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REGULATION OF THE MATRIX METALLOPROTEINASE MATRILYSIN IN HUMAN PROSTATE CARCINOMA IN VITRO

by

Mimi Suzanne Stratton

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Mimi Suzanne Stratton

entitled Regulation of the Matrix Metalloproteinase Matrilysin in Human Prostate Carcinoma In Vitro

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

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Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director G. Tim Bowden — Date June 27, 2001

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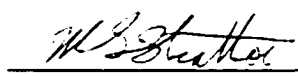
SIGNED: 

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DEDICATION

This dissertation is dedicated to my husband, Steven Paul Stratton. His love and his very existence have made my life magical and worthwhile. Steven is my life, my partner and my destiny. I will always strive to make him proud and to do what is best for him and for us. I could easily write chapters about his influence on every aspect of my life and his kindness, but I will save those words for him, alone. Thank you, my love.

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ABSTRACT

Prostatic carcinoma is the most frequently diagnosed cancer in men in the United States, however, its etiology is largely unknown. The mechanisms of invasion and metastases of prostate carcinoma are currently topics of intense study. Our research focused on IL-1β induced expression of the matrix metalloproteinase, matrilysin, in the prostate. Matrilysin is suspected to be involved with invasive and/or metastatic properties of prostate carcinoma cells and has been shown to be up regulated in prostate cancer. Inhibition of NFκB completely abrogated IL-1β induced promatrilysin expression, however, inhibition of protein synthesis with cyclohexamide completely blocked induction of IL-1β stimulated matrilysin mRNA which indicated that synthesis of one or more signaling factors was required for potentiation of promatrilysin expression by IL-1β. IL-1β also induced expression of IL-6 by LNCaP cells; and, recombinant IL-6 stimulated promatrilysin expression. Cyclohexamide did not inhibit induction of promatrilysin by IL-6 indicating that IL-6 induced promatrilysin expression was direct and did not require new protein synthesis. In addition, our data revealed that inhibition of IL-6 activity with a neutralizing antibody directed against the IL-6 ligand, blocked IL-1β induced promatrilysin expression. Further investigation of this pathway suggested that STAT3 acts downstream to regulate IL-6 induced matrilysin expression. Dominant negative STAT3 inhibited both IL-1β and IL-6 induced activity of a co-transfected matrilysin-luciferase reporter construct.

We next examined promatrilysin expression in co-cultures of two prostatic carcinoma cell lines and found that IL-6 secreted by DU-145 cells induced promatrilysin expression by LNCaP cells. DU-145 conditioned media stimulated promatrilysin by LNCaP cells; and, pretreatment of the DU-145 conditioned media with IL-6 neutralizing antibody completely abrogated promatrilysin expression.

Because of their relevance to prostate cancer, we next examined the effect of androgens on IL-1β induced promatrilysin expression. We found that the androgens, testosterone and dihydrotestosterone blocked IL-1β induced promatrilysin and IL-6 expression through inhibition of NFκB. Androgens showed no effect on IL-6 induced promatrilysin expression indicating that STAT3 is not regulated by androgens in our system. Therefore, our data indicate that IL-1β induced promatrilysin expression is regulated by NFκB mediated synthesis of IL-6 and STAT3 signaling; and, through inhibition of NFκB, androgens can block IL-1β induced promatrilysin.

Degradation of the extracellular matrix by MMPs is thought to play a role in prostate cancer invasion and metastatasis. These data provide evidence that IL-1β and IL-6 mediated expression of matrilysin may be involved in prostate cancer progression.

CHAPTER I.
INTRODUCTION

Prostate Cancer

Prostate adenocarcinoma is a major health concern in many populations. It is the most frequently diagnosed visceral cancer in men in the United States and accounts for 14 percent of all cancers, more that 25 percent of all male cancers and approximately 11 percent of male cancer deaths. Since the 1970s, 5-year survival statistics have improved, probably, in part, due to more accurate diagnostic indicators and efficacy of therapy for low grade disease (Boring, *et al.*, 1994). However, according to the most recent statistics published by the National Cancer Institute (Surveillance, Epidemiology, and End Results (SEER)) for the year 2000, despite the slight drop in prostate cancer incidence in the United States within the last few years (200 cases per 100,000 people in 1992 to 170 cases per 100,000 people in 1997), over the last four decades, overall frequency and mortality are on the rise. In the early 1970s, incidence of prostate cancer was approximately 60 cases per 100,000 people (Greenlee, *et al.*, 2001).

Epidemiology and risk factors

The etiology of prostate cancer remains largely a mystery. Epidemiological studies conducted over the past twenty years have revealed statististically significant differences of clinically apparent prostate carcinoma between several economically developed nations. The incidence of prostate cancer is not evenly distributed among men

of different racial background and ethnicity or geographical region. Data describing the epidemiology of prostate cancer from the 1930s to the present document a dramatic racial difference in incidence, survival, and mortality rates in American men. African American men have the highest incidence and mortality rates of prostate cancer in the world. Survival data have been related to access to medical care, genetic and environmental factors, and cultural differences, including diet and social habits. Most reports present conflicting data with no clear positive correlations, and conclusions are often speculative (Burks, *et al.*, 1992). In addition, while prostate cancer is one of the major malignant diseases in Western countries, in Japan, the incidence and mortality of prostate cancer is remarkably low by comparison; however, it is continuously increasing over time in Japan. The increase in incidence within the last ten years in the Japanese population is speculated to be attributed to the growth of the elderly population, a westernized diet in daily life and widespread environmental contamination by carcinogens. Also, the increase in incidence in of prostate cancer in Japan has been attributed to the improvement of screening techniques such as the serum PSA test (Imai, *et al.*, 1994). This makes the assumption that the low incidence of prostate cancer was always deceivingly low in this population because it was never detected. Epidemiological studies conducted by Imia and colleagues comparing the incidence of prostate cancer in the United States versus Japan suggested that the strikingly large difference in incidence was also skewed because the prostatic cancers diagnosed in men in the United States appeared to be more aggressive than those in Japanese men. The epidemiology of prostate cancer hints that its etiology is both environmental and genetic.

Androgenic stimulation over time, perhaps due to a high fat diet, has been suggested as a cause of prostate cancer (Imai, *et al.*, 1994; Sata, *et al.*, 2001; Imai, *et al.*, 1992).

The most apparent risk factor of prostatic carcinoma appears to be age. Ninety-five percent of all prostatic cancers occur in men ranging from 45 to 80 years of age. Before the age of 50, men are at relatively low risk. For men aged 50-54, incidence is approximately 30 cases in 100,000 men. However, beginning at 55 years of age, the potential for developing prostatic carcinoma is heightened dramatically and can even double with each successive 5-year age increment, resulting in an overall incidence of 1000 cases per 100,000 men aged 85 years and older (Guinan, *et al.*, 1981; Guinan, *et al.*, 1980).

More recently, genetic factors that contribute to prostate cancer have been examined. Research into the molecular genetics of prostate cancer to date has largely focused on the possible existence of one or several single-locus high-penetrance susceptibility genes and several candidate regions have been identified, but confirmatory studies of these regions have been inconclusive. Increasingly, attention has turned to identification of candidate genes that may increase prostate cancer risk because their products potentially play an important role in possible etiological pathways for prostate cancer (Xu, 2000; Ostrander, *et al.*, 2000). Of various pathways that have been suggested for prostate cancer, the best studied in terms of molecular genetics, is the androgen signaling pathway. Two genes in this pathway, the androgen receptor (AR) gene and the steroid 5-alpha reductase type II (SRD5A2) gene, have been under particular scrutiny

and, polymorphic markers in each of these genes, which reproducibly predict prostate cancer risk, have been identified. (Abate-Shen, *et al.*, 2000).

Prostate biology

The prostate is a variable sized gland located in the male pelvis, usually the size of a walnut measuring 3-4 centimeters long by 3-5 centimeters in width. On average, the gland weighs about 20 grams. It is found behind the pubic bone, in front of the rectum, and below the bladder, surrounded by the pelvic muscles. The prostate surrounds the urethra, which carries urine from the bladder to the penis and travels in the center of the gland. The seminal vesicles attach to the prostate and produce material that mixes with prostate fluid to form ejaculate (Aumuller, 1989).

The regions of the prostate gland are defined by concentric zones. These zones are termed: the anterior fibromuscular stroma, the peripheral zone, the central zone, and the transition zone. Almost all prostate cancers originate from the peripheral zone (Chang, *et al.*, 1998). The transition zone generally accounts for less than 5% of the total prostate volume. It is the exclusive site for the development of benign prostatic hyperplasia (BPH) a frequent differential diagnosis for prostatic carcinoma.. BPH can cause urinary symptoms including pain and urinary obstruction (Di Silverio, *et al.*, 1993). The central zone is involved with the connection of the seminal vesicles to the prostate and is rarely associated with any disease process. The anterior fibromuscular stroma is the anchoring point of the urethral sphincter that controls urination; it does not have any glands and therefore cancer or enlargement does not develop here (Aumuller, 1989). The prostate is

made up of several different cell types. Epithelial cells comprise the glandular portion of the prostate and stromal cells make up the surrounding muscle and connective tissues. Cancer of the prostate develops from the epithelial cells, but the interaction with the stromal cells is very important to the behavior and characteristics of prostate cancer (Chung, 1993; Wong, *et al.*, 2000).

Detection, staging and treatment

Adenocarcinoma of the prostate is recognized as a relatively slowly growing tumor but it can display an extremely variable rate and pattern of disease progression. A small primary and focal nodule may progress directly to a clinically detectable nodule; or, disease confined within the prostatic gland capsule may rapidly progress to lymph node and widespread bone metastases. The pattern and rate of progression for each individual patient is unpredictable. In addition, contrary to many other solid tumors, primary prostatic tumors are often multifocal and heterogeneous in nature (Whitmore, 1984).

The most critical factor in diagnosing prostate cancer is a properly performed digital rectal examination (DRE). Any area with irregularities including asymmetry, firmness or indurations should be considered suspicious for carcinoma and considered for biopsy. According to studies conducted by Guinan and colleagues, the sensitivity and specificity of a properly performed DRE are approximately 70 and 80 percent, respectively, with an overall accuracy of approximately 85 percent (Guinan, *et al.*, 1980).

Serial measurements of prostate-specific antigen (PSA) also provide a useful marker of prostatic carcinoma and for detection at early relapse following disease

response to after front line therapy. It is argued that PSA is more useful for detection of relapse and response to therapy and not reliable for detection of a primary cancer, in part, due to the differential diagnosis of BPH, where PSA can be elevated even though there is no known clinical link to prostate cancer (Killian, *et al.*, 1990; Killian, *et al.*, 1986; Killian, *et al.*, 1985).

Clearly, we are in dire need of a more accurate screening technique for prostate carcinoma. Several potential techniques are currently under clinical evaluation. The University of California, San Francisco is evaluating the feasibility of using three-dimensional magnetic resonance spectroscopic imaging as a relatively non-invasive technique for diagnosing and determining stage of prostatic carcinoma (Kurhanewicz, *et al.*, 1996; Scheidler, *et al.*, 1999; Kurhanewicz, *et al.*, 2000), however, although this technique is showing promise, currently DRE, PSA measurements and histologic examination of biopsy samples remain the gold standard for diagnosis. Currently, data evaluating using three-dimensional magnetic resonance spectroscopic imaging is premature and still requires significant clinical development.

There are several methods that are employed for staging of prostate carcinoma. Many systems for histological grading are used. The most frequently used for histopathology is the Gleason's Grade, which classifies tumors into well-differentiated (Grade I) to anaplastic (Grade V). The final Gleason's score, which is an indication of the overall malignant potential of the tumor, is the sum of the two most frequent patterns assigned to the tissue sections examined. Therefore, the score will range from two to ten. A higher number indicates a more progressed tumor (Schroeder, *et al.*, 1985). TNM

staging, which is used for most solid tumor types, classifies the stage of a tumor by 3 major characteristics. These include the tumor itself (T_{1-4}), size, whether or not is palpable by DRE and multifocal or beginning to invade. The second characteristic utilized by TNM staging is lymph nodes (N_{1-4}) and whether there is local and/or nodal disease. This can further be classified into disease in proximal or distal nodal disease. The last factor of TNM staging is metastases (M). Metastases are divided into juxtaregional and distal.

The American system, which has adopted the standard TNM nomenclature, classifies stages of prostatic carcinoma by a combination of clinical symptoms, laboratory findings and regions of the prostate and distant sites to which malignant invasion has occurred. At Stage A, latent prostate carcinoma, patients are asymptomatic and both laboratory and clinical findings are normal. Stage B patients are considered to be in the early stage of prostatic carcinoma. These patients may also be asymptomatic and have no abnormal laboratory findings; however, the primary tumor is palpable by DRE. At Stage C, patients frequently have urinary symptoms or urethral obstruction. Clinical findings can reveal extensive indurations, but no metastatic spread, and laboratory alkaline phosphatase is often abnormal. Advanced prostatic carcinoma is classified as Stage D. Patients experience urinary symptoms to varying degrees in addition to bone pain due to metastatic spread. Laboratory findings of both acid phosphatase and alkaline phosphatase are abnormal.

There is no universally acceptable treatment regimen for prostatic carcinoma. Since the introduction of hormone manipulation, recommendations for therapy are

generally based on stage and grade of the primary tumor at the time of diagnosis as well as the age and general health of the patients taking into account the potential side effects of each therapy. Often, particularly in an older man or a man in relatively poor health, no intervention (watchful waiting) is the best option. Removal of the prostate by surgery is still commonly performed. However, standard external beam radiotherapy and brachytherapy, which involves implantation of radioactive seeds into the prostate, are also viable options for patients. Many studies have indicated that surgery is more effective for disease control and cure (Bonin, *et al.*, 1997; Paulson, 1988; Kanamaru, *et al.*, 1996). However, radiation also shows a good clinical response and perhaps less incidence of unfavorable side effects, such as urinary dysfunction and impotency (Ling, *et al.*, 1996; Shrader-Bogen, *et al.*, 1997; Pollack, 2000).

Matrix Metalloproteinases

With most types of malignancies for which solid tumors are characteristic, including prostatic carcinoma, morbidity and mortality are caused primarily by metastatic spread of the primary tumor(s) to distant sites. Clinical studies have demonstrated that metastases originating from a certain tumor type tend to occur in specific target organs. The most common sites of prostate carcinoma metastatic spread are the local lymph nodes (Pajouh, *et al.*, 1991; Ichikawa, *et al.*, 1998) and bone tissues of the pelvis and spinal column (Koutsilieris, 1995).

The process by which metastases occur is comprised of a complex cascade of steps that are regulated by a multitude of molecular mechanisms. Once a primary tumor is established, alterations in cell adhesive properties, which can be regulated by integrins and cadherins, permit one or more cells to de-adhere from the primary tumor cells and adhere to local surrounding extracellular matrix. Subsequently, the cells must intravasate into local vasulature. Cells must then be transported through the vascular system and will form metastatic tumors successfully only if the host immune system is evaded. At a site distant from the primary tumor from which they were derived, tumor cells extravasate into the surrounding parenchyma and organs where new growth can be established.

The mechanisms by which tumor cells can intravasate into vasculature and extravasate into surrounding tissue are mediated, in part, by matrix metalloproteinases, which are a family enzymes that require zinc for catalytic activity and are responsible for degradation of the extracellular matrix (Koutsilieris, 1995). Non-specific MMP inhibitors are often designed to block the zinc-binding region, which all MMP family members share identity (Hernandez-Pando, *et al.*, 2000). Over 25 matrix metalloproteinases have been identified to date. Subclasses including stromelysins and collagenases have been classified according to substrate specificity, however, each MMP has multiple substrates, which it is capable of degrading. More recently, a membrane-bound subclass (MTMMP) has been identified (Koutsilieris, 1995; Tsunezuka, *et al.*, 1996; Sato, *et al.*, 1997).

The progression of prostatic carcinoma is modulated not only by a net increase in cell proliferation, but also by inhibition of sensitivity to signals that normally result in

programmed cell death, referred to as apoptosis. Furthermore, it has been postulated that growth of prostatic tumors is also mediated through up-regulation growth factors including epidermal growth factor (EGF) (Gil-Diez, *et al.*, 1998; Liu, *et al.*, 1993), transforming growth factor beta (TGFβ) (Liu, *et al.*, 1993) and insulin like growth factor (IGF) (Wang, *et al.*, 1998). Many of these cytokines and growth factors are known to contribute to regulation of MMPs. (Kondapaka, *et al.*, 1997; Nagakawa, *et al.*, 2000; Barille, *et al.*, 2000; Bennett, *et al.*, 2000; Kubota, *et al.*, 2000; Johansson, *et al.*, 2000).

Regulation of MMPs is complex. Expression of some MMP family members is up-regulated during various stages of embryonic development such as heart valve formation (Alexander, *et al.*, 1997), as well as in response to tissue injury (Corbel, *et al.*, 2000; La Fleur, *et al.*, 1996) or other stresses that require remodeling of the extracellular matrix. MMPs are secreted as inactive zymogens that require the removal of the amino-terminal prodomain to achieve catalytic activity. All secreted MMPs characterized to date share in common three structural domains including a leader or pre sequence, which is required for secretion of the inactive zymogen, a prodomain that inhibits catalytic activity of the uncleaved enzyme, and the catalytic domain which contains the zinc binding region. Two other regions include the hinge region and hemopexin binding domain.

It has been shown, both *in vitro* and *in vivo* that invasion of a primary tumor and metastatic processes are facilitated, in part, by MMPs (Nagakawa, *et al.*, 2000; Curran, *et al.*, 1999; Thomas, *et al.*, 1999; Powell, *et al.*, 1996). Thant and colleagues demonstrated that Ras mediated signaling plays a critical role in activation of MMP-2 and,

subsequently, in the invasiveness of src- transformed cells in vivo using a Boyden chamber assay (Thant, *et al.*, 1999). It has also been shown, in a melanoma cell line, that stable transfection with dominant negative type I collagenase (MMP-1) inhibited the invasive capacity of the cells through matrigel. Inhibition of MMP-1 activity was confirmed by zymography (Durko, *et al.*, 1997). Bu and colleagues examined MMP-2 expression in hepatocellular carcinoma. Tissue samples were examined using immunohistochemical analyses (Bu, *et al.*, 1997). They demonstrated a positive correlation between the amount of MMP-2 present in tumor tissues and surrounding perenchyma and the level of invasion of the cancer.

MMP inhibitors have been developed and are currently in phase I and phase II clinical trials in many tumor types. Marimistat, a broad spectrum MMP inhibitor is currently being tested in the clinic in breast cancer (Fornier, *et al.*, 1999) and pancreatic cancer (Jones, *et al.*, 1999). Phase II studies, which have used Marimistat alone or in combination with other cytotoxic agents, have produced encouraging results with improved survival. Phase III pivotal trials are now underway for the use of marimastat in advanced pancreatic cancer and as an adjuvant therapy in patients following resection of pancreatic cancer (Jones, *et al.*, 1999). The MMP inhibitor, Batimistat is also being tested in clinical trials for treatment of pleural effusion caused by increased expression of MMPs by stromal cells in the lungs of lung cancer patients and melanoma patients with lung metastases (Macaulay, *et al.*, 1999).

Matrilysin

Matrilysin (MMP-7, PUMP-1) is a member of the stromelysin subclass of MMPs. The gene for matrilysin has been mapped to chromosome 11q21-q22 (Knox, *et al.*, 1996). It is approximately 9.65kbp in length, consists of 6 exons, and encodes a fully processed mRNA of 1.2kb (Rudolph-Owen, *et al.*, 1998). Although the regulation of matrilysin expression has not been fully characterized, it has been hypothesized that it is regulated primarily at the level of transcription through binding of transcription factors to consensus elements that reside within the 5' promoter/enhancer region of the gene. Cis elements identified within the published promoter sequence includes one AP-1 consensus element (TRE) and two ETS consensus elements (PEA3) (Matrisian, *et al.*, 1994. The promoter region also contains multiple binding sites for STAT3 (Gaire, *et al.*, 1994). Similar to most MMPs, matrilysin is secreted as an inactive zymogen. Activation occurs when the 28kd inactive precursor is cleaved at the amino terminal 9kd prodomain. Cleavage can occur in response to a variety of stimuli including cellular stresses such as heat or freeze-thaw (Crabbe, *et al.*, 1992), or to other enzymes including trypsin, plasmin, leukocyte elastase or stromelysin-1 (MMP-4) (Imai, *et al.*, 1995). Promatrilysin is highly stable and exists almost exclusively in the uncleaved, inactive form *in vitro* (Knox, *et al.*, 1996).

Similar to the more recently identified MMP-23 and –26, matrilysin lacks the haemopexin-like binding domain that is present in other secreted MMPs. It has been hypothesized that this domain mediates interactions of the MMPs with the tissue inhibitors of matrix metalloproteinases (TIMPs). Stromelysin, a MMP in which the

haemopexin-like binding domain is present, has been shown to have a much higher affinity for TIMP-1 than matrilysin. It is of interest that a truncated form of stromelysin lacking the haemopexin-like binding domain shares similar TIMP-1 binding characteristics to matrilysin (Baragi, *et al.*, 1994).

The substrates that matrilysin degrades most efficiently is entactin, a protein that links type IV collagen to laminin. However, similar to most of the MMPs, matrilysin is capable of degrading a diverse group of extracellular matrix components including proteoglycans, fibronectin, gelatin, and elastin (Wilson, *et al.*, 1996). Other substrates include the elastase inhibitor α-1-antitrypsin (Sires, *et al.*, 1994) and β4 integrin (von Bredow, *et al.*, 1997). Matrilysin plays an additional role in degradation of the extracellular matrix by cleavage, and thus activation of other MMPs including procollagenase B (von Bredow, *et al.*, 1998) and procollagenase (Imai, *et al.*, 1995).

Like all MMPs, matrilysin has been shown to be expressed during normal developmental and physiological processes including in the cycling endometrium (Rodgers, *et al.*, 1993), the involuting rat prostate (Powell, *et al.*, 1996) and uterus (Woessner, *et al.*, 1988) and in developing mononuclear phagocytes (Busiek, *et al.*, 1992), mammary gland tissue, pancreas, liver and lung (Saarialho-Kere, *et al.*, 1995). However, overexpression of matrilysin has been observed in numerous tumor types including carcinomas of the colon (Ichikawa, *et al.*, 1998; Yamamoto, *et al.*, 1995), ovary (Tanimoto, *et al.*, 1999) and prostate (Knox, *et al.*, 1996; Hashimoto, *et al.*, 1998). Furthermore, several laboratories have shown that inhibition of matrilysin inhibits colon cancer cell invasion. Yamamoto and colleagues examined overexpression and inhibition

of matrilysin in colon cancer cells and found that overexpression increased *in vitro* invasive capacity; and, down regulation of matrilysin expression by all trans-retinoic acid or by introduction of anti-sense matrilysin in the colon cancer cells reduced invasive capacity (Yamamoto, *et al.*, 1995). In other studies conducted by Hasegawa and colleagues, blocking matrilysin with antisense oligonucleotides inhibited liver metastases by colon cancer cells in nude mice (Hasegawa, *et al.*, 1998). Matrilysin has also been shown to be overexpressed by ovarian cancer cells and may be a useful diagnostic or prognostic indicator for this tumor type (Tanimoto, *et al.*, 1999).

Matrilysin and Prostate Cancer

Matrilysin has been shown to be present in both inflamed ducts of the human acinar prostate and prostate carcinoma (Knox, *et al.*, 1996; Saarialho-Kere, *et al.*, 1995; Hashimoto, *et al.*, 1997). Previous work performed by our laboratory demonstrated that expression of matrilysin is strikingly focal in prostate carcinoma. Cells expressing high levels of message were found directly adjacent to cells negative for matrilysin mRNA. Furthermore, Western analyses of prostatic carcinoma tissue specimens revealed that the matrilysin present was predominantly in the active form (Knox, *et al.*, 1996).

Research conducted by Hashimoto and colleagues indicated that elevated matrilysin expression in prostate carcinoma, in a population of men in Japan, significantly correlated with prognostic and pathological indicators of grade and stage as well as vascular and lymphatic invasion (Hashimoto, *et al.*, 1997). The potential role of

matrilysin in potentiation of metastatic capacity was also demonstrated by stable transfection of a plasmid construct encoding the full-length human matrilysin cDNA driven by a CMV promoter into the cell line, DU-145, a prostatic carcinoma cell line that has been characterized as weakly invasive. Our laboratory demonstrated that DU-145 cells stably transfected with constitutively expressed human matrilysin had increased invasive capacity *in vitro* using matrigel assays and when injected into SCID mice intraperitoneally, compared to control cells stably transfected with the control parent vector. Furthermore, the increase in both *in vitro* and *in vivo* invasive capacity in matrilysin transfected cells was inhibited by the synthetic MMP inhibitor, Batimistat (BB-94, British Biotech). There was no effect on *in vitro* growth of these cells compared to mock transfected DU-145 cells (Knox, *et al.*, 1998). Together, these data suggest that matrilysin may play a role in the invasion and progression of prostate carcinoma. It is of interest that, unlike the gelatinases, which are expressed by the cells of the tumor stroma (Jeziorska, *et al.*, 1996; Autio-Harmainen, *et al.*, 1993), it is the cancer cells themselves that express matrilysin (Knox, *et al.*, 1996).

Cytokines

Cytokines are signaling factors (usually glycoproteins) of relatively low molecular mass. Cytokines often consist of a single chain peptide between 8 and 25kDa. They are known to play significant roles in regulation of biological processes including cell growth (Denburg, 1990; Cohen, *et al.*, 1991; Reiter, *et al.*, 1992; Matsue, *et al.*, 1993), immune cell activation (Callard, 1989; Fujimaki, *et al.*, 1993; Hendrie, *et al.*,

1991; Patel, *et al.*, 1992), inflammation (Sartor, 1996; Kono, *et al.*, 1991; Elias, *et al.*, 1990) and tissue repair (Pellegrini, *et al.*, 1996; Roesel, *et al.*, 1995; Zhang, *et al.*, 1997; Diehl, 2000). Some cytokines, such as interleukin-8, are also involved with chemotaxis and are thus referred to as chemokines (Gesser, *et al.*, 1996; Hammond, *et al.*, 1995; Loetscher, *et al.*, 1994). Although cytokines are considered to be a 'family' of factors, this is a functional rather than a structural concept. Most cytokines are structurally unrelated, however, there are groups of cytokine family members that share approximately 30% identity such as IL-α and IL-1β, TNFα and TNFβ and EGF and TGFβ. Within these groups exist subfamilies that share greater identitiy (approximately 80%) (Minasian, *et al.*, 1992).

Interleukins, a family of cytokines first characterized in the early 1980s, were defined as molecules that were produced by leukocytes. However, it has since been found that interleukins are also made by non-leukocytes and often act on non-leukocytes. Targets for interleukin action have been found to be quite variable ranging from immune cells to fibroblasts to cells of epithelial tissue. Dysregulation of cytokines is often observed in cancer (Wise, *et al.*, 2000; Eskandari, *et al.*, 2001; Kerbel, 1992; Blankenstein, *et al.*, 1990).

Interleukin-1 and Interleukin-6

The interleukin-1 (IL-1) family members were previously known as endogenous pyrogen, lymphocyte activating factor and catabolin (Gauldie, *et al.*, 1987; Dinarello, *et al.*, 1985). They are produced by many types of cells including endothelial cells

(Shanahan, *et al.*, 1989), B cells (Zimecki, *et al.*, 1988; Reinecker, *et al.*, 1993) and fibroblasts (Kawaguchi, 1994; Apte, *et al.*, 1992), but are most abundantly produced by macrophages (Roux-Lombard, *et al.*, 1989; Adams, *et al.*, 1994). IL-1 family members - α and β are involved in stimulation of T and B cell activation (Varga, *et al.*, 1999; Hoffmann, *et al.*, 1984), acute inflammatory processes (Hultner, *et al.*, 2000; Drelon, *et al.*, 1992), induction of degradative enzymes including the MMP, collagenase (Shingu, *et al.*, 1994); and, differentiation of cartilage to bone (Jacobsen, *et al.*, 1994; Amano, *et al.*, 1996). IL-1β has also been shown to upregulate expression of other interleukins. Sironi and colleagues demonstrated that recombinant IL-1β stimulated IL-6 production by a leukocyte cell line (Sironi, *et al.*, 1989). In addition, an IL-6 receptor antagonist was shown to inhibit IL-1 mediated osteoclast formation indicating that IL-6 signaling is necessary for IL-1 regulated bone differentiation (Devlin, *et al.*, 1998).

Interleukin-6 (IL-6) which was initially named B cell differentiating factor or hepatocyte stimulating factor, is produced by many cell types including T cells and B cells (Wong, *et al.*, 1998; O'Shaughnessy, *et al.*, 1996; McGee, *et al.*, 1995), macrophages (Wong, *et al.*, 1998; Bost, *et al.*, 1995), fibroblasts (Carty, *et al.*, 1991; Raap, *et al.*, 2000) and endothelial cells (Soderquist, *et al.*, 1998; Jirik, *et al.*, 1989). IL-6 is a pleiotropic cytokine and most cells are IL-6 responsive, however, it plays a particularly important role in differentiation of B cells to mature antigen presenting cells. In hepatic cells, IL-6 regulates expression of acute immune response phase proteins (Kopf, *et al.*, 1995; Dalziel, *et al.*, 1999); and, circulating levels of IL-6 have been shown to be elevated in patients with liver disease (Biro, *et al.*, 2000).

IL-1β signaling and NFκB

Transcription factors of the nuclear factor kappa B (NFκB) Rel family are critical regulators of genes that function inflammatory processes, cell proliferation, programmed cell death and cell differentiation (Verma, *et al.*, 1995; Verma, *et al.*, 1997). The prototype Rel family member, NFκB, is comprised of p50 (NFκB1) and p65 (RelA) (Baeuerle, *et al.*, 1997; Baichwal, *et al.*, 1997). Inactive NFκB resides in the cytoplasm when inactive, but enters the nucleus when activated in response to various stimuli including viral infection (Naumann, 2000), ionizing radiation (Mohan, *et al.*, 1994; Prasad, *et al.*, 1994) or stimulation by inflammatory cytokines (You, *et al.*, 2001; Knop, *et al.*, 1998). Activation of NFκB is regulated by an inhibitory subunit, IκB, which sequesters NFκB in the cytoplasm (Beg, *et al.*, 1992). Activation of NFκB requires activity of a cascade of phosphorylation and recruitment enzymes that results in sequential phosphorylation of two serine residues, ubiquitination of adjacent lysines (Brown, *et al.*, 1995) and degradation of the inhibitory IκB. This results in exposure of the nuclear localization subunit on p50. Once in the nucleus the active NFκB heterodimer can bind to the cis elements within the promoter region of target genes and regulate transactivation (Beg, *et al.*, 1992; DiDonato, *et al.*, 1995; Hershko, *et al.*, 1992).

IL-1β is known to elicit its downstream effects through activation of NFκB (Uehara, *et al.*, 1999; Bergmann, *et al.*, 1998). In order to better elucidate the acute immune response to Gram-negative bacteria and sepsis, Zhang and colleagues examined cytokine release in lipopolysaccharide stimulated monocytes using transient co-transfection experiments with dominant negative constructs of the IL-1β signaling

cascade. They found that IL-1β secretion induced NFκB transactivation activity and that NFκB transactivation activity was inhibited by co-transfection with dominant negative IκB. (Zhang, *et al.*, 1999). In has been shown in many cell types, that IL-1β induces NFκB dependent expression of IL-6 (Bankers-Fulbright, *et al.*, 1996). Miyazawa and colleagues demonstrated that mutation of the promoter region of the IL-6 gene within NFκB binding elements blocked IL-1β induced IL-6 expression in human rheumatoid fibroblast-like synoviocytes (Miyazawa, *et al.*,1998). It has also been shown that NFκB dependent IL-1β induced regulation of IL-6 expression mediates IL-6-dependent induction of type II acute-phase response genes in a hepatocellular carcinoma cell line (Shen, *et al.*, 2000).

IL-1β induced activation of NFκB has also been shown to be involved in regulation of several members of the MMP family including MMP-1 and -9 (Origuchi, *et al.*, 2000; Hayashi, *et al.*, 1997; Oleksyszyn, *et al.*, 1996; Yokoo, *et al.*, 1996; Shingu, *et al.*, 1995), as well as other MMPs (Zheng, *et al.*, 2000; Stearns, *et al.*, 1999; Dias, *et al.*, 1998). Furthermore, both MMP-1 (Vincenti, *et al.*,1998) and –9 (Farina, *et al.*,1999) are known to have NFκB binding elements residing within the 5' flanking region of their respective genes. However, contrary to the MMPs known to be regulated by NFκB, the sequenced promoter region of the matrilysin gene does not contain a known binding element for NFκB.

IL-6 signaling and STAT3

IL-6 frequently elicits its downstream effects by signaling through the transcription factor, signal transducer and activator of transcription 3 (STAT3). in the prostate carcinoma cell line, LNCaP binding of IL-6 to its receptor leads to activation of Janus kinases as well as two major downstream signaling components, STAT3 (Qiu, *et al.*, 1998; Chen, *et al.*, 1999) and the MAPK (Qiu, *et al.*, 1998; Chen, *et al.*, 1999) (Qiu, *et al.*, 1998; Chen, *et al.*, 1999; Spiotto, *et al.*, 2000a; Spiotto, *et al.*, 2000b). STAT3 has been shown to mediate neuroendocrine differentiation of LNCaP cells. In addition, in the differentiated cells showing neurite outgrowth and increased expression of the neuroendocrine markers, neuron specific enolase and chromagranin A that had undergone growth arrest, STAT3 remained active (Spiotto, *et al.*, 2000a). Chung and colleagues also demonstrated that STAT3 mediated IL-6 induced growth inhibition in LNCaP cells (Spiotto, *et al.*, 2000b). It is of interest that the more progressed and less differentiated prostatic carcinoma cell lines, PC3 and DU-145, express a constitutive level of secreted IL-6, however, those data also showed that the less progressed and hormone responsive LNCaP cells do not secrete any detectable IL-6 (Chung, *et al.*, 1999). In addition, it has been shown that the more progressed prostatic carcinoma cell lines lack a functional STAT3 pathway (Mori, *et al.*, 1999). Research conducted by Ni and colleagues demonstrated that cells derived from both rat and human prostate cancers have constitutively activated STAT3, and STAT3 activation has been directly correlated with malignant potential (Ni, *et al.*, 2000). Furthermore, inhibition of STAT3 transactivation activity by ectopic expression of a dominant-negative STAT3 in human prostate cancer

cells significantly suppressed their growth *in vitro* and their tumorigenicity *in vivo* (Ni, *et al.*, 2000). Furthermore, the Janus kinase inhibitor, tyrphostin AG490, inhibited the constitutive activation of STAT3 and suppressed the growth of human prostate cancer cells *in vitro*. These results indicate that activation of STAT3 signaling is essential in the progression of prostate cancer cells and suggest that targeting STAT3 signaling may yield a potential therapeutic intervention for prostate cancer (Ni, *et al.*, 2000).

Dysregulation of STAT3 has also been observed in other tumor types in including BRCA1 positive breast carcinoma (Gao, *et al.*, 2001), B cell lymphoma (Hirano, *et al.*, 2000) and promyelocytic leukemias (Yoshinari, *et al.*, 1999). More specifically, IL-6 mediated STAT3 regulation has been associated with many tumor types in addition to prostate carcinoma, some of which include myeloma; (Puthier, *et al.*, 1999; Tanaka, et al., 2000), breast carcinoma (Badache, *et al.*, 2001) (Gaemers, *et al.*, 2001) and pancreatic cancer (Watchorn, *et al.*, 2001).

Cytokine regulation of MMPs

Research conducted by Yamamoto and colleagues demonstrated that cytokine monocyte chemoattractant protein-1 (MCP-1), which is known to play a role in the pathogenisis of tissue fibrosis, affects gene expression of MMP-1 in primary human skin fibroblasts. MCP-1stimulation in fibroblasts can induce MMP-1 and –2 mRNA expression. Interestingly, TIMP-1 mRNA was also up-regulated by MCP-1 stimulation. In addition, MCP-1 strongly induced IL-1β mRNA expression in dermal fibroblasts in parallel with the induction of MMP- 1; and, preincubation with IL-1 receptor antagonist

almost completely abrogated MCP-1 induced expression of MMP-1 mRNA. Furthermore, transient transfection of primary skin fibroblasts with a MMP-1 promoter-reporter construct indicated a dose dependent increase in promoter activity by MCP-1 stimulation. These data demonstrate that MCP-1 up-regulates MMP-1 mRNA expression and synthesis in human skin fibroblasts at a transcriptional level and provide evidence that this is mediated by an IL-1 autocrine loop (Yamamoto, *et al.*, 2000). It has also been shown that IL-1β can induce IL-6 as well as MMP-1 and –3 expression in human fibroblasts, and lipoxin A4, which has been shown to have anti-inflammatory activity *in vivo*, blocked induction of both IL-6 and MMP-1 and -3 indicating that they may all rely on the same transcriptional regulation mechanism downstream of the IL-1 receptor (Sodin-Semrl, *et al.*, 2000).

Using an *in vivo* rheumatoid arthritis model in rats, Mentzel and colleagues showed that several inflammatory cytokines, including IL-1β and IL-6 induced expression of MMP-9 and increased cartilage and bone degradation. These data suggest that cytokine induced MMP expression may play a role in the pathogenesis of arthritis (Mentzel, *et al.*, 1998). MMP-2 and MMP-9 were also shown to be up regulated by IL-1α in a rabbit model of bone resorption (van der Zee, *et al.*, 1998).

In bone marrow stromal cells, IL-6 upregulates expression of MMP-1 and –2. Because IL-6 is known to play a role in the progression of multiple myeloma (Nilsson, *et al.*, 1990; Klein, *et al.*, 1992; Filella, *et al.*, 1996), and because bone destruction is characteristic of myeloma (Lari, *et al.*, 1986; Bataille, *et al.*, 1989), Barrile and colleagues have hypothesized that these data suggest that cytokine mediated expression

of MMP family members may play a significant role in the pathophysiology of multiple myeloma (Barille, *et al.*, 2000).

Cytokines and cancer

Some cytokines, such as IL-2 (Lotze, *et al.*, 1987; Thatcher, *et al.*, 1989; Koretz, *et al.*, 1991), IL-4 (Gallagher, *et al.*, 1992; Zaloom, *et al.*, 1993) and members of the interferon family of cytokines (Fierlbeck, *et al.*, 1996; Vaglini, *et al.*, 1994; Mareel, *et al.*, 1988; Brouckaert, *et al.*, 1986) are frequently used as standard course of therapy for several tumor types. In clinical studies that tested IL-2 therapy in advanced melanoma, an activation of the immune system was typically detected. Immune system activation was measured by an increase in lymphocyte populations, especially in activated natural killer cells. In addition, a tendency for higher numbers of cytotoxic cells has been found in patients with objective tumor responses (Thatcher, *et al.*, 1989; Dorval, *et al.*, 1992; Soubrane, *et al.*, 1994). IL-2 is also used for treatment of early and advanced stage renal cell carcinoma following surgical removal of the renal tumor (Louie, 1990; Sosman, *et al.*, 1991; Morita, *et al.*, 1992). Cytokines are used in cancer therapy with the intent of up-regulating immune factors that should allow the patients' immune response to better recognize and destroy tumor cells.

Unlike cytokines used for cancer therapy, up-regulation of some cytokines including IL-8 (Yokoe, *et al.*, 2000), IL-1 family members (Nakatani, *et al.*, 1998; Chyczewska, *et al.*, 1997) and IL-6 (Chung, *et al.*, 1999; Offner, *et al.*, 1995; Baba, *et al.*, 1995; Strassmann, *et al.*, 1992), are associated with cancer progression, and possibly poor

prognosis. It has also been shown that continuous elevation of peripheral IL-6 levels may indicate poor prognosis in patients with recurrent breast cancer that have undergone extensive chemotherapy. Yokoe and colleagues have postulated that combination therapy, including agents that reduce IL-6 levels, may be a new strategy for aggressively treating recurrent breast cancer (Yokoe, *et al.*, 2000).

Kossakowska and colleagues demonstrated that elevated IL-6 expression correlated with upregulation of MMPs-2 and –9 in tumor biopsy specimens from patients with non-Hodgkin's lymphoma (NHL). In addition, IL-6 stimulated expression of MMP-2 and –9, and significantly up-regulated transmigration in the Matrigel invasion assay by the lymphoid cell lines Raji, Jurkat and NC-37 (Kossakowska, *et al.*, 1999; Kossakowska, *et al.*, 1998). Those data suggested that IL-6 may play a role in determining aggressiveness of NHL by regulation of MMP production.

Using immunohistochemical analyses examining localization of IL-6 in benign, prostatic intraepithelial neoplasia (PIN) and malignant prostate tissue, it has been show that the pattern of expression is distinctly different. In all tissue types, IL-6 was found in the cytoplasm of epithelial cells. Very few secretory cells in benign tissue showed IL-6 expression, in contrast with carcinoma tissue, which showed a high level of expression of IL-6 in secretory cells (Hobisch, *et al.*, 2000). In a clinical study examining peripheral blood levels of IL-6, Drachenberg and colleagues showed that serum levels of IL-6 were significantly elevated in patients with clinically evident hormone refractory disease and statistical significance was seen when comparing the elevated serum IL-6 levels to those in normal controls or patients with an infection in the prostate gland (prostatitis) or BPH.

This suggests that serum IL-6 levels may be a valuable prognostic indicator for hormone-refractory prostate cancer patients and may be a surrogate marker of the androgen independent phenotype (Drachenberg, *et al.*, 1999).

Androgens

The word 'androgen' is a generic term used for a hormone that is involved in regulating activity of accessory male sex organs. Androgens are also involved in development of male sex characteristics (Ottinger, *et al.*, 1979). Androgens are involved in development of non-reproductive tissues such as skeletal muscle (Lu, *et al.*, 1997; Florini, 1970; Grigsby, *et al.*, 1976). Also, in studies conducted by Pochi and colleagues, a positive correlation was observed in teenagers between the presence of acne and elevated androgen levels in skin secretions (Pochi, *et al.*, 1977).

Testosterone is the most potent, naturally occurring androgen. It is formed in greatest quantities by the interstitial cells of the testes (Fenske, 1987; Paz, *et al.*, 1985; Garnier, *et al.*, 1970), and is also secreted by the ovary (Hilliard, *et al.*, 1974; Botella-Llusia, *et al.*, 1980) and adrenal cortex (Vinson, *et al.*, 1975; Turpin, *et al.*, 1985). Testosterone can also be produced by non-glandular tissue by metabolism of a precursor, androstenedione.

Androgens and the prostate

Androgens are critical for the normal development, growth and survival of the prostate gland (Buttyan, *et al.*, 2000; Lostroh, 1971). Edwards and colleagues conducted

a study in which rat ventral prostates were transplanted to the mammary fat pads of castrated male hosts or into intact or ovariectomized female syngeneic hosts. Implanted animals were given either testosterone proprionate with or without prolactin daily, or no treatment. Their data showed that daily treatment with testosterone proprionate was necessary and sufficient for maintenance of epithelial cell height and secretory activity of the implanted tissue. The addition of prolactin, a hormone involved in regulation of the secretion of breast milk in nursing women (Noel, *et al.*, 1974), did not affect implanted tissue structure (Edwards, *et al.*, 1980).

In men, low circulating testosterone is associated with prostatic atrophy. In addition, removal of the testes in rats results in massive atrophy of the prostate gland due to apoptosis of secretory epithelial cells, and normal function and cell growth patterns can be rapidly restored by testosterone (Barley, *et al.*, 1975; Greenstein, 1979). It has also been shown that it can reverse atrophy of accessory genital glands following castration (Tryphonas, *et al.*, 1979). However, although testosterone is involved with skeletal muscle development, testosterone cannot prevent atrophy of skeletal muscle induced by glucocorticoids (Capaccio, *et al.*, 1987).

Metabolism of testosterone

All hormones are metabolically derived from the same precursor, cholesterol (Kitao, 1972; Hume, *et al.*, 1978). Once it is metabolized, additional enzymes are required in order for testosterone to be converted to its active form, 5α-dihydrotestosterone, which is capable of binding the nuclear androgen receptors to elicit

downstream effects. In the prostate gland, 17- beta-ol-dehydrogenase and 5-α-reductase are responsible for metabolism of testosterone to 5α-dihydrotestosterone. The enzymes are not co-dependent and either enzyme is capable of testosterone metabolism alone, however, the presence of the enzymes appears to be site specific within the prostate gland (Shimazaki, *et al.*, 1965).

Delos and colleagues conducted a comparison of the presence of 5α-reductase and testosterone metabolism in primary cultures of epithelial cells and fibroblasts separated from BPH and prostate cancer tissues. Their data suggested that a higher level of prostatic carcinoma cells express 5α-dihydrotestone compared to BPH cells. In addition, 5α-reductase inhibitors, 4-MA (17 beta-(N,N-diethyl)carbamoyl-4-methyl-4-aza-5 alpha-androstan-3-one) and finasteride, inhibited 5α-dihydrotestosterone formation. Interestingly, the inhibitory action of 4-MA appeared to be preferential on epithelial cells of both carcinoma and BPH. In constrast, the inhibitory action of finasteride was targeted primarily towards fibroblasts from adenocarcinoma (Delos, *et al.*, 1995). Unlike other prostatic carcinoma cell lines, LNCaP cells express 5α-reductase, and are therefore capable of metabolism of testosterone to dihysrotestosterone (Negri-Cesi, *et al.*, 1998).

Hormone signaling

Synthetically derived steroid hormones, such as dexamethasone and cortisone, are frequently used for treatment of inflammatory conditions including rheumatoid arthritis (Balint, *et al.*, 1998; Cutolo, 1998), ulcerative colitis (Fedorak, *et al.*, 1995; Danielsson, *et al.*, 1992) and acute back pain (Blomberg, *et al.*, 1993; Koes, *et al.*, 1995). These

androgens have been hypothesized to elicit anti-inflammatory action, in part, by inhibition of NFκB (McKay, *et al.*, 1998; Ray, *et al.*, 1995). Using a yeast two hybrid system, studies conducted by Na and colleagues demonstrated that the steroid receptor coactivator-1 specifically binds to the transcription factor NFκB subunit p50, which inhibits nuclear transolcation of the active NFκB heterodimer subsequent to dissociation and degradation of the IκB subunit that sequesters NFκB in the cytoplasm (Na, *et al.*, 1998). In addition, in a clinical study examining the role of NFκB in inflammatory bowel disease caused either by ulcerative colitis or Crohn's disease, it was shown that NFκB DNA binding was increased in regions of inflamed bowel compared to normal colon tissue in the same patient; and, treatment with androgens alleviated their symptoms and decreased NFκB DNA binding (Ardite, *et al.*, 1998).

Many studies have shown that androgens can effect the expression of MMPs. Schroen and colleagues demonstrated that binding of hormones, including androgens and estradiol to their nuclear receptors, stimulates production of TGFβ, which, in turn, suppresses expression of matrilysin and other MMP family members. It is of interest that these studies found that inhibition of matrilysin occurred primarily, but not exclusively, by disruption of AP-1 sites. Nuclear receptors were found to form complexes on the DNA through direct interactions with AP-1 proteins. This caused sequestration of AP-1 family members and, because these proteins are regulated, in part, by a positive feedback loop, decreased transcription of the genes for these transcription factors. In addition, nuclear receptors and their ligands were found to inhibit MMP function through an indirect mechanism by upregulating transcription of TIMPs (Schroen, *et al.*, 1996).

MMP-9 and TIMP-1 expression have also been shown to be regulated by the gonadotropin-releasing hormone during early human implantation in the uterus. The hormone was shown to upregulate expression of MMP-9 and inhibit transcription of TIMP-1. Because of these data, it has been postulated that trophoblastic gonadotropin-releasing hormone may play an important role in placental tissue organization and in the early embryo-maternal interactions by enhancing trophoblast invasion through regulation of MMPs and TIMPs (Raga, *et al.*, 1999).

In a clinical study examining postmenopausal women undergoing hormone replacement therapy who were experiencing vaginal bleeding, it was found that expression of MMP-9 in endometrial tissue was increased and the expression of TIMP-1 was lower than postmenopausal women not receiving hormone replacement therapy who were not experiencing bleeding. These data suggest that breakdown of the endometrial extracellular matrix and blood vessels that causes the bleeding may be hormonally regulated (Hickey, *et al.*, 2001)

Androgens and prostate cancer

Androgens are implicated in the pathogenesis of prostate cancer. Androgen ablation is currently the most commonly used front line treatment (Garzotto, 2000; Culig, *et al.*, 2000). Nevertheless, this treatment may result in the development of androgen-independent cancers that are refractory to hormone-related treatment. It is therefore extremely important to elucidate the mechanism of action of the androgens in the etiology of prostate cancer. Although there is a known link between androgens and

prostate cancer progression, understanding the molecular mechanisms of these roles may facilitate identification of more specific drug targets that may prevent blocking some of the positive effects that androgens may elicit with regards to blocking tumor progression. Although it is a topic of intense study, currently the roles for androgens, specifically testosterone, in prostate cancer progression have not been defined. In a retrospective clinical study of tumor bank samples in Norway that consisted of serum from over 250 men, androgens were examined as an indicator of prostate cancer risk. Approximately 20% of the men who participated in the study subsequently developed prostate cancer; however, there was no association, positive or negative, between androgens measured in serum and the subsequent risk of developing prostate cancer (Vatten, *et al.*, 1997). *In vivo* animal studies, perhaps using the TRAMP mouse model, which does develop bone metastases may beinvaluable to studying the effects of androgens and androgen ablation therapy on prostate cancer disease progression.

The growth rates of fast growing prostatic carcinoma cell lines, such as DU-145 and PC3, that have been established from more progressed and less differentiated prostatic carcinoma metastatic lesions, are androgen independent (Davies, *et al.*, 2000; Webber, *et al.*, 1996). The more differentiated prostate carcinoma cell line, LNCaP, which is also less invasive than the more poorly differentiated cell lines, is androgen responsive. LNCaP cells express an abnormal androgen receptor, which harbors a mutation within the ligand binding domain (Veldscholte, *et al.*, 1992; Veldscholte, *et al.*, 1994). The mutated androgen receptor is promiscuous in nature and may be activated in response to many stimuli including estrogens and drugs specifically designed to inhibit

the androgen receptor (Veldscholte, *et al.*, 1992). Some known antagonists of the normal androgen receptor can act as agonists to the mutated LNCaP androgen receptor (Olea, *et al.*, 1990). It has also been demonstrated that LNCaP cells express an isoform of 5-α-reductase, which is capable of metabolizing testosterone to 5α-dihydrotestosterone (Negri-Cesi, *et al.*, 1998). At present it is clear that androgens are involved in prostatic cancer progression, however, the specific roles are not characterized. Androgens may be involved in cell growth, loss of differentiation or in one of the many processes that comprise the metastatic pathways. Elucidating the molecular roles of androgens in prostate tumor progression will broaden our knowledge of the disease. This could lead to development of improved treatment modalities and diagnostic indicators.

Statement of the Question and Specific Aims

Morbidity and mortality of prostatic carcinoma is caused by invasion and metastases of the primary tumor to distant sites. MMP family members most likely play a role in the metastatic processes of cancer that require degradation of the extracellular matrix including intravasation of tumor cells into the circulatory system and extravasation of tumor cells at distant tissue sites. Matrilysin has been shown to be overexpressed in several tumor types including prostate carcinoma. In addition, our laboratory demonstrated that stable transfection of matrilysin increased invasive capacity of the weakly invasive prostate cancer cell line, DU-145. We also showed that promatrilysin is expressed by the prostate carcinoma cell line, LNCaP, which was originally derived from a lymph node metastatic lesion of a prostatic carcinoma patient.

It was demonstrated that physiologically relevant doses of the cytokine, IL-1β, increased promatrilysin expression by LNCaP cells up to 100-fold.

However, the molecular mechanisms by which IL-1β induced promatrilysin expression potentiated have not been characterized. The primary goal of these studies was to elucidate the pathway by which IL-1β can induce expression of promatrilysin by LNCaP cells (Figure 1.1). In addition, given the heterogeneic nature of prostate cancer the second goal of these studies was to test the hypothesis that paracrine signaling between different cell types is critical for the progression of the disease. Although androgens are known to be involved in the progression of prostate carcinoma and androgen ablation therapies are the most commonly prescribed treatments, the actual roles of androgens in progression of prostate carcinoma have not been elucidated. The third goal of our studies was to examine the effect of androgens on IL-1β induced prostate promatrilysin expression in LNCaP cells with the hypothesis that regulation of MMPs may be one mechanism by which androgens regulate prostate carcinoma progression.

- **Specific aim #1:** To determine the pathway by which IL-1β induces expression of the matrix metalloproteinase, promatrilysin, in the prostate carcinoma cell line, LNCaP.

- **Specific aim #2:** Given the heterogeneic nature of prostate cancer, the second aim of these studies was to test the hypothesis that paracrine signaling between different cell types is critical for the progression of the disease

- **Specific aim #3:** To examine the effect of androgens on IL-1β induced prostate promatrilysin expression in LNCaP cells with the hypothesis that regulation of MMPs may be one mechanism by which androgens regulate prostate carcinoma progression.

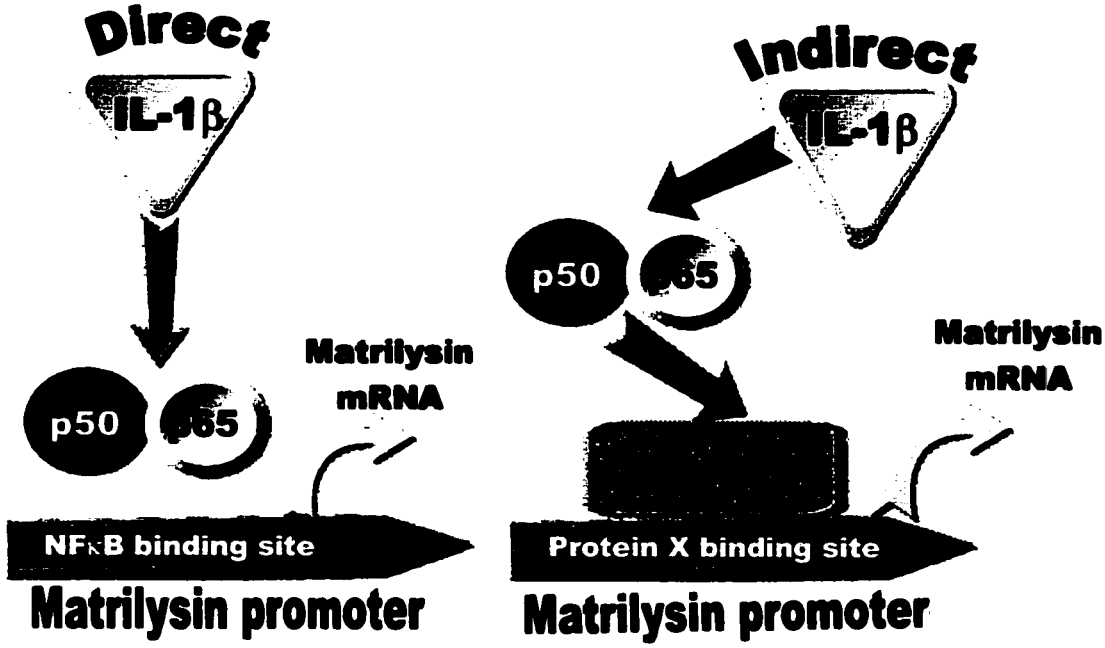

Figure 1.1. The goal of our specific aims was to elucidate the multistep process that regulates IL-1β induced promatrilysin expression in LNCaP cells. If the mechanism were direct, IL-1β would bind the matrilysin promoter directly and elicit transcription of the matrilysin gene. If the mechanism were indirect, IL-1β would bind the promoter region of one or more downstream target genes, which would then be responsible for matrilysin transcription. The second mechanism would require protein synthesis.

CHAPTER II.
MATERIALS AND METHODS

Cell Culture

Lymph node derived carcinoma cells of the prostate (LNCaP) were acquired from the American Type Culture Collection (ATCC, Manasass, VA). LNCaP cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 100μg/mL penicillin and 100μg/mL streptomycin, both from GIBCO-BRL (Rockville, MD). Cells were split 1:2 when they reached approximately 90% confluency (twice weekly). Passage numbers no higher than 40 were used for all experiments.

Cytokines

IL-1β and IL-6 were obtained from Calbiochem (La Jolla, CA). Cells were serum starved for approximately 16 hours prior to cytokine treatment in serum free DMEM. Cytokines were stored at -70^{o}C. Small aliquots of cytokines were made to avoid repeat freeze-thaw cycles.

Androgens

Tesosterone and 5α-dihydotestosterone were obtained from Sigma Chemical Co. (St. Louis, MO). Cells were treated with androgen 1 hour prior to cytokine stimulation. Androgen concentration remained constant during cytokine stimulation. Androgens were stored a 70% ethanol vehicle and stored at -20°C.

Plasmid Constructs

The plasmid construct used in the reporter assays to measure NFκB transactivation activity, NFκB-Luc, was generously provided by the laboratory of Zigang Dong (University of Minnesota, The Hormel Institute, Austin, MN). The construct encodes a 196bp fragment of the HIV promoter containing 2X NFκB binding elements driving a luciferase reporter gene. The parent vector, pgl2-basic (Promega, Madison, WI) was used as a control plasmid. To generate the heterologous human matrilysin promoter (HMAT-luc) construct used in these studies, 1192 base pairs of the sequenced human matrilysin promoter located directly upstream of the TATA box were amplified by polymerase chain reaction and subcloned into the pTAL-Luc parent vector (Promega, Madison, WI). Polymerase chain reaction amplification of the human matrilysin promoter (kindly provided by the laboratory of Lynn Matrisian, Vanderbilt University) was carried out using the following heterologous primers, which contained either Nhe1 (upstream primer) or Xho1 (downstream primer) restriction site sequences linked to

matrilysin promoter specific sequences (matrilysin specific sequences are underlined): upstream primer 5'-CGTCTTGTCATTGGCGAATTC-3', and the antisense downstream primer 5'-CCCCAGTGCAAGTGCAGGTGC-3'. The resultant 1217 base pair amplification product was digested with Nhe1/Xho, gel purified, and directionally cloned into Nhe1/Xho1 digested pTAL-luc vector directly upstream of the thymidine kinase minimal promoter. The resultant plasmid construct was confirmed with DNA sequencing. The pTAL-Luc parent vector was used as a control plasmid. The STAT3 dominant negative plasmid construct was generously provided by the laboratory of Ralph A. Bradshaw (University of California Medical School, Irvine, Irvine, CA). The double mutant STAT3 contains both Tyr→Phe and Ser→Ala mutations preventing phosphorylation at sites critical for STAT3 activity (Wu, *et al.*, 2000). The parent vector into which the STAT3 double mutant was cloned, pCMV-1, was used as a control plasmid. To ensure consistency of transient transfection efficiency, LNCaP cells were transfected with pIRES-EGFP (Promega, Madison, WI) and viewed under a fluorescent light microscope to visualize enhanced green fluorescent protein. A transfection efficiency of 40% was consistently and reproducibly obtained.

Cationic Lipid Transfection

LNCaP cells were seeded into 12.5cm^2 flasks with 1x10^6 cells per flasks, 24 hours prior to transfection. The cationic lipid DMRIE-DOPE™ (Vical, Inc., La Jolla, CA) was used as per standard procedures. Briefly, plasmid DNA (2.5μg/flask) and DMRIE-

DOPE™ (10μg/flasks) were incubated in a polystyrene tube for 30 minutes. The transfection complex was then added to cells in serum free DMEM (1.5mL/flask). Cells remained in transfection medium for approximately 16 hours at which time the cells were removed from the transfection medium and stimulated with cytokine under serum free conditions. For co-transfection experiments, 1.25μg of each plasmid were used per flask such that the total DNA content per flask was 2.5μg.

NFκB Inhibitors

Sulfasalazine and pyrrolidine dithiocarbamate (PDTC) were obtained from Sigma (St. Louis, MI). LNCaP cells were treated with 0.5-1mM sulfasalazine in serum free DMEM, 2 hours prior to IL-1β stimulation. NFκB SN50, Cell-Permeable Inhibitor Peptide and NFκB SN50M Inactive Control Peptide were obtained from Calbiochem®, (La Jolla, CA). LNCaP cells were treated with 18μM inhibitory or control peptide in serum free DMEM, 1 hour prior to stimulation with IL-1β as per manufacturer recommendation.

Quantification of Promatrilysin Expression by ELISA

Sandwich ELISA analyses were performed as described (Klein, *et al.*, 1997). The mouse monoclonal capture antibody (10D2) was coated onto a 96-well ELISA plate

overnight in sodium carbonate coating buffer. The 10D2 antibody can only detect the inactive zymogen of matrilysin (promatrilysin). Plates were rinsed thoroughly with PBS-Tween three times. Coated plates were blocked with 5% non-fat dry milk in PBS-Tween for at least 30 minutes on a shaker at room temperature, then rinsed with PBS-Tween twice. Conditioned media samples were measured in triplicate (50μL per well). Plates were incubated with samples for at least two hours at room temperature on a shaker. Following the two hour incubation period, wells were thoroughly rinsed with PBS-Tween three times. Subsequently, the detection (primary) antibody, which is a rabbit polyclonal antibody (RB2) that binds both active and inactive forms of matrilysin, was put into each well. Plates were treated with primary antibody for at least two hours on a shaker at room temperature. Following incubation with primary antibody, plates were rinsed thoroughly with PBS-Tween three times. Both the 10D2 and RB2 antibodies were produced by and obtained from the laboratory of Raymond Nagle (Arizona Cancer Center, Tucson, AZ). Horseradish peroxidase-conjugated goat anti-rabbit antibody (Pierce, Rockford, IL) was used to detect RB2. One molar hydrochloric acid (50μL per well) was used to stop the colorimetric reaction. Plates were read A_{450} (minus A_{650}) on a microplate reader (Biotek Instruments, Winooski, Vermont) . Purified promatrilysin was used to generate a standard curve within the linear range of 0.2-12.5ng/mL. Samples with more that 12.5ng/mL promatrilysin were diluted in PBS-Tween into the range of the assay.

Quantification of IL-6 Expression by ELISA

Sandwich ELISA analyses were performed by the UMAB Cytokine Core Laboratory (CCL) in Baltimore, Maryland. Conditioned media samples were diluted and shipped overnight on dry ice. Human IL-6 was measured by a two antibody ELISA using biotin-strepavidin-peroxidase detection. Polystyrene plates (96-well) were coated with capture antibody in PBS overnight at 25°C. The plates were washed 4 times with 50mM Tris, 0.2% Tween-20, pH 7.0-7.5 and then blocked for 90 minutes at 25°C with assay buffer (PBS containing 4% BSA (Sigma, St. Louis, MO) and 0.01% Thimerosal, pH 7.2-7.4. The plates were washed four times and 50μL assay buffer was added to each along with 50μL of sample prepared in assay buffer and incubated at 37°C for two hours. The plates were then washed four times and 100μL of biotinylated detecting antibody in assay buffer were added and incubated at 25°C for one hour. After washing the plate four times , strepavidin-peroxidase polymer in casein buffer (RDI, Flanders, NJ) was added and incubated at 25°C for 30 minutes. The plate was then washed four times and 100μL of commercially prepared substrate (TMB; DAKO) were added and incubated for 10-30 minutes at 25°C. The reaction was stopped with 100μL 2N HCL and the A_{450} (minus A_{650}) was read on a microplate reader (Molecular Dynamics, Queensland, Australia). Cytokine concentration in each sample was calculated from the standard curve equation. The range of the assay is 1.562-200pg/mL. A standard curve was generated using concentrations within this range and an internal control containing 50pg/mL was

analyzed in parallel. Conditioned media samples were diluted in assay buffer as necessary to bring the IL-6 concentration within range of the assay.

Collection of Cell Lysates

Media were aspirated from the flasks, then cells were rinsed with PBS. The cells were removed from each 12.5cm^2 flask with 300µg of lysis buffer (1% Triton-X 100, 25mM glycylglycine, 15mM magnesium sulfate, 4mM EGTA, 1mM DTT (DTT was added immediately before use) using a cell scraper. Cell lysates were transferred to 1.5mL Eppendorf tubes, then centrifuged for one minute to separate cell debris from lysate. Lysates were transferred to clear Eppendorf tubes.

Measurement of Luciferase

Cell lysates were sometimes frozen and stored at -20°C before analyses were performed. Total protein concentration of each cell lysate sample was determined using the DC Bio-Rad Protein Assay (Bio-Rad, Cambridge, MA). Ten to 30µg total protein of each sample were used for the luciferase assays. This assay utilizes the enzymatic reaction of firefly luciferase with its substrate, luciferin (Promega, Madison, WI), which emits visible light at 560nm. A TD 20/20 luminometer (Turner Designs, Pharmingen,

San Diego, CA) was used to quantify the emitted light, which is directly proportional to the amount of luciferase present in the sample.

Northern Analyses

Total RNA was extracted using RNEasy® (Qiagen, Valencia, CA) 8-10 hours following IL-1β or 2 hours following IL-6 stimulation. Twenty micrograms of total RNA were subjected to electrophoresis on a 1% agarose, 3-{N-morpholino}propane-sulfonic acid/formaldehyde gel. The RNA was transblotted to a nylon membrane (GeneScreen®, NEN Research Products, Boston, MA) and crosslinked with ultraviolet light (GS Genelinker®, Biorad, Hercules, CA). The membranes were then hybridized with a ^{32}P-labeled random primed probe generated from cDNA for the mRNA of interest using the TS RadPrime® DNA labeling system (Gibco BRL, Gaithersburg, MD) and washed according to the membrane manufacturer instructions. The membranes were then exposed to a storage phosphorscreen (Molecular Dynamics, Sunnyvale, CA). A Molecular Dynamics phosphorimager equipped with ImageQuant package was used for obtaining and analyzing digital images. As a control for loading and transfer of RNA, membranes were stripped by boiling on 0.1% SDS for 30 minutes and re-probed for glyceraldehyde-3'-phosphate-dehydrogenase (GAPDH) as described with a probe generated from an 800bp Xba1-Pst1 fragment from pHcGAP (ATCC, Rockville, MD).

CHAPTER III.

INTERLEUKIN-1β INDUCED PROMATRILYSIN EXPRESSION IS MEDIATED BY NFκB REGULATED SYNTHESIS OF INTERLEUKIN-6

Abstract

Previously, our laboratory showed that IL-1β secreted by lipopolysaccharide activated monocytes induces promatrilysin expression in the prostate carcinoma cell line, LNCaP. We now demonstrate that IL-1β induced promatrilysin expression is mediated by an indirect mechanism that requires NFκB dependent synthesis of IL-6. Inhibition of protein synthesis with cyclohexamide blocked IL-1β mediated induction of matrilysin mRNA suggesting that synthesis of one or more additional factors is required for IL-1β induced promatrilysin protein expression. Blockage of NFκB transactivation activity abrogated IL-1β induced promatrilysin expression to baseline levels suggesting that NFκB transactivation activity is necessary. Inhibition of IL-6 activity attenuated IL-1β induced promatrilysin, but not NFκB transactivation activity indicating that IL-6 acts downstream of NFκB in potentiation of IL-1β mediated promatrilysin expression. Inhibition of protein synthesis with cyclohexamide did not alter IL-6 induced induction of matrilysin mRNA indicating that, contrary to the mechanism by which IL-1β regulates promatrilysin expression, IL-6 mediated matrilysin mRNA expression does not require new protein synthesis. Transient transfection with dominant negative STAT3 inhibited IL-1β and IL-6 induced promatrilysin. These data provide evidence that NFκB-mediated

IL-6 synthesis is required for IL-1β induced promatrilysin expression; and, IL-6 signaling through STAT3 plays a role in IL-1β induced promatrilysin expression.

Introduction

The processes by which tumor cells can invade through the basal lamina as well as intravasate into the vasculature and extravasate into surrounding tissues are mediated, in part, by matrix metalloproteinases (MMPs) (Curran, *et al.*, 1999; Thomas, *et al.*, 1999). MMPs are a family of secreted zinc-dependent proteolytic enzymes responsible for degradation of the extracellular matrix (Baramova, *et al.*, 1995; Cockett, *et al.*, 1994; Matrisian, *et al.*, 1994). MMP activity has been implicated in matrix degradation during both normal physiological processes, such as embryonic development, the cycling endometrium and wound healing, and pathological conditions such as cancer. The MMP, matrilysin (MMP-7, PUMP-1), which, like other MMPs, is secreted in an inactive form (promatrilysin), is unique in that it is expressed primarily in glandular epithelial cells. Overexpression of promatrilysin has been associated with multiple types of malignancies including gastric, (Adachi, *et al.*, 1998) esophageal, (Yamamoto, *et al.*, 1995; Hasegawa, *et al.*, 1998) colon (Powell, *et al.*, 1993) and prostate carcinomas (Knox, *et al.*, 1996; Hashimoto, *et al.*, 1997). In addition, high levels of promatrilysin expression have been observed by our laboratory in inflamed prostatic ducts and glands of the human acinar prostate (Knox, *et al.*, 1996), cycling

endometrial tissues (Gaire, *et al.*, 1994), and in the involuting rat prostate (Powell, *et al.*, 1996).

Previously, our laboratory demonstrated that physiologically relevant doses of the inflammatory cytokine, interleukin-1β (IL-1β), could induce promatrilysin protein expression up to 100-fold over baseline expression levels in the prostate carcinoma cell line, LNCaP (Klein, *et al.*, 1997). IL-1β stimulates pleiotropic effects that regulate the acute immune response. Downstream effects of IL-1β include stimulation of T cell (Bismuth, *et al.*, 1985; Siese, *et al.*, 1999; Zieleniewski, *et al.*, 1995) and B cell (Greenbaum, *et al.*, 1988) proliferation and, expression of other signaling factors such as tumor necrosis factor-α, interleukin-6 (IL-6) and IL-8 (Kitamura, *et al.*, 1998). Because of our *in vitro* finding that IL-1β can induce promatrilysin expression, we proposed that IL-1β signaling could also be responsible for the overexpression of promatrilysin in inflamed glandular epithelial tissues and prostatic carcinomas.

Several mechanisms of matrilysin regulation have been characterized. Induction of matrilysin gene expression by epidermal growth factor (EGF) and phorbol esters have previously been attributed to the activity of activator protein-1 (AP-1) and serum response protein transcription factors (Sundareshan, *et al.*, 1999). However, the molecular mechanism(s) involved in regulation of IL-1β induced promatrilysin expression have not been elucidated.

IL-1β induced activation of NFκB has been shown to be involved in regulation of several other members of the MMP family including MMP-1 and -9 (Origuchi, *et al.*,

2000; Hayashi, *et al.*, 1997; Oleksyszyn, *et al.*, 1996; Yokoo, *et al.*, 1996; Shingu, *et al.*, 1995), as well as other MMPs (Zheng, *et al.*, 2000; Stearns, *et al.*, 1999; Dias, *et al.*, 1998). Furthermore, both MMP-1 (Vincenti, *et al.*, 1998) and -9 (Farina, *et al.*, 1999) are known to have NFκB binding elements residing within the 5' flanking region of their respective genes. But, contrary to the MMPs that are known to be regulated by NFκB, the sequenced promoter region of the matrilysin gene does not contain a known binding element for NFκB. However, because IL-1β frequently elicits its downstream effects through NFκB transactivation activity, we conducted experiments to determine whether NFκB plays a role in IL-1β induced expression of promatrilysin in LNCaP cells. We also examined whether the mechanism of promatrilysin induction by IL-1β is direct, whereby IL-1β induces NFκB mediated transactivation of the matrilysin gene independent of synthesis of additional signaling factors or, indirect, whereby synthesis of one or more intermediate signaling factors is required. Since there has been no NFκB binding element identified within the sequenced region of the human matrilysin promoter, we hypothesized that IL-1β induced NFκB mediated transcription of an intermediate signaling factor(s) is required for potentiation of promatrilysin expression.

Our laboratory has previously demonstrated that overexpression of promatrilysin increases invasive capacity of the prostatic carcinoma cell line, DU-145 (Powell, *et al.*, 1993). In addition, Hashimoto and colleagues showed a significant correlation between the level of promatrilysin expression and the grade and stage of prostatic carcinoma indicating that matrilysin may play an important role in tumor invasion and metastasis (Hashimoto, *et al.*, 1998). These data suggested that control of matrilysin expression may

be a key to preventing prostatic carcinoma progression; and, upstream regulatory factors, including the cytokine, IL-1β be a useful targets in developing a therapeutic strategy for treatment of prostate cancer at early stage, to block invasion and metastases; or, at later stage, to prevent further progression.

Our data have characterized a signal transduction pathway by which IL-1β regulates promatrilysin expression through an indirect mechanism requiring NFκB dependent synthesis of IL-6. This pathway demonstrates, for the first time, regulation of a MMP by a secreted signaling factor (IL-1β) through an indirect pathway that requires synthesis of an additional secreted protein (IL-6). In addition, we show that STAT3 appears to play a role in the signal transduction pathway downstream of IL-6.

Results

NFκB mediates IL-1β induced promatrilysin expression

NFκB is known to mediate transcription of IL-1β induced genes including the cytokines IL-6 and IL-8 (Yoon, *et al.*, 1999). To determine whether NFκB transactivation activity is necessary for potentiation of IL-1β induced promatrilysin expression in LNCaP cells, we used a variety of inhibitors of NFκB activation or nuclear localization to block NFκB transactivation activity in IL-1β treated cells. These inhibitors, which are both known to inhibit activation of NFκB, included the antioxidant and metal chelator, pyrrolidine dithiocarbamate (PDTC), as well as sulfasalazine, a drug

commonly prescribed for inflammatory disorders including ulcerative colitis and rheumatoid arthritis (Ferran, *et al.*, 1995; Liptay, *et al.*, 1999). We also used the NFκB SN50 Cell-Permeable Inhibitor Peptide (Calbiochem, San Diego, CA), which specifically binds to and blocks the nuclear localization site on the p50 subunit of NFκB, thereby inhibiting nuclear translocation of the active NFκB heterodimer following degradation of IκB. The NFκB SN50M Cell permeable Inactive Control Peptide is scrambled and does not have the ability to bind p50 (Lin, *et al.*, 1995).

We first demonstrated that the inhibitors were effective in blocking NFκB transactivation activity in LNCaP cells using a luciferase reporter assay. LNCaP cells were transfected with a plasmid construct encoding a minimal HIV promoter containing 2X NFκB cis elements driving a luciferase reporter gene. Transfected cells were pretreated with inhibitor for one hour prior to stimulation with 200pg/mL IL-1β. Inhibitor concentration remained constant throughout the duration of IL-1β stimulation. Transcription through NFκB cis elements was determined by quantification of luciferase expression in whole cell lysates. IL-1β induced transcription through NFκB cis elements was significantly abrogated by pretreatment with NFκB inhibitors PDTC (Figure 3.1, top), sulfasalazine (center) and NFκB SN50 Cell-Permeable Inhibitor Peptide (bottom, column 3), but not by NFκB SN50M Cell Permeable Inactive control peptide (bottom, column 4).

Inhibition of NFκB transactivation activity also blocked IL-1β induced promatrilysin expression (Figure 3.2). Incubation and treatment times were identical to

the preceding experiment measuring NFκB transactivation activity. Twenty-four hours following IL-1β stimulation, promatrilysin expression in conditioned media were quantified using ELISA analyses. In agreement with the pattern observed for inhibition of transcription through NFκB cis elements, IL-1β induced promatrilysin expression was blocked by NFκB inhibitors, PDTC (Figure 3.2, top), sulfasalazine (center) and NFκB SN50 Cell-Permeable Inhibitor Peptide (bottom, column 3), but not by NFκB SN50M Cell Permeable Inactive control peptide (bottom, column 4).

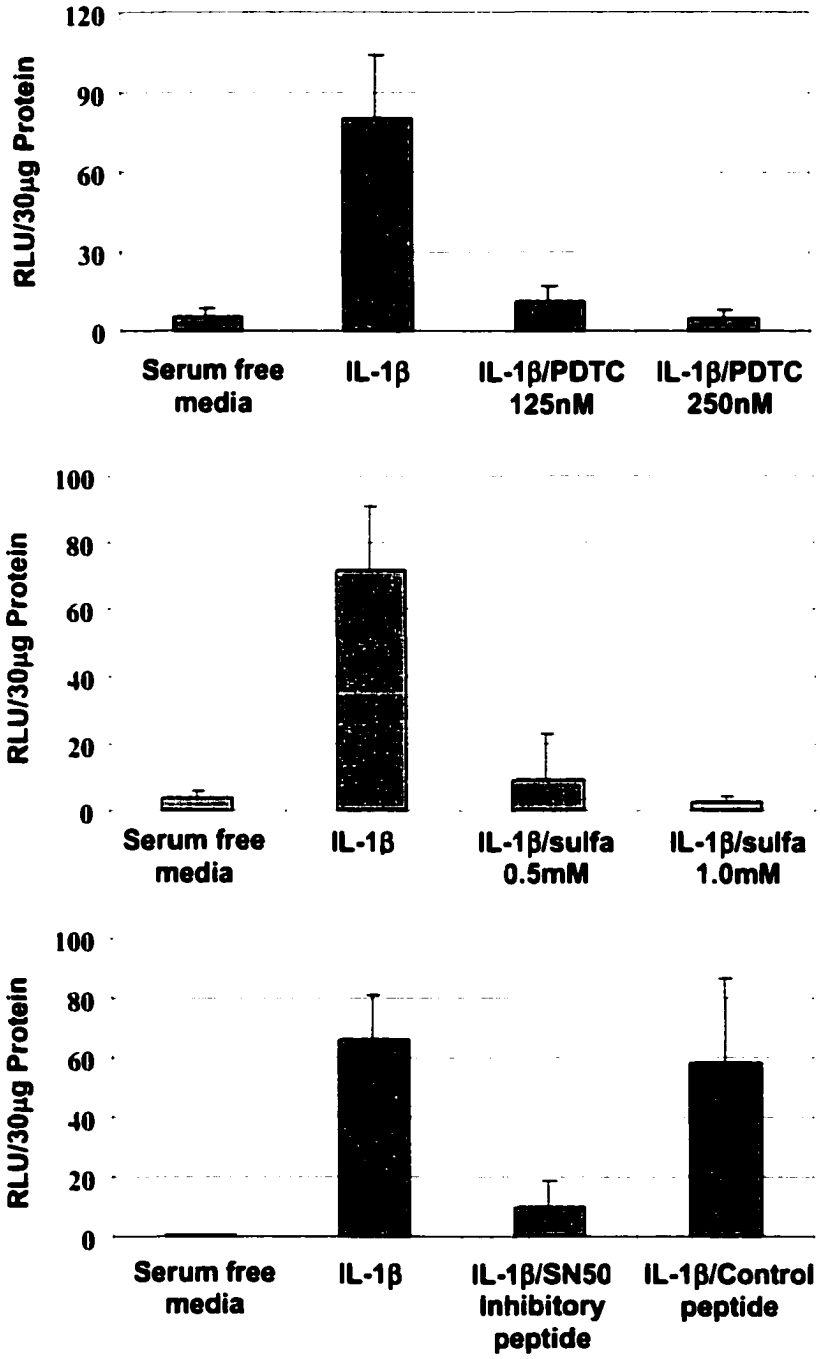

Figure 3.1. NFκB mediates promatrilysin expression in LNCaP cells. LNCaP cells were transiently transfected with a plasmid construct encoding a minimal HIV promoter containing 2X NFκB binding elements driving a luciferase reporter gene. Transfected cells were treated with either PDTC (125 or 250nM), sulfasalazine (0.5 or 1.0mM), SN50 NFκB inhibitory peptide or SN50B inactive control peptide (18µM) 1 hour prior to stimulation with IL-1β. Twenty-four hours following IL-1β stimulation, whole cell lysates were collected and analyzed for luciferase expression

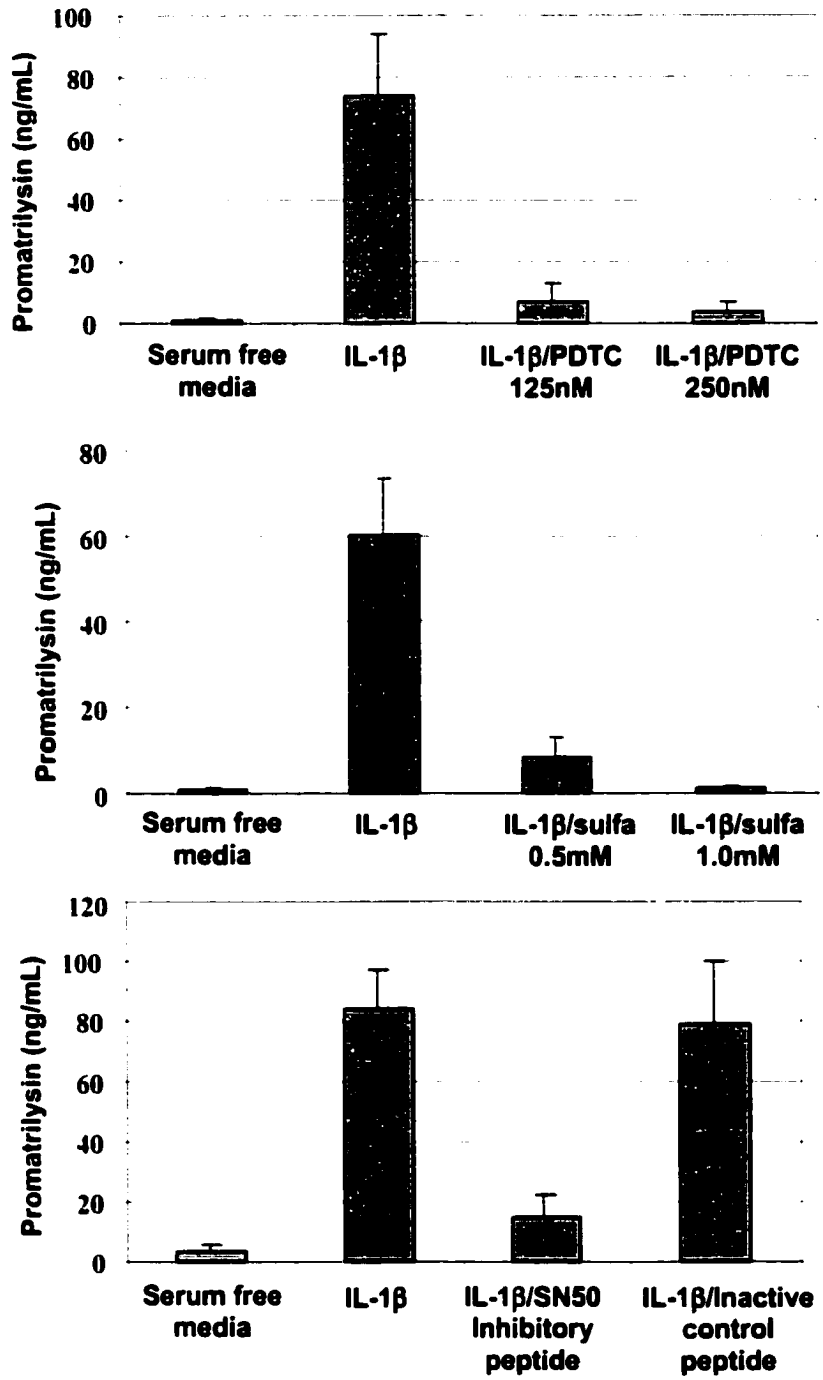

Figure 3.2. NFκB mediates promatrilysin expression in LNCaP cells. LNCaP cells treated with either PDTC (125 or 250nM), sulfasalazine (0.5 or 1.0mM), SN50 NFκB inhibitory peptide or SN50B inactive control peptide (18μM) 1 hour prior to stimulation with IL-1β. Twenty-four hours following IL-1β stimulation, promatrilysin expression in conditioned media was quantified using ELISA analyses. The results shown represent the means and standard deviations of three experiments each performed in triplicate.

Cyclohexamide blocks IL-1β induced matrilysin mRNA

The next step was to determine whether the induction of promatrilysin expression by IL-1β is indirect and dependent on synthesis of one or more intermediate signaling factors, or whether IL-1β acts directly to up-regulate promatrilysin expression in LNCaP cells without synthesis of one or more intermediate signaling factors. LNCaP cells were concurrently treated with cyclohexamide (10μg/mL) and stimulated with IL-1β. Northern analyses for matrilysin messenger RNA were performed 8 hours after IL-1β stimulation. The 8 hour timepoint was used because that is when peak matrilysin message is observed (data not shown). A strong induction of matrilysin message was apparent following treatment with IL-1β alone (Figure 3.3, Lane 1) as compared to cells not stimulated with IL-1β (Figure 3.3, Lane 2). Concurrent treatment with cyclohexamide blocked IL-1β induced expression of matrilysin mRNA (Figure 3.3, Lane 3) indicating a requirement for new protein synthesis for the induction of promatrilysin expression by IL-1β. Inhibition of protein synthesis was confirmed by quantification of ^{35}S-methionine uptake with the dose of cyclohexamide used (data not shown). Reprobing of the stripped blots for GAPDH mRNA demonstrated equal loading of RNA samples (Figure 3.3, lower panel).

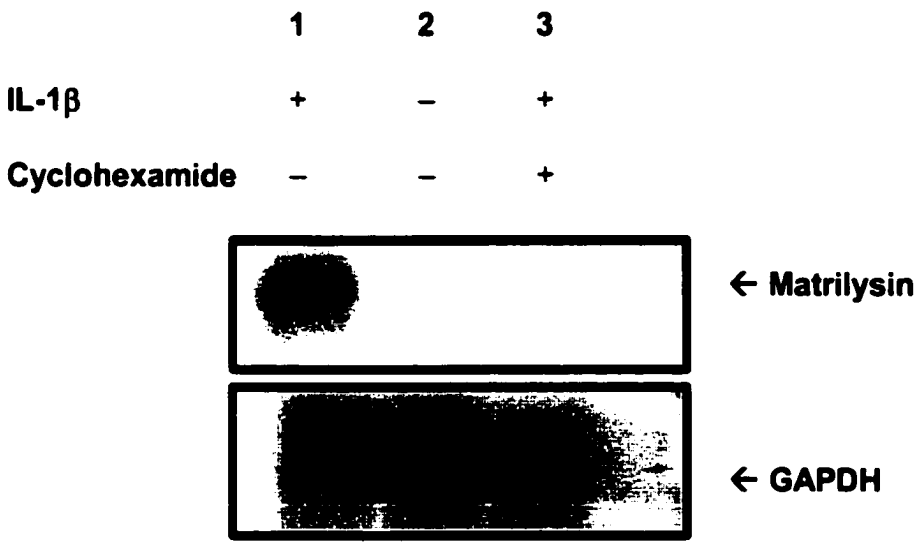

Figure 3.3. Cyclohexamide blocks IL-1β induced matrilysin mRNA. LNCaP cells were serum starved for 16 hours then were treated with either 200pg/mL IL-1β (Lane 1); serum free DMEM (Lane 2) or; IL-1β (200pg/mL) and cyclohexamide (10μg/mL) simultaneously (Lane 3). Total RNA was collected 8 hours following IL-1β stimulation. RNA samples were subjected to 1% agarose gel electrophoresis and transferred and crosslinked to a nylon membrane. The membrane was probed for matrilysin mRNA. Blots were stripped and reprobed for GAPDH (lower panel). The blot shown is representative of five repeats of this experiment.

IL-1β induces expression of IL-6 by LNCaP cells

IL-1β has been shown to induce synthesis of the cytokine IL-6 through NFκB transactivation activity in numerous cell types (Lin, *et al.*, 1995; Heyen, *et al.*, 2000). In addition, multiple IL-6 responsive consensus elements (NFIL6) have been identified within the published region of the human matrilysin promoter sequence (Gaire, *et al.*, 1994). It had previously been established that LNCaP cells do not constitutively express IL-6 (Okamoto, *et al.*, 1997); however, the ability of IL-1β to induce IL-6 expression in LNCaP cells had not been tested.

We determined that IL-1β does stimulate high levels of expression of IL-6 in LNCaP cells. Cells were stimulated with IL-1β, then ELISA analyses were used to quantify IL-1β induced IL-6 expression at various time points. IL-1β induced IL-6 secretion was substantially increased within three hours following stimulation with IL-1β, and peaked at approximately 5ng/mL (Figure 3.4). The secretion of IL-6 protein induced by IL-1β precedes the IL-1β induced increase in matrilysin mRNA expression, which begins approximately four hours after stimulation and peaks at approximately eight hours. Inhibition of NFκB completely blocked IL-1β induced expression of IL-6 (data not shown).

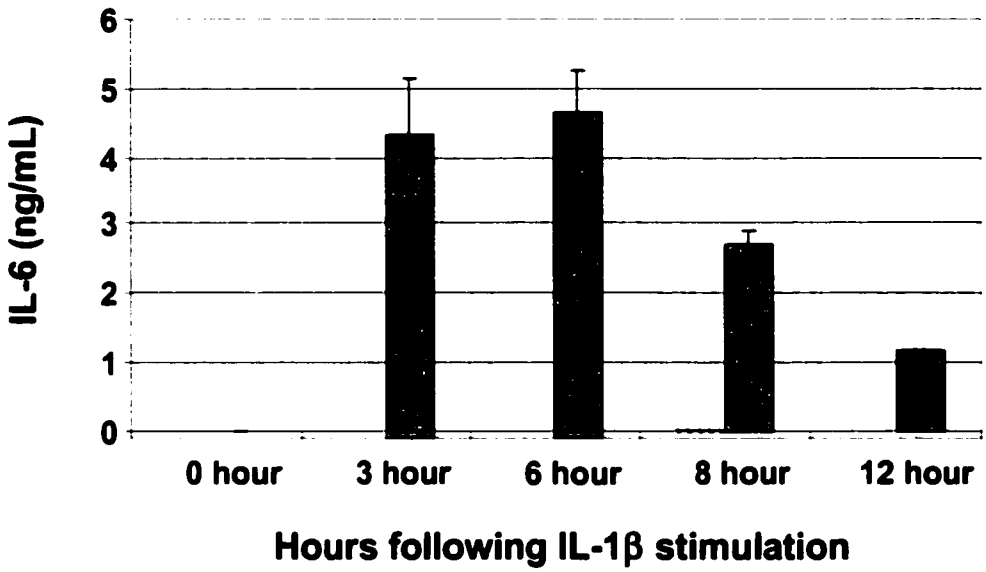

Figure 3.4. IL-1β induces expression of IL-6 in LNCaP cells. LNCaP cells were serum starved for 16 hours then they either remained in serum free DMEM or were stimulated with IL-1β in serum free DMEM. Conditioned media samples were collected at the indicated time points after IL-1β stimulation and analyzed for IL-6 concentration using ELISA analyses. Unstimulated control cells (the first bar of each timepoint) did not secrete a measurable amount of IL-6. IL-1β stimulated cells (the solid bar) demonstrate induction of IL-6 expression. The results shown represent the means and standard deviations of three experiments each performed in triplicate.

IL-6 induces promatrilysin expression in LNCaP cells

Previous studies have shown that IL-6 induces expression of several matrix metalloproteinases including MMPs-2 and –9 (Kossakowska, *et al.*, 1999). In order to determine whether IL-6 is an intermediate required for potentiation of IL-1β induced promatrilysin expression, we first measured promatrilysin expression in LNCaP cells treated with escalating dose levels of recombinant IL-6. Promatrilysin expression in conditioned media were quantified using ELISA analyses 24 hours following IL-6 stimulation. Our data demonstrate that IL-6 induces promatrilysin expression in a dose dependent manner (Figure 3.5 Panel A). The induction of promatrilysin with 10ng/mL recombinant IL-6 was equivalent to the induction observed with 200pg/mL IL-1β stimulation. Induction of promatrilysin observed using the concentration of IL-6 present in medium from IL-1β treated cells (5ng/mL), was approximately 35% less than the level achieved with IL-1β stimulation.

To determine whether NFκB plays a role in IL-6 induced promatrilysin expression, we quantified NFκB transactivation activity in IL-6 stimulated cells. LNCaP cells were transiently transfected with a plasmid construct encoding a minimal HIV promoter with 2X NFκB cis elements. Transfected cells were stimulated with increasing doses of IL-6. Luciferase activity in whole cell lysates was quantified 8 hours following cytokine stimulation. IL-6 did not significantly induce NFκB transactivation activity (Figure 3.5 Panel B), indicating that IL-6 induced promatrilysin expression is not mediated by NFκB transactivation activity.

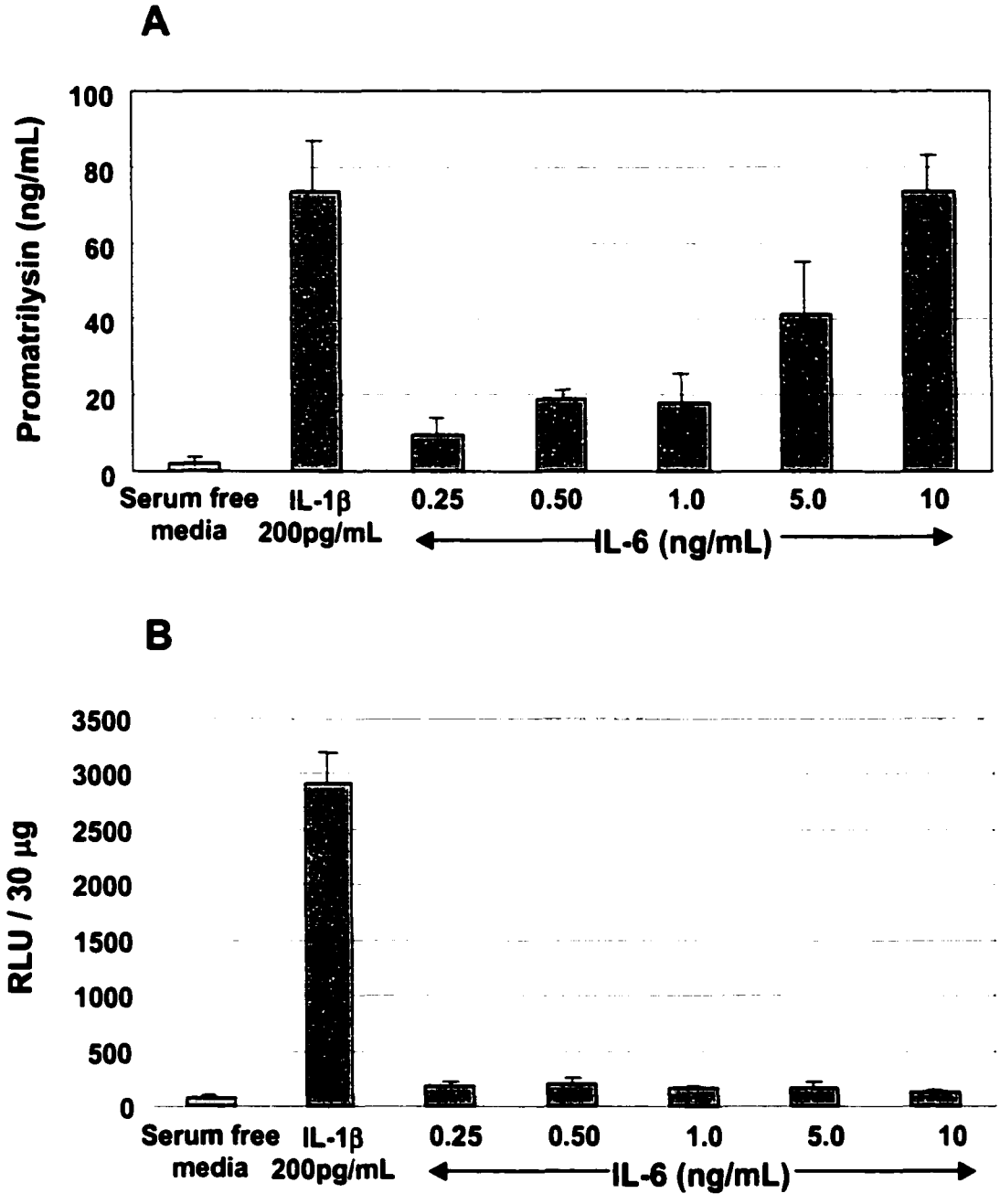

Figure 3.5. IL-6 induces promatrilysin expression in LNCaP cells. LNCaP cells were transiently transfected with a plasmid construct encoding a minimal HIV promoter containing 2X NFκB binding elements driving a luciferase reporter gene. Transfected cells were treated with escalating doses of recombinant IL-6. Twenty-four hours following cytokine stimulation, **A**) conditioned media were analyzed for matrilysin expression using ELISA analyses, and **B**) luciferase expression in whole cell lysates were quantified. The results shown represent the means and standard deviations of at least three experiments each performed in triplicate.

IL-6 mediates IL-1β induced promatrilysin expression

We then conducted studies to test whether inhibition of IL-6 activity could block IL-1β induced promatrilysin expression. LNCaP cells were treated with recombinant IL-1β alone or IL-1β plus either an IL-6 neutralizing antibody directed against the IL-6 ligand or, to control for a non-specific antibody effect, an antibody directed against an unrelated human protein (Nm23). Cells were pretreated with 10μg/mL antibody for one hour then, in the presence of antibody, stimulated with recombinant IL-1β (200pg/mL) or IL-6 (5ng/mL) in serum free conditions. An additional 10μg/mL of each respective antibody was added to the media 4 hours subsequent to cytokine stimulation. The dose of 5ng/mL recombinant IL-6 was chosen in order to simulate the concentration of IL-6 produced by LNCaP cells in response to treatment with 200pg/mL IL-1β. Using ELISA analyses, secreted promatrilysin expression in conditioned media was measured 14 hours following stimulation with either IL-1β or IL-6 (Figure 3.6). The first column shows the basal level of promatrilysin expression in the absence of cytokine stimulation. The second and third columns demonstrate the induction of promatrilysin with 200pg/mL of IL-1β and 5ng/mL IL-6, respectively. As anticipated, the rabbit polyclonal antibody directed against an unrelated human protein had no effect on IL-1β or IL-6 induced promatrilysin expression (columns 4 and 5). As shown in column 6, IL-6 neutralizing antibody significantly blocked IL-1β induced promatrilysin expression (approximately 60%). Inhibition of IL-6 activity by the neutralizing antibody was confirmed by demonstrating virtually complete inhibition of IL-6 induced promatrilysin expression by

the IL-6 neutralizing antibody (column 7). Higher concentration of IL-6 neutralizing antibody did not enhance the inhibitory effect (data not shown).

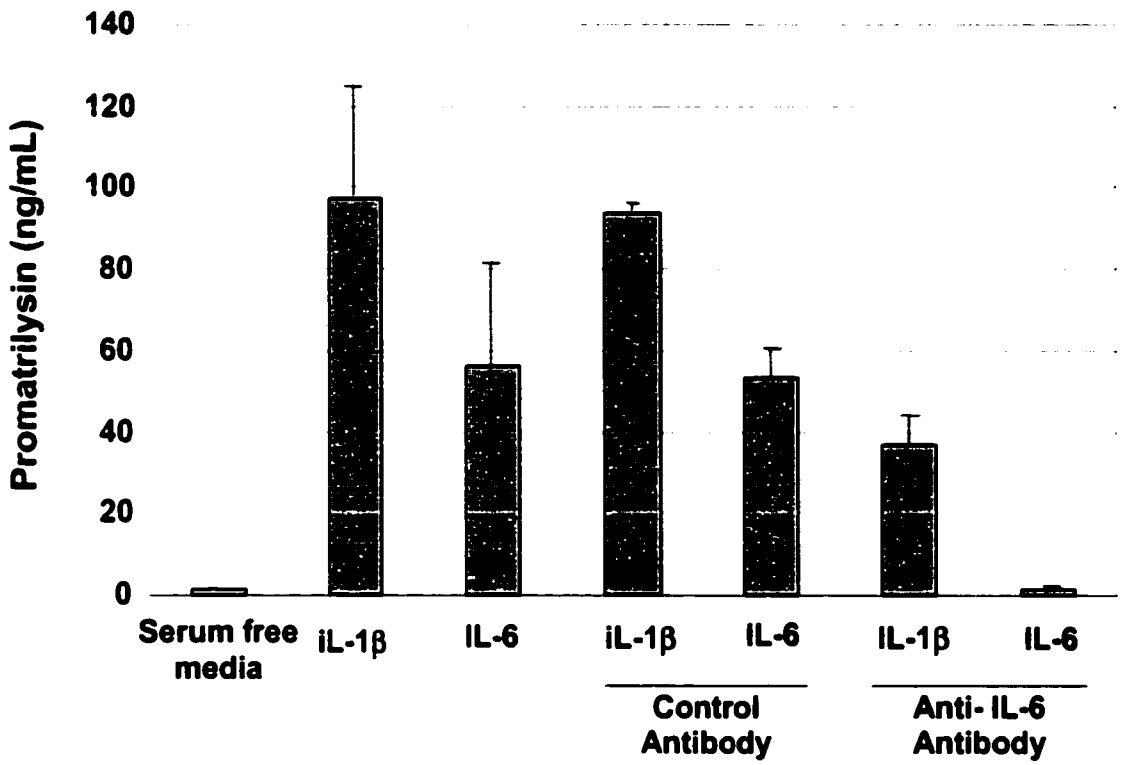

Figure 3.6. IL-6 mediates IL-1β induced promatrilysin expression. LNCaP cells were serum starved for 16 hours then stimulated with either IL-1β or IL-6 in the presence of 10μg/mL of an antibody against either IL-6 or an antibody against an unrelated human protein. An additional 10μg/mL of the respective antibody was added to the medium four hours following the first antibody treatment and cytokine stimulation. Using ELISA analyses, promatrilysin expression was measured 10 hours following IL-6 stimulation. The results shown represent the means and standard deviations of at least three experiments each performed in triplicate.

Cyclohexamide does not block IL-6 induced matrilysin mRNA

Similar to the approach taken to characterize the pathway by which IL-1β induces promatrilysin expression, the next step was to determine whether the induction of promatrilysin by IL-6 is indirect, and dependent on synthesis of one or more intermediate signaling factors, or whether IL-6 acts directly to up-regulate promatrilysin expression in LNCaP cells without synthesis of one or more intermediate signaling factors. LNCaP cells were concurrently treated with cyclohexamide (10μg/mL) and stimulated with IL-6. Northern analyses for matrilysin messenger RNA were performed 2 hours after IL-6 stimulation. The two hour time point was chosen because we observed that matrilysin message peaked 2 hours following stimulation with IL-6 (data not shown). A significant induction of matrilysin message was apparent following treatment with IL-6 alone (Figure 3.7, lane 2) as compared to unstimulated cells. It is of interest that concurrent treatment of cyclohexamide with IL-6 stimulation did not block IL-6 mediated transcription of matrilysin mRNA (Lane 3) indicating that the mechanism by which IL-6 induces promatrilysin expression is direct and does not require synthesis of new proteins. Inhibition of protein synthesis was confirmed by quantification of ^{35}S-methionine uptake with the dose of cyclohexamide used (data not shown). Reprobing of the stripped blots for GAPDH mRNA demonstrated equal loading of RNA samples (Figure 3.7, lower panel).

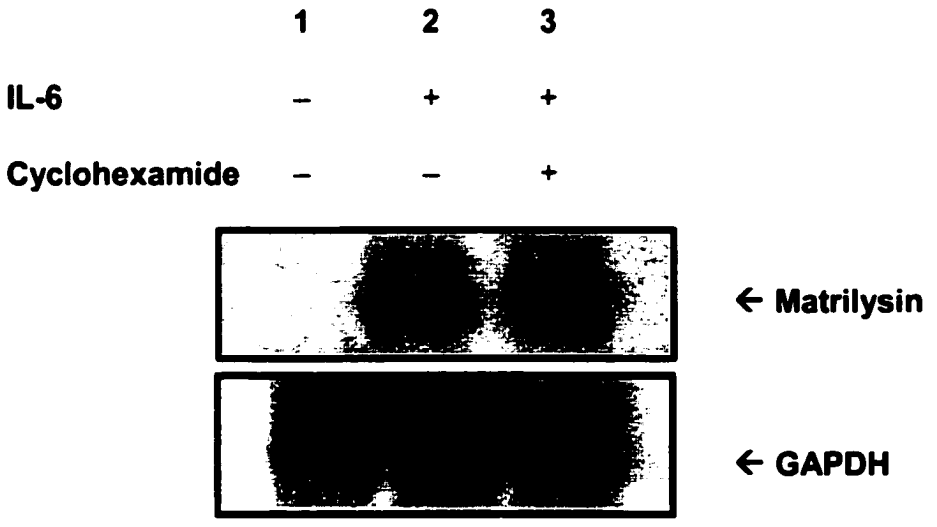

Figure 3.7. Cyclohexamide does not block IL-6 induced matrilysin mRNA. LNCaP cells were serum starved for 16 hours then were treated with either serum free DMEM (Lane 1), 5ng/mL IL-6 (Lane 2); or; IL-6 (5ng/mL) and cyclohexamide (10μg/mL) simultaneously (Lane 3). Total RNA was collected 2 hours following IL-1β stimulation. RNA samples were subjected to 1% agarose gel electrophoresis and transferred and crosslinked to a nylon membrane. The membrane was probed for matrilysin mRNA. Blots were stripped and reprobed for GAPDH (lower panel). The blot shown is representative of at least five repeats of this experiment.

STAT3 plays a role in IL-6 induced promatrilysin expression

We previously showed that transcriptional enhancer elements present in the human matrilysin promoter region are responsive to IL-1β (Klein, *et al.*, 1997). We next demonstrated that transcriptional enhancer elements present in the human matrilysin promoter region are also responsive to IL-6 stimulation. To study the downstream transcription factor(s) involved in the induction of matrilysin transcription by IL-1β and IL-6, STAT3 was examined for several reasons. First, many studies have shown that IL-6 signals directly through STAT3. For example, Villavicencio and colleagues demonstrated that autocrine and paracrine interactions of IL-6 family cytokines causes STAT3 activation in the angiotensin II pathway in rat hepatocytes (Villavicencio, *et al.*, 2000). STAT3 activation has also been shown to be responsible for IL-6-dependent T cell proliferation through preventing apoptosis (Takeda, *et al.*, 1998). Furthermore, Chung and colleagues have shown, that IL-6 directly activates STAT3, which then regulates growth inhibition and differentiation in LNCaP cells (Spiotto, *et al.*, 2000a; Spiotto, *et al.*, 2000b; Chung, *et al.*, 1999). In addition, the published human matrilysin promoter sequence is known to contain numerous cis elements including NF-IL6 elements to which STAT3 may be capable of binding.

LNCaP cells were transiently co-transfected with a plasmid construct encoding 1.2kb of the human matrilysin promoter driving a luciferase reporter gene and either, a plasmid construct encoding a dominant negative STAT3 containing point mutations at critical phosphorylation sites, or pCMV-1, the parent vector into which the STAT3

double mutant was cloned. The ratio of promoter construct to dominant negative or parent vector was 1:1. Columns 1 and 2 (Figure 3.8) show baseline luciferase activity in unstimulated cells. Both IL-1β and IL-6 induced activity of the 1.2kb human matrilysin promoter as demonstrated by induction of luciferase activity (columns 3 and 4). It is of interest that the fold induction of promoter activity achieved with IL-1β versus IL-6 agrees with the promatrilysin protein expression levels observed with the respective cytokines as measured by ELISA analyses shown in Figure 3.6. Co-transfection with the STAT3 dominant negative abrogated the fold increase of IL-1β induced promoter activity by approximately 45%. In addition, dominant negative STAT3 inhibited IL-6 induced promoter activity to baseline levels. These results also concur with the results presented in Figure 3.6, where neutralization of IL-6 inhibited IL-1β induced promatrilysin expression by approximately 60%. A higher ratio of dominant negative STAT3 plasmid to promoter construct (2:1) did not cause further inhibition of IL-1β induced matrilysin promoter activity (data not shown).

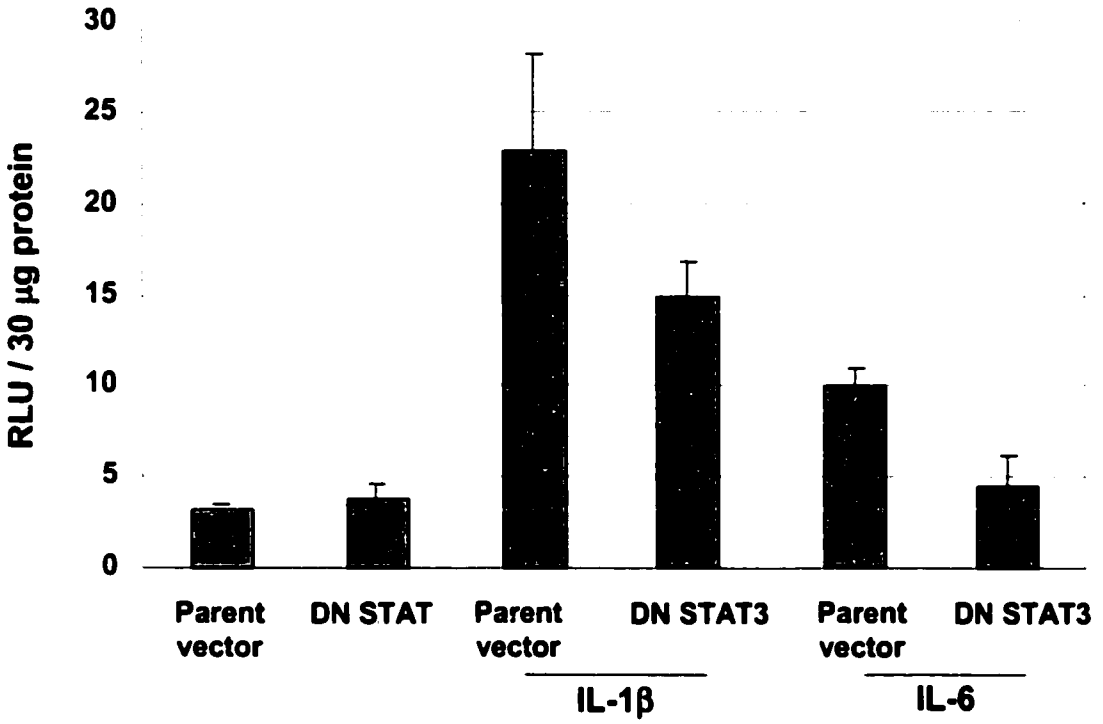

Figure 3.8. STAT3 mediates IL-6 induced promoter activity. LNCaP cells were transiently co-transfected with a plasmid construct encoding 1.1kb of the human matrilysin promoter driving a luciferase reporter gene and either, a plasmid construct encoding a dominant negative STAT3 containing point mutations at critical phosphorylation sites or, pCMV-1, the parent vector into which the STAT3 double mutant was cloned. Transfected cells were treated IL-1β or IL-6, and then luciferase activity in whole cell lysates was quantified 24 hours following cytokine stimulation. The results shown represent the means and standard deviations of at least three experiments.

Discussion

Our data demonstrate that IL-1β induced promatrilysin expression in LNCaP cells is dependent on NFκB mediated synthesis of IL-6. In addition, we demonstrate that STAT3 plays a role in the pathway downstream of IL-6. Previously, using immunohistochemical staining of paraffin embedded serial sections of primary prostate tumor tissue, our laboratory demonstrated that high levels of promatrilysin were detected adjacent to dilated ducts or atrophic glands that were surrounded by inflammatory cell infiltrates, which presumably contain a high concentration of cytokines, including IL-1β and IL-6 (Knox, *et al.*, 1996). Subsequently, we showed that LNCaP cells secrete a high level (70 to 100 fold increase over baseline expression levels) of promatrilysin in response to stimulation with recombinant IL-1β. We now reveal that IL-1β induced promatrilysin expression is potentiated through a multi-step pathway that requires NFκB mediated IL-6 synthesis and downstream signaling by STAT3.

IL-6 is particularly relevant to prostatic carcinoma for several reasons. Adler and colleagues have shown that patients with metastatic and hormone refractory prostatic carcinoma have a high level of IL-6 circulating in their peripheral blood (Adler, *et al.*, 1999). Furthermore, both IL-6 (Suda, *et al.*, 1995; Udagawa, *et al.*, 1995) and matrilysin (Busiek, *et al.*, 1992) are known to play a role in differentiation of bone, the tissue to which prostatic carcinoma characteristically metastasizes; and, circulating

levels of IL-6 have been associated with bone metastasis in patients with prostate carcinoma (Adler, *et al.*, 1999). In addition, other studies have demonstrated that the more progressed and less differentiated hormone independent prostatic carcinoma cell lines, PC3 and DU-145, express a constitutive level of secreted IL-6, however, those data also showed that the less progressed and hormone responsive LNCaP cells did not secrete any detectable IL-6 (Chung, *et al.*, 1999).

IL-6 has been shown to regulate the expression of several MMP family members (Louis, *et al.*, 2000; Solis-Herruzo, *et al.*, 1999). Kossakowska and colleagues demonstrated that elevated IL-6 expression correlated with upregulation of MMPs-2 and –9 in tumor biopsy specimens from patients with non-Hodgkin's lymphoma (NHL). In addition, IL-6 induced expression of the MMPs-2 and –9, and significantly up-regulated transmigration in the Matrigel invasion assay by the lymphoid cell lines Raji, Jurkat and NC-37 (Kossakowska, *et al.*, 1999; Kossakowska, *et al.*, 1998). Those data suggested that IL-6 may play a role in determining aggressiveness of NHL by regulation of MMP production. In addition, our laboratory demonstrated that stable transfection of the prostatic carcinoma cell line, DU-145, with the full length cDNA of the human matrilysin gene enhanced invasive capacity of DU-145 cells both *in vitro* and *in vivo* (Powell, *et al.*, 1993).

In the initial studies conducted with LNCaP cells, a dose of 200 pg/mL of IL-1β was sufficient to elicit a measurable induction of promatrilysin (Klein, *et al.*, 1997). However, this dose was insufficient for induction of a measurable level of promatrilysin by the other cytokines tested, including IL-6. It has since been determined that

IL-6 requires a much higher concentration (ng/mL range) in order to elicit its observed downstream effects. Interestingly, elevated levels (ng/mL ranges), of IL-6 have been shown to be clinically relevant in the peripheral blood of patients with hormone refractory prostatic carcinoma (Drachenberg, *et al.*, 1999). For the first time, we demonstrate that LNCaP cells have the capacity to secrete IL-6 in response to IL-1β through a NFκB dependent mechanism.

Research conducted by Okamoto and colleagues demonstrated that IL-6 is constitutively expressed by the prostatic carcinoma cell lines, DU-145 and PC3, but not by the prostatic carcinoma cell line, LNCaP. Interestingly, IL-1β and IL-6 have been reported to inhibit the growth of LNCaP cells, but not DU-145 or PC-3 cells. It is possible that the inhibitory effect of IL-1β on the growth of LNCaP cells is mediated by the induction of IL-6 expression in these cells. Spiotto and Chung have recently determined that the inhibition of growth in LNCaP cells caused by IL-6 is due to activation of the STAT3 pathway (Spiotto, *et al.*, 2000b). Furthermore, they determined that this pathway is not functional in DU-145 and PC-3 cells. However, the defect in the pathway has not been characterized.

We previously determined that IL-1β does not induce promatrilysin in DU-145 or PC-3 cells (data not shown); and, these cells have no basal expression of promatrilysin, although they are known to secrete IL-6 constitutively (Okamoto, *et al.*, 1997). There is, therefore, a correlation between the ability of IL-6 to induce promatrilysin and the presence of an intact STAT3 pathway. In addition, new protein synthesis is not required for transcription of matrilysin mRNA by IL-6; and, dominant

negative STAT3 inhibited IL-1β and IL-6 induced matrilysin promoter activity. Thus, STAT3 is strongly implicated in the transcriptional regulation of promatrilysin expression by IL-6. This suggests that IL-6 downstream signaling through STAT3 initiates transcription of the matrilysin gene.

In our proposed model (Figure 3.9), NFκB is activated in response to IL-1β ligand binding to its receptor. NFκB subsequently Tran locates to the nucleus and stimulates transcription of the IL-6 gene. Once secreted, IL-6 binds to its receptor, which, in turn, results in activation of STAT3. We hypothesize that IL-1β induced expression of promatrilysin may be regulated by both autocrine and paracrine pathways of IL-6 ligand binding. Prostatic carcinoma tumors are heterogeneous in nature, therefore, paracrine signaling mechanisms may be the most relevant *in vivo*, and secretion of a cytokine, like IL-6, or a MMP, like matrilysin, could potentially elicit multiple effects on the different types of cells that are present within a tumor. Degradation of the extracellular matrix by promatrilysin secreted by an epithelial cell may enhance invasive capacity of neighboring cells.

It is of interest that, although IL-6 is appears to be the major factor responsible for promatrilysin expression in response to IL-1β, these data suggest that an additional signaling factor(s) may be involved. In our system, approximately 5ng/mL IL-6 is secreted by LNCaP cells following stimulation with 200pg/mL IL-1β and, promatrilysin expression is increased up to 100 fold over baseline expression levels. However, stimulation with 5ng/mL of exogenously added recombinant IL-6 induces only a 50 to 60 fold induction of promatrilysin. Furthermore, concurrent treatment of

LNCaP cells with IL-1β and IL-6 neutralizing antibody reduces IL-1β induced promatrilysin by 50 to 60 percent, not to baseline expression levels. Inhibition of IL-1β and IL-6 induced matrilysin promoter activity by dominant negative STAT3 agreed with the levels of inhibition observed with the IL-6 neutralizing antibody. Thus, IL-1β may induce one or more factors in addition to IL-6 that upregulate promatrilysin expression, and the total induction of promatrilysin observed upon stimulation with IL-1β could be the result of an additive or synergistic effect of multiple factors. However, because inhibition of NFκB transactivation activity completely abrogated IL-1β induced promatrilysin expression, the other factor(s) involved must also be regulated by NFκB.

Induction of matrilysin gene expression by factors including EGF and phoebe esters is attributable to AP-1 and other serum response factor regulated transcription factors (Sundareshan, *et al.*, 1999). However, until now, the pathway by which the inflammatory cytokine, IL-1β, induces promatrilysin expression has been uncharacterized. Our data show a novel pathway by which NFκB mediated synthesis of IL-6 is required for potentiation of IL-1β mediated transcription of the matrilysin gene via STAT3 transactivation activity downstream of IL-6. These data reveal a novel pathway for MMP regulation. Examination of paracrine interactions between different cells types and MMP expression may provide a valuable model to help elucidate invasive and metastatic mechanisms that occur *in vivo* in cancer progression.

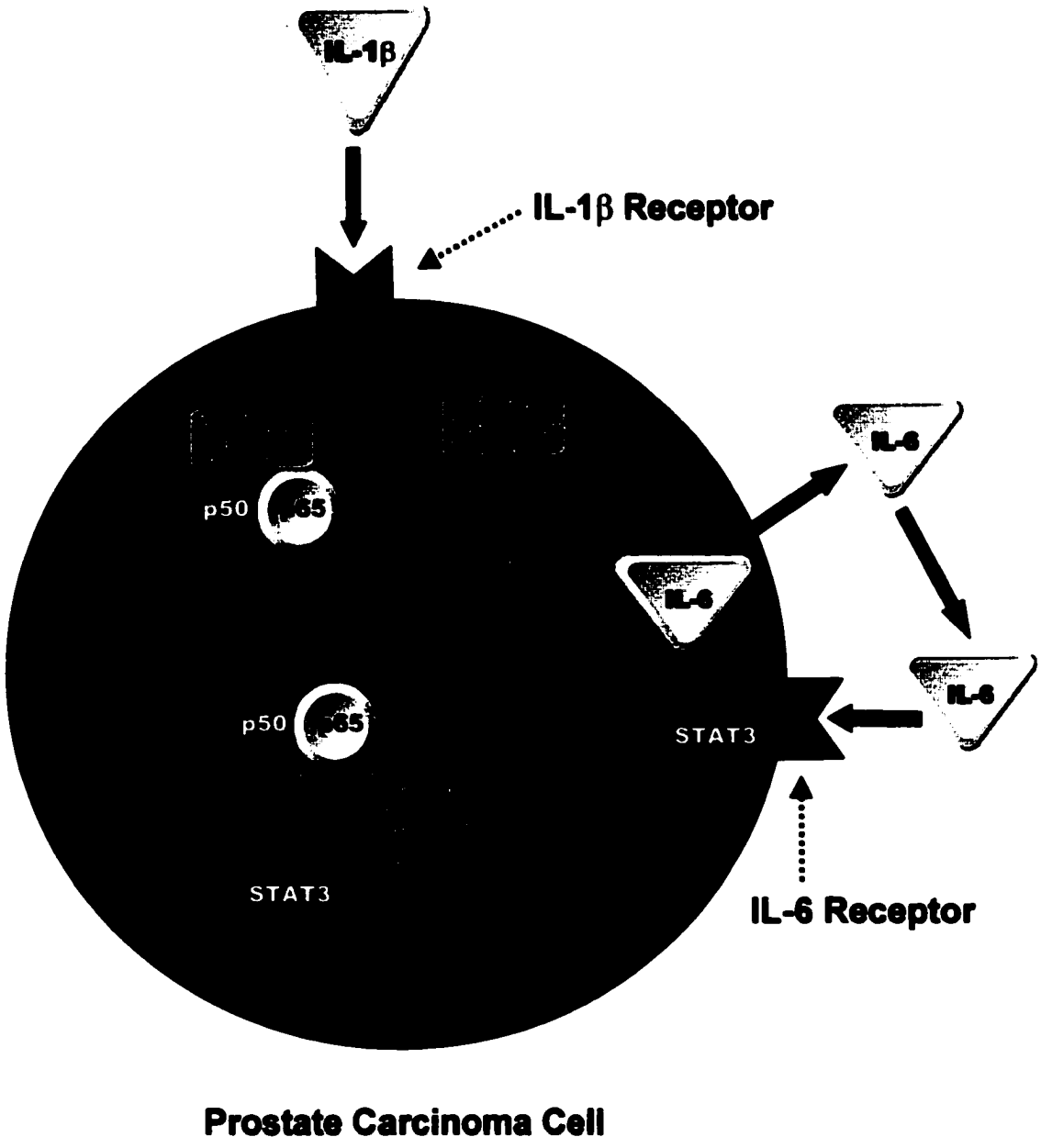

Figure 3.9. Proposed IL-1β induced matrilysin expression signaling model in LNCaP cells. IL-1β induced promatrilysin expression is mediated by synthesis of IL-6 through a NFκB dependent pathway. In this model, IL-1β ligand binding to its receptor initiates the signaling cascade that results in degradation of IκB allowing nuclear translocation of the active NFκB heterodimer. Once in the nucleus, NFκB binds cis elements and induces transcription of IL-6 and other downstream effectors, which, in turn, result in transcription of the matrilysin gene. IL-6 mediated promatrilysin expression may be mediated through both autocrine and paracrine pathways. STAT3 is activated in response to IL-6; and, dominant negative STAT3 inhibits IL-6 mediated promatrilysin expression to baseline levels which strongly indicates that STAT3 is involved in transcriptional regulation of IL-1β and IL-6 induced promatrilysin expression.

CHAPTER IV.

EXPRESSION OF THE MATRIX METALLOPROTEINASE, PROMATRILYSIN, IN CO-CULTURE OF PROSTATE CARCINOMA CELL LINES

Abstract

Matrix metalloproteinases (MMPs) are involved in tumor progression. Matrilysin (MMP-7) has been shown to be upregulated in prostatic carcinomas and can increase the invasive capacity of DU-145 cells. Because of the heterogeneous nature of prostatic tumors, we examined promatrilysin expression in co-cultures containing two different prostatic carcinoma cell lines, DU-145 and LNCaP. Using ELISA analyses, promatrilysin expression was measured in DU-145/LNCaP co-cultures and conditioned media cross-cultures. The effects of blocking IL-6 on promatrilysin expression were examined by pretreating conditioned media with IL-6 neutralizing antibody. A significant induction of promatrilysin expression was observed in DU-145/LNCaP co-cultures compared to LNCaP cells alone. In addition, DU-145 conditioned medium induced the same fold induction of promatrilysin as was observed in the co-cultures. LNCaP cell conditioned medium did not induce promatrilysin expression in DU-145 cells. Neutralization of IL-6 with neutralizing antibody abrogated DU-145 conditioned media induced promatrilysin expression to baseline levels. IL-6 secreted by DU-145 cells can induce promatrilysin expression in LNCaP cells. IL-6, *in vivo*, may act as a paracrine signaling factor that regulates matrix metalloproteinase expression. Therefore, IL-6 may play a role in invasive metastastic processes of a prostate carcinoma.

Introduction

Prostatic carcinoma tumors are known to be heterogeneous in nature. Thus, paracrine interactions between different types of cells within a tumor may play a key role in signaling the events that cause invasion and metastases. Therefore, examination of prostatic carcinoma cell line co-culture systems may be valuable in elucidating the processes that occur *in vivo* during metastatic invasion of a prostatic carcinoma.

Matrix metalloproteinases (MMPs), a family of zinc-and calcium-dependent proteolytic enzymes responsible for degradation of the extracellular matrix (Baramova, *et al.*, 1995; Cockett, *et al.*, 1994; Matrisian, *et al.*, 1994), have been shown to play a role in invasion and metastases (Curran, *et al.*, 1999; Thomas, *et al.*, 1999). Matrilysin (MMP-7) is over expressed in multiple types of malignancies including breast, colon and prostate carcinomas (Powell, *et al.*, 1996; Hashimoto, *et al.*, 1997). Our previous studies indicated at both mRNA and protein level the expression of matrilysin was strikingly focal in prostatic carcinoma tissue, but not in normal prostate tissue (Knox, *et al.*, 1996). In addition, our laboratory previously demonstrated *in vivo* and *in vitro*, that stable transfection with the full-length matrilysin cDNA driven by a constitutively active β-actin promoter significantly increased the invasive capacity of DU-145 cells, which are generally considered to be weakly invasive (Knox, *et al.*, 1993).

In this report, we show that IL-6 secreted by DU-145 cells can induce promatrilysin expression by LNCaP cells. These findings may provide insight into paracrine signaling mechanisms of prostate carcinoma cells within a tumor *in vivo*.

Results and Discussion

Promatrilysin expression is induced in DU-145/LNCaP co-cultures

Promatrilysin is expressed in co-cultures containing DU-145 and LNCaP cells (Figure 4.1). LNCaP cells secreted a low level of promatrilysin (column 1) and DU-145 cells did not secrete a measurable amount of promatrilysin (column 2); however, expression of promatrilysin in DU-145/LNCaP co-cultures was more than 7-fold higher than LNCaP cells alone (column 3).

DU-145 cells secrete a factor that induces promatrilysin expression in LNCaP cells

We then determined that DU-145 cells secrete a factor that could induce promatrilysin expression in LNCaP cells (Figure 4.2). LNCaP cells alone expressed a low level of promatrilysin. However, LNCaP cells treated with DU-145 conditioned media secreted elevated levels of promatrilysin and the fold induction was the same as the increase in expression observed in DU-145/LNCaP co-cultures. DU-145 cells treated with LNCaP conditioned media did not secrete promatrilysin. The low level of promatrilysin present was the same as the amount typically found in LNCaP conditioned media.

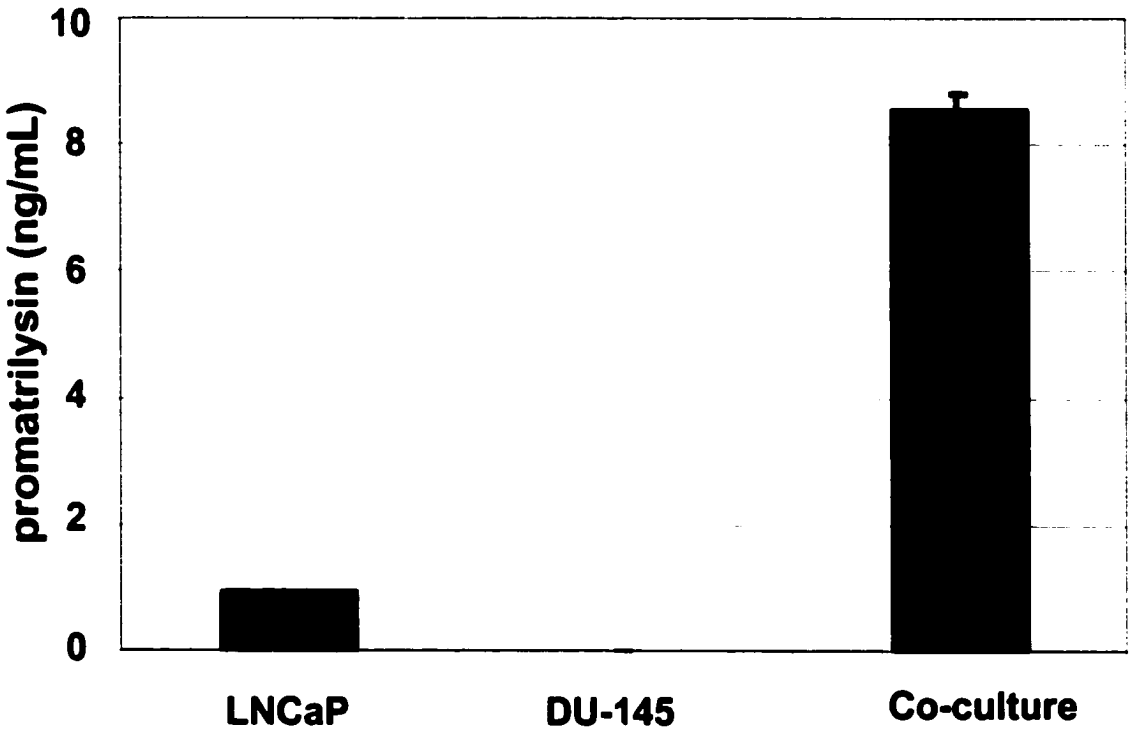

Figure 4.1. DU-145/LNCaP co-cultures express elevated levels of promatrilysin. Using ELISA analyses, promatrilysin expression was measured in conditioned media collected from LNCaP cells, DU-145 cells or equal numbers of LNCaP and DU-145 cells in co-culture.

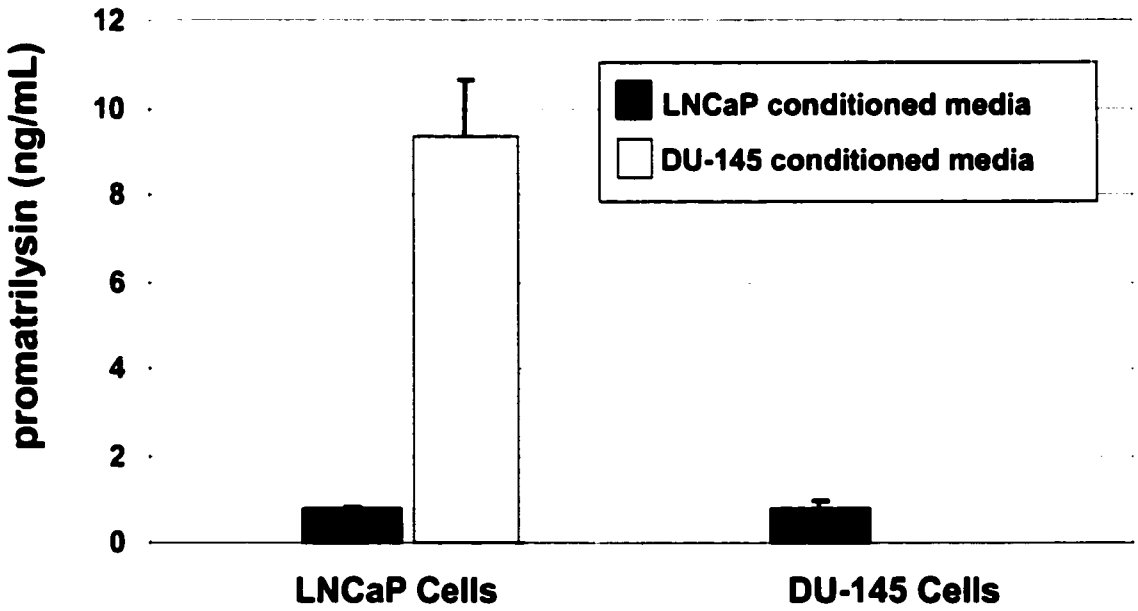

Figure 4.2. DU-145 cells secrete a factor(s) that induces promatrilysin expression by LNCaP cells. Conditioned media were collected from confluent LNCaP and DU-145 cultures. The effect of LNCaP conditioned media (■) and DU-145 conditioned media (□) on promatrilysin expression by LNCaP and DU-145 cells was examined.

IL-6 secreted by DU-145 cells induces promatrilysin expression in LNCaP cells

The next step was to identify the factor(s) secreted by DU-145 cells that were regulating promatrilysin expression in LNCaP cells. IL-6 has been implicated in playing a role in progression of prostatic carcinoma (Chung, *et al.*, 1999). In addition, it is known that DU-145 cells constitutively express IL-6 (Okamoto, *et al.*, 1997). In our system, conditioned media collected from confluent DU-145 cells contained approximately 1ng/mL IL-6 (data not shown). We examined the effect of neutralization of IL-6 on DU-145 conditioned media induced promatrilysin expression in LNCaP cells. Conditioned media collected from DU-145 cultures were pretreated with either an IL-6 neutralizing antibody against the IL-6 ligand and independent of IL-6 receptor activity, or a control antibody (Figure 4.3). The control antibody had no effect on DU-145 conditioned media induced promatrilysin expression. However, pretreatment with IL-6 neutralizing antibody abrogated DU-145 conditioned media induced promatrilysin expression to basal expression levels. Treatment with 1ng/mL of recombinant IL-6 induced the same level of promatrilysin expression as the level achieved with DU-145 conditioned media (data not shown).

In this report we demonstrate that DU-145 cells secrete IL-6, which can induce promatrilysin expression in LNCaP cells. In previous studies, our laboratory demonstrated *in vivo* and *in vitro*, that stable transfection with the full-length matrilysin cDNA driven by a constitutively active β-actin promoter significantly increased the invasive capacity of DU-145 cells, which are generally considered to be weakly invasive.

In addition, our previous studies indicated, at both mRNA and protein, the level the expression of matrilysin was strikingly focal in prostatic carcinoma tissue, but not in tissue from normal prostate (Knox, *et al.*, 1996). It is also of interest that IL-6 is known to be significantly overexpressed in the peripheral blood of prostate carcinoma patients (Drachenberg, *et al.*, 1999) and is a potential prognostic indicator (Nakashima, *et al.*, 2000). These facts suggest that IL-6 induced promatrilysin expression may play a role in prostate tumor cell invasion.

It is of interest that IL-6 both secreted by DU-145 cells and recombinant IL-6 induces promatrilysin expression in LNCaP cells and not in DU-145 cells. However, the effects of IL-6 on each of these cell types is remarkably different. It has been shown that addition of exogenous IL-6 elicits a stimulatory growth response on the androgen independent prostatic carcinoma cell line, DU-145 (Hobisch, *et al.*, 1998), however, IL-6 induces growth arrest (Abolhassani, *et al.*, 1995) and differentiation on LNCaP cells (Spiotto, *et al.*, 2000a; Spiotto, *et al.*, 2000b), which are known to be androgen responsive, however, both cells types express the IL-6 receptor (Hobisch, *et al.*, 1998). It has not been determined why DU-145 cells do not secrete promatrilysin in response to IL-6 in an autocrine interaction, however, our laboratory has treated DU-145 cells with numerous cytokines and growth factors and none were able to induce promatrilysin expression (unpublished data). The phenomenon may be indicative of the heterogeneity of prostatic carcinoma tumors and further supports the importance of using co-cultures as study models for prostatic carcinoma. Paracrine signaling may largely be responsible for regulation of invasion and metastasis of a primary prostatic tumor. This model may be

representative of a regulatory mechanism for matrix metalloproteinase expression *in vivo*. In fact, the original co-culture experiments performed by our laboratory lead to the discovery that IL-1β secreted by lipopolysaccharide (LPS) activated monocytes (THP-1 cells) stimulated promatrilysin expression by LNCaP cells. Furthermore, because IL-1β is a secreted factor, cell-cell contact was not necessary for potentiation of the signal.

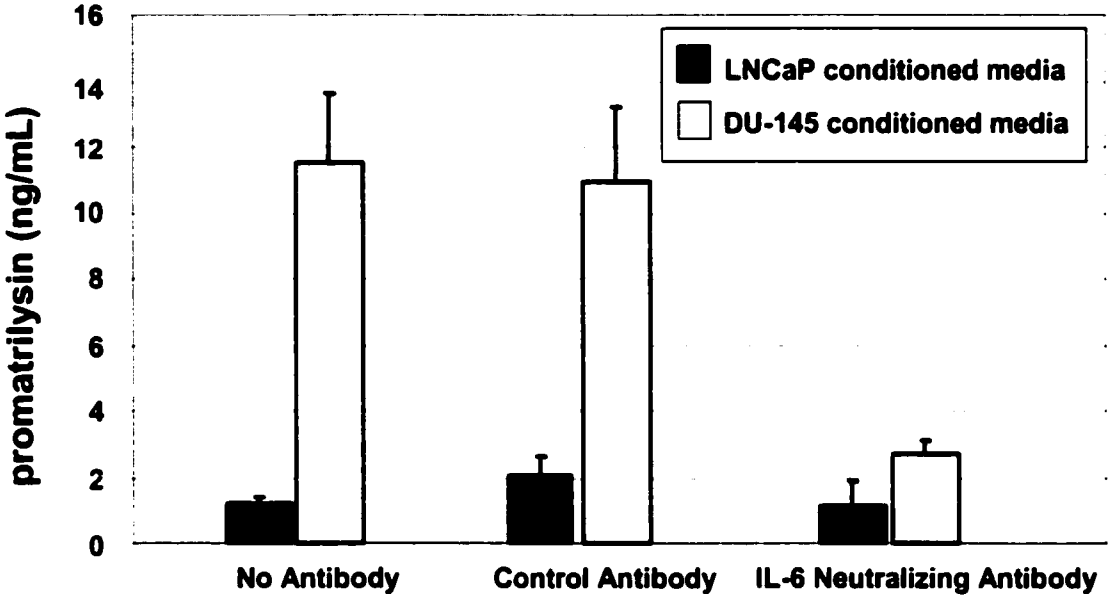

Figure 4.3. IL-6 secreted by DU-145 cells induces promatrilysin expression by LNCaP cells. LNCaP cells were treated with either LNCaP (■) or DU-145 (□) conditioned media pretreated with IL-6 neutralizing antibody against the IL-6 ligand or control antibody. Conditioned media were collected 24 hours following treatment and analyzed for promatrilysin expression.

CHAPTER V.

ANDROGENS BLOCK IL-1β INDUCED PROMATRILYSIN EXPRESSION IN PROSTATE CARCINOMA CELLS

Abstract

We have shown that IL-1β induced promatrilysin expression is mediated by an indirect pathway that requires NFκB dependent synthesis of IL-6 and STAT3 signaling. We now demonstrate that IL-1β induced promatrilysin expression can be blocked by androgens in the prostate carcinoma cell line, LNCaP. Treatment with testosterone prior to IL-1β stimulation blocked IL-1β induced promatrilysin expression, NFκB transactivation activity, and induction of IL-6 expression. However, testosterone and dihydrotestosterone did not have an inhibitory effect on IL-6 induced promatrilysin expression. In the absence of IL-1β testosterone had no effect on constitutive promatrilysin expression or NFκB transactivation activity. From these data, we conclude that testosterone blocks IL-1β induced promatrilysin expression by inhibition of NFκB transactivation activity that, in turn, blocks IL-6 expression.

Introduction

Removal of the testes in rats results in massive atrophy of the prostate gland due to apoptosis of secretory epithelial cells, and normal function is restored by testosterone (Barley, *et al.*, 1975; Greenstein, 1979). It is of interest that castration of male rats leads to a transient increase in matrilysin in the involuting rat ventral prostate. An increase in the steady-state

levels of matrilysin messenger RNA was observed 5 days after castration, and the levels began to decline by 8 days following castration (Powell, *et al.*, 1996). Androgens are also implicated in the pathogenesis of prostate cancer. Androgen ablation is currently the most commonly used treatment (Garzotto, 2000; Culig, *et al.*, 2000). Nevertheless, this treatment results in the development of androgen-independent cancers that are refractory to hormone-related treatment. It is therefore extremely important to elucidate the mechanism of action of the androgens in the etiology of prostate cancer.

An early stage in the metastasis of prostate cancer is the penetration of the basement membrane through a process that involves the proteolysis of the extracellular matrix proteins (Nakashima, *et al.*, 2000). The matrix metalloproteinases (MMPs) are a family of enzymes whose expression is elevated in many forms of cancer (Pajouh, *et al.*, 1991; Hashimoto, *et al.*, 1998; Hashimoto, *et al.*, 1997; Klein, *et al.*, 1997), and there is evidence that matrilysin (MMP-7) is overexpressed in prostate cancer cells (Knox, *et al.*, 1996; Hashimoto, *et al.*, 1998). Although it is known that testosterone plays a role in progression of prostate cancer, it is not known in which stage testosterone is involved. Androgen signaling could be responsible for an early event or a later stage of progression. Therefore, at present, it is not known specifically whether testosterone is involved in prostate cancer metastasis. In addition, it is known that testosterone has inhibitory effects on NFκB (Keller, *et al.*, 1996). Therefore, it was decided to test the effects of testosterone on the expression of promatrilysin in LNCaP cells, an *in vitro* model of prostate cancer. We showed that the cytokine interleukin-1β (IL-1β) induces promatrilysin expression in LNCaP cells through NFκB dependent expression of IL-6

and STAT3 signaling (Maliner-Stratton, *et al.*, 2001). We now demonstrate that testosterone inhibits IL-1β induced promatrilysin through NFκB dependent expression of IL-6. Our data demonstrate that testosterone can inhibit IL-1β but not IL-6 induced promatrilysin expression because IL-6 acts downstream of NFκB and therefore does not require NFκB transactivation activity to elicit promatrilysin expression directly.

Results and Discussion

Testosterone inhibits IL-1β but not IL-6 induced promatrilysin expression.

Cells were pretreated with physiologically relevant doses of testosterone 1 hour prior to stimulation with IL-1β or IL-6. Conditioned media were collected 24 hours following cytokine stimulation and analyzed for promatrilysin using ELISA analyses. Pretreatment with relatively low doses of testosterone inhibited IL-1β-induced promatrilysin expression (Figure 5.1, Panel A). The effect of testosterone on IL-1β induced promatrilysin expression was dose related and complete inhibition was observed at doses as low a $5x10^{-10}$M. Testosterone inhibited IL-1β induced promatrilysin expression. Pretreatment with relatively low doses of testosterone inhibited IL-1β induced promatrilysin expression. Testosterone had no effect on basal promatrilysin expression levels (data not shown). Testosterone does not inhibit IL-6 induced promatrilysin expression (Figure 5.1, Panel B). These data suggested that the inhibitory effect of testosterone on the IL-1β signaling pathway was upstream of IL-6. 5α-dihydrotestosterone

elicited the same effect on IL-1β induced promatrilysin expression as testosterone (data not shown).

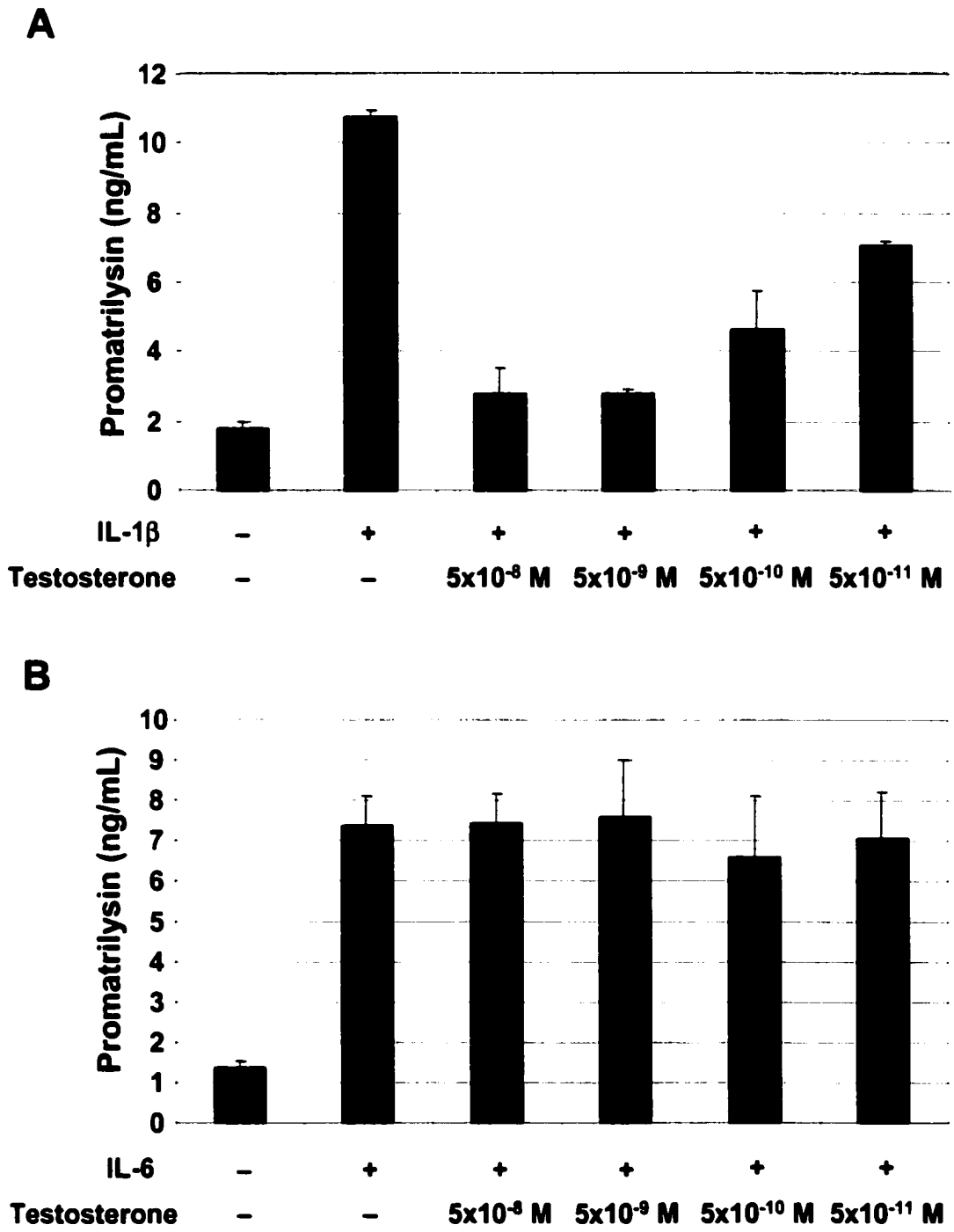

Figure 5.1. Testosterone inhibits IL-1β but not IL-6 induced promatrilysin expression. Cells were pretreated with testosterone 1 hour prior to stimulation with IL-1β (50 pg/mL) or IL-6 (1 ng/mL). Conditioned media were collected 24 hours following cytokine stimulation and analyzed for promatrilysin using ELISA analyses. **A)** Inhibition of IL-1β induced promatrilysin expression by testosterone. **B)** Testosterone does not inhibit IL-6 induced promatrilysin expression.

Testosterone inhibits IL-1β induced transcription through NFκB cis elements.

Cells were transiently transfected with a plasmid construct encoding a minimal HIV promoter containing 2X NFκB cis elements driving a luciferase reporter gene. Transfected cells were preteated with testosterone prior to stimulation with IL-1β. Whole cell lysates were collected 24 hours following cytokine stimulation and analyzed for luciferase activity. Testosterone blocked IL-1β induced transcription through NFκB cis elements in a dose responsive manner similar to that observed for the effect of testosterone on IL-1β induced promatrilysin expression (Figure 5.2). Testosterone did not have an effect on basal NFκB transactivation activity (data not shown).

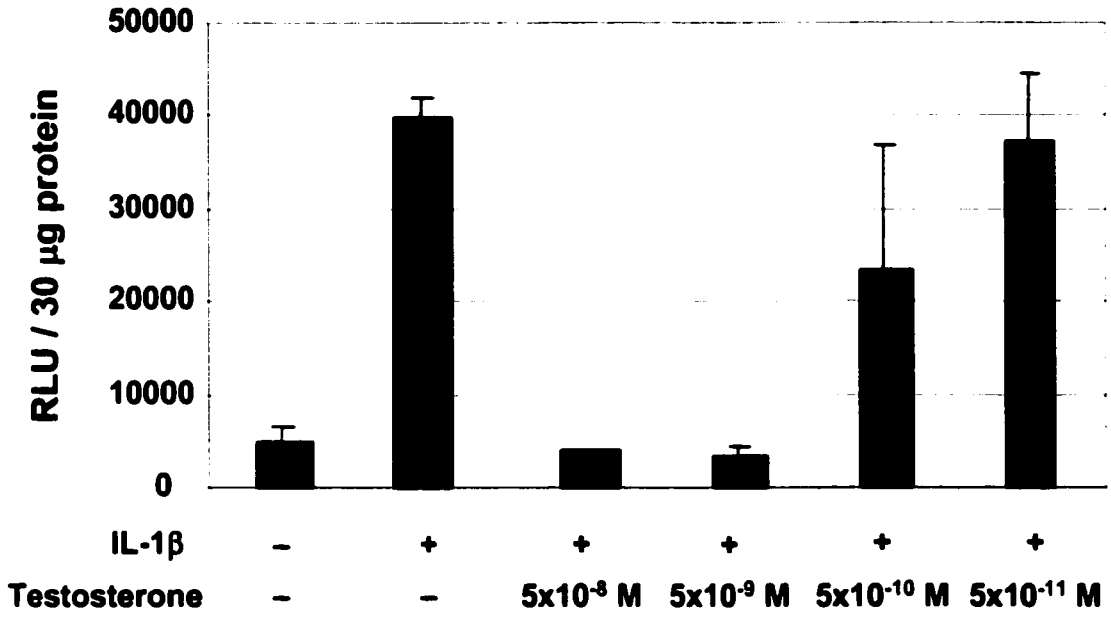

Figure 5.2. Testosterone inhibits IL-1β induced transcription through NFκB cis elements. Cells were transiently transfected with a plasmid construct encoding a minimal HIV promoter containing 2X NFκB cis elements driving a luciferase reporter gene. LNCaP transfected cells were pretreated with testosterone prior to stimulation with IL-1β (50 pg/mL). Whole cell lysates were collected 24 hours following cytokine stimulation and analyzed for luciferase activity.

Testosterone inhibits IL-1β induced expression of IL-6 by blocking accumulation of IL-6 mRNA.

We have previously shown that IL-1β can stimulate expression of IL-6 in LNCaP cells; and, IL-1β induced expression of IL-6 is dependent on NFκB transactivation activity. Peak expression of IL-6 in response to IL-1β was observed 6 hours following stimulation. Therefore, we asked whether testosterone would inhibit IL-1β induced expression of IL-6. Cells were pretreated with testosterone 1 hour prior to stimulation with IL-1β. Conditioned media were collected 6 hours following cytokine stimulation and analyzed for expression of IL-6 using ELISA analyses. Testosterone blocked IL-1β induced expression of IL-6 in a dose responsive manner resembling the pattern observed for inhibition of IL-1β induced promatrilysin expression and NFκB transactivation activity (Figure 5.3). The effect of testosterone on IL-1β induced accumulation of IL-6 mRNA was also examined. Cells were pretreated with testosterone or vehicle (70%ethanol) 1 hour prior to stimulation with IL-1β. RNA were collected at various timepoints ranging from 30 minutes to 24 hours following cytokine stimulation and probed by northern analyses for IL-6 mRNA. IL-6 mRNA was detectable within one hour following cytokine stimulation (Figure 5.4) and remained elevated 24 hours following cytokine stimulation (data not shown). Thus, testosterone blocked IL-1β induced accumulation of IL-6 mRNA.

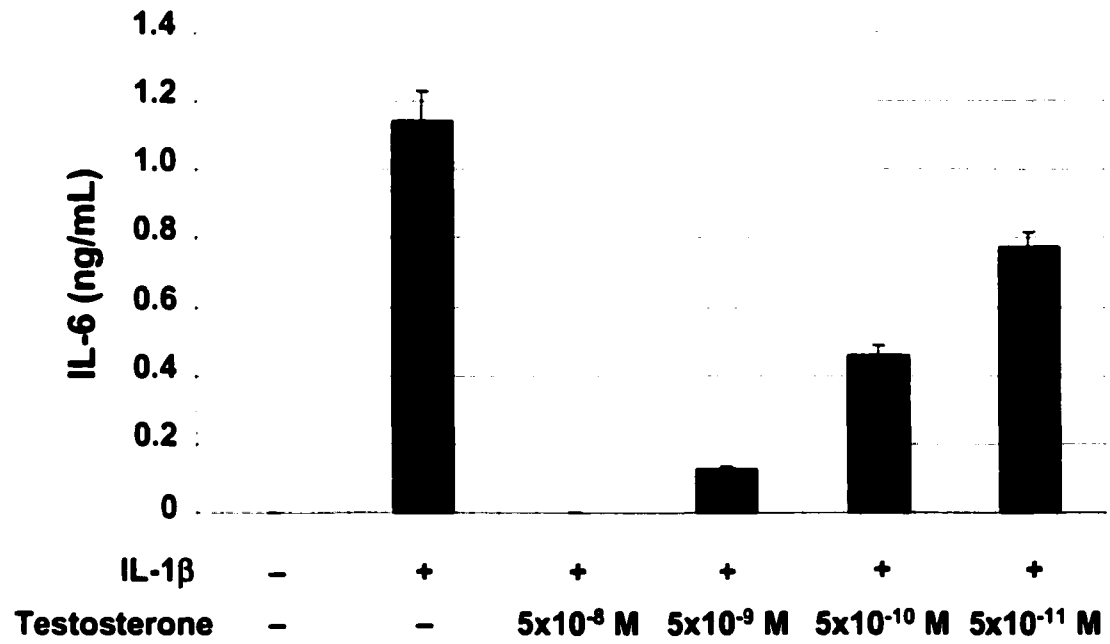

Figure 5.3. Testosterone inhibits IL-1β induced expression of IL-6. Cells were pretreated with testosterone 1 hour prior to stimulation with IL-1β (50 pg/mL). Conditioned media were collected 6 hours following cytokine stimulation and analyzed for expression of IL-6 using ELISA analyses.

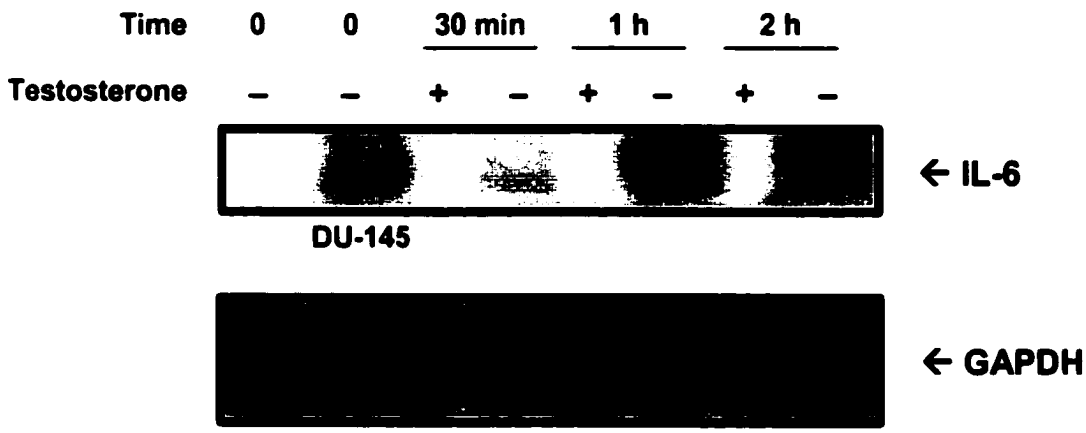

Figure 5.4. Testosterone blocks accumulation of IL-6 mRNA. Total RNA was isolated from LNCaP cells following stimulation with IL-1β or IL-1β and testosterone and analyzed for IL-6 mRNA. The first lane demonstrates that LNCaP cells do not express any steady state IL-6 mRNA. Lane 2 contains mRNA isolated from untreated DU-145 cells, which are known to constitutively express IL-6. The next three sets of lanes show testosterone treated and vehicle treated LNCaP cells at 30 minutes, one hour and 2 hours following treatment.

Proposed model: Testosterone inhibits IL-1β induced promatrilysin expression through inhibition of NFκB transactivation activity.

Our data show that androgens can inhibit IL-1β induced expression of promatrilysin by blocking NFκB transactivation. In the absence of testosterone, when IL-1β binds to its extracellular receptor, a cascade of events occurs. IκB, which sequesters NFκB in the cytoplasm, becomes phosphorylated and subsequently ubiquitinated. It then detaches from the NFκB heterodimer and is degraded by the 26S proteosome. This unmasks the nuclear localization sequence on the p50 subunit of the active NFκB heterodimer allowing it to translocate to the nucleus where it can bind and regulate its target genes (Lentsch, *et al.*, 1999; Li, *et al.*, 1999). We have shown that IL-1β, through the NFκB pathway, induces expression of IL-6. When IL-6 is secreted and binds to its extracellular receptor, STAT3 becomes activated (Spiotto, *et al.*, 2000a; Spiotto, *et al.*, 2000b). STAT3 can then translocate to the nucleus and regulate transcription of target genes. We have shown that this pathway induces expression of promatrilysin in LNCaP cells. It has been shown that testosterone blocks activation of NFκB by maintaining a high steady state level of IκB (Keller, *et al.*, 1996). Therefore, in our model (Figure 5.5) testosterone blocks NFκB transactivation activity, which blocks expression of IL-6, thereby blocking promatrilysin expression.

Steroid hormones, through the action of their receptors, are known to affect the expression of the matrix metalloproteinases (MMPs), and, the effect is generally negative. The glucocorticoid receptor (GR), for example, down-regulates MMP expression through an inhibitory interaction with AP-1, a transcription activator (Xu, *et al.*, 2001). Progesterone

receptor (PR) regulates MMP expression in the ovary (Robker, *et al.*, 2000) and in the uterine endometrium (Matrisian, *et al.*, 1994; Rodriguez-Manzaneque, *et al.*, 2000). There is evidence that testosterone and its androgenic metabolite 5α-dihydrotestosterone (DHT) down-regulate the expression of the MMPS, collagenase-1, and stromelysin-1 in prostatic cell line LNCaP cells through an interaction with Ets-related transcription factors (DiBattista, *et al.*, 1991). It has recently been reported that progesterone blocks the interleukin-Iα (ILα)-1-mediated release of MMP-3 in human endometrial stromal cells (Keller, *et al.*, 2000).

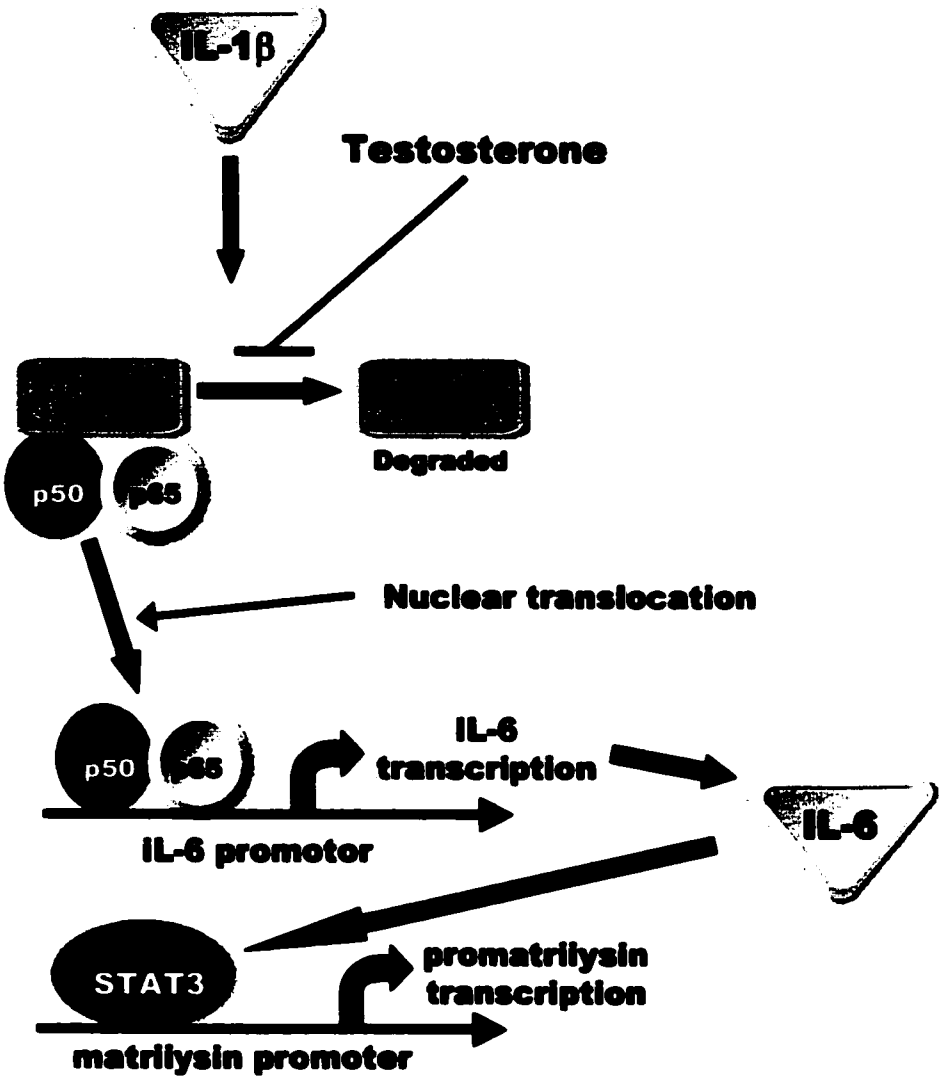

Figure 5.5. Proposed model. Testosterone inhibits IL-1β induced promatrilysin expression through inhibition of NFκB transactivation activity. In the absence of androgen, IL-1β stimulates IL-6 synthesis by an NFκB dependent mechanism. IL-6 then activates STAT3, which regulates transcription of its target genes, including matrilysin. In our proposed model, androgens block IL-1β induced promatrilysin expression by inhibition of NFκB dependent synthesis of IL-6.

Keller and colleagues have shown that androgens inhibit IL-6 synthesis by maintenance of high IκBα levels, which blocks NFκB activity by inhibiting nuclear translocation of the active p50-p65 heterodimer (Keller, *et al.*, 1996). In our system, we have previously shown that IL-1β induced promatrilysin expression is regulated by NFκB dependent expression of IL-6 and signaling by STAT3. We now demonstrate that testosterone inhibits IL-1β induced expression of promatrilysin by blocking NFκB transactivation activity which results in inhibition of IL-1β induced IL-6 expression. We also show that testosterone has no effect on IL-6 induced promatrilysin expression. From these data, we hypothesize that the mechanism by which testosterone inhibits IL-1β induced promatrilysin expression is through inhibition of NFκB transactivation activity.

Androgen hormones can regulate expression of numerous genes including genes involved in cell growth, differentiation and the immune response. Both androgen inducible and androgen repressed genes have been described. Chemical and surgical androgen ablation is standard, front line therapy for prostate carcinoma. Although this type of therapy is initially successful in the majority of cases that are diagnosed at an early stage of disease, over time, tumor cells become resistant and can grow in the absence of androgens.

Our data show that androgens can inhibit IL-1β induced expression of promatrilysin by blocking NFκB transactivation. These data suggest that androgens may block some of the factors that promote invasion and metastases; and, blocking androgens may, in fact, promote some of these processes. Although androgen ablation is currently standard practice for treatment of prostate carcinoma, while developing new therapeutic strategies, it may be essential to consider the pleiotropic effects of androgens. Identification of novel therapeutic

targets downstream of the androgen receptor in order to selectively block factors that promote growth of prostate carcinoma without blocking the beneficial signaling pathways downstream of the androgen receptor may be a key to successful therapy.

CHAPTER VI
CONCLUSIONS

The inflammatory response has been suggested to play a key role in development and progression in prostatic carcinoma. Okada and colleagues examined inflammation in needle biopsy specimens in both normal and carcinoma prostatic tissue in order to reveal the possible contribution of histological inflammation within the prostate to the abnormal elevation of serum prostate-specific antigen (PSA) levels. Their data revealed that the presence of histological inflammation within the prostate correlated significantly with serum PSA levels. Because of these results, Okada and colleagues have hypothesized that the assessment of inflammation in needle biopsy specimens, in conjunction with PSA levels, may be a useful diagnostic or prognostic indicator for prostatic carcinoma (Okada, *et al.*, 2000). Other studies have concurred with these data. Irani and colleagues conducted studies assessing the prognostic value of stromal tissue inflammation in surgically treated localized prostate carcinoma for biochemical recurrence-free survival. Biochemical recurrence based on serum prostate-specific antigen (PSA) level was defined as two successive PSA measurements greater than 1 ng/mL. Stromal prostatic inflammation grading was studied in patients who underwent radical prostatectomy for prostatic cancer without lymph node involvement and who did not receive preoperative or postoperative radiotherapy or hormonal therapy until recurrence. Inflammation was graded as high-grade inflammation if glandular epithelium disruption associated with interstitial inflammatory infiltrate was present and as low-grade inflammation otherwise. The results of the study indicated that patients with high-grade inflammation surrounding

malignant glands in radical prostatectomy specimens had significantly more postoperative biochemical recurrence than patients with low-grade inflammation. It is of interest that the inflammatory processes occurred primarily in the tissue surrounding the tumor and not in the malignant tissue itself (Irani, *et al.*, 1999).

In the studies that comprise this dissertation, we elucidate a molecular pathway by which the acute immune response cytokine, IL-1β, stimulates promatrilysin expression in the prostate carcinoma cell line, LNCaP. Our data demonstrate that the pathway by which IL-1β induced promatrilysin expression is potentiated indirectly and requires NFκB dependent synthesis of IL-6, another cytokine involved in the immune response. Once IL-6 is secreted from the cell through either autocrine or paracrine interactions, the IL-6 ligand binds its receptor and elicits its downstream effects through STAT3 signaling. Because the matrilysin gene contains numerous cis elements to which STAT3 may bind including NFIL6 sites and a STATX element, we hypothesize that STAT3 binds the matrilysin promoter directly and regulates matrilysin transactivation.

It is of interest, that, in addition to IL-6, there is at least one more additional factor(s) involved in IL-1β induced promatrilysin expression. IL-6 is responsible for approximately 65% of the IL-1β mediated induction of promatrilysin. The additional factor(s) may act additively or synergistically with IL-6 to achieve the full IL-1β mediated induction. However, blockage of NFκB transactivation activity completely inhibited the IL-1β induced expression of promatrilysin indicating that the induced expression of the additional factor(s) is also mediated by NFκB. We suspect that the additional factor may be another secreted cytokine. This speculation is supported

because NFκB is known to be involved in many cytokine mediated inflammatory processes. However, since NFκB subunits are known to be promiscuous and capable of binding to other transcription factor family members, including members of the AP-1 family of proteins, the unknown factor(s) could also be an additional transcription factor with which NFκB may interact. These studies may provide one mechanism by which inflammatory factors participate in progression of prostatic carcinomas.

We also examined promatrilysin expression in a co-culture of the prostatic carcinoma cell lines, LNCaP and DU-145. Our data demonstrated that LNCaP cells secrete elevated levels of promatrilysin in the presence of DU-145 cells; and, cell-cell contact is not required. DU-145 conditioned media collected from DU-145 cells cultured alone elicited the same induction of promatrilysin expression as was observed in the LNCaP-DU-145 co-culture. LNCaP conditioned media did not stimulate DU-145 cells to secrete promatrilysin. In fact, to date, DU-145 cells have never been shown to express promatrilysin under any condition. Our data clearly indicated that a factor secreted by DU-145 cells into conditioned medium was stimulating promatrilysin expression in LNCaP cells. The factor was found to be IL-6. Pretreatment of DU-145 conditioned media with IL-6 neutralizing antibody abrogated promatrilysin expression to baseline levels. In addition, 1ng/mL of recombinant IL-6, the concentration present in DU-145 conditioned media, elicited the same fold induction of promatrilysin as did the DU-145 conditioned media in LNCaP cells.

The majority of solid tumors are not heterogeneous and develop from a single foci under most circumstances. However, prostate carcinomas are unique in that they are

typically heterogeneous in nature and can often be multi-focal. Although studying prostate carcinoma cells *in vitro* does not accurately reflect *in vivo* conditions, we hypothesize that examining interactions of different prostatic carcinoma cell lines in co-culture experiments may contribute to our understanding of *in vivo* progression and metastatic mechanisms. *In vivo*, LNCaP cells are usually noninvasive to weakly invasive and DU-145 cells are characterized as weakly invasive. Our laboratory previously demonstrated that forced overexpression of the matrilysin gene in DU-145 cells increased their invasive capacity both *in vivo* and *in vitro* (Powell, *et al.*, 1993). Now we have shown that interaction between DU-145 cells and LNCaP cells, two different types of prostate carcinoma cells, results in promatrilysin expression by LNCaP cells. To further develop these observations, similar to the experiments performed to study invasion by matrilysin expressing DU-145 cells, it may be of interest to perform invasion assays with the DU-145 and LNCaP cells in co-culture both *in vivo* and *in vitro*. It may also be of interest to inject SCID mice with LNCaP cells and subsequently treat them with IL-6 to observe whether the presence of the cytokine would enhance invasive capacity by upregulation of promatrilysin expression.

Androgens are known to be important steroid factors in prostate cancer. Therefore, we next examined the effect of androgens on IL-1β and IL-6 induced promatrilysin expression in LNCaP cells. Interestingly, we found that pretreatment with relatively low dose levels of either testosterone or 5α-dihydrotestosterone completely abrogated IL-1β induced promatrilysin expression, but had no effect on IL-6 induced promatrilysin expression. It had been reported that testosterone could block activation of

NFκB thereby inhibiting NFκB transactivation activation through maintenance of IκB levels (Keller, *et al.*, 1996). Using a NFκB luciferase reporter assay, we confirmed that testosterone did, indeed, block NFκB transactivation activity in LNCaP cells. Furthermore, because NFκB transactivation activity is required for IL-1β induced expression of IL-6, IL-6 expression was also abrogated. However, IL-6 promatrilysin expression was unaffected by testosterone suggesting that testosterone does not affect IL-6 STAT3 signaling.

The defined roles of androgens in prostate carcinoma have not been elucidated, yet hormone therapies using anti-androgens and chemical or surgical castration are the most commonly prescribed non-surgical therapy regimens for prostate cancer. Usually, initial response is positive and patients can undergo shrinkage of the tumor and even experience apparent, temporary remission following these therapies. However, relapse of prostate carcinoma with tumor cells that are not hormone responsive is strikingly common. It is hypothesized that hormone ablation therapy kills the population of tumor cells that are responsive to it. However, this allows a selection process to occur and results in growth of cells that are hormone independent. It is of interest that some prostatic carcinoma cell lines that have been isolated from advanced tumors that are poorly differentiated, do not express the androgen receptor. It is also of interest that LNCaP cells, which are considered to be less progressed and more differentiated, express a mutant androgen receptor. The mutation resides within the ligand binding domain and allows the receptor to be promiscuous in nature. In fact, it has been shown that some

compounds that have been designed to block the androgen receptor and the female hormone, estradiol, are capable of stimulating the androgen receptor on LNCaP cells.

Our data show that androgens inhibit IL-1β induced expression of promatrilysin by blocking NFκB transactivation. These data suggest that androgens may block some of the factors that promote invasion and metastases; and, blocking androgens may, in fact, promote some of these processes. Although androgen ablation is currently standard practice for treatment of prostate carcinoma, while developing new therapeutic strategies, it may be essential to consider the pleiotropic effects of androgens. Identification of novel therapeutic targets downstream of the androgen receptor in order to selectively block factors that promote growth of prostate carcinoma without blocking the beneficial signaling pathways downstream of the androgen receptor may be a key to successful therapy.

Prostate cancer is a major health problem in the US. Currently, adequate diagnostic techniques are not available. Digital rectal exams and serial PSA measurements may be helpful especially for detecting early relapse of prostate carcinoma following treatment, however, the accuracy of the assay is limited largely because of the differential diagnosis of benign prostatic hypertrophy, which while non-malignant, can also cause elevation of PSA levels and positive digital rectal exams. If the carcinoma is detected before invasion through the capsule, surgical intervention or radiotherapy with of without hormone ablation may offer the patient a positive prognosis. However, the therapies that are currently available have considerable side effects including urinary and bowel dysfunction and impotence. If diagnosed at a later stage of disease, prognosis for the patient worsens. Successful therapeutic strategies for metastatic

prostate carcinoma are not currently available. Clearly, improved diagnostic techniques and treatment are needed.

Matrix metalloproteinases are potentially involved in the processes of invasion and metastasis. Characterization of the pathways by which they are regulated may provide useful information for the development of both diagnostic techniques and therapy. Although MMP inhibitors, such as Batimistat and Marimistat have not shown success in clinical trials, other targets in the signal transduction pathways upstream of MMPs may prove to be superior.

IL-6 is known to be overexpressed in prostatic carcinoma patients. Furthermore, both IL-6 and matrilysin are known to be critical for differentiation of bone, the tissue to which prostate carcinoma characteristically spreads. There is published evidence presented in the Introduction of this dissertation that matrilysin plays a role in progression of prostate cancer (Knox, *et al.*, 1996; Saarialho-Kere, *et al.*, 1995; Hashimoto, *et al.*, 1997), and the research conducted in our studies have characterized a pathway whereby IL-6 is involved in significant upregulation of matrilysin. From our studies, we hypothesize that peripheral IL-6 may be a valuable diagnostic and/or prognostic indicator for prostate carcinoma. Furthermore, if IL-6 induced matrilysin expression is involved with progression of disease, it may be a viable therapeutic target for successful therapeutic intervention.

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