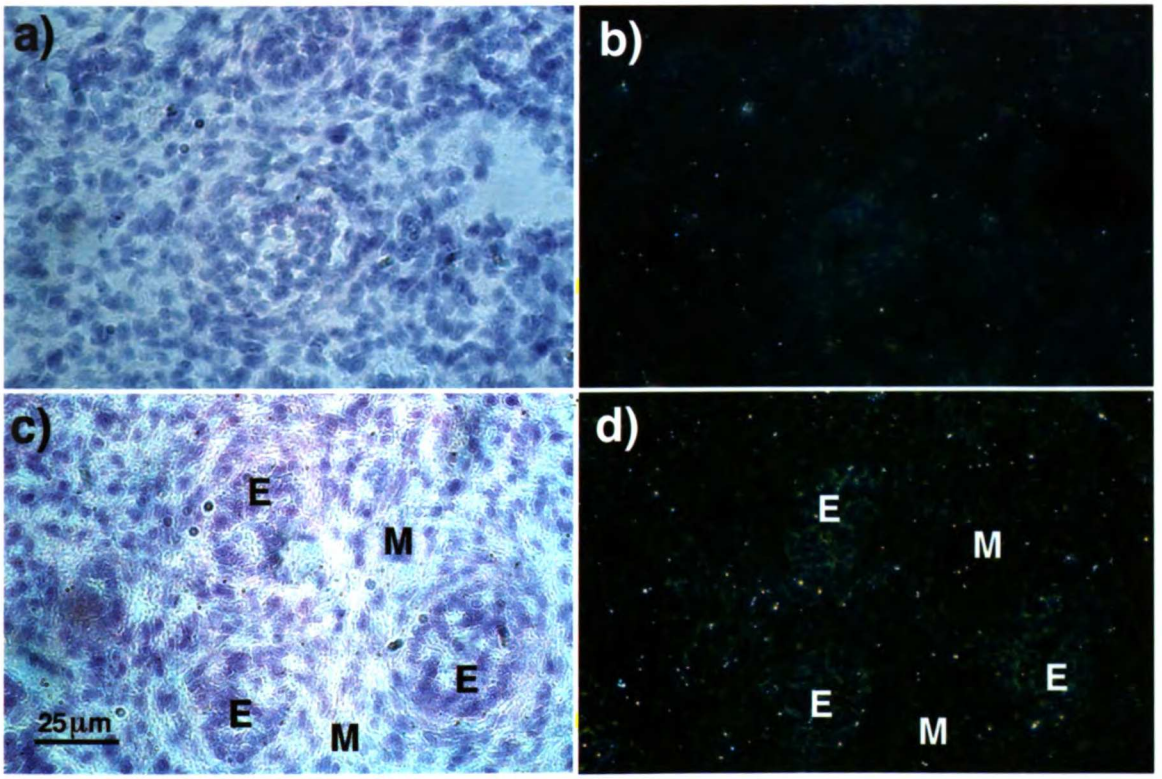


Figure 2.3

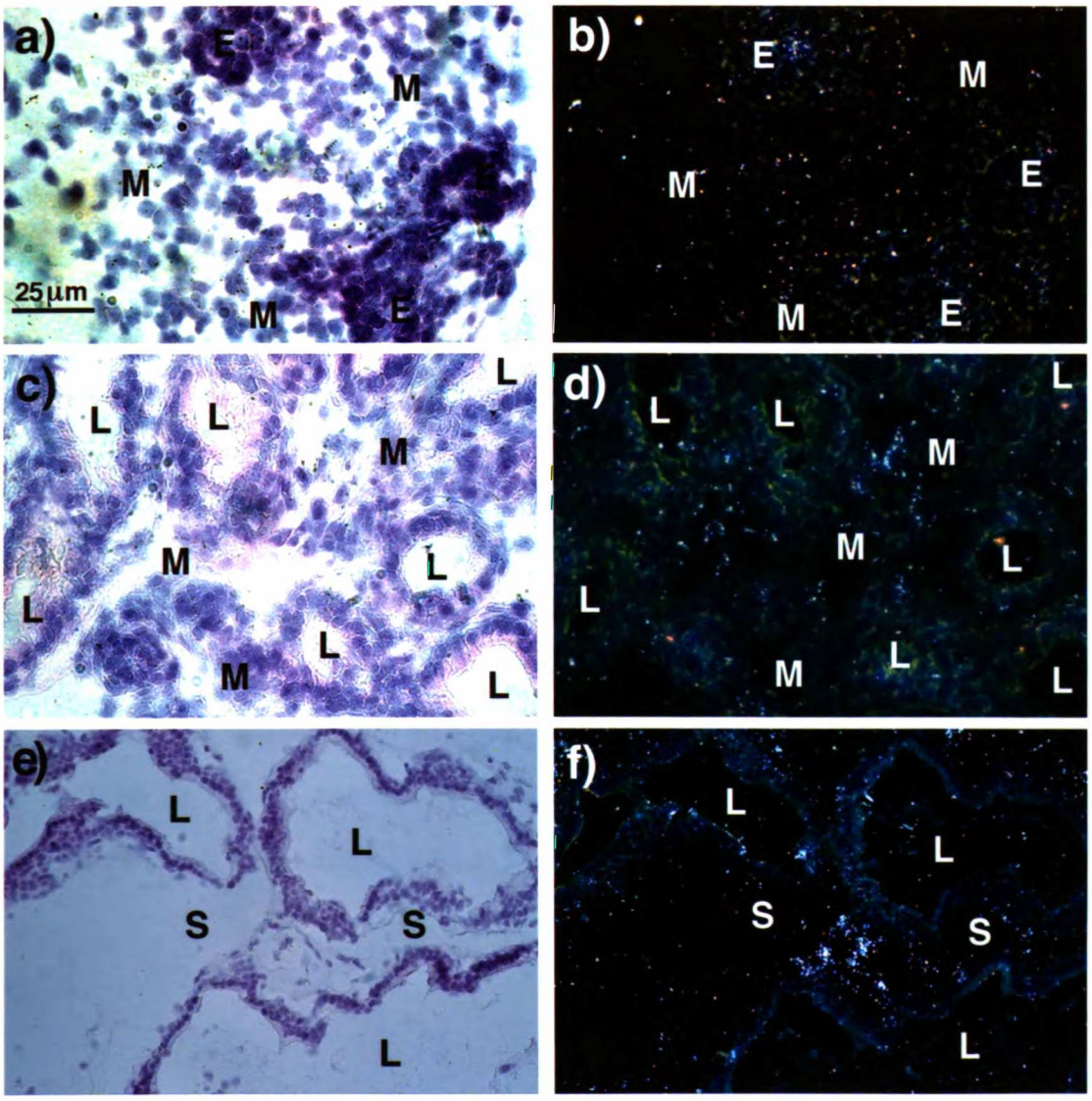
***In situ* hybridization for uPA mRNA in developing rat ventral prostates**

Distribution of uPA mRNA in 3 day old, 14 day old, and adult rat ventral prostates, as detected by *in situ* hybridization. Seven mm thick frozen tissue sections of developing and adult rat VPs were hybridized with ^{35}S-labled sense riboprobes for uPA mRNA (not shown), or ^{35}S-labled anti-sense uPA mRNA riboprobes (c, d, e). Sense and anti-sense probes were prepared, and hybridizations were performed as described in Materials and Methods. Tissue sections were viewed by bright field (a, c, e) or dark field (b, d, f). "E" and "M" in (c) indicate epithelial and mesenchymal tissue, respectively. Compared to the weak diffuse signal obtained in all ages with the sense probe, hybridization with the anti-sense probe gave a strong signal for uPA mRNA in the mesenchyme as well as in smooth muscle of 3 day old and 14 day old rat VPs (b, d). In adult rat VPs a positive signal was restricted to the stroma and was detected in the interstitial fibroblasts and smooth muscle (f). Sections were counterstained with hematoxylin/eosin. Bar, 25 mm.

Figure 2.3

In situ hybridization for uPA mRNA in developing rat ventral prostates

a) 3 day old, bright field, anti-sense; b) 3 day old, dark field, anti-sense
c) 14 day old, bright field, anti-sense; d) 14 day old, dark field, anti-sense
e) adult, bright field, anti-sense; f) adult, dark field, anti-sense
M: mesenchyme; E: epithelium
S: stroma; L: lumen



Frozen sections gave a signal for uPA mRNA that was localized throughout the stroma when hybridized with ^{35}S-labled anti-sense riboprobes (fig. 2.3d).

In VPs from adult rats, canalized epithelial ducts are highly branched and contain polarized tall columnar luminal epithelial cells as well as dendritic basal cells (fig. 2.3e). The stroma contains well differentiated smooth muscle sleeves intimately surrounding each epithelial duct and interstitial fibroblasts between ducts (fig. 2.3e). Frozen sections gave a signal for uPA mRNA that was localized to stromal cells when hybridized with ^{35}S-labled anti-sense riboprobes (fig. 2.3f). Thus, uPA mRNA is expressed exclusively in the mesenchyme of developing rat VPs and in the stroma of adult rat VPs.

The localization of activated uPA protein in adult rat ventral prostates

Biotinylated ecotin M84R/M85R binds to activated uPA and can be used to localize uPA protein in tissue sections of adult rat VP. By this method, activated uPA was localized to the epithelium, epithelial basement membrane, and most abundantly to the peri-epithelial smooth muscle tissue (fig. 2.4a, c, e, see arrows). Staining with biotinylated ecotin was abolished by a 1000-fold excess of unlabeled ecotin M84R/M85R (fig. 2.4b, d, f). Thus, active uPA protein appeared to localize to the epithelium, basement membrane, and smooth muscle of adult VPs.

Figure 2.4

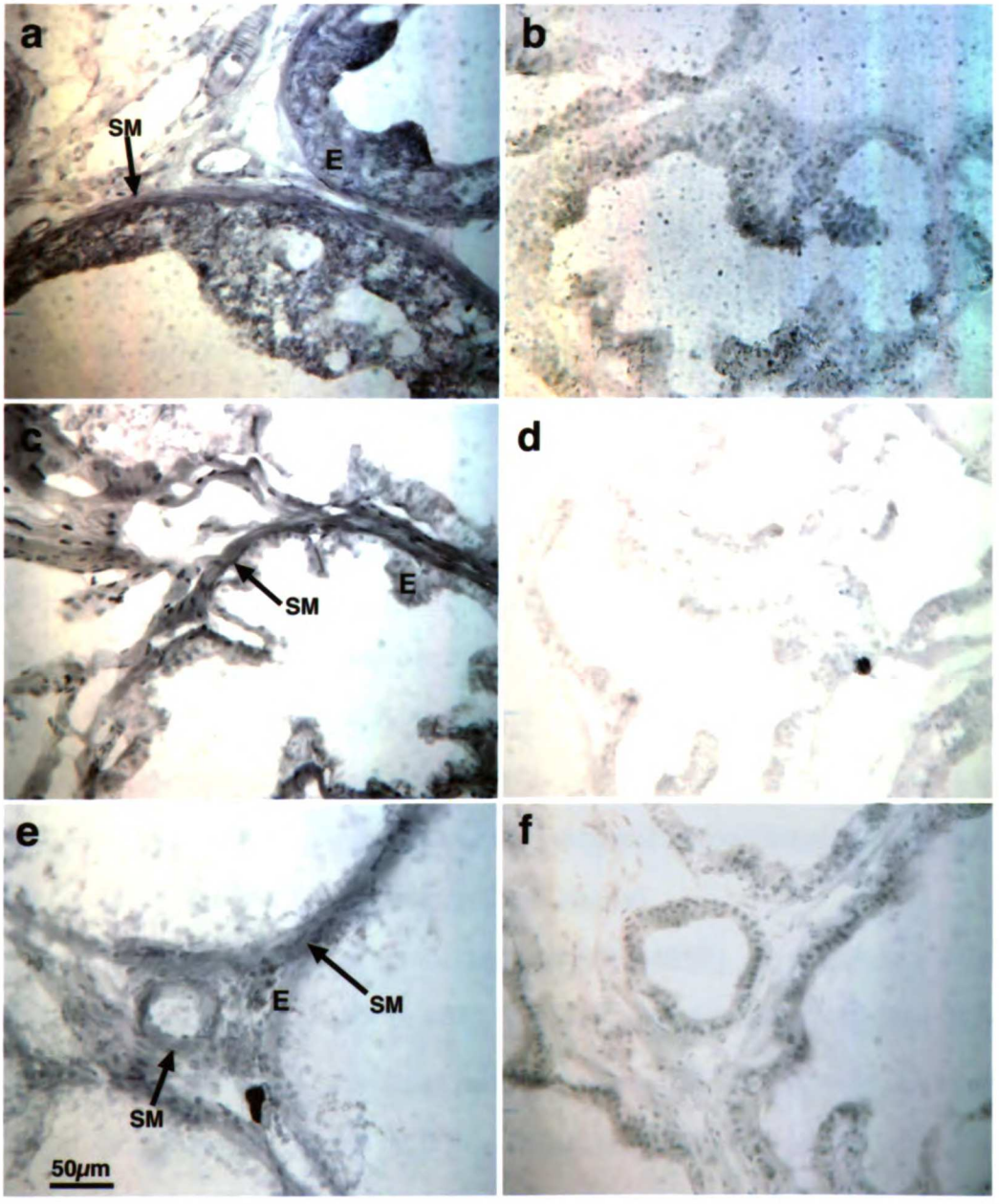
Localization of activated uPA in adult rat ventral prostates with biotinylated ecotin M84R/M85R

Biotinylated mutant ecotin M84R/M85R was used to determine the localization of active uPA in frozen tissue sections of adult rat ventral prostates. Shown are three separate experiments performed as described in Materials and Methods. Biotinylated ecotin M84R/M85R was localized most intensely in the smooth muscle tissue (arrows) and to a minor degree in the epithelium (E) (a, c, e). Nonspecific binding of biotinylated ecotin M84R/M85R was determined by the binding of labled ecotin in the presence of 1000X unlabeled ecotin M84R/M85R (b, d, f). Bar, 50 μm.

Figure 2.4

Localization of activated uPA in adult rat ventral prostates with biotinylated ecotin M84R/M85R

a,c,e) 10ug/ml biotinylated ecotin M84R/M85R
b,d,f) 10ug/ml biotinylated ecotin M84R/M85R
+ 1000 fold excess of unlabeled ecotin M84R/M85R
SM: smooth muscle tissue E: epithelium



2) Function of uPA

The requirement for uPA during development of newborn rat ventral prostates

At initiation of culture, the newborn rat VP has approximately 8 ductal tips. Following one week of serum-free culture in the presence of 10^{-8}M T, newborn rat VPs form approximately 50 terminal ductal tips. *In vitro* ductal branching morphogenesis induced by T is associated with ductal growth, ductal branching, and epithelial differentiation which includes ductal canalization and differentiation of columnar luminal epithelial cells as well as dendritic basal cells. As the epithelium differentiates, mesenchymal differentiation proceeds with the formation of dense peri-ductal smooth muscle sleeves and segregation of fibroblasts to the interstitial area between ducts. In the absence of exogenous androgens, ductal branching morphogenesis and histodifferentiation of cultured newborn rat VP were severely retarded. In one study, newborn rat VPs grown without T demonstrated significantly reduced growth and ductal branching morphogenesis, generating only 18.0 ± 1.7 terminal epithelial ductal tips, compared to 41.5 ± 2.2 terminal epithelial ductal tips observed in organs cultured with 10^{-8} M T, a significantly different value (n=12, p-value<0.0001) when analyzed with a unpaired t-test (not shown). Epithelial and mesenchymal histodifferentiation in newborn rat VPs cultured without T was also retarded (not shown).

In a second study, mutant ecotin (ecotin M84R/M85R), a specific uPA inhibitor, perturbed development of rat VP *in vitro*. Ecotin M84R/M85R at 10μg/ml (300nM) dramatically altered ductal branching morphogenesis and significantly reduced the number of ductal tips. Newborn rat VPs cultured with 10^{-8}M T generated 55.2 ± 3.6 terminal epithelial ducts compared to 14.2 ± 2.5 terminal ductal tips in VPs treated with

10^{-8}M T plus 10 μg/ml ecotin M84R/M85R, a significantly different value (n=18, p-value<0.0001) when analyzed with a unpaired t-test (fig. 2.5). The shape of ductal tips and the branching pattern of newborn rat VPs treated with ecotin M84/M85R was also abnormal. Also, control VPs cultured with T alone demonstrated extensive ductal arborization with slender terminal ducts extending to the peripheral mesenchyme. In contrast, VPs treated with T plus ecotin M84R/M85R had an aberrant truncated ductal branching pattern with ducts that did not extend into the peripheral mesenchyme. The effect of ecotin M84R/M85R was dose-dependent. The lowest concentration to disrupt ductal morphogenesis was 1.25μg/ml (40nM). A dose-dependent disruption of ductal morphogenesis was observed through 20μg/ml (600 nM) (fig. 2.6). At doses higher than 20μg/ml, ductal architecture was completely obliterated and identification of epithelial and mesenchymal tissues was difficult when viewed in whole mounts.

In VPs treated with ecotin M84R/M85R, histodifferentiation was dramatically different from VPs treated with T alone. The canalization of the epithelial ducts was greatly impaired in VPs treated with T plus ecotin M84R/M85R. Few ducts contained polarized luminal columnar epithelial cells and the proximal mesenchyme was dense with fibroblasts, while peri-ductal smooth muscle differentiation was reduced (fig. 2.7).

Transfer of VPs grown for 3 days in the presence of ecotin M84R/M85R to media containing T only, resulted in renewed ductal branching morphogenesis (fig. 2.8e). Therefore, ecotin M84R/M85R disrupted the growth, ductal branching morphogenesis, and histodifferentiation of newborn rat VP as a biological antagonist, rather than as a toxin. Furthermore, addition of ecotin M84R/M85R after 3 days of growth in T-containing media resulted in a rapid loss of ductal architecture and tissue integrity (fig. 2.8f). Thus, ecotin M84R/M85R perturbation of VP ductal morphogenesis was dose-dependent, reversible, and effective when added mid-culture. These results suggest that uPA, or a uPA-like enzyme is required for development of rat VPs.

Figure 2.5

Bar graphs showing number of terminal epithelial tips in newborn rat ventral prostates cultured with the uPA antagonist ecotin M84R/M85R.

Bar graph showing mean number of terminal epithelial tips ± standard errors of newborn rat ventral prostates grown serum-free for one week in the presence or absence of 10 μg/ml mutant ecotin (ecotin M84R/M85R) (p-value<0.0001, n=18).

Figure 2.5

Bar graph showing number of terminal epithelial tips in newborn rat ventral prostates cultured with a uPA antagonist

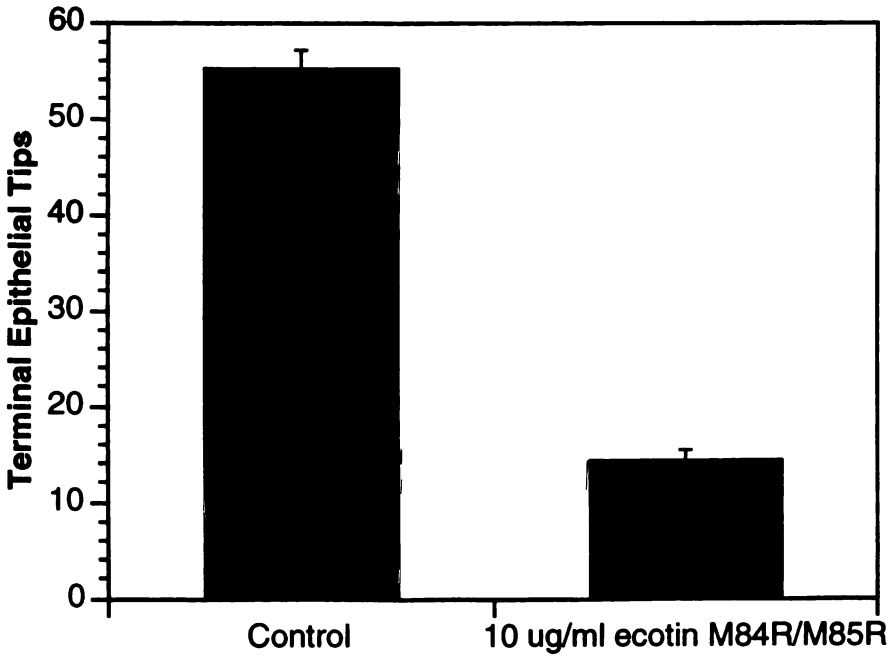


Figure 2.6

The effect of various doses of ecotin M84R/M85R on ductal branching morphogenesis of newborn rat ventral prostates

The effect of various concentrations of mutant ecotin (ecotin M84R/M85R) on newborn rat ventral prostates cultured for one week, serum-free. Newborn rat VPs were cultured and imaged as described in the Materials and Methods. (a) Testosterone (T) only, (b) T plus 1.25 μg/ml ecotin M84R/M85R, (c) 2.5 μg/ml ecotin M84R/M85R, (d) 5.0 μg/ml ecotin M84R/M85R, (e) 7.5 μg/ml ecotin M84R/M85R, (f) 10 μg/ml ecotin M84R/M85R, (g) 15 μg/ml ecotin M84R/M85R, (h) 20 μg/ml ecotin M84R/M85R.

Figure 2.6

The effect of various doses of ecotin M84R/M85R on ductal branching morphogenesis of newborn rat ventral prostates

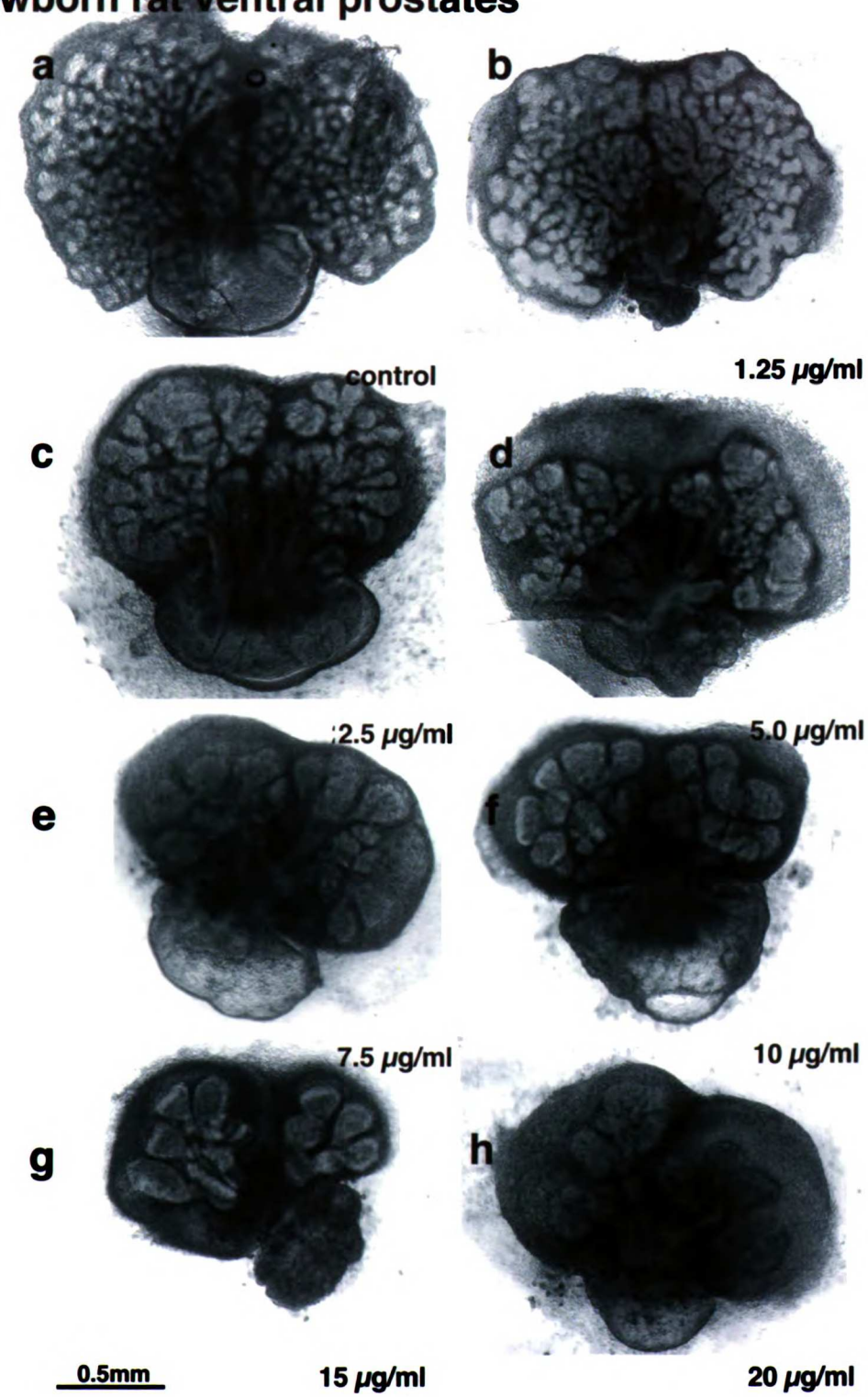


Figure 2.7

The effect of ecotin M84R/M85R on histodifferentiation of rat ventral prostates

Hematoxylin/eosin staining of frozen tissue section of newborn rat ventral prostates cultured with and without mutant ecotin (ecotin M84R/M85R). At lower magnification, fewer epithelial ducts can be seen in rat ventral prostates treated with 10 μg/ml ecotin M84R/M85R (fig. 2.7a) compared to VPs cultured with human uPAR antagonists (fig. 2.7b). At higher magnification, ecotin M84R/M85R-treated rat ventral prostates appear to have less mesenchymal differentiation in the form of circumferential peri-ductal fibroblasts, compared with control cultures (fig. 2.7 c, d). Bar, 50 μm.

Figure 2.7

The effect of ecotin M84R/M85R on histodifferentiation of rat ventral prostates

Hematoxylin/eosin staining of newborn rat ventral prostates cultured for one week with 10μg/ml ecotin M84R/M85R

a,b) control; c,d) ecotin M84R/M84R

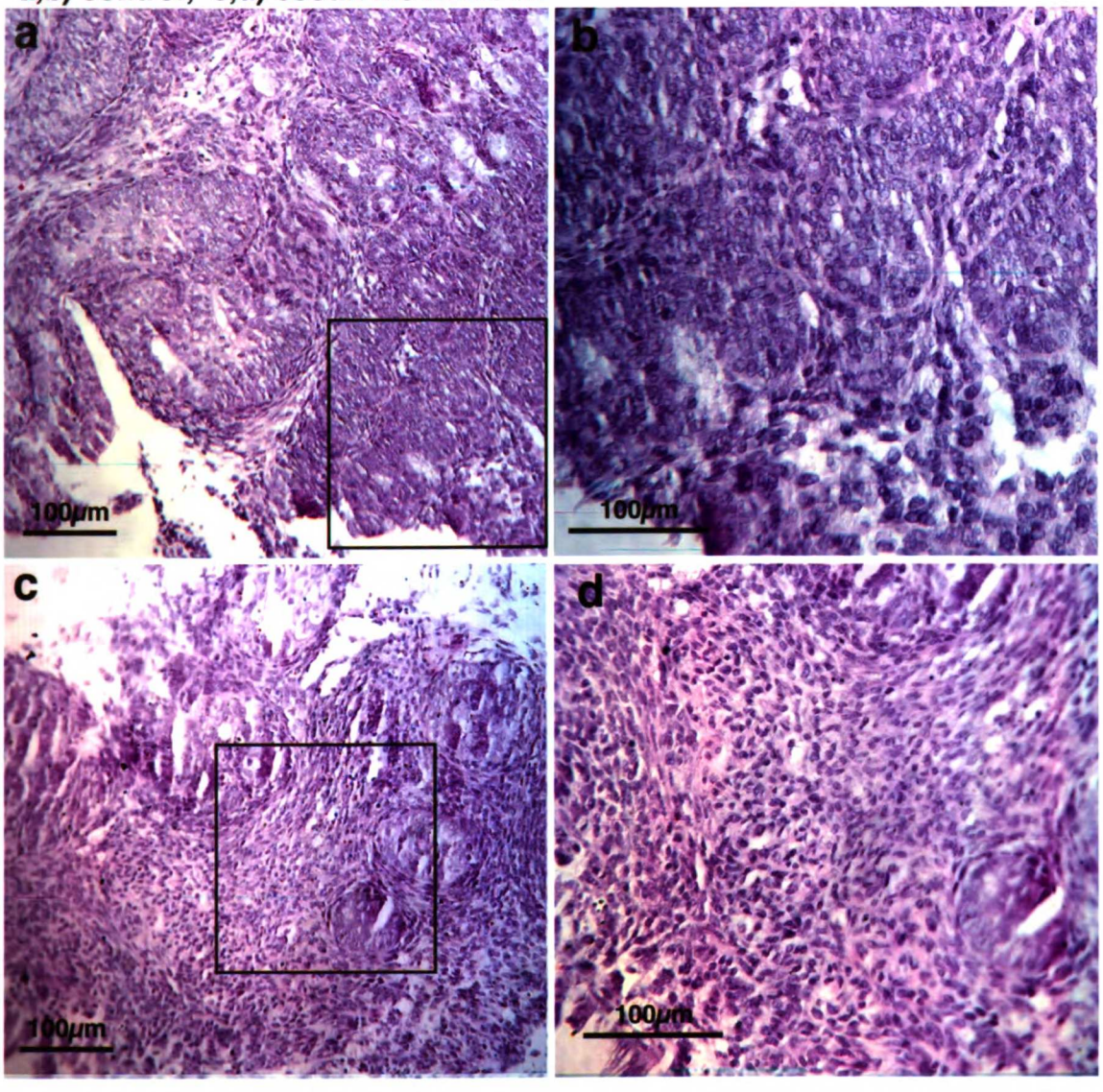


Figure 2.8

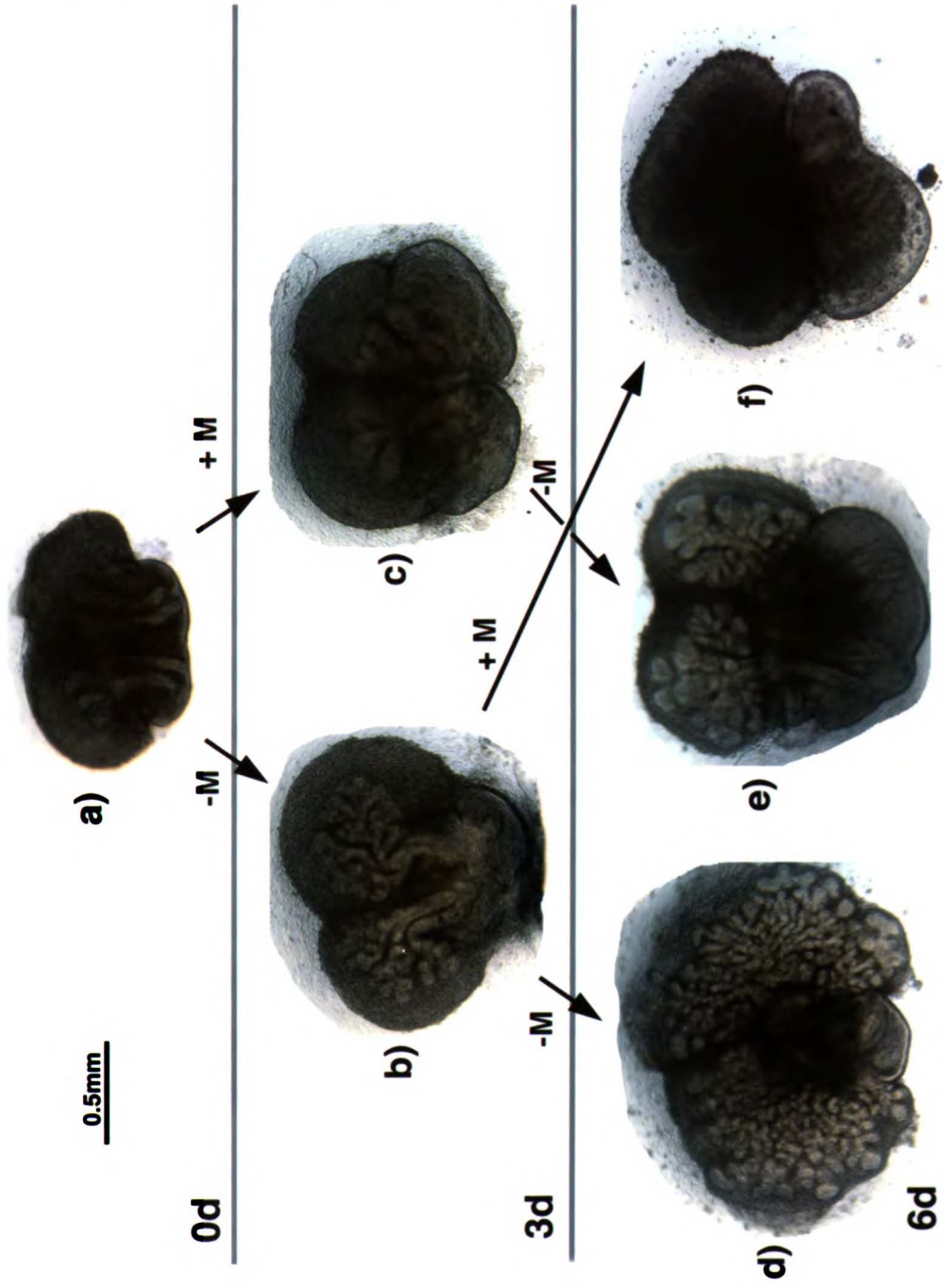
The effect of delayed dosage and rescue of newborn rat ventral prostates treated with ecotin M84R/M85R

Newborn rat ventral prostates were cultured plus T with and without 10 μg/ml mutant ecotin (ecotin M84R/M85R) for three days, then ecotin treatment was begun or discontinued for an additional three days. Organs were cultured and imaged as described in Materials and Methods. Arrows going from left to right indicate treated organs and arrows going from right to left indicate untreated organs. Bar, 0.5mm.

Figure 2.8

The effect of delayed dosage and rescue of newborn rat ventral prostates treated with ecotin M84R/M85R

Newborn rat ventral prostates cultured with testosterone for 3 days and then to 6 days +/- 10 μg/ml ecotin M84R/M85R (M)



Amiloride is a chemical that inhibits uPA as well as blocks ion-channels. Although we currently cannot specify which of these was affected, amiloride at concentrations >600nM also inhibited ductal branching morphogenesis (not shown). Wild-type ecotin shares with ecotin M84R/M85R, a nanomolar avidity for the chymotrypsins elastase and trypsin, and clotting Factor X. Ecotin M84R/M85R has 2800-fold greater avidity for the class of chymotrypsins that includes uPA and thrombin. Ecotin M84R/M85R was ~20-fold more potent than wild-type ecotin in perturbing the development of cultured newborn rat VP (not shown).

3) Expression and function of plasminogen

Plasminogen mRNA expression in newborn rat ventral prostates and the requirement for plasminogen during development of newborn rat ventral prostates

RT-PCR was performed on total RNA isolated from newborn rat VPs as described in Materials and Methods. By agarose gel electrophoresis, a 416 bp sized fragment of amplified DNA corresponding to plasminogen mRNA was expressed by newborn rat liver (fig. 2.9, lane 2), but not by newborn rat VPs (fig. 2.9, lane 5). The integrity of VP mRNA was verified by the generation of a 448 bp sized DNA fragment corresponding uPAR mRNA (not shown). Also, mRNA from newborn kidney which expressed mRNA for uPA and uPAR, did not express mRNA for plasminogen. Further, 200μg/ml of aprotinin, a specific and potent inhibitor of plasmin, had no effect on theductal branching morphogenesis of newborn rat VPs cultured serum-free for one week (not shown). Therefore, plasminogen is not necessary for prostatic development.

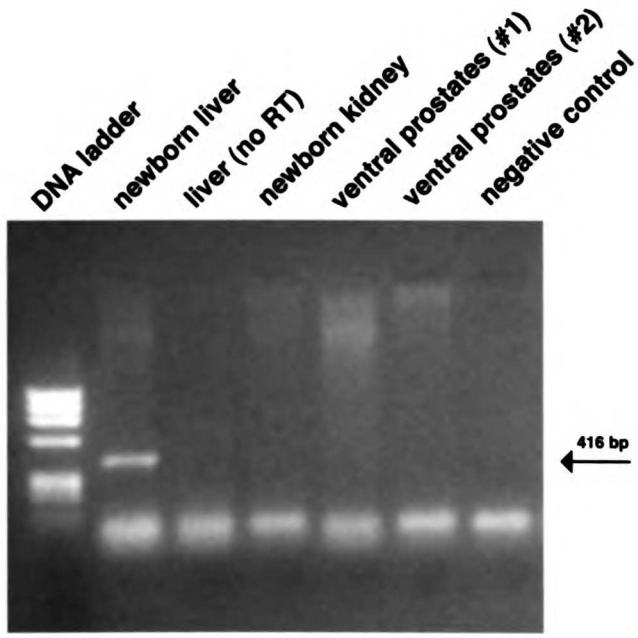
Figure 2.9

RT-PCR for plasminogen mRNA in newborn rat ventral prostates

RT-PCR was performed on isolated RNA from newborn rat ventral prostates as described in Material and Methods. Agarose gel electrophoresis showed: lane 1, DNA ladder; lane 2, the presence of a 416bp fragment corresponding to plasminogen mRNA in the newborn rat liver; lane 3, liver RNA without RT; lane 4, newborn rat kidney; lanes 5 and 6, the absence of signal for plasminogen mRNA in two preparations of newborn rat ventral prostates; lane 7, negative control containing the products of PCR from RT of VP mRNA, performed without RT.

Figure 2.9

RT-PCR for plasminogen mRNA in newborn rat ventral prostates



2.5 Discussion

The mouse urogenital sinus epithelium and mesenchyme express uPA (Timme et al., 1994). During prostatic organogenesis, uPA is expressed in the neonatal rat prostatic complex, which is composed of the prostatic urethra, peri-urethral fat, ventral, dorsolateral, and anterior prostatic lobes, as well as in the pubertal ventral, anterior and dorsolateral lobes (Wilson et al., 1988; Wilson, 1995). However, the expression and role of uPA during ductal branching morphogenesis of the newborn rat VP by itself, are unknown. The current study has demonstrated by zymography that uPA protein was expressed in newborn rat VPs and was localized to both the epithelium and the mesenchyme. In contrast, by *in situ* hybridization uPA mRNA was expressed exclusively by the mesenchyme of newborn and developing rat VPs. In adult rat VPs, uPA mRNA was synthesized exclusively by smooth muscle and interstitial fibroblasts. The stromal localization of uPA mRNA in adult rat VPs, the localization of some uPA protein to the epithelium, and the previously reported expression of uPA protein in adult rat VPs epithelium (Andreasen, 1990), raises the question that uPA may traffic between stromal and epithelial tissues.

Newborn rat VPs cultured in the absence of T demonstrated reduced growth, fewer epithelial ductal tips, as well as retarded epithelial and mesenchymal differentiation. In the current study we have reported that despite reduced development, CM from androgen-deprived cultured newborn rat VP contains equivalent amounts of uPA protein compared to CM's from VPs grown in the presence of added androgens. It has been speculated that androgens repress uPA expression in the adult rat (Andreasen, 1990; Rennie, 1984). Perhaps the regulation of uPA expression in developing rat VPs, compared to the adult, is mediated by alternative or supplementary factors.

uPA is a degradative, mitogenic and motogenic ligand that binds to a signaling receptor. However, the developmental function of the uPA system is unclear. In the current study, we demonstrated that disruption of uPA action results in a significant

inhibition of epithelial growth and perturbation of ductal branching morphogenesis of the newborn VP, compared to untreated control VPs cultured for one week. In neonatal rat VPs cultured with the uPA inhibitor ecotin M84R/M85R, significantly fewer terminal epithelial ductal tips formed. The shape and branching pattern of ductal tips were severely altered by treatment with ecotin M84R/M85R. Histodifferentiation was also perturbed as demonstrated by reduced canalization of ducts, fewer polarized columnar epithelial cells, and reduced circumferential organization of mesenchymal cells. Therefore, uPA or a uPA-like enzyme is necessary for one or more of the processes that are central to prostatic ductal morphogenesis and histodifferentiation.

uPA is one of many paracrine factors required for ductal branching morphogenesis of parenchymal organs. It is presently unclear which of uPA's described functions are necessary for prostatic development: ECM-degradation, mitogenesis, motogenesis, or cytokine activation? Mesenchyme can regulate organogenesis through cytokines and remodeling of the extracellular matrices. The mesenchyme specifies patterns of ductal branching morphogenesis in mouse mammary glands (Kratochwil, 1987; Sakakura, 1987), mouse salivary glands (Bernfield et al., 1984) and rat VPs (Cunha et al., 1987). Development of rat VPs occurs via epithelial-mesenchymal interactions (Cunha et al., 1987). Prostatic development requires the action of androgens which act via mesenchymal androgen receptors (Cunha et al., 1987). ECM-degradation such as basement membrane (BM) turnover mediates change in epithelial shape during ductal branching morphogenesis of the mouse salivary gland. Bernfield et al. have observed that glycosaminoglycan turnover in basement membranes is increased in growing ductal tips, not in clefts, with the mesenchyme being the origin of BM remodeling enzymes (Bernfield et al., 1984). Fukada et al. have observed that mesenchymal fibrillar collagen type III in the clefts of developing salivary glands are necessary for branching morphogenesis of the mouse salivary gland (Fukuda et al., 1988). In the current study, we have observed that uPA mRNA is synthesized by the mesenchyme of developing rat

VPs as well as the stroma of adult rat VPs. Also, activated uPA is localized in smooth muscle and in the epithelium of the adult rat VP. Since mesenchyme plays a central role in ductal branching morphogenesis, we propose that uPA represents one of the mesenchymal factors that is necessary for BM turnover during prostatic ductal morphogenesis. TGF-β_1 (Timme et al., 1994) and HGF/SF (Rubin et al., 1989) are potent regulators of mitogenesis and are morphogens made in the mesenchyme that bind to epithelial receptors in many developing organs, including rat VPs. Both require activation by proteolysis and are substrates of plasmin. Moreover, uPA is directly motogenic to vascular endothelium, as well as being mitogenic to vascular smooth muscle cells, dermal fibroblasts and prostatic cancer cells. Therefore, either ECM degradation, cytokine activation, or direct cytokine-type activity by uPA may be required for development of rat VPs.

Mesenchyme specifies the lobe-specific patterning of ductal branching morphogenesis in the each of the lobes of the rat prostate as well as the seminal vesicle. Interestingly, ecotin M844R/M85R also disrupts the growth and development of newborn rat seminal vesicles. 10μg/ml ecotin M84R/M85R severely disrupted the development of newborn rat seminal vesicles cultured for one week serum-free (Elfman, Takeuchi, Craik, Shuman, and Cunha, unpublished observation). Therefore, uPA appears necessary for morphogenic programs besides branching morphogenesis because though the seminal vesicle undergoes a fundamentally different developmental program compared to the ventral prostate, both require uPA. Ecotin M84R/M85R appeared to halt and reverse ductal branching morphogenesis as well as histodifferentiation when added to established neonatal rat VP cultures. Thus, uPA appears to be necessary for the continued growth of multiple organogenic programs, in addition to branching morphogenesis of rat VPs. How uPA regulates tissue development or maintenance is unclear.

The requirement for uPA in the development of the rat VP contrasts with the developmental normalcy of uPA knockout mice (Carmeliet et al., 1994). The disparity of

uPA requirement in the two systems may be explained by the difference in the ages of the uPA functional perturbation. When uPA is disrupted from the onset of embryonic gene expression in the blastocyst in uPA-knockout mice, redundant pathways may be utilized to compensate for the loss of uPA. This has apparently occurred in the uPA knockout mouse. Unfortunately, it is not known when compensation occurred and in what tissue or germ layer in the knockout mouse. Therefore, the normalcy of the uPA knockout mice renders the model uninformative as to the role of uPA in prostatic development. uPA function appears critical for ductal branching morphogenesis as well as epithelial histodifferentiation of VPs because growth and development are disrupted if uPA is perturbed in the newborn rat, (19 days after conception) beyond when compensation is apparently possible. In addition to inhibiting uPA, ecotin M84R/M85R also has nanomolar avidity to thrombin. We have not yet determined if the effect of ecotin M84R/M85R is due to the inhibition of uPA, thrombin, or the inhibition of a homologous uPA-like chymotrypsin. Thrombin has been reported to be mitogenic, and its receptor is expressed in fibroblasts of developing organs (S. Coughlin, personal communication). Studies are underway to determine the thrombin receptor expression in developing rat VPs.

While uPA is necessary for ductal branching morphogenesis and histodifferentiation of newborn rat VPs, its action appears to be independent of plasminogen. This conclusion is derived from the fact that the newborn rat VP culture system is serum-free and presumably devoid of plasminogen. Also, transcripts for plasminogen mRNA were not detected in the newborn rat VP by RT-PCR. Therefore, the availability and requirement for plasminogen must be questioned in our rat VP organ culture system. Either, plasminogen is deposited in the ECM of newborn rat VP from previous fetal vascular contact such that it is not rate-limiting during the one week culture period which is improbable given the limited half life of plasminogen (immunohistochemical measurement of plasminogen, in progress), or more likely and

most interesting, plasminogen is *not* necessary for VP development. Thus, the results of the current study suggest that during neonatal rat prostatic development, uPA is necessary, but acts through a plasminogen-independent mechanism. With regard to the lack of a role for plasminogen in normal development, the results and conclusions of the current study are consistent with the results and conclusions of the recently reported plasminogen/fibrinogen double knockout mice (Bugge et al., 1996). Whether uPA activates similar plasminogen homologues during prostatic development as well as during development of the plasminogen knockout mouse, is an intriguing question.

CHAPTER THREE

UROKINASE PLASMINOGEN ACTIVATOR RECEPTOR IS NECESSARY FOR DEVELOPMENT OF THE RAT VENTRAL PROSTATE

3.1 Abstract

The plasma membrane receptor for urokinase plasminogen activator (uPAR) localizes and increases uPA-mediated degradation of the extracellular matrix. uPAR can participate in signaling across the plasma membrane and mediates mitogenesis as well as motogenesis. However, the role of uPAR during prostatic development is unclear. In the current study we have examined the expression of uPAR mRNA in newborn Fisher 344 rat ventral prostates (VPs). Also, the role of uPAR in prostatic organogenesis was assessed in serum-free organ cultures of rat VPs grown for one week in the presence or absence of uPAR-antagonists. We report that the newborn rat VP expresses uPAR mRNA. Also, newborn rat VPs grown with 10^{-8} M testosterone (T) only or T plus a control uPA peptide underwent extensive branching morphogenesis and histodifferentiation. In contrast, active rodent uPA antagonists perturbed ductal branching morphogenesis in newborn rat VPs. Altered ductal morphology and significantly fewer terminal ductal tips were observed when newborn rat VPs were cultured with T plus either a peptide containing mouse uPA amino acids 1-138 or a peptide containing mouse uPA amino acids 1-48. The effect was dose-dependent and partially reversible. Histodifferentiation of both the epithelium and mesenchyme was impaired in newborn rat VPs treated with either of the two rodent uPAR antagonists, as measured by the immunohistochemical staining of cytoskeletal components. These observations suggest that uPAR is necessary for ductal branching morphogenesis and histodifferentiation of newborn rat VPs.

3.2 Introduction

Urokinase plasminogen activator (uPA) is a serine proteinase that is a member of the chymotrypsin family. It is secreted as an inactive single chain protein (scuPA) with a molecular weight of 48 kD in rodents (Dano, 1985). uPA is cleaved by plasmin at Arg_{156}-Phe_{157} or Lys_{158}-Ile_{159} into a disulfide linked, enzymically active two-chain molecule (tcuPA). The 20kD A chain (Ser_1-Lys_{158}) contains an epidermal growth factor-like (EGF)/transforming growth factor alpha-like domain, which mediates the binding of uPA to the uPA receptor (uPAR) (Blasi, 1993), followed by a kringle domain which mediates the binding of uPA to other proteins and to cell surfaces (Dano, 1985; Kwaan, 1992). The B chain contains the catalytically-active serine residue. uPA degrades a variety of substrates including proteinases as well as cytokines, in addition to mediating both motogenesis and mitogenesis. tcuPA binds to uPAR at nanomolar avidity, as does scuPA. scuPA is activated when bound to uPAR (Manchanda, 1991) and the activity of tcuPA is enhanced when bound to uPAR (Reinartz et al., 1994). uPAR localizes and enhances plasmin-mediated degradation of the ECM at cell surfaces (Dano, 1985; Alexander, 1991; Blasi et al., 1987). uPAR mediates activation, inactivation, internalization, and localization of uPA (Blasi, 1993). In addition, uPAR signals, with and without uPA (Resnati et al., 1996). Given the presumed importance of uPAR in tissue remodeling, it is surprising that uPAR-knockout mice appear to develop normally (Bugge, 1995b).

A 55kd heavily glycosylated protein, uPAR is linked to the plasma membrane by a glycosyl-phosphatidylinositol moiety which can be released by phospholipase C (Del Rosso et al., 1992) and D (Metz et al., 1994). Expression of uPAR mRNA is regulated by cytokines (Lund et al., 1995). uPAR has been localized to the calveolae of a human melanoma cell line (Stahl, 1995) and in rodents is expressed by normal embryonic and adult fibroblasts (Dano, 1985), migrating epithelial cells (Kristensen et al., 1991),

leukocytes (Manchanda, 1991), vascular endothelium (Fibbi et al., 1986), and kidneys (Kanalas, 1995). uPA bound to uPAR is localized to focal contacts, which are specialized areas of the plasma membrane where integrins link to the cytoskeleton via their β_1 subunits (Pollaen, 1988). uPAR binds to the ECM-glycoprotein vitronectin (Moser et al., 1995; Kanse et al., 1996). Integrin avidity and adhesion are modulated by uPAR (Wei et al., 1996). In association with c-src or even without uPA, uPAR can signal (Resnati et al., 1996). Lastly, uPAR mediates mitogenesis as well as motogenesis in dermal fibroblasts (Anichini, 1994). Therefore, uPAR mediates a range of activities including signaling, migration, and mitogenesis independent of plasmin. uPAR is expressed in the stroma of metastatic human colon adenocarcinomas (Pyke et al., 1993) and the stroma of metastatic human mammary adenocarcinomas (Bianchi et al., 1994). A role for uPAR in prostate cancer was suggested by the observation that a peptide containing human urokinase plasminogen activator (uPA) amino acid residues 1-138 coupled to a human IgG Fc domain (human uPA/ATF-Ig) inhibited 95% of native uPA binding to uPAR in the human prostate cell line PC3, and inhibited metastasis of these cells when injected into the rat tail vein (Crowley et al., 1993).

In the presence of androgens, the prostate originates from solid epithelial outgrowths (prostatic buds) that emerge from the embryonic urogenital sinus (UGS) immediately below the developing bladder (Cunha et al., 1987). The prostatic buds grow, elongate, invade into urogenital sinus mesenchyme (UGM), and arborize to form a complex ductal network (Sugimura et al., 1986a). The components and mechanisms that mediate tissue remodeling during prostate organogenesis are poorly understood. Though uPAR is postulated to regulate tissue remodeling, its expression and function during development of the rat ventral prostate are unknown. In the current study, we report that uPAR mRNA is expressed in newborn rat VPs, as detected by RT-PCR. The role of uPAR was examined during ductal branching morphogenesis of newborn rat VPs by perturbing it with specific peptide antagonists in a serum-free system that allows for the

culture of newborn rat VPs for one week (Sugimura et al., 1986a). We addressed the differential effects of two truncated mouse uPA ligands on ductal branching morphogenesis of cultured newborn rat VPs: uPA/amino terminal fragment (ATF)-IgG containing mouse uPA n-terminal through amino acid 138 and uPA/EGF-IgG containing mouse uPA n-terminal through amino acid 48. Neither peptide, therefore, contained the uPA catalytic domain so that the structural difference between the two peptides was the presence or absence of the kringle domain. The precise *in vivo* function of this domain is unclear, and to date this is the first study to address this issue during ductal branching morphogenesis. In the current study we report that both truncated mouse uPA peptides inhibited the ductal branching morphogenesis and histodifferentiation of newborn rat VPs.

3.3 Materials and Methods

Inbred Fisher 344 rats (Simenson Inc., Gilroy, CA.) were sacrificed by barbiturate overdose and bilateral thoracotomy in accordance with NIH guidelines. Specimens for histology were embedded in OCT compound (Sakura Inc., Torrence, CA.) and frozen on dry ice. Frozen sections were cut on a Jung Frigocut 2800N cryostat and thaw-mounted onto presialized Fisher-plus microscope slides (Fisher Inc., Springfield, NJ.)

RT-PCR of newborn rat VPs was performed as described previously using a 1st strand cDNA synthesis kit for RT-PCR (AMV), Boehringer Mannheim (Mannheim, Germany). mRNA was isolated from newborn rat VPs using RNA-STAT 60 reagent (Tel-Test, Inc., Friendswood, TX.). Primers for uPAR were constructed commercially (Beringer Mannhiem-UCSF Cell Culture Facility custom synthesis) according to sequences described previously (Kanalas, 1995). Primers were constructed for mouse uPAR sense (5'-AAAGACCAACAGGACCATGAG-3') and anti-sense (5'-TGTTCCCCTCACAGCTGTAACA-3') for a product of 448bp. The primer sets spanned at least one intron according to published gene sequences (Kristensen et al., 1991). No products were obtained from genomic DNA with these primers when RT was omitted before PCR.

Newborn Fisher 344 rat pups under 12 hours old were utilized to establish organ cultures. Individual VPs were isolated by dissection and transferred with micro-forceps to 25mm square floating Millipore-CM membrane rafts (Millipore Inc., Bedford, MA.) in 0.5ml of growth medium contained in four well plates (Nunc. Inc., Roskilde, Denmark). Culture medium was DME H-16 /F-12 (1/1) with 50μg/ml gentamycin (UCSF Cell Culture Facility) supplemented with insulin (10μg/ml), transferrin (50μg/ml) with and without testosterone 10^{-8} M (Sigma Inc., St Louis MO.). This method allows free

exchange of nutrients and treatments such as antibodies or peptides through the filter. Organs were cultured for one week with media changes every two days. At the termination of culture, unfixed glands were imaged as whole mounts on a Zeiss microscope using a Leaf Systems Lumina scanner system (Southborough, MA.). Images were collected on an Apple Power Macintosh 8100 (Apple Computer, Cupertino, CA.) using Adobe Photoshop software (Adobe Systems, Mountain View, CA). Statistical analysis was performed with Statview software (Abacus Concepts Inc., Berkeley, CA.). Peptides containing mouse or human urokinase plasminogen activator (uPA) amino acids 1-138 fused to an immunoglobulin G Fc domain uPA/ATF-Ig) were kindly provided by Robert L. Cohen and Genentech Inc. (South San Francisco, CA.). A peptide containing mouse uPA amino acids 1-48 fused to an immunoglobulin G Fc domain (uPA/EGF-Ig) was kindly provided by Steve Rosenberg and Hye Yeong Min of Chiron Corp. (Emeryville, CA.).

Antibodies for immunohistochemistry against smooth muscle α-actin, vimentin, and keratin 5 were purchased commercially (Sigma Inc., St Louis, MO. and Dako Inc., Carpenteria, CA.). Biotinylated anti-rabbit and anti-mouse IgG (Amersham Inc. Arlington Hts, IL.) and peroxidase linked avitin/biotin complex reagents were purchased from Vector Labs (Burlingame, CA.). Frozen sections were immunostained using the ABC amplified, cobalt intensified, immunoperoxidase method as previously described (Brody and Cunha, 1989). Formalin fixation and paraffin embedding of tissue were according to standard protocols as were the hematoxylin and eosin staining of fixed and -20°C acetone treated-frozen sections.

For analysis of conditioned media, a modification of the methods of Wilson was used (Wilson, 1988). Conditioned media (CM) of cultured VPs were concentrated with Centriprep-10 concentrators (Amicon Inc., Bedford, MA). Protein content of extracts and CMs were determined by the BCA method (Pierce Inc, Rockford, IL). Tissue extracts or CMs containing equal amounts of protein were mixed with 4X Lameli electrophoresis

sample buffer and loaded onto SDS PAGE gels containing 10% polyacrylamide (acrylamide, ammonium persulfate, and SDS, Bio-Rad Inc., Richmond, CA.) mixed with 1 mg/ml casein (Sigma Inc., St. Louis, MO.) and 50μg human plasminogen (American Diagnostica Inc., Greenwich, CT.) and electrophoresed on a mini-gel apparatus (Hoefer Inc., San Francisco, CA.). Following constant current electrophoresis with prestained molecular weight markers, the slabs were washed in 2.5% Triton X-100 (Sigma Inc., St. Louis, MO.) in Tris-HCL buffer in order to remove SDS. Next, the Triton X-100 was washed out, and the slabs were incubated in a Tris-HCL buffer at pH 7.5-8.0 for 18 hours at 37^0 C. Gels were stained with coomassie blue (Bio-Rad Inc., Richmond, CA.), destained, fixed, rubbed with glycerol (Sigma, St. Louis, MO.), mounted and dried in Biodesign gel wrap (Biodesign Inc., Carmel, NY.).

3.4 Results

uPAR mRNA expression in newborn rat ventral prostates

In order to observe the expression of uPAR in newborn Fisher 344 rat ventral prostates (VPs), RT-PCR was performed as described in Materials and Methods using 5' and 3' primer sequences specific for mouse uPAR (Kanalas, 1995). The predicted 448bp fragment of amplified cDNA representing uPAR mRNA was generated from newborn rat VPs as well as kidneys and observed by agarose gel electrophoresis. No products were observed when RT was omitted (fig. 3.1).

The requirement for uPAR during development of newborn rat ventral prostates

Ductal branching morphogenesis

The role of uPAR in ductal branching morphogenesis was evaluated by perturbing the function of uPAR with competitive inhibitors. Peptides containing truncated non-catalytic uPA domains, which compete with native endogenous uPA for binding to uPAR, are effective uPAR antagonists. Human uPA does not bind to rodent uPAR and visa versa. Thus, a peptide containing a human uPA domain serves as a control reagent for homologous mouse uPA domain peptides. The effect of uPAR antagonists on the ductal branching morphogenesis and histodifferentiation of newborn rat VPs was examined in two separate studies by quantitating terminal epithelial ductal tips and observing ductal patterning in organ cultures.

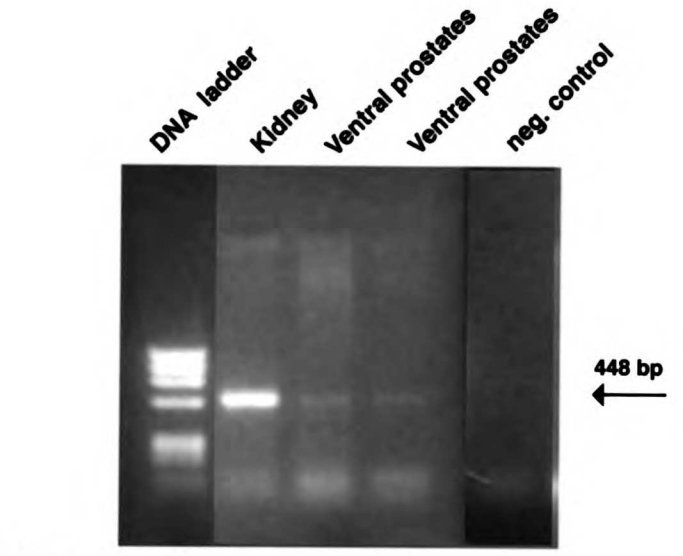
Figure 3.1

RT-PCR for uPAR mRNA in newborn rat ventral prostates

RT-PCR was performed on isolated RNA from newborn rat ventral prostates as described in Material and Methods. Lane 1, DNA ladder; lane 2, the presence of a 448bp fragment corresponding to uPAR mRNA in the newborn rat kidney; lane 3, 4, the presence of a 448bp fragment corresponding to uPAR mRNA in two preparations of newborn rat ventral prostates; lane 5, negative control containing the products of PCR from VP mRNA performed without RT.

Figure 3.1

RT-PCR for uPAR mRNA in newborn rat ventral prostates



In the first study, newborn rat VPs were cultured: (a) with androgens; (b) without androgens; with androgens plus either (c) a human uPA control peptide or (d) a peptide containing mouse uPA amino acids 1-138 (uPA/ATF) coupled to an IgG domain. Newborn rat VPs cultured with 10^{-8}M testosterone (T) generated 36.8 ± 1.9 terminal ductal tips while newborn rat VPs cultured for one week in the absence of androgens generated only 16.5 ± 1.7 terminal ductal tips (the values were significantly different, n=10, p-value<0.0001, when analyzed by an unpaired t-test). Newborn rat VPs cultured with 10^{-8}M T plus 100μg/ml of human control peptide generated 40.1 ± 3.8 terminal ductal tips. VPs cultured for one week with 10^{-8}M T plus 100μg/ml of a peptide containing mouse uPA/ATF-Ig generated only 20.3 ± 2.3 terminal ductal tips (the values were significantly different, n=13, p-value=0.004, when analyzed by an unpaired t-test) (fig. 3.2a). In contrast to control cultures, VPs grown with uPAR antagonists demonstrated dramatically altered ductal branching patterning. The epithelium exhibited less growth and contained broad, poorly branched ducts (fig. 3.3d, f).

In the second study, newborn rat VPs were cultured: (a) with androgens; (b) without androgens; with androgens plus either (c) a human uPA control peptide or (d) a peptide containing mouse uPA amino acids 1-48 (uPA/EGF) coupled to an IgG domain. Newborn rat VPs cultured with 10^{-8}M testosterone (T) generated 41.5 ± 17.6 terminal ductal tips (not shown). Newborn rat VPs cultured for one week in the absence of androgens generated 18.0 ± 1.7 terminal ductal tips (the values were significantly different, p-value<0.0001, when analyzed by an unpaired t-test). Newborn rat VPs cultured with 10^{-8}M T plus 100μg/ml of human control peptide generated 57.7 ± 2.1 terminal ductal tips, while VPs cultured for one week with 10^{-8}M T plus 100μg/ml of a peptide containing mouse uPA/EGF-Ig generated only 25.7 ± 1.7 terminal ductal tips (the values were significantly different, n=18, p-value<0.0001, when analyzed by an unpaired t-test) (fig. 3.2a). In contrast to control cultures, rat VPs treated with uPAR antagonists demonstrated dramatically altered ductal branching patterning. uPAR antagonist-treated

Figure 3.2

Bar graphs showing number of terminal ductal tips in newborn rat ventral prostates cultured with the uPAR antagonists

a) Study I. Bar graph showing mean number of terminal epithelial tips and standard errors of newborn rat ventral prostates grown serum-free for one week in the presence of control 100 μg/ml human or 100 μg/ml mouse uPA/ATF-Ig. Organ cultures and imaging were as described in Materials and Methods. Shown are the mean number of terminal epithelial tips ± standard error (p-value<0.005, n=22).

b) Study II. Bar graph showing mean number of terminal epithelial tips and standard errors of newborn rat ventral prostates grown serum-free for one week in the presence of 100 μg/ml human or 100 μg/ml rat uPA/EGF-Ig. Organ cultures and imaging were as described in Materials and Methods. Shown are the mean number of terminal epithelial tips ± standard error (p-value<0.0001, n=16).

Figure 3.2

Bar graphs showing number of terminal epithelial tips in newborn rat ventral prostates cultured with uPAR antagonists

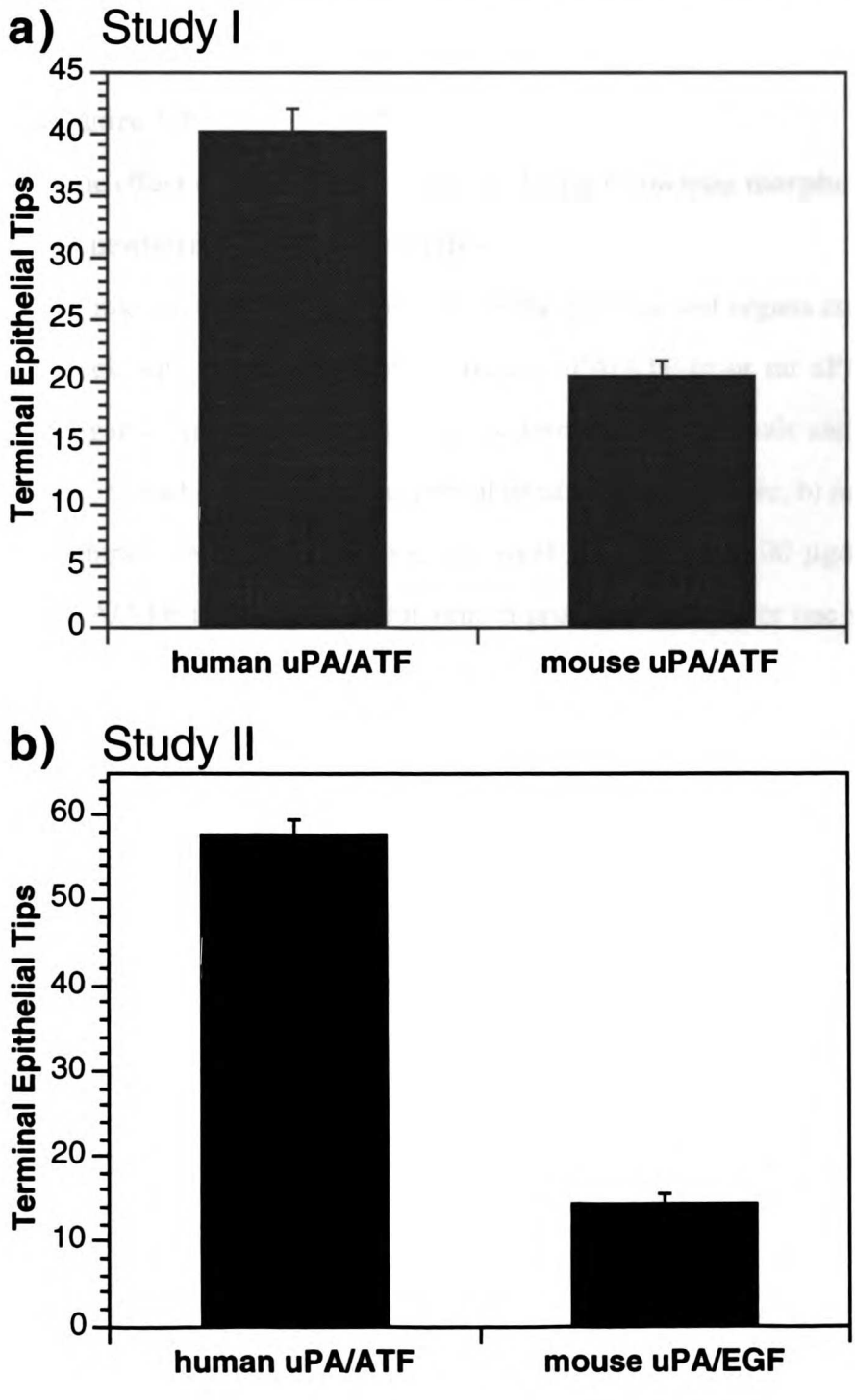


Figure 3.3

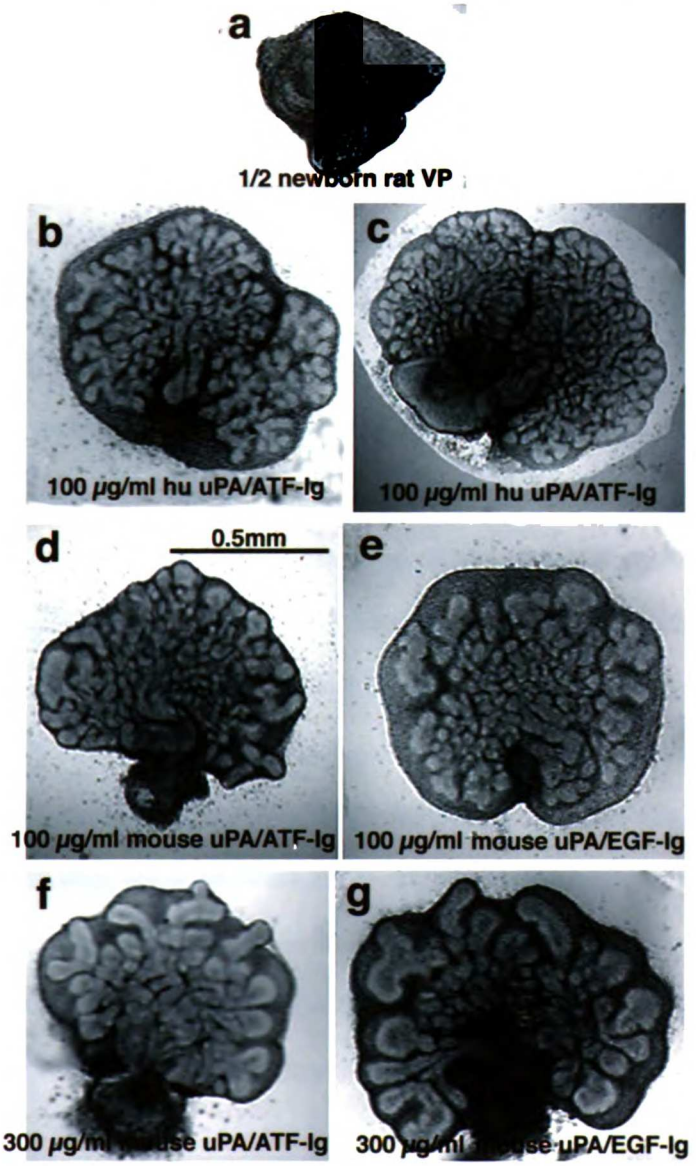
The effect of uPAR antagonists on ductal branching morphogenesis of newborn rat ventral prostates

Whole mounts of a newborn rat ventral prostate and organs cultured one week with various amounts of mouse uPA/ATF-Ig or rat uPA/EGF-Ig. Organ culture and imaging were as described in Materials and Methods. a) one half of a newborn rat ventral prostate before culture, b) newborn rat ventral prostate cultured for one week with control 100 μg/ml human uPA/ATF-Ig, c) newborn rat ventral prostate cultured for one week with control 100 μg/ml human uPA/ATF-Ig, d) newborn rat ventral prostate cultured for one week with 100 μg/ml mouse uPA/ATF-Ig, e) newborn rat ventral prostate cultured for one week with control 100 μg/ml mouse uPA/EGF-Ig, f) newborn rat ventral prostate cultured for one week with 300 μg/ml mouse uPA/ATF-Ig, g) newborn rat ventral prostate cultured for one week with 300 μg/ml mouse uPA/EGF-Ig. Bar, 0.5mm.

Figure 3.3

The effect of uPAR antagonists on ductal branching morphogenesis of newborn rat ventral prostates

a) 1/2 newborn rat VP
b,c) rat VP cultured one week with human uPA/ATF-Ig
d,f) rat VP cultured one week with mouse uPA/ATF-Ig
e,g) rat VP cultured one week with mouse uPA/EGF-Ig



organs also exhibited less growth and contained broad, poorly branched ducts (fig. 3.3d, f). In both studies, a higher dose of 300mg/ml of uPAR antagonists resulted in further disruption of ductal branching morphogenesis (fig. 3.3g, and h). Withdrawal of treatment resulted in a partial rescue of branching morphogenesis (not shown). Moreover, cultures treated with uPAR antagonists appeared healthy, and histology confirmed that the uPAR antagonists were not toxic as necrosis was rarely observed. Both uPA/ATF-Ig and uPA/EGF-Ig had identical effects on the ductal branching morphogenesis of newborn rat VPs (fig. 3.3). Therefore, uPA amino acids 49-138 do not provide additional inhibition of uPAR.

Histodifferentiation

At birth, the VP contains 4 main epithelial ducts containing zero, one, or two branch points. The epithelial ducts are solid and uncanalized. The mesenchyme is dense with fibroblasts. There is sparse smooth muscle directly adjacent to the epithelium (Hayward et al., 1996a). Following one week of culture in the presence of 10^{-8}M testosterone (T), newborn rat VPs form approximately 50 terminal ductal tips in a highly arborized pattern that extends to the outer edge of the mesenchyme. T-induced ductal branching morphogenesis leads to ductal canalization and epithelial differentiation. Polarized columnar luminal epithelial cells differentiate and express cytokeratin 8, while dendritic basal cells express cytokeratin 5. In addition, mesenchymal differentiation occurs with the formation of peri-ductal circumferentially oriented sheaths of alpha-actin-positive smooth muscle cells as well as a reduced expression of vimentin in the mesenchyme.

In the current study we report that newborn rat VPs cultured in the absence of androgens exhibit retardation of ductal branching morphogenesis and histodifferentiation, as observed previously (Sugimura et al., 1986a). uPAR antagonists significantly disrupted the histodifferentiation of epithelial and mesenchymal tissues in cultured

newborn rat VPs. VPs treated with either mouse uPAR antagonist demonstrated altered epithelial morphology. Ducts were thicker and included larger, uncanalized and mostly devoid of polarized tall columnar epithelial cells. In addition, the histomorphology of the mesenchyme is altered to resemble that of the newborn: Peri-ductal fibroblasts were not flattened and were arranged in concentric rings around the ducts, while mesenchyme away from the ducts was densely cellular (fig. 3.4).

Immunohistochemical staining of cytoskeletal components also demonstrated that uPAR antagonists retarded histodifferentiation of both epithelial and mesenchymal tissue in newborn rat VPs. Delayed mesenchymal differentiation was demonstrated in VPs treated with uPAR antagonists as demonstrated by reduced immunohistochemical staining for smooth muscle alpha actin in peri-epithelial mesenchyme (fig. 3.5). Staining for vimentin was increased in uPAR-treated organs compared to VPs treated with human uPA/ATF-Ig (fig. 3.6). VPs treated with both rodent uPAR antagonists also demonstrated delayed differentiation of epithelium, with reduced segregation of K5 to the basal layer of epithelial cells (fig. 3.7). Thus, disruption of the uPAR results in a retardation of histodifferentiation in both epithelial and mesenchymal tissues of rat VP.

Zymography for displaced uPA in conditioned media from uPA/ATF-Ig-treated rat ventral prostates

Substrate zymography was utilized to assess whether conditioned media (CM) from uPAR antagonist-treated cultured VPs contained displaced native rat uPA. CM from newborn rat VP cultured for one week under serum-free conditions with 10^{-8}M T plus a control human uPA peptide expressed a 44 kD plasminogen activator previously identified as uPA (Elfman, submitted) (fig. 3.8, lane 2). CM from newborn rat VPs cultured for one week under serum-free conditions with 10^{-8}M T plus 100μg/ml mouse uPA/ATF-IgG contained increased levels of a uPA (fig. 3.8, lane 3). At a dosage of 300 ug/ml mouse uPA/ATF, the amount of uPA in CM was further increased (fig. 3.8, lane

Figure 3.4

The effect of uPAR antagonists on histodifferentiation of newborn rat ventral prostates

Paraffin embedded six mm sections of newborn rat ventral prostate cultured for one week in the presence of T plus a) 100 μg/ml human uPA/ATF-Ig, b) 100 μg/ml mouse uPA/ATF-Ig, c) 100 μg/ml uPA/EGF-Ig. Bar, 50μm.

Figure 3.4

The effect of uPAR antagonists on differentiation of newborn rat ventral prostates

Hematoxylin/eosin staining of tissue sections from newborn rat ventral prostates cultured for one week

a) 100 μg/ml human uPA/ATF (arrow: epithelial duct)
b) 100 μg/ml mouse uPA/ATF (arrow: epithelial duct)
c) 100 μg/ml mouse uPA/ATF (arrow: mesenchyme)

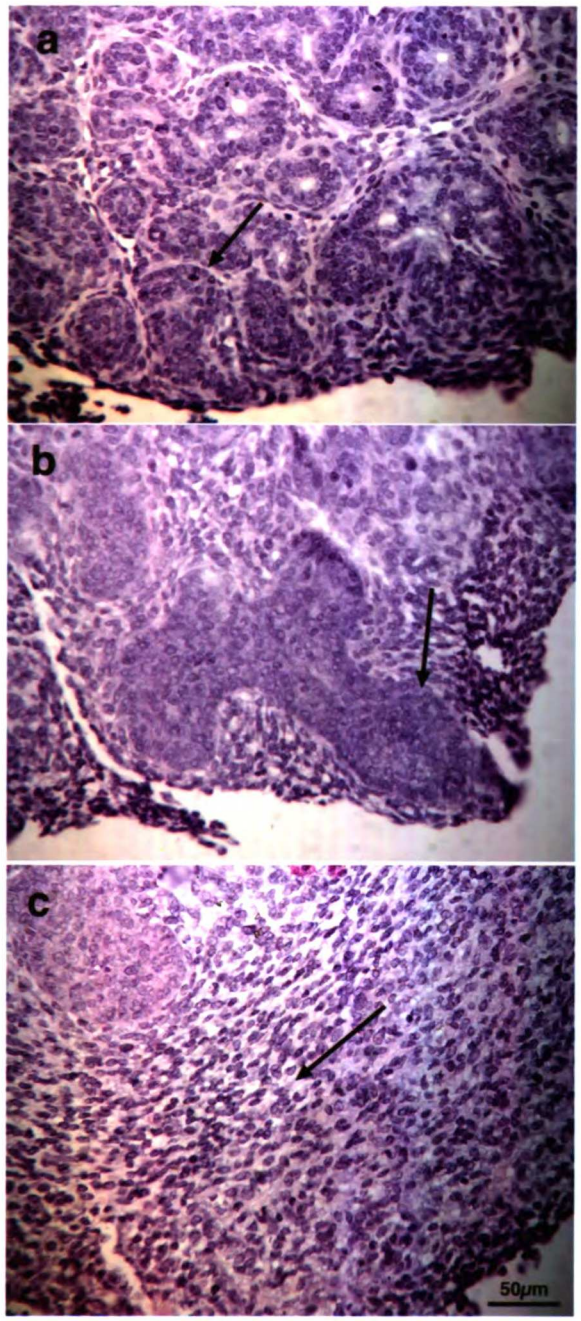


Figure 3.5

The effect of uPAR antagonists on mesenchymal differentiation in newborn rat ventral prostates as measured by staining for smooth muscle alpha actin

Immunohistochemical staining for alpha smooth muscle actin in newborn rat ventral prostates cultured for one week with T plus uPAR antagonists. Organ culture and immunohistochemistry were performed as described in Materials and Methods. a) newborn rat ventral prostate cultured for one week with control 100 μg/ml human uPA/ATF-Ig, b) newborn rat ventral prostate cultured for one week with 100 μg/ml mouse uPA/ATF-Ig, c) newborn rat ventral prostate cultured for one week with 100 μg/ml mouse uPA/ATF-Ig, d) newborn rat ventral prostate cultured for one week with 100 μg/ml rat uPA/EGF-Ig. Bar, 100 μm.

Figure 3.5

The effect of uPAR antagonists on mesenchymal differentiation in newborn rat ventral prostates as measured by staining for smooth muscle alpha actin

Immunohistochemistry staining for smooth muscle alpha actin (arrows) in newborn rat ventral prostates cultured for one week with uPAR antagonists
a) 100ug/ml human uPA/ATF-Ig
b,c)100ug/ml mouse uPA/ATF-Ig
d) 100ug/ml mouse uPA/EGF-Ig

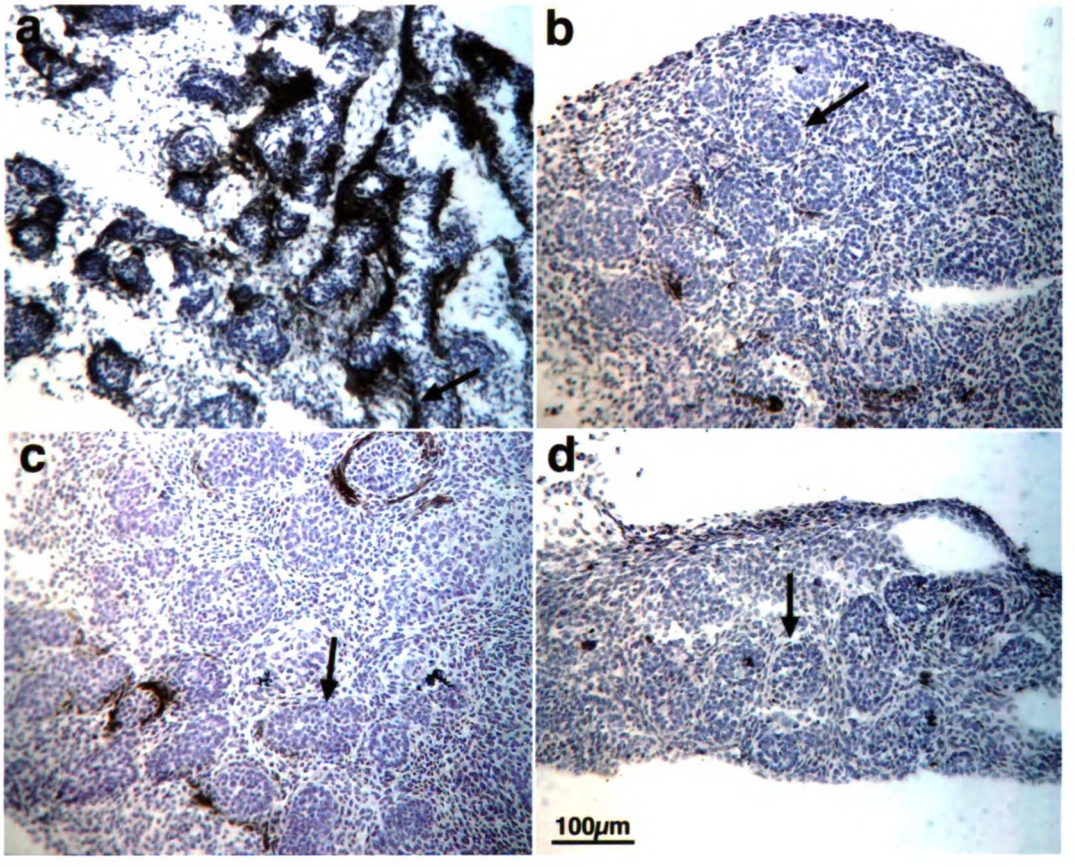


Figure 3.6

The effect of uPAR antagonists on mesenchymal differentiation in newborn rat ventral prostates as measured by staining for vimentin.

Immunohistochemical staining for vimentin in newborn rat ventral prostates cultured for one week with T plus uPAR antagonists. Organ culture and immunohistochemistry were performed as described in Materials and Methods. a) newborn rat ventral prostate cultured for one week with control 100 μg/ml human uPA/ATF-Ig, b) newborn rat ventral prostate cultured for one week with 100 μg/ml human uPA/ATF-Ig, c) newborn rat ventral prostate cultured for one week with 100 μg/ml mouse uPA/ATF-Ig, d) newborn rat ventral prostate cultured for one week with 100 μg/ml rat uPA/EGF-Ig. Bar, 100 μm.

Figure 3.6

The effect of uPAR antagonists on mesenchymal differentiation of newborn rat ventral prostates as measured by staining for vimentin

Immunohistochemical staining for vimentin (arrows) in newborn rat ventral prostates

a) 100 μg/ml human uPA/ATF-Ig
b) 100 μg/ml human uPA ATF-Ig
c) 100 μg/ml mouse uPA/ATF-Ig
d) 100 μg/ml mouse uPA/EGF-Ig

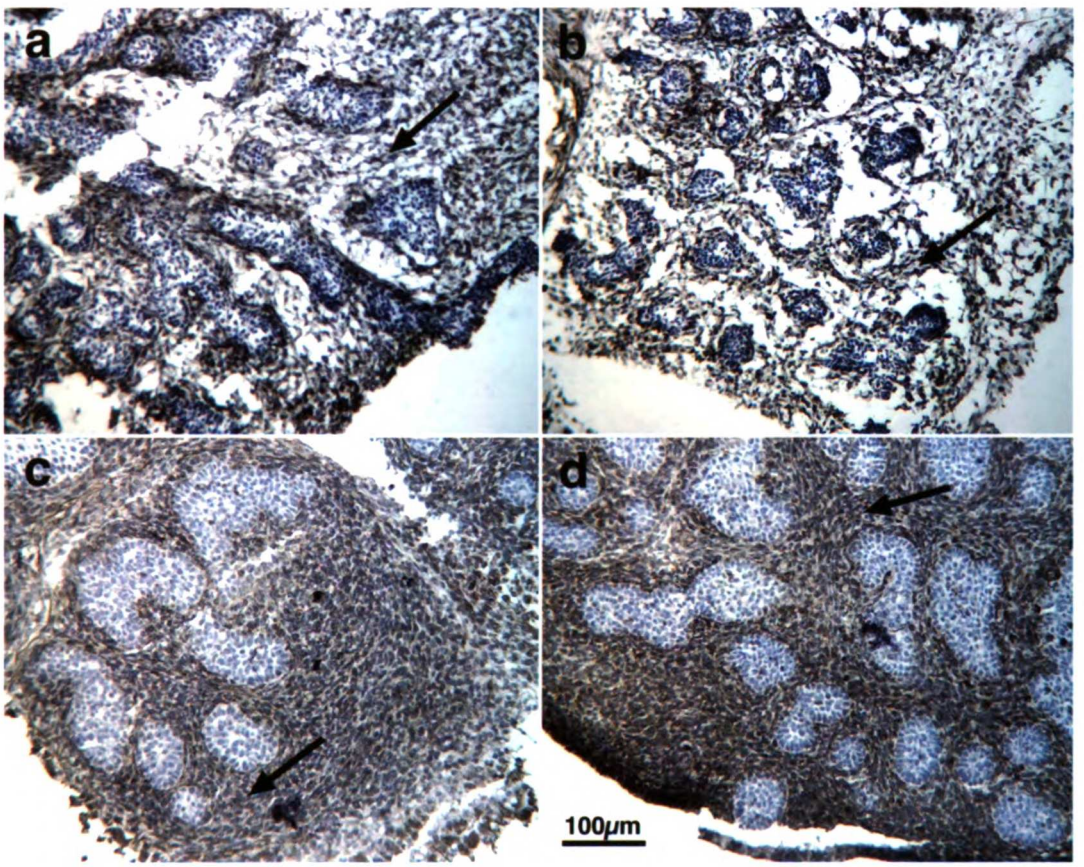


Figure 3.7

The effect of uPAR antagonists on epithelial differentiation

in newborn rat ventral prostates as measured by staining for cytokeratin 5. Immunohistochemical staining for cytokeratin 5 in newborn rat ventral prostates cultured for one week with T plus uPAR antagonists. Organ culture and immunohistochemistry were performed as described in Materials and Methods. a) newborn rat ventral prostate cultured for one week with control 100 μg/ml human uPA/ATF-Ig, b) newborn rat ventral prostate cultured for one week with 100 μg/ml mouse uPA/ATF-Ig, c) newborn rat ventral prostate cultured for one week with 100 μg/ml rat uPA/EGF-Ig. Bar, 100 μm.

Figure 3.7

The effect of uPAR antagonists on epithelial differentiation in newborn rat ventral prostates as shown by staining for cytokeratin 5

Immunohistochemical staining for cytokeratin 5 (arrows) in newborn rat ventral prostates cultured for one week with uPAR antagonists

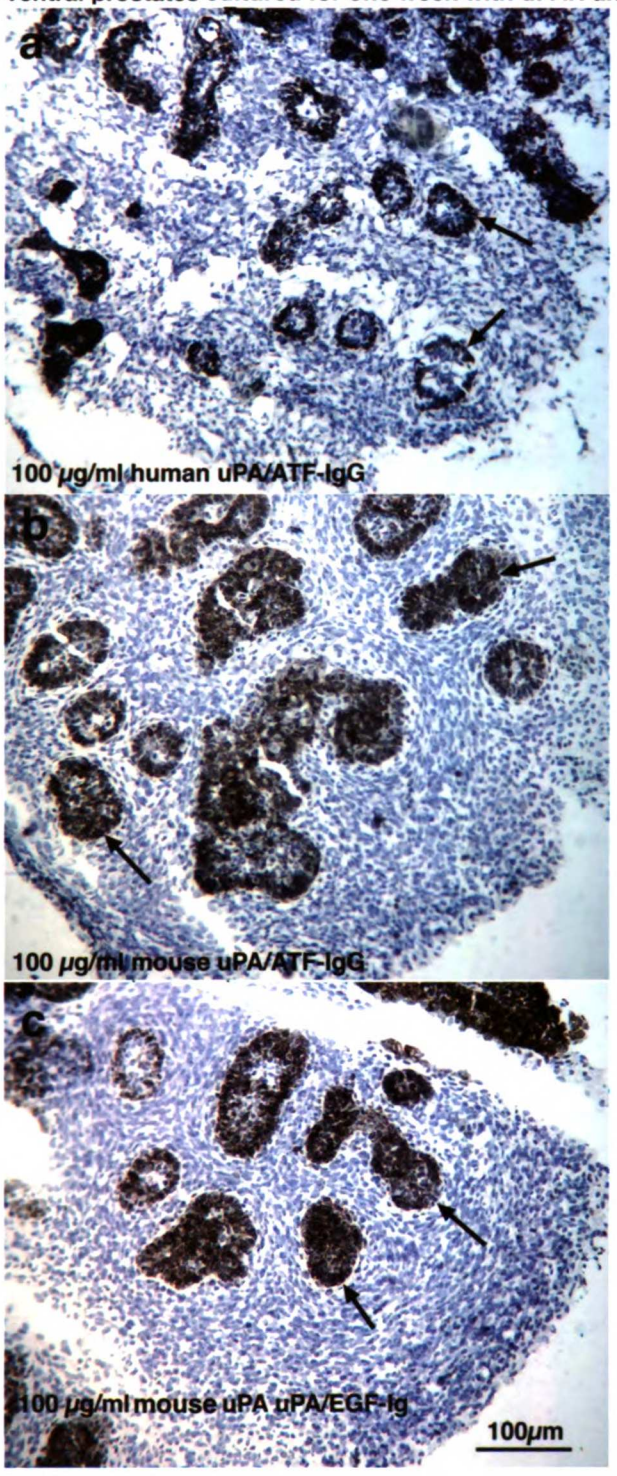


Figure 3.8

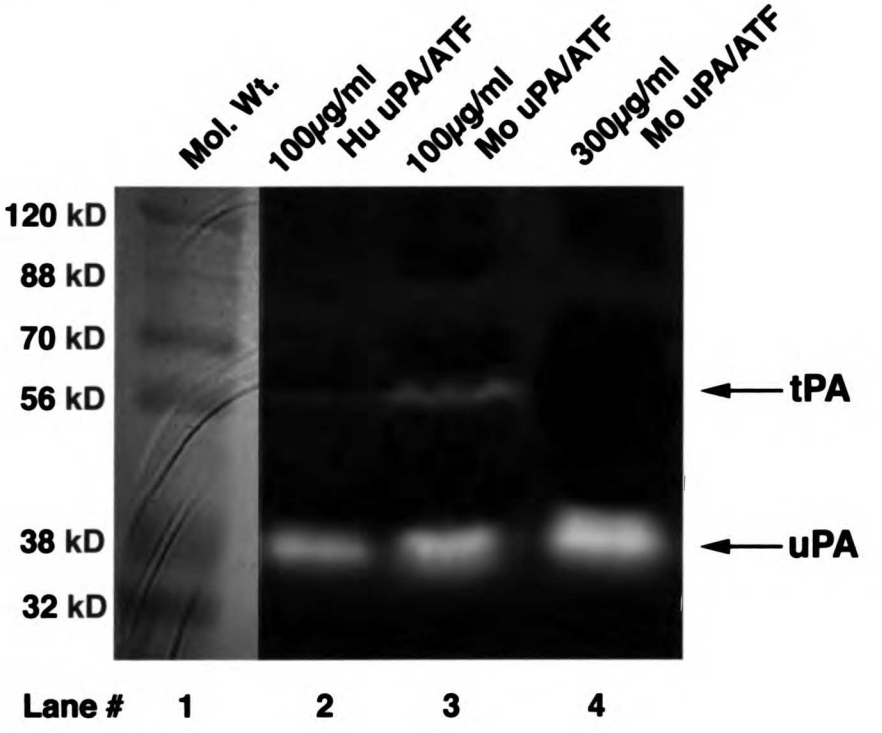
Displacement of uPA into conditioned media by uPAR antagonists

Substrate zymography for plasminogen activators in concentrated conditioned media from newborn rat ventral prostates cultured for one week with varying amounts of uPAR antagonists. Organ culture, processing of conditioned media, and zymography were performed as described in Materials and Methods. Lane 1, molecular weight standards; lane 2, concentrated conditioned medium from newborn rat ventral prostate cultured for one week with 100 μg/ml human uPA/ATF-Ig; lane 3, concentrated conditioned medium from newborn rat ventral prostate cultured for one week with 100 μg/ml mouse uPA/ATF-Ig; and lane 4, concentrated conditioned medium from newborn rat ventral prostate cultured for one week with 300 μg/ml mouse uPA/ATF-Ig.

Figure 3.8

Displacement of uPA into conditioned media by uPAR antagonists

Plasminogen/casein zymography of concentrated conditioned media from rat ventral prostates cultured with uPAR inhibitory peptides



4). Therefore, mouse uPA/ATF-Ig competes with native rat uPA for binding to uPAR in cultured rat VP tissue, displacing uPA into the culture media. When incubated with amiloride, a specific uPA inhibitor, zymographic activity of the 44 kD plasminogen activator was lost, suggesting further that the 44 kD plasminogen activator that was observed, was uPA. (not shown).

3.5 Discussion

In the newborn rat VP, uPA mRNA is localized in the mesenchyme, while uPA protein is localized in both the epithelium and mesenchyme. Specific uPA antagonists severely disrupt the ductal branching morphogenesis of newborn rat VPs, suggestive of a requirement for uPA or a uPA-like enzyme (Elfman et al., submitted). However, the expression and function of uPAR in prostatic development are unknown. In the current study, we demonstrated by RT-PCR that uPAR mRNA was expressed by newborn rat VPs. Further, uPAR antagonists disrupted ductal branching morphogenesis of newborn rat VPs *in vitro* as demonstrated by severe alteration in ductal patterning and a significant reduction in the number of ductal tips in newborn rat VPs treated with uPAR antagonists. The histodifferentiation of newborn rat VPs was also retarded in rat VPs treated with uPAR antagonists as exhibited by reduced polarization of epithelial cell and nascent ductal canalization. By immunohistological staining, the differentiation of K5-positive basal epithelial cells was retarded. Mesenchymal differentiation into smooth muscle was delayed as demonstrated by reduced condensation of fibroblasts into peri-ductal circumferential sheathes, and reduced immunohistological staining for smooth muscle alpha actin, and increased staining for vimentin in peri-ductal mesenchyme.

Ductal branching morphogenesis and histodifferentiation of the rat ventral prostate includes complex integration of many cellular process including: ECM turnover, cellular migration, proliferation, and differentiation. Further, prostatic organogenesis requires androgens that act via mesenchymal-epithelial interactions. Androgen receptors (AR) in the prostatic mesenchyme induce the differentiation of epithelial ARs. This epithelial differentiation subsequently induces smooth muscle differentiation. Smooth muscle-epithelial interactions are necessary for the continued development and maintenance of normal adult structure and function. Newborn rat VPs grown in the absence of androgens demonstrate retarded mesenchymal differentiation mimic previous

studies have demonstrated the concomitant maintenance of vimentin staining with reduced in staining for smooth muscle alpha actin in peri-epithelial areas (Hayward et al., 1996a). Thus, the disruption of uPAR with competitive inhibitors appears to fundamentally disrupt this histodifferentiation in a manner similar to that observed in androgen-deprived cultures. In the current study we reported that disruption of the uPA/uPAR system results in a retardation of androgen-dependent ductal branching morphogenesis as well as impaired epithelial and mesenchymal cytodifferentiation that is similar to the effects of androgen deprivation. Keratinocyte growth factor (KGF) and basic fibroblast growth factor (bFGF) are produced by the mesenchyme, bind to the epithelium, and suggested to be downstream of mesenchymal ARs during ductal branching morphogenesis of rat VPs (Sugimura et al., 1986b; McKeehan, 1991). Perhaps uPAR acts cooperatively with bFGF and KGF by regulating proteolysis of the ECM which affects the presentation of glycosaminoglycan tethered cytokines and the generation of migratory ligands, as well as by directly or indirectly regulating mitogenesis.

uPA/EGF can serve as a morphogen for cultured human endothelial cells (Schnaper et al., 1995). In contrast, the entire proteolytically active uPA is necessary for uPAR signaling as well as mitogenesis in other studies (Del Rosso et al., 1993) and appears to be required for normal VP organogenesis. uPA and not uPA/EGF-associated to uPAR, mediates chemotactic and mitogenic responses in human dermal fibroblasts (Anichini, 1994). Therefore, it is unclear how uPA's structure relates to uPAR-mediated functions. Thrombin, a chymotrypsin closely related to uPA, is the only other reported mitogenic ligand that requires a proteolytic domain in order to signal. Because thrombin signals through a 7-spanner-type receptor that is structurally unlike uPAR (Coughlin), their mechanisms appear analogous, not homologous.

In the current study, differential effects of the two uPAR antagonists were not observed on ductal branching morphogenesis of cultured rat VPs even though uPA/ATF-

IgG contains mouse uPA amino acids 1-138 and uPA/EGF-IgG contains mouse uPA amino acids 1-48. The structural difference between the two peptides is the presence or absence of the kringle domain. The uPA kringle domain (amino acids 49-138) present in uPA/ATF-Ig is suggested to mediate the binding of uPA to plasminogen and PAI-1. Based on data presented in the current study, this domain is not necessary for uPA/EGF-inhibition of ductal branching morphogenesis of the newborn rat VP.

We have shown that uPAR-associated uPA is required for optimal ductal branching morphogenesis and histodifferentiation of the newborn rat VP *in vitro*. A recent study has shown that uPA is also necessary for optimal ductal branching morphogenesis and histodifferentiation of the newborn rat VP (Elfman et al., submitted). Surprisingly, in both studies the availability and requirement of plasminogen is unclear. Plasminogen may be preloaded in the ECM of newborn VP from the fetal vasculature such that it is not rate-limiting during the one week culture period, which is unlikely given the half-life of plasminogen. Plasminogen maybe synthesized *in situ* which is very unlikely since plasminogen transcripts were undetectable by RT-PCR in newborn rat VPs (Elfman et al, submitted). Preliminary studies suggest that the plasminogen inhibitor aprotinin does not perturb ductal branching morphogenesis of the newborn rat VP. Taken together, these data suggest that plasminogen is not required for growth and development of the newborn rat VP and that the principle role of uPA and uPAR in prostatic development is not linked to the activation of plasminogen. Independent of plasminogen, uPAR regulates adhesion, signaling, and motogenesis. It is unclear which of these processes are affected by uPA/uPAR antagonists during the development of rat VPs.

Disruption of uPA/uPAR function by peptide antagonists might also inhibit the development of rat VPs by impairing direct uPA mediated mitogenesis or indirectly by impairing uPA's activation of HGF/SF and TGF-β_1. TGF-β_1 and HGF/SF both require activation by proteolysis and HGF/SF is a substrates of uPA. Thus, independent of plasminogen, uPAR could also indirectly regulate migration and proliferation by directed

proteolysis of ECM, by activation of MMP-2, or through the localized cell-surface activation of cytokines such as TGF-β_1 and HGF/SF, which are morphogens that regulate mitogenesis and are expressed during prostatic development. Both are expressed by the mesenchymal cells and are thought to act in a paracrine manner by binding to signaling receptors on epithelial cells. uPA/uPAR may mediate the localized activation of cytokines in a spatially restricted pattern such that mitogenic cytokines (HGF/SF) are activated at sites where DNA synthesis are required (on ductal tips), while inhibitory cytokines (TGF-β) are activated in sites (proximal ducts) that are growth quiescent. Studies are underway to quantitate how various uPA/uPAR functions: ECM degradation, cellular proliferation, cytokine activation, cellular signaling, or migration, are affected by uPAR perturbation with peptide antagonists.

CHAPTER FOUR

EXPRESSION AND ACTIVATION OF METALLOPROTEINASE TWO IN DEVELOPMENT OF THE RAT VENTRAL PROSTATE AND MOUSE BULBOURETHRAL GLAND

4.1 Abstract

Metalloproteinase two (MMP-2) is suggested to mediate the remodeling of basement membranes during organogenesis, angiogenesis, and metastasis. MMP-2 degrades collagen types IV, V, gelatin, and elastin. In the current study, the tissue localization of MMP-2 mRNA and protein were examined in the developing rat ventral prostate (VP) and the mouse bulbourethral gland (BUG). *In situ* hybridization demonstrated that MMP-2 mRNA was expressed exclusively in the mesenchyme of developing rat VP. In the adult rat VP, MMP-2 mRNA was expressed in smooth muscle and interstitial fibroblasts. Equal amounts of MMP-2 protein were expressed in both the epithelium and mesenchyme of newborn rat VPs as demonstrated by zymography. Equal proportions of activated enzyme were found in each tissue. Activated MMP-2 was observed during organogenesis of the rat ventral prostate and mouse BUG. The expression and activation of MMP-2 during development of the rat ventral prostate and mouse BUG suggests that MMP-2 is among the mesenchymal factors that influence epithelial ductal branching.

4.2 Introduction

Metalloproteinase two, 72kD and 76kD-type IV collagenase, gelatinase A (MMP-2) is a secreted metalloproteinase that is suggested to mediate the degradation of the extracellular matrix (ECM) during dynamic tissue remodeling in development, disease, inflammation, and wound healing.

MMP-2 is a member of the matrixin family that is suggested to mediate the remodeling of basement membranes during development and metastasis. MMP-2 degrades collagen types IV, V, elastin, and gelatin (Alexander, 1991; Matrisian, 1992). Transcription of rat MMP-2 mRNA is regulated by cytokines. Treatment of mouse lung organ cultures with epidermal growth factor (EGF) or transforming growth factor-alpha (TGF-α) increases expression of MMP-2 (Ganser, 1991). Production of MMP-2 in human keratinocytes is also increased by TGF-β_1 (Salo et al., 1991).

MMP-2 mRNA has been detected by *in situ* hybridization in the mesenchyme of the mouse branchial arches, eye, heart, kidney, and lung (Reponin, 1992). Also, MMP-2 mRNA is expressed in both the epithelium and mesenchyme of newborn mouse submandibular glands (Reponin, 1992) and rat tracheal glands (Lim, 1995). By immunohistochemistry, MMP-2 protein has been detected in both the epithelium and mesenchyme of embryonic rat salivary gland as well as postnatally in the stroma of rat submandibular glands (Reponin, 1992), tracheal glands (Lim, 1995), and mammary glands (Talhouk et al., 1991). MMP-2 protein is expressed in the developing neonatal rat prostatic complex, which is composed of the prostatic urethra, peri-urethral fat, ventral, dorsolateral, and anterior prostatic lobes. The rodent prostate is composed of three lobes: The anterior prostate, the dorsolateral prostate, and the ventral prostate (VP). MMP-2 protein is expressed in each of these lobes in adult rats. Further, neonatal rat prostatic complex and pubertal rat VPs express larger amounts of total MMP-2 protein, compared to their adult counterparts (Wilson, 1992).

In the current study, the tissue localization of MMP-2 mRNA and protein were examined in the developing Fisher 344 rat ventral prostate (VP) and Balb/c mouse bulbourethral gland (BUG). The tissue localization of MMP-2 mRNA in developing rat VPs was evaluated with *in situ* hybridization. We report that MMP-2 mRNA was expressed exclusively by the mesenchyme of developing rat VPs and in the stroma of adult rat VPs. MMP-2 protein was measured in developing rat VPs and mouse bulbourethral glands (BUGs) with zymography. MMP-2 protein, in latent and activated form, was localized to both the epithelium and mesenchyme of newborn rat VP. In rat VPs and mouse BUGs, the amount of activated MMP-2 declines with age. Activated MMP-2 was undetectable in growth quiescent adult rat VPs and mouse BUGs.

4.3 Materials and Methods

Inbred Fisher 344 rats and Balb/c mice (Simenson Inc., Gilroy, CA.) were sacrificed by barbiturate overdose and bilateral thoracotomy in accordance with NIH guidelines. Specimens for histology were embedded in OCT compound (Sakura, Inc., Torrence, CA.) and frozen on dry ice. Frozen sections were cut on a Jung Frigocut 2800N cryostat and thaw mounted onto presialized Fisher-plus microscope slides (Fisher, Springfield, NJ).

In situ hybridization for MMP-2 transcripts was performed on developing rat VPs using frozen tissue sections as described previously (Lim, 1995). Frozen sections were hybridized with ^{35}S-labeled anti-sense and sense riboprobes for MMP-2 mRNA according to standard protocols (Robinson et al., 1991). Following hybridization, the slides were washed at high stringency and digested with RNAase. The positive hybridization signal was detected autoradiographically, and the sections were counterstained. Nonspecific hybridization was assessed by using a labled sense riboprobe.

For analysis of tissue extracts, a modification of the methods of Wilson was used (Wilson et al., 1988). Newborn rat VPs or mouse BUGs are either unbranched or have only few branch points. The epithelium and mesenchyme of newborn rat VPs were separated surgically with fine gauge needles. All visible traces of mesenchyme were removed from the epithelium and collected. The separated epithelium and mesenchyme were then extracted with detergent. Tissues were homogenized in a glass mortar and pestle in the presence of 0.25% Triton X-100 (Sigma Inc., St. Louis, MO.) at 4° C with ~100 strokes for 30 minutes, then centrifuged in Eppendorf tubes at 12,000g for 2 minutes. Supernatants were removed and frozen on dry ice. Protein content of extracts was determined by the BCA method (Pierce Inc, Rockford, IL). Tissue extracts or conditioned media containing equal amounts of protein were mixed with 4X Lameli

electrophoresis sample buffer and loaded onto SDS PAGE gels containing 10% polyacrylamide (acrylamide, ammonium persulfate, and SDS, (Bio-Rad Inc., Richmond, CA.)) mixed with 1 mg/ml gelatin (Sigma Inc., St. Louis, MO.) and electrophoresed on a mini-gel apparatus (Hoefer Inc., San Francisco CA.). Following constant current electrophoresis with prestained molecular weight markers, the PAGE slabs were washed in 2.5% Triton X-100 (in Tris-HCL buffer) in order to remove SDS. Next, the Triton X-100 was washed out, and the slabs were incubated in a Tris-HCL buffer at pH 7.5-8.0 for 24 hours at 37^0 C. Gels were stained with coomassie blue (Bio-Rad Inc., Richmond, CA.), destained, fixed, rubbed with glycerol (Sigma, St. Louis, MO.), mounted, and dried in Biodesign gel wrap (Biodesign Inc., Carmel, NY.).

4.4 Results

MMP-2 mRNA expression in developing and adult rat ventral prostates

At birth, the epithelial "ducts" of rat VPs are mostly unbranched, solid, and uncanalized (fig. 4.1a, c). The mesenchyme is dense with fibroblasts. Little peri-ductal smooth muscle has differentiated (fig. 4.1a, c). Frozen tissue sections from all ages of rat VP exhibited a diffuse, weak background signal for MMP-2 mRNA when hybridized with ^{35}S-labeled sense riboprobes (fig. 4.1b). By contrast, sections gave a strong signal for MMP-2 mRNA that was localized homogeneously throughout the mesenchyme when hybridized with an ^{35}S-labeled anti-sense riboprobe. The hybridization signal did not vary in relationship with distance from the epithelium (fig. 4.1d).

In VPs from 3 day old rats, the solid, uncanalized epithelial ducts are branching (fig. 4.2a). Ducts are surrounded by circumferentially oriented fibroblasts. Peri-ductal smooth muscle is beginning to differentiate (fig. 4.2a). Frozen sections gave a signal for MMP-2 mRNA that was localized to fibroblasts throughout the mesenchyme, without preference for peri-epithelial mesenchymal cells undergoing differentiation into smooth muscle, when hybridized with ^{35}S-labled anti-sense riboprobes (fig. 4.2b).

In VPs from 14 day old rats, the canalized epithelial ducts are highly branched with polarized tall columnar epithelial cells (fig. 4.2d). The stroma contains well differentiated smooth muscle sleeves circumferentially surrounding each epithelial duct and interstitial fibroblasts between adjacent ducts (fig. 4.2c). Frozen sections gave a signal for MMP-2 mRNA that was localized to fibroblasts throughout the stroma, as well as to smooth muscle cells immediately surrounding the epithelial ducts when hybridized with ^{35}S-labeled anti-sense riboprobes (fig. 4.2d).

In VPs from adult rats, the canalized epithelial ducts are highly branched with polarized tall columnar epithelial cells (fig. 4.2e). The stroma contains well differentiated

Figure 4.1

***In situ* hybridization for MMP-2 mRNA in newborn rat ventral prostates**

Distribution of MMP-2 transcripts in newborn rat ventral prostates as detected by *in situ* hybridization. The 7 μm thick frozen tissue sections of newborn rat VPs were hybridized with sense MMP-2 mRNA ^{35}S-labled-riboprobes (a, b), or anti-sense MMP-2 mRNA ^{35}S-labled riboprobes (c, d). Sense and anti-sense probes were prepared and hybridizations were performed as described in Materials and Methods. Tissue sections were viewed by bright field (a, c) or dark field (b, d). Compared to the weak diffuse signal obtained with the sense probe (b), hybridization with the anti-sense probe gave a strong signal for MMP-2 mRNA in the mesenchyme indicating a positive signal (d). Sections were counterstained with hematoxylin/eosin. Bar, 25 μm.

Figure 4.1

In situ hybridization for MMP-2 mRNA in newborn rat ventral prostates

a) bright field, sense probe; b) dark field, sense probe
c) bright field, anti-sense probe; d) dark field, anti-sense probe
E: epithelium; M: mesenchyme

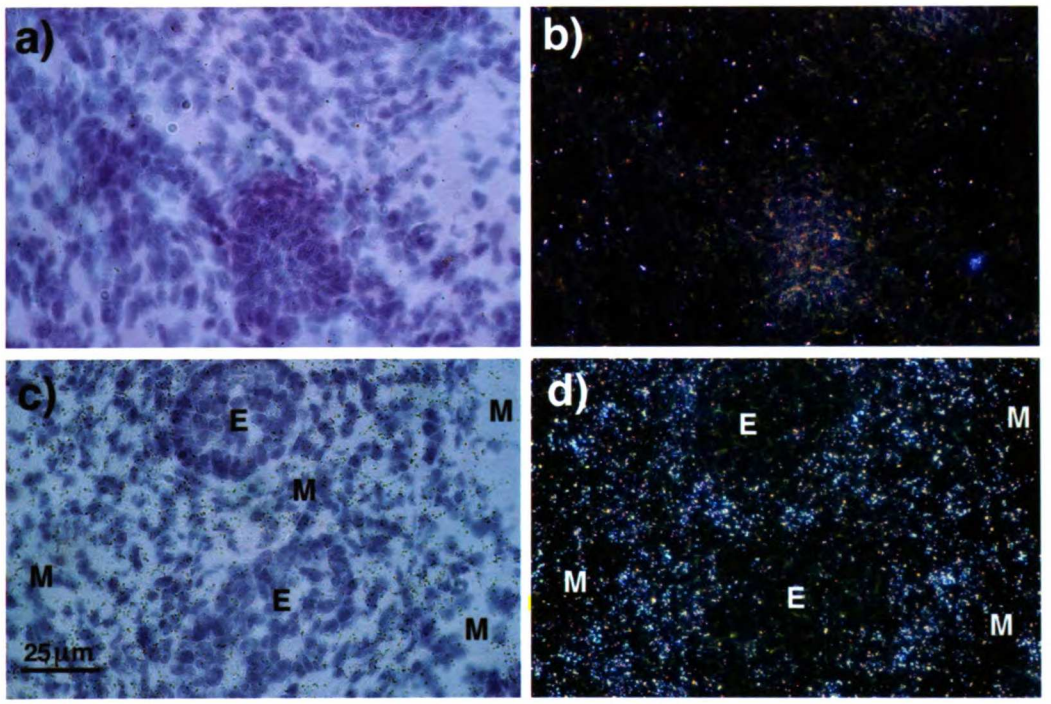


Figure 4.2

***In situ* hybridization for MMP-2 mRNA in developing rat ventral prostates**

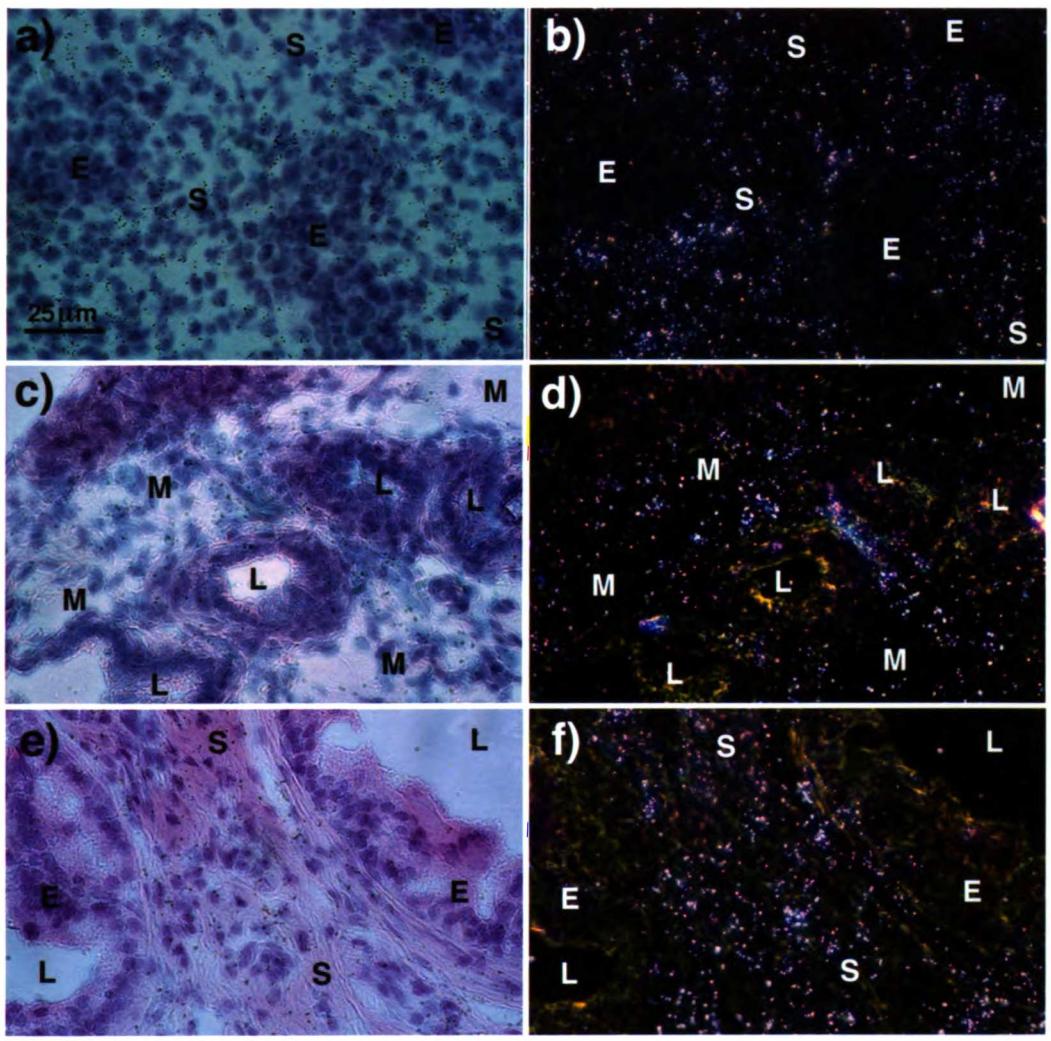
Distribution of MMP-2 mRNA in 3 day old, 14 day old, and adult rat ventral prostates, as detected by *in situ* hybridization. 7 μm thick frozen tissue sections of developing and adult rat VPs were hybridized with ^{35}S-labled sense riboprobes for MMP-2 mRNA (not shown), or ^{35}S-labled anti-sense MMP-2 mRNA riboprobes (c, d, e). Sense and anti-sense probes were prepared and hybridizations were performed as described in Materials and Methods. Tissue sections were viewed by bright field (a, c, e) or dark field (b, d, f). Compared to the weak diffuse signal obtained in all ages with the sense probe (not shown), hybridization with the anti-sense probe gave a strong signal for MMP-2 mRNA in the mesenchyme as well as in smooth muscle of 3 day old and 14 day old rat VPs (b, d) In adult rat VPs, a positive signal was restricted to the stroma and was detected in the interstitial fibroblasts and smooth muscle (f). Sections were counterstained with hematoxylin/eosin. Bar, 25 μm.

Figure 4.2

In situ hybridization for MMP-2 mRNA in developing rat prostates

a) 3 day old, bright field, anti-sense; b) 3 day old, dark field, anti-sense
c) 14 day old, bright field, anti-sense; d) 14 day old, dark field, anti-sense
e) adult, bright field, anti-sense; f) adult, dark field, anti-sense

E: epithelium; M: mesenchyme
L: lumen; S: stroma



smooth muscle sleeves circumferentially surrounding each epithelial duct and interstitial fibroblasts between ducts (fig. 4.2e). Frozen sections gave a signal for MMP-2 mRNA that was localized to fibroblasts throughout the stroma as well as to smooth muscle when hybridized with ^{35}S labeled anti-sense riboprobes also (fig. 4.2f). Thus, MMP-2 mRNA was localized to mesenchymal fibroblasts in the developing rat VPs and to interstitial fibroblasts and smooth muscle in adult rat VPs.

The expression and activation of MMP-2 protein in developing rat ventral prostates

Newborn rat VPs were isolated and epithelial and mesenchymal tissues were separated by microdisection, extracted with detergents, and electrophoresed on gelatin substrate zymography, as described in the Materials and Methods. 70kD, 68kD, 62kD, and 60kD gelatinases were expressed in both the epithelium and the mesenchyme of newborn Fisher 344 rat VPs at equal levels of activation, as measured with detergent extract zymography (fig. 4.3a, lanes 6, 7). A slight signal was also seen in each tissue at 70kD. Evidence that the observed gelatinases were latent and active MMP-2 was suggested by their co-migration with MMP-2 standards (fig. 4.3b, lanes 8, 9). Thus, unlike MMP-2 mRNA, MMP-2 protein was localized in both the mesenchymal and epithelium of newborn rat VPs. The amount of activated MMP-2 expressed by rat VPs declines with age. Activated MMP-2 was not detected in adult rat VPs (not shown).

The expression and activation of MMP-2 protein during ductal branching morphogenesis of the Balb/c mouse bulbourethral gland

The expression and activation of MMP-2 was associated with ductal branching morphogenesis of the mouse BUG. Amounts of latent and activated MMP-2 were highest

Figure 4.3

Gelatinases in developing rat ventral prostates and mouse bulbourethral glands

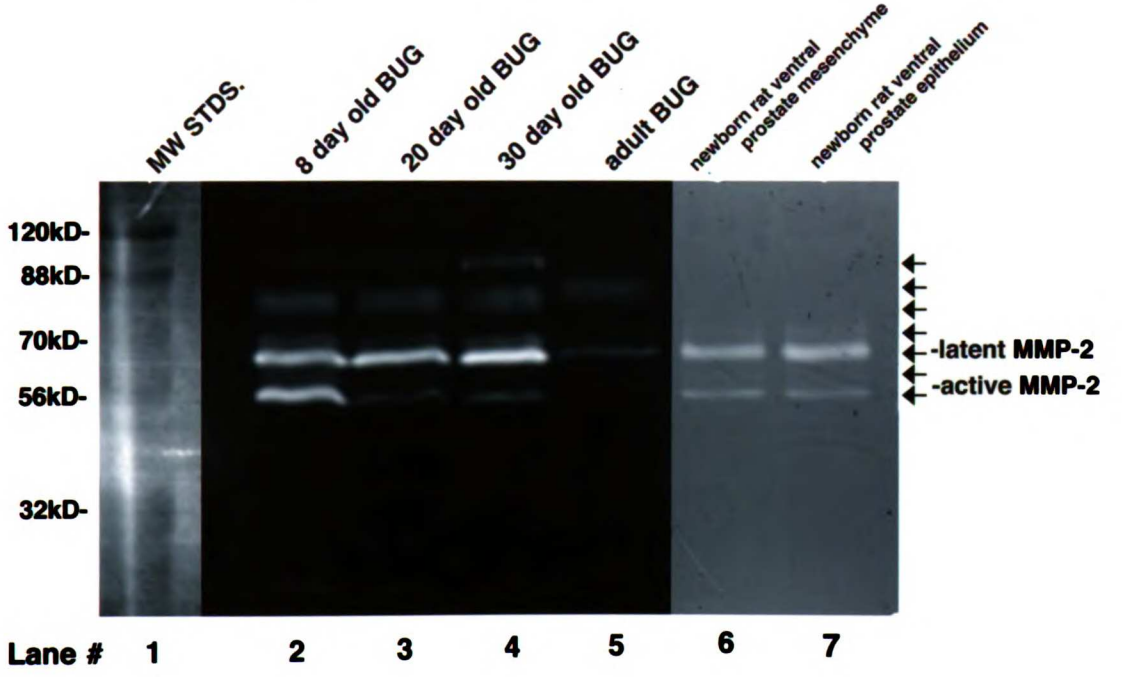
a) Gelatin substrate gel zymography of detergent extracts from mouse bulbourethral glands (BUGs) of various ages and separated epithelial and mesenchymal tissues from newborn rat ventral prostates (VPs). Extraction and zymography were performed as described in Materials and Methods. Lane 1, molecular weight standards; lane 2, 8 day old mouse BUG; lane 3, 20 day old mouse BUG; lane 4, 30 day old mouse BUG; lane 5, adult mouse BUG; lane 6, newborn rat ventral prostate mesenchyme; lane 7, newborn rat ventral prostate epithelium.

b) Substrate zymography of BUG gelatinases treated with EDTA and MMP-2 standards. Detergent extracts of mouse BUGs of various ages were generated and electrophoresed as described in Materials and Methods. Lane 1, molecular weight standards; lanes 2, 3, 4, untreated BUG gelatinases, lanes 5, 6, 7, extracted BUG gelatinases treated with 10mM EDTA in Ca^{++}-free; lanes 8, MMP-2 positive control, APMA-treated conditioned media from 3T3 fibroblasts, containing latent and activated MMP-2; lane 9, purified latent MMP-2.

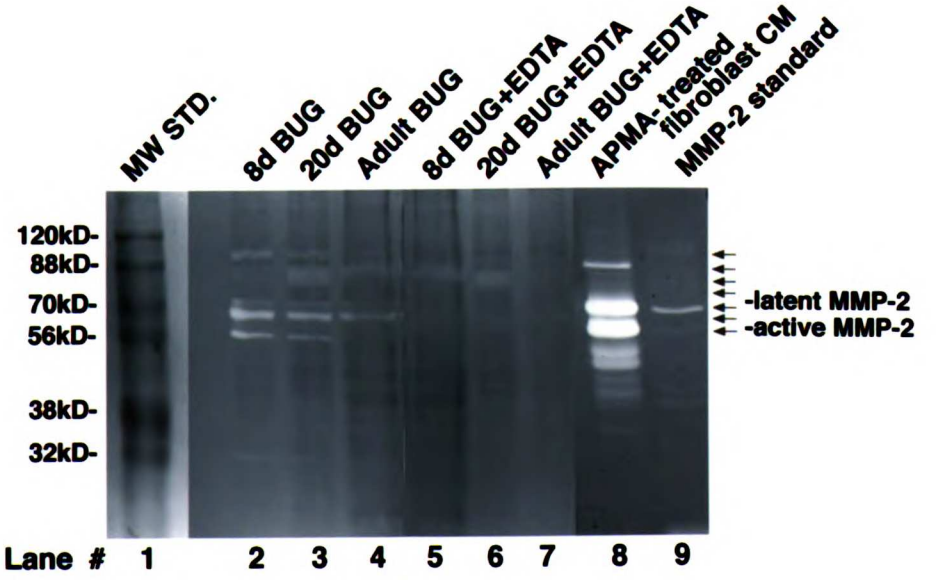
Figure 4.3

Gelatinases in developing rat ventral prostates and mouse bulbourethral glands

a) Gelatin zymography of mouse bulbourethral gland (BUG) and rat ventral prostate



b) Gelatin zymography of MMP-2 standards and BUG extracts with EDTA



in the newborn and decreased with age, when observed in a series of whole-organ BUG detergent extracts from birth to adult. In the BUGs of 8 day old mice, sharp signals for gelatinases were seen at 92kD, 82kD, 70kD, 68kD, 62kD, 60 kD, with a broader signal seen at 84kD (fig. 4.3a, lane 2). In the 20 day old mouse BUG, virtually the same pattern was seen except that the signal at 62 kD was not detectable, and the 60kdD was significantly reduced (fig. 4.3a, lane 3). In the 30 day old mouse BUG, the gelatinase signals were identical to that of the 20 day old BUG, although the 92kD signal was slightly stronger (fig. 4.3a, lane 4). In the adult mouse BUG, 92kd gelatinase activity was not detectable, nor were 70kd, 62kD, or 60kD gelatinases detectable. Only a faint 68kD gelatinase and a broad signal at 86kD were observed (fig. 4.3a, lane 5). Thus, activated MMP-2 was not observed in the adult mouse BUG.

Extracts from 8 day old, 20 day old, and adult mouse BUGs demonstrated an age-dependent loss of 60kD gelatinase during mouse BUG development, as reported earlier (fig. 4.3b, lanes 1, 2, 3). The same electrophoresed BUG samples were treated with 10mM ETDA in Ca^{++}-free buffer to detect metalloproteinases. Gelatinase signal was lost was in all but the broad 84kD-86kD bands after Ca^{++} celation, suggestive of metalloproteinases (fig. 4.3b, lanes 4, 5, 6). Gelatinases at 68kD and 60kD from BUG extracts also co-migrated with MMP-2 latent and active positive controls (fibroblast conditioned media containing latent and active MMP-2 (fig. 4.3b, lane 8) and purified MMP-2 (fig. 4.3b, lane 9), kindly provided by Zena Werb). Thus, the identities of the 68kD and 60kD gelatinases observed in fig 4-5a, were confirmed to be latent and active MMP-2.

4.5 Discussion

Previously, latent and active MMP-2 were observed in the neonatal rat prostatic complex, which is composed of the prostatic urethra, peri-urethral fat, ventral, dorsolateral, and anterior prostatic lobes. The entire prostatic complex contains glands undergoing branching morphogenesis, as well as other structures which do not undergo branching morphogenesis. In the current study, we report that MMP-2 mRNA transcripts were restricted to the mesenchyme of neonatal Fisher 344 rat VPs as assessed by *in situ* hybridization. In the adult prostate, MMP-2 mRNA is expressed by smooth muscle and interstitial fibroblasts. By zymography newborn rat VPs expressed MMP-2 protein in both the epithelium and the mesenchyme. Further, the ratio of latent to activated MMP-2 was equivalent in each of the two tissues. Levels of activated MMP-2 protein in isolated VPs declined with age in Fisher 344 strain rats, as has been observed in the entire prostatic complex of Sprague-Dawley strain rats (Wilson, 1992). We also report that in BUGs of Balb/c mice, the highest levels of activated MMP-2 were measured in the newborn and remained elevated during branching morphogenesis. Levels of activated MMP-2 decline with age as branching morphogenesis declines, becoming undetectable in growth-quiescent adult mouse BUGs. Thus, activation of MMP-2 correlates temporally with branching morphogenesis of parenchymal organs such as the rat lung, mouse mammary glands, mouse BUGs, and rat VPs. In the Fisher rat bladder activated MMP-2 was localized to the mesenchyme and declined with age (Sutherland et al., 1997). In the case of the bladder, activation of MMP-2 correlates with the organ growth and not branching morphogenesis, since the bladder has a planar epithelium lining the basal lamina lumen. Therefore, despite the marked difference in patterning between UGS derivatives having a branched ductal parenchymal (VPs and BUGs) versus having a planar epithelium (bladder), each express activated MMP-2 during development.

Apparently, expression and activation of MMP-2 does not correlate with branching morphogenesis per se but instead may be associated with growing organs whose epithelia are expanding, necessitating remodeling of epithelial and stromal ECMs. However, only newborn rat VP epithelium and not bladder epithelium localize MMP-2 protein. Therefore, the epithelial localization of MMP-2 appears to correlate with ductal branching morphogenesis while the activation of MMP-2 occurs during the growth of both planar and branched parenchymal epithelium. It is intriguing to speculate how MMP-2, synthesized by the mesenchyme, may affect the growth, patterning and histodifferentiation of both androgen-dependent ductal branching morphogenesis of rat VPs, mouse BUGs, and the androgen-independent planar growth of the rat bladder. Interestingly, a 92kD gelatinase (putative gelatinase B/MMP-9/type IV collagenase) was expressed during development of the mouse BUG, but not during development of the rat VP.

Mouse salivary glands have been used as a model system to study ductal branching morphogenesis since the pioneering studies of Grobstein in 1953 (Grobstein, 1953). These studies established the concept that mesenchyme induces and specifies patterns of during ductal branching morphogenesis. Bernfield et al. further established that basement membrane (BM) turnover regulates change in epithelial shape during ductal branching morphogenesis of mouse salivary glands (Bernfield and Banerjee, 1982; Bernfield et al., 1984). BM turnover was shown to be increased in growing ductal tips and was required for ductal branching morphogenesis. Glycosaminoglycan turnover in the BM occured at a higher rate in ductal tips versus the clefts between lobules, and was thought to be due to the action of BM remodeling enzymes in the mesenchyme (Bernfield and Banerjee, 1982). Fukada et al. observed that mesenchymal fibrillar collagen in the clefts of developing mouse salivary glands are necessary for branching morphogenesis of the mouse salivary gland (Fukuda et al., 1988), however, epithelial proliferation was not (Nakanishi, 1987). In addition, epidermal growth factor (EGF) and Matrigel can

substitute for mesenchyme in mouse salivary gland (Nogawa, 1991) and lung development (Ganser, 1991). Further, the EGF-regulated, mesenchymally-derived basement membrane component nidogen, complexed with basement membrane laminin, is required for ductal branching morphogenesis of the mouse salivary gland (Kadoya et al., 1995). Previously, Cunha expanded the model of instructive mesenchyme during ductal branching morphogenesis to include the androgen-dependent development of the rodent UGS. Prostatic development requires the action of androgens whose morphogenic effects are elicited via the androgen receptors in the mesenchyme (Cunha et al., 1987). Development of the rat VP requires both epithelium and mesenchyme (Cunha, 1994). The mesenchymal synthesis of MMP-2 mRNA along with the mesenchymal as well as epithelial localization of MMP-2 protein suggests that MMP-2 traffics through the BM and likely regulates BM turnover. Since turnover of epithelial BM collagen type IV and mesenchymal interstitial collagens are required for ductal branching morphogenesis, we propose that MMP-2 is a mesenchymal factor that is necessary for prostatic ductal branching morphogenesis.

Activation of MMP-2 has been associated with tissue remodeling during ductal branching morphogenesis in the lung (Ganser, 1991), rat mammary gland (Talhouk et al., 1991), neonatal rat prostatic complex, and pubertal prostatic lobes (Wilson, 1992). An unknown mechanism for trafficking and perhaps activation is suggested by the observation that MMP-2 mRNA was localized exclusively to the mesenchyme, yet latent and activated MMP-2 protein were also localized to the epithelium. Whether MMP-2 is activated by autocrine or paracrine factors is also unknown. The current study does not address whether MMP-2 is activated in an autocrine or paracrine manner. MT-MMP 1 mRNA and protein are co-localized with MMP-2 during mouse embryogenesis (Kinoh et al., 1996). MT-MMP 1 activates MMP-2 and is co-expressed during embryonic development of the mouse as measured with in situ hybridization and immunohistochemistry (Kinoh et al., 1996). Studies are underway to measure the

expression of MT-MMP 1 mRNA in developing VP. Basbaum and Werb have recently speculated that a furin-like enzyme, in association with MT-MMP 1, may activate MMP-2 during secretion (Basbaum and Werb, 1996).

Although synthesized throughout life, MMP-2 is activated only during development. Activated MMP-2 has been observed in prostatic and bladder cancers. Increased levels of activated MMP-2 correlate with higher grades of malignancy in human bladder carcinomas (Elfman, Baskin, and Cunha, unpublished observations). Also, increased levels of activated MMP-2 also correlate with experimentally induced bladder outlet obstruction in female rats (Sutherland et al., 1997). Thus, activation of MMP-2 in the adult human and rat bladders is coincidental with increased tissue remodeling during disease. Whether active MMP-2 mediates BM remodeling during development in a similar manner as during carcinogenesis is an interesting question that merits investigation. Novel specific MMP-2 inhibitors have been reported. Preliminary studies are underway to assess the effect of MMP-2 inhibitors on the ductal branching morphogenesis of newborn rat VPs. We predict that adding such inhibitors to newborn rat VP cultures will result in perturbed ductal branching morphogenesis, similar to the results obtained by adding uPA/uPAR antagonists (Elfman et al., submitted). Studies are also underway to define the morphological changes in the BM during VP organogenesis. Development of neoepitope-specific reagents that quantitate substrate degradation (Basbaum and Werb, 1996) and metalloproteinase activation (L. Matrisian, personal communication), would greatly facilitate these studies.

CHAPTER FIVE

SUMMARY

"...still, all they can offer you is slash, burn, or poison (surgery, radiation, or chemotherapy)..."

a breast cancer patient, 1996...

As suggested by the quoted patient, cancer therapies based on traditional methods of tissue destruction are often ineffective. Novel anti-cancer therapies based on compounds that biologically interact with cancerous tissues (biotherapeutics) may offer improved efficacy with reduced side-effects, compared to current cytotoxic methods of cancer treatment. Extracellular proteinases such as uPA and MMP-2 have been extensively studied as candidates for biotherapeutic intervention because they are key regulators of tissue remodeling. Although extensive data suggest that uPA and MMP-2 mediate tissue remodeling *in vitro*, little is known regarding the role of these enzymes in prostatic cancer. Ductal branching morphogenesis and histodifferentiation of newborn rat ventral prostates provides a useful model system because mutual paracrine interactions between epithelium and smooth muscle are common to both prostatic cancer and normal prostatic development. Prostatic development and maintenance of adult prostatic structure and function require androgens and interactions between epithelial and mesenchymal/stromal tissues. Disrupted paracrine interactions between stroma and epithelium are postulated to regulate the progression of prostatic adenocarcinoma (Cunha, 1994; Hayward et al., 1996b). The factors that mediate these paracrine interactions are unknown, but cytokines as well as proteinases are postulated to be among them. The current study has investigated the tissue expression of uPA, uPAR, and MMP-2 during development of the newborn rat VP. In addition, the function of uPA and uPAR during development of the newborn rat VP was assessed by perturbation with specific peptide inhibitors.

The rodent prostate is composed of three lobes: The anterior prostate, the dorsolateral prostate, and the ventral prostate (VP). Previous studies have demonstrated that MMP-2 protein is expressed in the developing neonatal rat prostatic complex, which is composed of the individual prostatic lobes, prostatic urethra, and surrounding peri-urethral fat. In pubertal rats, MMP-2 protein was expressed in the anterior, dorsolateral, and ventral prostates (Wilson, 1992), while activation of MMP-2 correlates with ductal branching morphogenesis.

In the current study, *in situ* hybridization was used to demonstrate that MMP-2 mRNA was expressed exclusively by fibroblasts throughout the mesenchyme of developing rat VPs as well as by interstitial fibroblasts and smooth muscle of adult rat VPs. MMP-2 protein was expressed in both the mesenchyme and the epithelium of newborn rat VPs, with each tissue expressing equivalent amounts of activated MMP-2. Further, the highest levels of activated MMP-2 protein were observed in newborn rat VP, with progressively less activated MMP-2 observed with age. Activated MMP-2 was undetectable in the adult rat VP. In Balb/c mice, activated MMP-2 was expressed at high levels in developing neonatal BUGs. Levels of activated MMP-2 decline with age and were undetectable in the adult mouse BUG. Previously, MMP-2 activation has been shown to correlate with developmental growth of the rat bladder (Sutherland et al., 1997). Therefore, all of the derivatives of the rodent male urogenital sinus: the bladder, the prostate, and the BUG, each share the general pattern of higher levels of activated MMP-2 correlating with early development. Interestingly, this pattern of MMP-2 activation was shared by structures with diverse developmental programs. The bladder epithelium undergoes a planar developmental program forming an epithelium that lines a cavity, while in prostate and BUGs the epithelium undergoes ductal branching morphogenesis. Thus, activation of MMP-2 appears to correlate with expansive epithelial growth during organogenesis of each of the UGS derivatives, regardless of developmental program.

Studies of the mouse salivary gland and other model systems have established the concept that mesenchyme induces and specifies the pattern of ductal branching morphogenesis of the epithelium. For example, when embryonic mouse mammary epithelium is associated with salivary gland mesenchyme, the glands that form exhibit the ductal branching pattern distinctive of the salivary gland even though the epithelium remains functional mammary gland and produces casein (Sakakura, 1987). Thus, mesenchyme determines the pattern of ductal branching morphogenesis. In embryonic mouse salivary glands, basement membrane (BM) turnover, which is increased in growing ductal tips, regulates change in epithelial shape during ductal branching morphogenesis. In particular, glycosaminoglycan turnover in the BM occurs at a higher rate in ductal tips versus the clefts between lobules, and is thought to be due to mesenchymal enzymes (Bernfield and Banerjee, 1982; Bernfield et al., 1984). Further, fibrillar collagens in mesenchymal clefts, but not epithelial proliferation (Nakanishi, 1987 ; Spooner et al., 1989) are necessary for branching morphogenesis. Most recently, the mesenchymally-derived BM component nidogen, when complexed with BM laminin has been demonstrated to be required for ductal branching morphogenesis of mouse lung (Ekblom et al., 1994) and salivary glands (Kadoya et al., 1995). Further, nidogen expression is regulated by epidermal growth factor (EGF). Thus, both ECM and cytokine components of the mesenchyme regulate ductal branching morphogenesis.

While salivary gland mesenchyme can determine patterns of branching morphogenesis, UGM can instructively induce various endodermal epithelium to undergo specific patterns of ductal branching and can instructively induce complete changes in epithelial cytodifferentiation and functional expression (Cunha et al., 1987). During androgen-dependent prostatic organogenesis, androgens elicit prostatic growth and ductal branching morphogenesis via the androgen receptors in the mesenchyme, the inducer of epithelial development (Cunha et al., 1980). Thus, the development of the rat VP involves the exchange of paracrine factors between the epithelium and mesenchyme.

One important paracrine cytokine involved in prostatic development is KGF (Sugimura et al., 1996). The mesenchymal synthesis of MMP-2 mRNA along with the mesenchymal as well as epithelial localization of MMP-2 protein, suggest that MMP-2 traffics through the epithelial BM and therefore is likely to regulate BM turnover. Since the regulation and turnover of the MMP-2 substrate type IV collagen is required for ductal branching morphogenesis, we propose that MMP-2 represents one of the mesenchymal factors that are necessary for prostatic ductal branching morphogenesis. It remains unclear how is MMP-2 protein, which is found activated only in developing rat VPs and not in the adult, moves from mesenchyme to the epithelium. In addition, where MMP-2 is activated? It is intriguing to speculate how MMP-2 may regulate BM morphology during ductal branching morphogenesis. The recently characterized MT-MMP 1/TIMP-2 complex is a leading candidate for an activator of MMP-2 during development. Studies are underway to measure the expression of MT-MMP 1 mRNA in developing rat VPs.

By zymography, increased levels of activated MMP-2 correlated with induced bladder obstruction in female rats (Sutherland et al., 1997). Also, increased levels of activated MMP-2 correlate with grades of malignancy in human bladder carcinomas (Elfman, Baskin, and Cunha, unpublished observations). Human bladder obstructive disease and induced obstruction in adult rats result in an increased mass of stromal ECMs whose levels may be regulated in part by the activation of MMP-2 that occurs during tissue remodeling in both bladder disease and development. Whether activated MMP-2 mediates BM remodeling in a similar manner during bladder development, bladder carcinogenesis, or bladder obstruction is unclear. The role of MMP-2 during ductal branching morphogenesis could be examined with the newborn rat VP culture model system (Sugimura et al., 1986a). Novel specific synthetic MMP-2 inhibitors have been reported and their preliminary trials with newborn rat VPs are underway. We predict the addition of such inhibitors to cultured newborn rat VPs will result in similar disruption of ductal branching morphogenesis as has been reported in our uPA/uPAR inhibition

studies. Studies are also underway to define the morphological changes that occur in the BM during VP organogenesis. Neoepitope-specific reagents to quantitate substrate degradation, as described recently in a review by Basbaum and Werb (Basbaum and Werb, 1996), and antibodies that discriminate active and latent metalloproteinases isoforms (L. Matrisian, personal communication) would greatly facilitate these studies by demonstrating the localization of these enzymes in prostatic organogenesis.

In rodents, expression of uPA can be initially detected at the two blastomere stage when activation of embryonic genome begins (Zhang, 1994). uPA is also synthesized during organogenesis of the avian heart (McGuire, 1992), mammary glands (Talhouk et al., 1991), mouse urogenital sinus (Timme et al., 1994), and rat prostatic complex. uPA protein is expressed in the entire neonatal prostatic complex, as well as the pubertal anterior, dorsolateral, and ventral prostates (Wilson et al., 1988; Wilson, 1995). Our studies show for the first time that the isolated rat VP produces uPA protein throughout development and in lesser abundance in the adult. The other lobes of the rodent prostate do not express uPA in the adult (Wilson et al., 1988). It has been speculated that expression of uPA in the adult rat VP is related to luminal secretions, but this is unclear (Andreasen, 1990). Castration of the adult rat results in massive organ regression and an increase in the level of uPA mRNA and protein (Rennie, 1984). In the current study, we have reported that uPA mRNA is expressed exclusively by the mesenchyme of developing neonatal rat VPs. uPA protein is localized in both the epithelium and mesenchyme of newborn VP. Thus, the localization of uPA mRNA and protein localization reported here suggest a paracrine mechanism of uPA synthesis and localization during prostatic organogenesis. uPA mRNA is also expressed by the stroma of adult rat VPs.

uPA is a degradative, mitogenic, and motogenic ligand that binds to a signaling receptor. However, the functions of uPA/uPAR during prostatic development are unclear. In the current study, we demonstrated that disruption of uPA or uPAR protein

activity by peptide antagonists during *in vitro* development of newborn rat VPs resulted in a significant disruption of ductal branching morphogenesis. In newborn rat VPs treated with uPA or uPAR antagonists, ductal branching morphogenesis was severely retarded as demonstrated by abnormal ductal patterning as well as significantly fewer ductal tips. Newborn rat VPs treated with uPA antagonists demonstrated retarded histodifferentiation as evidenced by fewer canalized ducts and a reduction in polarized columnar epithelial cells. In addition, the mesenchyme remained densely fibroblastic with impaired organization of circumferentially oriented sheaths of smooth muscle cells. In newborn rat VPs treated with uPAR antagonists, histodifferentiation was also delayed as evidenced by fewer canalized ducts that contained fewer polarized columnar epithelial cells. Moreover, the mesenchyme remained densely fibroblastic with impaired organization of circumferentially oriented sheaths of smooth muscle cells. Further, in newborn rat VPs treated with uPAR antagonists, immunohistochemical staining showed that the differentiation of luminal epithelial cells and cytokeratin 5-positive basal epithelial cells was impaired. Also, smooth muscle differentiation, as demonstrated by staining for alpha smooth muscle actin, was reduced in peri-epithelial mesenchyme, while the staining for vimentin in the same region was increased. Previous studies have demonstrated the concomitant loss of vimentin staining with an increase in staining for smooth muscle alpha actin in peri-epithelial mesenchyme during normal androgen-dependent development of the newborn rat VPs (Hayward et al., 1996a). Thus, disruption of uPA or uPAR resulted in a retardation of ductal branching morphogenesis and histodifferentiation in both epithelium and mesenchyme that is similar to that observed in growth under androgen-deprived conditions.

While uPA was necessary for ductal branching morphogenesis and histodifferentiation of newborn rat VPs, its action appears to be independent of plasminogen. This conclusion is derived from the fact that the newborn rat VP culture system was serum-free and presumably devoid of plasminogen quite early into culture.

Also, transcripts for plasminogen mRNA were not detected in the newborn rat VP by RT-PCR (Elfman et al., submitted). Therefore, the availability and requirement for plasminogen must be questioned. Either plasminogen was deposited in the ECM of newborn rat VP from previous fetal vascular contact such that it is not rate-limiting during the one week culture period, or more likely and most interesting, plasminogen is *not* necessary for VP development. Thus, uPA/uPAR are necessary for organogenesis of the newborn rat VP, but given the limited half-life of plasminogen, uPA/uPAR probably acts through a plasminogen-independent mechanism. We speculate that uPA/uPAR regulates ductal branching morphogenesis and histodifferentiation of the rat ventral prostate by mediating four kinds of processes:

1) uPA/uPAR activates MMP-2 and proteinases related to plasminogen, which mediate the turnover of basement membranes and other ECMs.

uPA/uPAR increases cell-surface proteolysis, localizing the degradation of basement membrane and mesenchymal interstitial ECMs by activating MMP-2 and perhaps plasminogen-like enzymes. ECM degradation breaks physical barriers and results in the presentation of motogenic ligands, which affect cell-ECM adhesion and in turn cellular adhesion, migration as well as proliferation. We speculate that in newborn rat VPs treated with uPA or uPAR antagonists, ECM turnover is surely perturbed which could impair ductal branching morphogenesis. This suggests that uPA constitutes part of the soluble, mesenchymally-derived cascade of peptide factors that are required for ductal branching morphogenesis. Studies are planned to measure the degradation rate of labled substrate in rat VPs treated with uPA/uPAR antagonists. In addition, we will examine the turnover of basement membranes during ductal branching morphogenesis of the rat VP.

2) uPA/uPAR activate HGF/SF and TGF-β 1 which in turn regulate cellular mitogenesis and differentiation.

The ECM binds, localizes and releases cytokines such as transforming growth factor beta-one (TGF-β_1). TGF-β_1 and hepatocyte growth factor/scatter factor(HGF/SF) are mitogens as well as morphogens that have been observed in many developing organs including the rat prostate (Timme et al., 1994; Rubin et al., 1989). TGF-β_1 and HGF/SF are each expressed by the mesenchymal cells and thought to act in a paracrine manner by binding to signaling epithelial cell receptors. Further, TGF-β_1 induces smooth muscle differentiation (Gabbiani, 1992; Gabbiani, 1994). Both cytokines require activation by proteolysis by enzymes such as plasmin (Blasi, 1993). Therefore, uPA/uPAR may mediate the localized activation of cytokines in a spatially restricted pattern such that mitogenic cytokines (HGF/SF) are activated at sites where DNA synthesis are required (on ductal tips), while inhibitory cytokines (TGF-β_1) are activated in sites (proximal ducts) that are growth quiescent. Thus, we further speculate that disruption of uPA/uPAR impairs the localized activation of TGF-β_1 and HGF/SF, which leads to retarded ductal branching morphogenesis and histodifferentiation in developing rat ventral prostates.

3) uPAR-bound uPA is directly mitogenic.

uPA is a mitogenic ligand for vascular smooth muscle cells (Okada et al., 1995; Noda-Heiny and Sobel, 1995), endocardial mesenchymal cells (McGuire, 1993), dermal fibroblasts (Anichini, 1994), and prostate cancer cells (Hoosein, 1991). uPAR can signal through c-src and without bound uPA (Resnati et al., 1996). Therefore, uPAR can mediate signaling and mitogenesis independent of plasmin. We speculate that disruption of uPA/uPAR function may retard direct mitogenesis mediated by uPA/uPAR, which is necessary for ductal branching morphogenesis of the rat VP. Studies are underway to

measure the effect of uPA/uPAR antagonists on the mitotic rate of various tissues in cultured newborn rat VPs.

4) uPAR regulates ECM adhesion and cellular migration.

uPA bound to uPAR is motogenic for vascular endothelium (Pepper et al., 1993) and prostate cancer cells (Hoosein, 1991). uPAR binds to ECM-glycoproteins such as vitronectin (Moser et al., 1995) and is localized to focal contacts (Pollaen, 1988). uPAR has been shown to modulate integrin avidity and adhesion (Wei et al., 1996). In leukocytes, uPAR co-localizes with other receptors for chymotrypsin enzymes that may modulate migration such as the receptor for complement factor three (Xue et al., 1994); (Simon et al., 1996). We speculate that disruption of uPA/uPAR results in impaired cellular adhesion to uPA and non-uPA ligands, leading to the subsequent failure of cells to migrate to their proper positions which is required for ductal branching morphogenesis and histodifferentiation. Studies are planned to inject lipophilic dyes into the cells of developing rat VPs and assess the affect of uPA/uPAR perturbation on individual cell migration.

We propose that during ductal branching morphogenesis, the first three processes are organized around the fourth process. Though degradation of the ECM and cellular mitogenesis are necessary, it is movement which regulates tissue remodeling during ductal branching morphogenesis, and uPA/uPAR are central molecules in cellular migration (Blasi, 1993). Migration is a multi-step process that includes: 1) Extracellular factors such as the ECM; *uPA regulates ECM turnover by activating MMP-2 (and plasminogen when vascularized) that degrade basement membranes.* 2) Transmembrane factors that mediate adhesion, cell shape change, signaling and migration; *uPAR regulates migration by binding to multiple adhesive and migratory ligands, localizing to focal contacts, modulating integrin adhesion, and signaling with c-src.* 3) Intracellular factors downstream of cytokine signaling such as the phosphorylation of kinase cascades

and gene transcription; *uPA can act as a cytokine and can activate the morphogens HGS/SF and TGF-β1 which regulate cell cycle.* Therefore, uPA/uPAR are involved with the entire process of cellular movement.

If movement is necessary for prostatic ductal organogenesis, then the disruption of several of movement-related, uPA/uPAR mediated functions by peptide inhibition, would explain the impaired rat VP development. For example, differentiation such as the linkage of epithelial cells into polarized sheets, the turnover of basement membrane, as well as movement and differentiation of mesenchymal cells into circumferentially oriented peri-ductal smooth muscle sheathes, each require cellular migration. The basal epithelium-BM-smooth muscle interface is a precisely ordered structure that regulates organogenesis and requires the coordination of cellular proliferation, differentiation, adhesion, ECM synthesis, in addition to ECM-degradation. We propose that cellular movement of epithelial and mesenchymal cells is central to BM turnover and therefore ductal morphogenesis and tissue differentiation. Accordingly, we conclude that uPA regulates cellular movement during prostatic organogenesis.

Does proliferation precede and regulate movement, or visa versa? The results of the current study suggest that cellular movement and cellular proliferation are at least linked, perhaps via uPA. Two observations in the current study support this idea. First, uPAR mRNA was expressed by newborn rat VPs, and uPA mRNA was expressed exclusively by the mesenchyme of developing rat VPs as well as the stroma of adult rat VP. uPA protein was detected in both the epithelium and the mesenchyme of newborn rat VPs. Second, uPA and uPAR are necessary for the growth and ductal branching morphogenesis of cultured newborn VP. Therefore, we speculate that uPA synthesized by mesenchymal cells must be localized, activated, and available for cells in the developing epithelial-smooth muscle interface in order to mediate precise cellular migration. Disruption of these processes by peptide inhibitors of uPA/uPAR resulted in impaired cellular migration as well as retarded growth, ductal branching morphogenesis

and tissue differentiation. Though we have not directly measured the impairment of cellular migration in this system, uPA has been required for the migration of cells in other systems such as endothelial cells and tumor cells (Blasi, 1993).

Vascularization is important in prostatic cancer (Weidner et al., 1992); (Weidner et al., 1993). The process of vascularization is complex and mediated cellular migration is mediated by many factors including integrins (Enenstein et al., 1992; Enenstein and Kramer, 1994; Brooks et al., 1994) and proteinases such as uPA and MMP-2 (Mignatti and Rifkin, 1996). Recently, a fragment of plasminogen, angiostatin, is suggested to inhibit angiogenesis and metastasis of Lewis lung tumors (O'Reilly et al., 1994). Further, three human prostate cancer cell lines apparently express serine proteinase activity that cleaves plasminogen into angiostatin (Gately et al., 1996). uPAR mediates the growth and metastasis of prostate cancer cell lines (Hoosein, 1991). A role for uPA/uPAR in vascularization was suggested by the observation that vascular endothelial growth factor (VEGF), a mitogenic cytokine produced by tumors that acts on vascular endothelium, up regulates the expression of uPA and uPAR in vascular endothelium (Pepper et al., 1993). Also, HGF/SF, which is mitogenic and motogenic for vascular endothelium, is activated by uPA (Naldini et al., 1992). Evidence that uPAR may be important for the vascularization of tumors was also suggested by a recent study in which uPAR appeared to regulate the growth rate of prostate cancer cell lines. The growth and metastasis of the Dunning tumor cell line MATLyLu R-3327 grafted into the host Copenhagen strain rat was dramatically reduced by the perturbation uPAR function. Compared to control transfections, clonal cell lines that expressed catalytically inactive uPA formed smaller primary tumors and fewer lung metastasis. Primary tumors from cells with inactive uPA had a significantly lower vascular density as measured by immunohistochemistry for factor VIII. The growth rates of the control and inactive uPA producing cell lines were equal in cell culture. Therefore, we speculate that the inhibition of tumorigenesis and metastasis observed in this study were due to a paracrine inhibition of host vascular

endothelial cell uPAR that resulted in impaired cellular movement and vascularization of the tumor cells overproducing catalytically inactive uPA (Evans, Elfman, et al., submitted Cancer Research, 1997).

Thus, uPAR appears to regulate the migration of cells during both ductal branching morphogenesis of the rat VP and during angiogenesis of prostate cancer cells. The mechanism by which uPAR facilitates migration during ductal branching morphogenesis and angiogenesis during cancer may be related and need to be clarified. Obviously, disruption of vascularization cannot explain the inhibition of rat VP ductal branching morphogenesis by uPAR antagonists, *in vitro*.

We have reported that uPA/uPAR, independent of plasminogen, regulates ductal branching morphogenesis of the newborn rat VP. This observation and the developmental normalcy of plasminogen knockout mice (Bugge, 1995a) does not preclude a role for plasminogen during wound healing (Romer et al., 1996), disease (Bugge et al., 1996), or vascular development (Mignatti and Rifkin, 1996). Indeed, the influx of large quantities of blood-derived plasminogen could greatly increase proteolysis, ECM degradation, cytokine activation, and mitogenesis. Given that plasminogen is not necessary for organogenesis, uPA/uPAR activation of plasminogen in pathological circumstances could profoundly impact tissue remodeling by regulating angiogenesis. Thus, contrary to developmental angiogenesis or vascularization (Sage and Vernon, 1994; Sage, 1995), the activation of plasminogen during pathological angiogenesis may be important. The stromal origin of uPA and MMP-2 reported in the current study is intriguing to interpret in the context of vascularization. Does the presence of these enzymes in the normal adult stroma imply that uPA and MMP-2 are utilized by endothelial cells during the remodeling of BMs during angiogenesis in prostatic cancer? During angiogenesis in prostate cancer is the expression of uPA and MMP-2 increased in the prostatic stroma or just in the stroma of vascular endothelium?

Thus, the current study suggests that uPA/uPAR and perhaps MMP-2 play an important role in development of the newborn rat VP. We have reported that uPA and MMP-2 are expressed throughout VP development and in the adult rat VP. uPAR mRNA is expressed by newborn rat VPs. uPA and MMP-2 mRNA are localized in the mesenchyme or stroma, while uPA and MMP-2 proteins are localized to both epithelial and mesenchymal tissues. Levels of activated MMP-2 were increased during the development of several UGS derivatives (the prostate, bulbourethral glands, and bladder). uPA and uPAR antagonists severely perturb the development of rat VPs. From these observations we conclude that: the mesenchyme as well as the stroma are the sites of synthesis for uPA and MMP-2 and that these enzymes traffic to the epithelium in newborn rat VPs; uPA and uPAR are necessary for prostatic organogenesis and appear not to proceed through the classic model of plasminogen activation.

Thus, the primary function of uPA must be re-evaluated. Data presented in the current study support the hypothesis that during prostatic organogenesis, ECM-degrading enzymes mediate tissue remodeling by more than just degradation of the ECM by regulating cellular migration, proliferation, as well as differentiation. Defining the paracrine regulation and functions of uPA and MMP-2 in prostatic development may provide the basis for new therapeutic strategies for prostatic cancer.

CHAPTER 6

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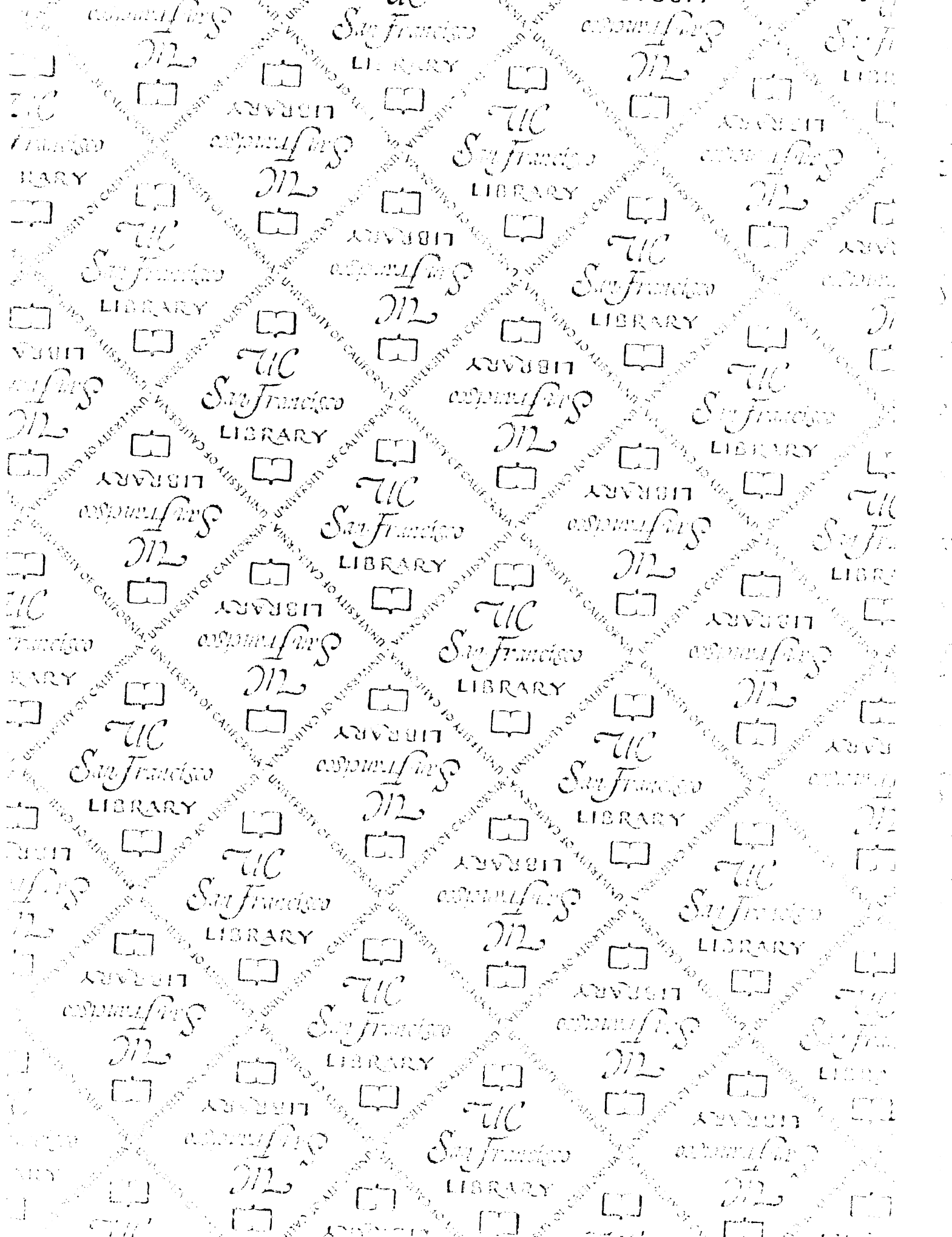
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