The Regulation of Parathyroid Hormone Related Peptide (PTHRP) Gene Expression by Androgens in Prostate Cancer

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Abstract

Our study aimed at understanding the regulation of parathyroid hormone related peptide (PTHRP) by androgens in prostate cancer. Although PTHRP has been identified as a factor responsible for malignancy- associated hypercalcemia, it is known to be widely expressed in normal tissues where it regulates cellular growth and differentiation. PTHRP binds with equal affinity and activates the membrane-embedded G protein-coupled PTH/PTHRP receptor present on the surface of target cells such as bone and kidney. Upon receptor binding several signaling pathways are activated including the adenylyl cyclase and the phospholipase C pathways.

A number of growth factors and steroid hormones have previously been shown to regulate PTHRP production. To study the regulation of PTHRP by androgens, we used a late stage human prostate cancer cell line that is androgen non- responsive, PC-3 cells. In addition, we used an androgen responsive cell line, transfected with a functional androgen receptor, PC-3T cells. Results obtained by Northern blots and immunoradiometric assays demonstrated that androgens inhibit PTHRP expression in this human prostate cancer system. Male Balb/c nu/nu mice, injected subcutaneously with PC-3 cells, developed larger tumors than experimental animals inoculated with PC-3T cells. Moreover, castration of the host animals resulted in the substantial increased in PC-3T tumor volumes. Immunohistochemical studies indicated that PTHRP expression was inhibited in experimental PC-3T tumors and that castration of animals increased PTHRP production. In conclusion, our study demonstrated that androgens down regulate PTHRP production in prostate cancer cells *in vitro* and that prostate tumor cells producing less PTHRP exhibited a reduction in tumor volume *in vivo*.

Résumé

Notre étude vise à comprendre la régulation du peptide apparenté à l'hormone parathyroïdienne (PTHRP) par des androgènes dans le cancer de la prostate. Bien que la PTHRP fut identifiée comme le facteur causal de l'hypercalcémie observée dans certains cancers malins, la protéine semble jouer un rôle dans les processus de prolifération et de différenciation cellulaires. La PTHRP se lie au récepteur PTH/PTHRP, couplé aux protéines G, sur la surface des cellules cibles telles que celles des os et du rein. La liaison du récepteur, mène à l' activation de plusieurs signaux cellulaires tels que l'adénylate cyclase et la protéine kinase C.

Plusieurs études ont démontré qu'un grand normbre de facteurs de croissance et d'hormones stéroïdes peuvent régler la production de la PTHRP. Afin d'étudier la régulation de la PTHRP par les androgènes, nous avons utilisé une lignée cellulaire humaine de cancer avancé de la prostate, les cellules PC-3, qui ne répondent pas aux androgènes. De plus, nous avons utilisé une lignée cellulaire, transfectée avec le récepteur de l'androgène, qui est sensible aux androgènes, les cellules PC-3T. Les résultats obtenus, suite à des analyses radio-immunologiques et par technique de "northern", démontrent que les androgènes inhibent l'expression de la PTHRP dans ce système cellulaire. Des souris mâles, Balb/c nu/nu, injectées sous-cutané avec des cellules PC-3, ont développé de plus grosse tumeur que les souris expérimentales inoculées avec des cellules PC-3T. De plus, la castration des souris a augmenté le volume des tumeurs PC-3T. Des études immunohistochimiques ont démontré que la production de la PTHRP est inhibée dans les tumeurs expérimentales PC-3T et que la

castration des souris a augmenté la production de la PTHRP. En conclusion, notre étude a démontré que les androgènes inhibent l'expression de la PTHRP *in vitro* et que les cellules tumorales du cancer de la prostate, qui produisent moins de PTHRP, présentent des tumeurs de plus petit volume, *in vivo*.

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Abbreviations

cAMP	cyclic adenosine 3,5- monophosphate
C-terminal	carboxyl-terminal
DHT	dihydrotestosterone
EGF	epidermal growth factor
FBS	fetal bovine serum
FGF	fibroblast growth factor
FLU	flutamide
G-protein	guanine nucleotide-binding protein
HM	hypercalcemia of malignancy
IGF-I	insulin-like growth factor I
IP3	phosphoinositol 1,4,5-triphosphate
MAP kinase	mitogen activated protein kinase
N-terminal	amino-terminal
PI 3-kinase	phosphoinosite 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PTH	parathyroid hormone
PTHRP	parathyroid hormone-related peptide
SRE	serum response element
TGF	transforming growth factor
TNF	tumor necrosis factor

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Chapter 1

Introduction

The Discovery of Parathyroid Hormone Related Peptide

Parathyroid hormone related peptide (PTHRP) was discovered as the primary factor in the pathogenesis of hypercalcemia in association with malignancy but it is now known to be expressed by a variety of normal fetal and adult tissues. The best characterized paraneoplastic syndrome results from ectopic secretion of a polypeptide hormone by the tumor. Hypercalcemia of malignancy (HM) is the most common paraneoplastic syndrome and it has been described in association with a number of malignancies (Halloran and Nissenson 1992).

In the 1920s, it was observed that cancer could be associated with elevated levels of serum calcium and low phosphorus concentrations (Zondek et al. 1923). It was initially assumed that this syndrome arose exclusively from cancer cells metastasizing to bone. However, later evidence of hypercalcemia in the absence of bone metastasis and parathyroid dysfunction led to the theory that a parathyroid hormone-like compound might be produced by the tumor (Albright, 1941). The biochemical properties of the putative factor were defined in the early 1980s using adenylyl cyclase bioassays (Stewart et al., 1983) and together with high performance liquid chromatography (HPLC) a major bioactive compound was isolated (Nissenson et al., 1981; Rodan et al., 1983). A protein with biological activities similar to parathyroid hormone (PTH) was purified from serum-free culture medium obtained from the BEN cell line which was established from a patient with hypercalcemia who had squamous cell carcinoma of the lung (Moseley et al.,

1987). Amino acid sequence analysis of the material purified by HPLC revealed that 8 of the 16 amino- terminal residues were identical with those of human PTH. However, antibody raised to a corresponding synthetic peptide recognized the PTH-related material but showed almost no cross-reactivity with human PTH amino-terminal peptides indicating the uniqueness of this mediator of the syndrome of malignancy-associated hypercalcemia (Burtis et al., 1987; Moseley et al., 1987; Strewler et al., 1987). These findings eventually led to the cloning of the cDNA (Mangin et al., 1988; Suva et al., 1987) and then the gene for PTHRP.

Parathyroid Hormone Related Peptide and Hypercalcemia of Malignancy

PTHRP is expressed in a large number of tumors and is the mediator of the parathyroid hormone-like effects seen in hypercalcemia of malignancy (HM). This phenomenon typically occurs in patients with solid tumors and in the absence of extensive bone metastasis (Mackenzie et al., 1994). HM is characterized by increased levels of circulating PTHRP, serum calcium and nephrogenous cAMP and decreased levels of serum phosphorus, PTH and 1, 25- dihydroxyvitamin D3 (Stewart et al., 1980). The serum calcium levels reach a value of 2.9 mmol/L, which is significantly greater than the normal range of 2.1-2.6 mmol/L (Mundy and Martin, 1982). HM occurs more frequently in advanced malignancy and seldom in early stages of cancer. Ectopic production of PTHRP by tumors and its systematic distribution to target organs leads to osteoclastic bone resorption and retention of calcium by the kidneys subsequently resulting in kidney malfunction from the excess calcium load (Mundy and Guise, 1997; Mundy and Martin, 1982).

Defining the pathophysiology of HM has been possible with the use of animal models. The rat H-500 Leydig tumor cell model is a well-characterized animal model of HM. Animals bearing H-500 tumors were shown to rapidly develop the biochemical abnormalities associated with HM including excess PTHRP production and they eventually die of complications arising from the disease (Sica et al., 1983). Tumor-bearing animals inoculated with PTHRP neutralizing antibodies reversed the biochemical signs of HM and delayed the onset of hypercalcemia (Henderson et al., 1990; Sato et al., 1993). Studies using this model demonstrated the major role of PTHRP in HM.

Parathyroid Hormone Related Peptide Gene

The gene encoding human PTHRP (Mangin et al., 1988; Yasuda et al., 1989b) is a complex transcriptional unit which uses multiple promoters to differentially transcribe messenger RNA (mRNA) with distinct 5` ends. Furthermore, due to alternative exon splicing, mRNAs encoding three different translation products result. The PTHRP gene is similar in organization and nucleotide sequence to the PTH gene. The two genes are located within a cluster of related genes on homologous arms of chromosomes 11 and 12, respectively (Martin and Suva, 1989). The PTH and PTHRP genes are thought to have arisen from a common ancestral gene through an ancient chromosomal duplication event. With its three promoters and complex pattern of alternative exon splicing (Mangin et al., 1988), the human PTHRP gene evolved to be far more complex than the gene encoding PTH.

The human PTHRP gene comprises at least seven exons and spans more than 15 kilobases of genomic DNA (Mangin et al., 1988; Suva et al., 1987; Yasuda et al., 1989b).

Exons I and II encode different 5`untranslated regions of the gene. Exon III encodes the prepro coding region of the protein and exon IV contains most of the mature peptide sequence. Exon VI encodes 36 additional amino acids and a stop codon; furthermore, exon VII encodes two extra amino acids and another stop codon which serve as termination sites during translation. Depending on the promoters used the gene gives rise to different 5`transcriptional messenger RNA (mRNA) products. By the use of alternative splicing of exons, a multitude of human PTHRP mRNA transcripts can occur, giving rise to three different protein isoforms of 139, 141, and 173 amino acids in length which are identical up to amino acid 139.

The human PTHRP gene uses both TATA and GC- rich promoter elements. Promoters P1 and P3 contain classical TATA boxes (Suva et al., 1987; Yasuda et al., 1989b). Unlike the other two PTHRP gene promoters, the P2 promoter is located in a GC- rich region enriched in Sp1 binding sites and does not possess classical TATA or CAAT sequences (Vasavada et al., 1993). The majority of normal and neoplastic human tissues contain PTHRP mRNA transcripts initiating from both the up-stream (P1) and down-stream (P2) human PTHRP promoters. The two TATA promoters P1 and P3 direct the majority of the expression of the PTHRP gene in human tissues (Vasavada et al., 1993).

The PTHRP gene has been isolated from different species including the rat, mouse and chicken (Karaplis et al., 1990; Mangin et al., 1990; Schermer et al., 1991; Thiede and Rutledge, 1990; Yasuda et al., 1989b). The non-human genes have a considerably simpler organization than their human counterpart. They consist of only four or five exons and possess only a single promoter, that is homologous with the

downstream P3 promoter in the human gene (Karaplis et al., 1990; Mangin et al., 1990). The simpler organization of the rat gene predicts, in mammals, the predominant use of a single promoter and generation of a 141-amino acid peptide as the major molecular form. The organization of the mouse and chicken gene includes a single 3' exon and an apparent single 3' splicing pathway, leading to a mRNA encoding a 139-amino acid mature PTHRP. (Mangin et al., 1990; Schermer et al., 1991). In the mouse, the 139-amino acid mature protein is synthesized rather than the 141 amino acid isoform because of a 6- base pair deletion corresponding to amino acids 130 and 131 of the human and rat protein. The chicken gene can encode two isoforms, one of 139 amino acids and a predominant form of 141 amino acids. This conservation throughout a large evolutionary range suggests that PTHRP is of essential biological importance.

Regulation of the Parathyroid Hormone Related Peptide Gene

The PTHRP gene is widely expressed in normal and neoplastic tissues. Protein secretion generally occurs via the constitutive pathway. Therefore regulation of gene expression and intracellular processing appear to be important mechanisms for determining the rate of PTHRP production and secretion. The PTHRP gene is induced by a number of growth factors and cytokines and it is thought to belong to the family of early response genes. (Allinson and Drucker, 1992; Holt et al., 1994; Thiede et al., 1991) This family of genes include cytokines, transcription factors and nuclear proto-oncogenes.

Early response genes exhibit low basal expression; however, in the presence of growth factors and cytokines they undergo a rapid induction of gene expression which

occurs independently of new protein synthesis (Herschman, 1991). Moreover, this induction of gene expression is transient and their mRNA transcripts have short half-lives. These characteristics also hold true for the PTHRP gene. Cytokines and growth factors induce a rapid and transient PTHRP gene expression (Ikeda et al., 1993; Liu et al., 1993; Sebag et al., 1994; Werkmeister et al., 1993); moreover, PTHRP mRNA is known to have a short half-life of 90-120 minutes (Allinson and Drucker, 1992; Ikeda et al., 1990). Studies suggest that many early response genes possess an AU motif, located in the 3`untranslated region of the mRNA that confers instability (Shaw and Kamen, 1986; Wilson and Treisman, 1988). PTHRP mRNA also contains multiple copies of this instability motif encoded by the exon which specify the 3`untranslated region (Ikeda et al., 1990).

The transcription of many early response genes is known to be induced by protein synthesis inhibitors (Greenberg et al., 1986). This results from an increase in gene transcription and/or in an increase in the stability of the mRNA. Accordingly, through both transcriptional and post-transcriptional mechanisms, PTHRP mRNA expression is induced by the protein synthesis inhibitor cyclohexamide (Allinson and Drucker, 1992; Falzon, 1996b; Ikeda et al., 1990). This pattern of PTHRP expression, typical of genes encoding cytokines, transcription factors and growth factors, therefore suggests that PTHRP may play an important role in cell proliferation and differentiation.

The regulation of PTHRP gene expression has been studied in a variety of tissues and cells. Generally, serum and growth factors induce a common pattern of PTHRP gene expression described as a rapid and transient induction which peaks within a few hours. Expression in response to these factors, appear to have both a transcriptional and post-

transcriptional component. (Falzon, 1996b; Kremer et al., 1991; Merryman et al., 1994). More specifically, epidermal growth factor (EGF) has been shown to increase PTHRP expression in keratinocytes (Allinson and Drucker, 1992), human mammary epithelial cells (Sebag et al., 1994) and rat Leydig tumor cells (Liu et al., 1993). Transforming growth factor-β (TGF-β) has also been shown to stimulate PTHRP expression in keratinocytes (Allinson and Drucker, 1992; Werkmeister et al., 1993), squamous carcinoma cells (Merryman et al., 1994) and uterine smooth muscle cells (Shenberger et al., 2001). Moreover, induction of PTHRP expression has been demonstrated in keratinocytes treated with insulin and insulin like growth factor type I (IGF-I) (Sebag et al., 1994) and fibroblast treated with interleukin-1α (IL–1α) and tumor necrosis factor-α (TNF-α) (Yoshida et al., 2001).

PTHRP expression is particularly high in the lactating mammary gland. Prolactin concentrations rise dramatically in response to suckling. This rise in prolactin coincides with a rapid increase in PTHRP expression in the early phase and more sustained increased in the later stage of lactation (Thiede and Rodan, 1988). Consequently, PTHRP mRNA in lactating mammary tissue is stimulated by prolactin although the exact mechanism of this effect is unknown. These findings suggest that PTHRP plays a physiological role in lactation, possibly as a hormone for the mobilization or transfer of calcium to the milk (Yamamoto et al., 1992a).

Intrauterine occupancy in preterm myometrium causes increased PTHRP expression in the uterus (Thiede et al., 1990). Elevated levels of PTHRP persist throughout pregnancy and a large peak in both mRNA and protein levels are observed in the myometrium 48 hours before parturition. Mechanical stretch has also been shown to

induce PTHRP in the urinary bladder (Yamamoto et al., 1992b). Histochemical studies indicated the presence of PTHRP immunoreactivity in smooth muscle cells of distended bladder tissue. PTHRP mRNA levels change in response to the stretch of the bladder wall. Since PTHRP is a potent smooth muscle relaxant its induction by mechanical stretch may be linked to this function (Martin et al., 1997). In addition, estrogens regulate PTHRP expression in the myometrium immediately prior to parturition. Estrogen administration was able to induce a rapid and transient increase in PTHRP mRNA in the uterus of ovariectomized rats (Thiede et al., 1991). In other tissues including the pituitary and the hypothalamus, PTHRP expression is also subject to regulation by estrogens (Holt et al., 1994).

PTHRP gene transcription is suppressed by 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the active metabolite of vitamin D_3. A sustained inhibition of basal PTHRP gene transcription by $1,25(OH)_2D_3$ was observed in various cell lines and tissues including keratinocytes (Allinson and Drucker, 1992; Werkmeister et al., 1993), squamous cell carcinoma (Abe et al., 1998), epithelial cells (Sebag et al., 1994) and tumor Leydig cells (Liu et al., 1993). Modulation of PTHRP gene transcription by $1,25-(OH)_2D_3$ is mediated by the vitamin D receptor (VDR) interacting with identified vitamin D response elements (VDREs) in the 5'- flanking sequence of the gene (Falzon, 1996a). Transfection of PTHRP promoter- reporter constructs in normal human keratinocytes in addition to mobility shift assays and mutational analysis identified an inhibitory VDRE within the PTHRP promoter (Kremer et al., 1996).

Glucocorticoids, such as dexamethasone, and anabolic steroid hormones such as testosterone and dihyrotestosterone have both been shown to cause a dose dependent

inhibition of serum induced PTHRP gene expression (Allinson and Drucker, 1992; Glatz et al., 1994; Liu et al., 1993). Steroids exert their effects on gene expression through nuclear receptors that interact directly or indirectly with response elements located in the promoter region of the PTHRP gene. Although putative response elements for glucocorticoids and for steroid hormones can be identified within the PTHRP promoter sequence by promoter sequence analysis, the existence of these elements have yet to be determined (Glatz et al., 1994).

Understanding the regulation of the PTHRP gene will further our knowledge as to why this protein becomes aberrantly expressed in cancer. A number of different mechanisms may govern PTHRP overexpression in malignancy. These may include increased mRNA stability, change in methylation status leading to increased promoter activity or dysregulation of trans- activating factors in the promoter regions leading to constitutive expression of PTHRP.

Tumor suppressor genes and oncogenes play an essential role in the multi step process of carcinogenesis and tumor progression. To define the mechanisms involved in the deregulation of PTHRP production a number of studies have examined the regulation of PTHRP expression by oncogenes. Cellular transformation by oncogenes correlated with increased PTHRP expression. Transfection of fibroblasts by the oncogenes Ha-Ras and v-src are associated with a marked increase in PTHRP gene expression (Li and Drucker, 1994). In a later study, PTHRP expression was induced after co- transfection of oncogenic Ras and p53 genes into normal rat embryo fibroblasts (Motokura et al., 1995). Similarly, it was reported that the p53 oncogene increased the level of PTHRP expression in squamous carcinoma cells (Foley et al., 1996). These studies demonstrate the

important role that oncogenes and cellular transformation play in the overexpression of PTHRP in malignancy.

DNA methylation has been studied as a mechanism that could lead to overproduction of PTHRP in malignancy (Holt et al., 1993). Using cultured lung cells as a model system to study the methylation status of CpG dinucleotides in the 5' region of the PTHRP gene showed that in normal cells the CpG islands were completely unmethylated. In the BEN squamous carcinoma cell line, two-thirds of the CpG island were substantially methylated (Ganderton and Briggs, 1997). Methylation is usually associated with inhibition of gene activity; however, this heavy methylation did not prevent expression of any of the three PTHRP gene promoters. Unexpectedly, malignancy is associated with increased DNA methylation in the 5' region of the PTHRP gene. Hence methylation of the 5' non-coding region of the PTHRP gene may not play a role in the regulation of adjacent promoters (Ganderton and Briggs, 2000). Overall these studies suggest that the overexpression of the PTHRP gene in cancer cells must be mediated through mechanisms other than DNA methylation.

Biosynthesis and Processing of Parathyroid Hormone Related Peptide

Three protein products, of 139, 141, and 171 amino acids in length, are translated from the PTHRP gene. These PTHRP isoforms are identical in the first 139 amino acids and differ only in their extended C-terminal end (Mangin et al., 1988; Yasuda et al., 1989a) Adding another degree of complexity, the PTHRP protein products undergo extensive post-translational modifications. After translation, PTHRP enters the classical secretory pathway (Deftos et al., 1993). Signal peptide mediated entrance to the Golgi

apparatus is followed by entry to the trans Golgi network and finally entry into the vesicles of the constitutive secretory pathway or less commonly to the granules where it is subject to regulated secretion. PTHRP uses mainly constitutive but also to some extent the regulated secretory pathways (Plawner et al., 1995). A constitutive release of newly synthesized PTHRP occurs in cell types that do not have a system of secretory granules such as keratinocytes, chondrocytes, osteoblasts and vascular smooth muscle cells (Kremer et al., 1991; Rabbani et al., 1993; Rodan et al., 1989). On the other hand, PTHRP expressed in neuroendocrine cells that contain the regulated pathway including pancreatic islet, parathyroid, adrenal and pituitary cells, is secreted in a regulated fashion (Asa et al., 1990; Drucker et al., 1989; Plawner et al., 1995).

The first 36 amino acids of the PTHRP sequence are predicted to serve as the prepro- sequence. The N- terminal signal peptide has charged amino acid residues on either sides of a hydrophobic core of 10 to 15 amino acids. This hydrophobic region is essential in allowing for the docking and transport of the nascent peptide from the cytoplasmic compartment into the rough endoplasmic reticulum. Signal peptidase is thought to cleave the initial PTHRP chain at amino acid Gly^{-13} as it enters the rough endoplasmic reticulum. Cleavage of the signal peptide releases the pro- sequence starting at Arg^{-12}, which is thought to guide the mature protein through the Golgi apparatus to the secretory pathway and to facilitate proper folding. The pro-hormone sequence of PTHRP contains basic amino acid residues Arg-Leu-Lys-Arg, which is a typical substrate sequence for the pro-hormone convertase, furin (Liu et al., 1995). The mature PTHRP protein begins with alanine at position 1 (Moseley et al., 1987; Strewler et al., 1987).

As with other endocrine or neuroendocrine proteins including somatostatin, proopiomelanocortin (POMC) and atrial natriuretic peptide (ANP), it is likely that PTHRP serves as a pro-hormone from which a group of secreted forms are derived. PTHRP is endoproteolytically processed, at clusters of basic amino acids, to yield a family of mature secretory forms. The generation of three peptides possibly with their own distinct physiological functions can result. These include an amino-terminal, a mid-region, and a carboxyl-terminal form. The existence of an N- terminal PTHRP (1-36) peptide results from the cleavage, on the carboxyl- terminal side, of the monobasic residue Arg^{37}. Mid- region fragments beginning at amino acid 38 and terminating at amino acids 94, 95 and 101 have been identified, both within and outside cells following secretion, by mass spectroscopic analysis (Wu et al., 1996). Consequently, C- terminal peptides have been detected in circulation of patients with renal failure and HM (Orloff et al., 1993). Therefore, PTHRP may function as a polyhormone precursor of a number of biologically active fragments with distinct biological actions.

Sequences within PTHRP may function to direct the protein to a ubiquitin proteosome degradation system. The ubiquitin proteolytic pathway is responsible for the degredation of misfolded or aberrant proteins in the cell. In an *in vitro* translation system, ubiquitin was shown to bind and degrade prepro- PTHRP in an ATP dependent and proteosome inhibitor sensitive manner (Meerovitch et al., 1997). In transfected cells, degradation of PTHRP was shown to be protosome-dependent. Proteosomal degradation of PTHRP is thought to involve the binding of a chaperon protein, BiP, which facilitates the reverse transport of PTHRP out of the lumen of the endoplastic reticulum into the cytosol where it accesses the proteosome (Meerovitch et al., 1998).

PTHRP is able to enter the nucleus directly after translation (Henderson et al., 1995; Massfelder et al., 1997). The cluster of basic residues 88-91 and 102-106 which serve as putative sites for cleavage by prohormone convertases, are also homologous with nuclear targeting sequences. These nuclear localization sequences are found in viral and eukaryotic transcription factors such as c-Jun and c-Fos and in growth factors such as members of the fibroblast growth factor family. PTHRP has also been identified in the nucleus by immunocytochemistry; furthermore, its nuclear localization was found to be dramatically increased in mitotic cells (Massfelder et al., 1997). Deletion of the putative nuclear targeting multibasic motifs decreased PTHRP staining in the nucleus and deletion of the signal peptide, which directs the protein to the secretory pathway, but leaving the multibasic motifs intact increased the staining of PTHRP in the nucleus (Henderson et al., 1995).

Receptors for Parathyroid Hormone Related Peptide

Both PTH and PTHRP bind with equal affinity and activate the same membrane-embedded G protein-coupled receptor present on the surface of target cells such as bone and kidney. The receptor contains an extracellular amino terminus, seven transmembrane-spanning helices and an intracellular carboxy-terminal tail. Along with the receptor for calcitonin, glucagon and several other peptide hormones, the PTH/PTHRP receptor belongs to the class II family of G protein-coupled receptors. The receptor was originally cloned from the classical PTH targets of rat and human bone cells and human kidney cells (Abou-Samra et al., 1992; Juppner et al., 1991; Schipani et al., 1993). The PTH\PTHRP receptor has also been characterized in other cells such as

fibroblasts (Pun et al., 1988), vascular smooth muscle cells (Nickols et al., 1990) and human keratinocytes (Henderson et al., 1991). It is probable that PTHRP signaling through the receptors in these tissues account for its autocrine or paracrine actions.

The organization of the PTH/PTHRP receptor gene is highly homologous in the three mammalian species including the rat, human and mouse. This gene extends over 22 kilobases and contains at least 15 exons and 14 introns (Kong et al., 1994). The cDNA of the PTH/PTHRP receptor isolated from different mammalian species encode homologous proteins that range between 585-593 amino acids in length.

PTH and PTHRP possess significant sequence homology within the amino-terminal region and this sequence conservation reveals the functional importance of the amino acid residues in receptor binding and signaling. Studies on ligand interactions with the PTH/PTHRP receptor focused mainly on amino terminal peptide hormone analogs including PTH (1-34) and PTHRP (1-34) as there is no evidence that the mid- or the carboxy terminal regions interact with this receptor. Nonetheless, the 15-34 region within the C-terminal region of PTH (1-34) and PTHRP (1-36) are critical for high affinity binding to the PTH/PTHRP receptor. The mechanism of receptor interaction for this domain is largely unknown. Photoaffinity cross-linking data predicted that residues 23, 27, 28, and 33 of native PTHRP are each near different regions of the N-terminal extracellular domain of the PTH/PTHRP receptor (Gensure et al., 2001). Mutations as well as photoaffinity cross-linking studies have identified two regions of the PTH/PTHRP receptor important in ligand binding, one in the extracellular N-terminal domain and the other is the third extracellular loop (Mannstadt et al., 1998; Zhou et al., 1997). A polyclonal antiserum, directed against the N-terminal residues 88-97 of the

human PTH/PTHRP receptor, revealed that these residues are involved, either directly or indirectly, in agonist binding of the receptor (Fukayama et al., 1998).

The PTH/PTHRP receptor expressed in COS cells, was reported to activate two second messenger systems. The first system involves the adenylyl cyclase pathway which leads to increases in intracellular cAMP levels and in the activation of protein kinase A. The second signaling system includes the phospholipase C pathway which results in increases in inositol trisphosphate and calcium concentrations and in the activation of protein kinase C (Abou-Samra et al., 1992).

The activation of the PTH/PTHRP receptor has also been shown to interact with the mitogen activated protein (MAP) kinase pathway. PTH/PTHRP receptor, belongs to a growing number of G-protein coupled receptors that can interact with the MAP kinase pathway. It is possible that activation of MAP kinases by the receptor may mediate its actions on cellular processes such as cell growth and differentiation. In primary rat bone marrow cells, PTHRP increased MAPK activity and PTHRP-induced osteogenic cell proliferation (Miao et al., 2001b). The finding that PTHRP can activate these pathways might explain how PTHRP production in malignancy may lead to the growth and progression of tumors. In human osteosarcoma cells, results demonstrate that acting via its G protein-coupled receptor, PTHRP can induce indexes of osteoblast differentiation by utilizing multiple signaling pathways including the MAPK pathway (Carpio et al., 2001).

By activating MAP kinases, PTHRP can further modify its own expression, as well as the expression of other genes that are under the control of MAPK pathways. Results demonstrate that the Ras oncogene can stimulate PTHRP gene expression in

Fr3T3 fibroblasts *in vitro*. In transplantable rat Leydig tumors, PTHRP is induced via both Raf-ERK and Rac-JNK mediated pathways, effects which can be blocked by chemical inhibitors and dominant-negative mutants of these pathways *in vitro* and *in vivo* (Aklilu et al., 2000). These findings have important implications for the overexpression of PTHRP in disease and may provide the molecular basis for the autocrine action of this protein in cancer.

PTHRP undergoes extensive post-translational processing which produces different secretory forms. It is possible that the mid- and the C-terminal region peptides have there own unique receptors. A mid-region fragment of PTHRP, which is intensively conserved across species, has been identified as a secretory product of several different cell types, including keratinocytes and squamous carcinomas. Recent data suggest that a mid-region PTHRP fragment may be biologically active and that it interacts with unique cell surface receptors that mediate autocrine or paracrine actions. PTHRP is an important regulator of fetal blood calcium and placental calcium transport. The bioactivity of PTHRP for placental calcium transport is specified by a mid-molecular region that does not use the PTH/PTHRP receptor (Kovacs et al., 1996). Elevations in intracellular calcium were observed in squamous carcinoma cells exposed to human PTHRP (67- 86) (Orloff et al., 1996). Activation of the phospholipase C pathway by PTHRP (67-86) was confirmed by measuring the accumulation of inositol trisphosphate. In contrast, PTHRP (67- 86) did not stimulate adenylyl cyclase. PTHRP (67-86) may activate phospholipase C-dependent pathways through a receptor distinct from that activated by PTHRP (1-36) in the same cells. As a mid-region secretory fragment of PTHRP has been partially purified from several different cell types, this receptor may have broad biological

significance (Orloff et al., 1996). C-terminal PTHRP (107-139) inhibits growth and various functions of osteoblasts and osteoclasts apparently through PTHRP-specific receptors. In osteosarcoma cells and lymphocytes, this C-terminal region has been shown to increase intracellular calcium and to stimulate membrane-associated protein kinase C activity (Fenton et al., 1991) (Whitfield et al., 1994). Like the mid- region, a C- terminal receptor has yet to be identified.

Actions of Parathyroid Hormone Related Peptide

In addition to the actions of PTHRP in hypercalcemia of malignancy, the abundant expression of PTHRP in fetal tissue, suggests its role as a developmental factor. Moreover, PTHRP and its receptors are present in many normal tissues where it appears to be involved in a diverse array of physiological effects including the transepithelial transport of calcium in renal tubules, placenta and mammary gland. PTHRP is also a smooth muscle relaxant and a regulator of cellular proliferation, differentiation and apoptosis.

Through the direct consequences of its actions in kidney and on bone, it has been established that PTHRP causes HM. PTHRP increases calcium retention and bone resorption by interacting with the classical PTH/PTHRP receptor. Renal actions of PTHRP are thought to account for the initial rise in serum calcium in the early stages of HM. PTHRP was shown to inhibit sodium-phosphate cotransport, to increase nephrogenous cAMP excretion and reduce the fractional excretion of calcium in renal cortical membranes isolated from hypercalcemic rats bearing Leydig H-500 tumors (Sartori et al., 1988) as well as *in vivo* (Rabbani et al., 1986). PTHRP has also been

shown to stimulate adenylyl cyclase activity and cAMP production in rat and human renal membranes (Orloff et al., 1991).

In addition to its effects in the kidney, the action of PTHRP on bone is an essential component of HM. PTHRP has been shown to act on osteosarcoma cells to increase expression of osteocalcin, and cytokines. In other studies, production of collagen, osteopontin and alkaline phosphatase were shown to be suppressed by PTHRP (Kano et al., 1992). PTHRP also interacts with its receptors in bone to promote the proliferation and differentiation of osteoclast precursors resulting in increased bone resorption. Their activation by PTHRP is thought to occur indirectly through stimulation of osteoblastic cells which produce osteoclast- activating cytokines notably the RANKL system, because osteoclasts do not posses the PTH/PTHRP receptor (Weir et al., 1993). The combined actions of PTHRP on bone and kidney are responsible for the advanced stages of hypercalcemia.

The most convincing evidence for the physiologic role of PTHRP and its role as a modulator of chondrocytic proliferation and differentiation implicated in skeletal development comes from knockout studies (Amizuka et al., 1994; Karaplis et al., 1994). PTHRP gene ablation in mice was found to be lethal in the immediate postnatal period from respiratory failure due to profound abnormalities in endochondral bone formation. Marked skeletal deformities arising from impaired proliferation and premature differentiation of chondrocytes in the epiphyseal growth plate resulted in a mouse phenotype with short limbs due to rapid maturation of the skeleton (Amizuka et al., 1996; Amizuka et al., 1994; Lee et al., 1996). The complex processes resulting in normal endochondral bone development involve additional factors such as the hedgehog

signaling pathway with which PTHRP interacts (Lanske et al., 1996; Vortkamp et al., 1996). Further studies of the regulation of these pathways will enable a more in depth understanding of the pathogenesis underlying metabolic bone diseases and new directions for therapeutic interventions.

Chondrocyte-specific overexpression of PTHRP, using the mouse type II collagen promoter, induces a form of chondrodysplasia characterized by short-limbed dwarfism and a delay in endochondral ossification. A profound delay in chondrocyte differentiation gives rise to mice born with a cartilaginous endochondral skeleton (Weir et al., 1996). Jansens's metaphyseal chondrodysplasia is a rare autosomal dominant form of short limb dwarfism characterized by asymptomatic hypercalcemia and skeletal deformities (Calvi and Schipani, 2000). This rare disorder is caused by a constitutively activated PTH/PTHRP receptor leading to ligand-independent cAMP accumulation. An activating mutation of the PTH/PTHRP receptor was found in patients with Jansen's disease. Analysis of patient genomic DNA identified missense mutations that resulted in either substitution to arginine of a conserved histidine residue at position 223 in the receptor's first intracellular loop (Schipani et al., 1995) or in changes of threonine at position 410, to proline in the receptor's sixth membrane-spanning region (Schipani et al., 1996). The expression of constitutively active PTH/PTHRP receptors in kidney, bone, and growth-plate chondrocytes provides a genetic explanation for mineral-ion abnormalities and metaphyseal changes in patients with this disease. An inactivating mutation in the PTH/PTHRP receptor gene result in a rare and lethal form of dwarfism known as Blomstrand chondrodysplasia. Blomstrand chondrodysplasia is a genetic disorder characterized by extremely advanced endochondral bone maturation. This alteration

changes a strictly conserved proline residue at position 132 in the PTH/PTHRP receptor's amino terminal extracellular domain to leucine. These findings suggest that the Blomstrand form of human short-limbed dwarfism arises from defective PTH/PTHRP receptor signaling and confirms the importance of receptor signaling in human fetal skeletal development (Karaplis et al., 1998; Zhang et al., 1998).

There is strong evidence that PTHRP is the major regulator of placental calcium transport (Abbas et al., 1989; Rodda et al., 1988). The normal calcium concentration in fetal blood is higher than the maternal level. This calcium gradient is maintained by active transport of maternal calcium across the placenta. Produced by the fetal parathyroid glands, PTHRP has been identified as the active component that maintains the calcium gradient. Furthermore, the placenta is another source of PTHRP responsible for calcium transport in the early stages of gestation when the parathyroids are not fully functional. The calcium gradient is maintained primarily by the action of a placental calcium pump located in the basal plasma membrane of the trophoblast. It is suggested that the activity of this pump is stimulated by a mid-molecular fragment of PTHRP(38-94 amide), produced in the placenta and also in the parathyroid, as a result of post translational processing (Care, 1997; MacIsaac et al., 1991). Gene knockout studies, demonstrated the absence of the fetal- maternal calcium gradient in mice lacking a functional PTHRP gene. Infusion of PTHRP peptides (1-86), (67-86), but not (1-34) was able to increase calcium transport in these mice (Kovacs et al., 1996). In mice that lacked the classical PTH/PTHRP receptor gene, placental calcium transport was even more efficient than in the wild type littermates. This indicated that the bioactivity of PTHRP

for placental calcium transport is specified by a mid-molecular region that does not use the PTH/PTHRP receptor (Kovacs et al., 1996).

PTHRP present in mammary tissue before parturition and in milk, may assist in the development of the mammary gland during pregnancy (Rakopoulos et al., 1992). These high levels of PTHRP suggest that it may be involved in the transport of calcium to milk. It is likely that acting through an endocrine pathway, PTHRP promotes resorption of maternal bone and the retention of calcium by the kidneys, for milk production (Grone et al., 1994; Rakopoulos et al., 1992). Studies measuring bone density in lactating women revealed that women lose bone during lactation but gain bone after weaning; consequently, lactation may not result in a net bone loss (Kalkwarf and Specker, 1995).

The role of PTHRP in relieving muscle contraction has been investigated in smooth muscle of the arteries, of the uterus and of the urinary bladder. Mechanical stretch of cultured rat aortic smooth muscle cells induces a marked increase in gene expression of the vasorelaxant, PTHRP (Noda et al., 1997). Vasoconstrictors including angiotensin II, serotonin and bradykinin stimulated PTHRP expression, whereas the vasodilator atrial natriuretic peptide did not (Pirola et al., 1993). These results suggest that PTHRP acts locally in SMC, possibly to oppose the vasoactive and/or growth-promoting effects of vasoconstrictor agents.

PTHRP also induces uterine smooth muscle relaxation during gestation. In the rat uterus, PTHRP increases rapidly during the last stages of pregnancy when fetal growth is rapid (Thiede et al., 1990). Results were reproduced using uterine smooth muscle segments subjected to mechanical stretch in culture (Daifotis et al., 1992). The PTHRP N-terminal peptide, but not a mid-region peptide, was able to induce smooth muscle

relaxation in the uterus suggesting that this action of PTHRP is possible mediated through the PTH/PTHRP receptor (Barri et al., 1992). In the urinary bladder, PTHRP production directly correlates with bladder volume and the infusion of the N-terminal peptide was found to induce relaxation of bladder muscle (Yamamoto et al., 1992b). These observations implicate a physiological role for PTHRP in mechanical distension and in the regulation of smooth muscle.

In addition to its known role in chondrocytes, PTHRP can modulate proliferation and differentiation in a variety of other cell types. Using antisense RNA technology, PTHRP was found to induce cell proliferation and to suppress the expression of keratinocyte differentiation markers in non-malignant human keratinocyte cells (Kaiser et al., 1994). Supported by transgenic studies, the role of PTHRP in cellular differentiation and morphogenesis was studied. Targeted overexpression of PTHRP in the developing epidermis resulted in the arrest of ventral hair follicle development and reduced density of dorsal hair follicles (Wysolmerski et al., 1994). In the mammary gland, overexpression of PTHRP has been documented to prevent ductular proliferation and elongation in addition to the absence of terminal ductule formation during early pregnancy (Wysolmerski et al., 1995). These results suggest that PTHRP may determine morphology and development in these organs.

The growth promoting effects of PTHRP are often observed in malignant systems. Inhibiting the actions of PTHRP with receptor antagonists decreased the growth of human renal carcinoma cells (Burton et al., 1990). Blocking PTHRP synthesis by antisense RNA technology in a rat Leydig tumor cell model resulted in a significant reduction in cell proliferation *in vitro*, and decreased the growth of transplanted tumors *in*

vivo (Rabbani et al., 1995). Expression of PTHRP has frequently been shown to accelerate with tumor progression in human carcinomas implying the possibility that PTHRP may be involved in promoting tumor progression (Hidaka et al., 1998).

PTHRP has also been described as an anti-apoptotic agent. It has been suggested that PTHRP prevents apoptotic death, in differentiating chondrocytes, by inducing the expression of the Bcl-2 gene, an anti-apoptotic gene. The process of hypertrophied chondrocytes undergoing apoptosis, prior to calcification, was disrupted in transgenic mice with targeted overexpression of PTHRP. The retention of a large proportion of hypertrophic chondrocytes in the developing bones of these transgenic mice were observed (Amling et al., 1997; Weir et al., 1996). Collectively, these findings make a convincing argument that PTHRP plays multiple physiological and pathophysiologic roles.

Parathyroid Hormone Related Peptide and Prostate Cancer

The biological and clinical importance of PTHRP in cancer has become well established. PTHRP is the main factor responsible for hypercalcemia of malignancy. PTHRP expression has been demonstrated in tumors associated with hypercalcemia of malignancy, such as squamous cell carcinoma, renal cell carcinoma and breast carcinoma. PTHRP has also been found in tumors not typically associated with hypercalcemia such as colon, gastric, melanoma, and prostate cancer.

Prostate cancer is the second leading cause of cancer- related deaths and the most commonly diagnosed cancer in men in the western world (Coffey, 1993; Wingo et al., 1995). Prostate cancer also has a very high propensity to metastasize to bone. Treatment

of advanced prostate cancer aims at inhibiting cancer growth by suppression of endogenous androgen production or action (Catalona, 1994). However, almost all tumors eventually progress, due to the growth of androgen-independent cells and the development of hormone-refractory disease. A cascade of genetic alterations caused by the activation of oncogenes and the inactivation of tumor suppressor genes leads to the transition from hormone-dependent to hormone-independent tumorgenesis (Knudson, 1993).

The male accessory sex organ, the prostate gland, is strongly dependent on androgens for its normal development and physiological functions. It has become apparent that additional factors, including growth factors, neuroendocrine peptides, and cytokines may also play important roles in the development of the prostate and in maintaining its normal functions. Aberrant expression or derangement of these factors or of their receptors may be involved in the malignant transformation of prostatic epithelial cells (Steiner, 1995).

Androgens, primarily dihydrotestosterone (DHT) are responsible for the stimulation of prostate cancer growth. The testes secrete 90% of the testosterone produced in adult men; in addition, the adrenals provide the remaining 10% of the testosterone produced. Testosterone is converted to the metabolically more active DHT by the enzyme 5alpha-reductase in peripheral tissues (Ross et al., 1998). A large portion of DHT present in benign and malignant tissues is produced within the prostate gland from testicular testosterone. Androgen suppression can be obtained by elimination of the primary source of circulating androgens via orchiectomy and by direct inhibition of androgen synthesis and/or receptor binding at a cellular level.

Prostate tumor embodies heterogeneous subpopulations of cells with varying characteristics. Some of these cells are completely hormone- dependent and die when deprived of androgens. Other cells are hormone- sensitive, and grow faster when activated by androgens; these cells enter the resting phase (G_0) of the cell cycle when androgens are absent. Still other tumor cells are hormone- independent, and grow in the complete absence of androgen stimulation. The proportion of each of these three cell types greatly influences the initial response to androgen deprivation therapy. The tumor cell type controls whether remission, stabilization of the disease, or continued tumor progression will occur. The relative number of each cell type changes progressively in reaction to hormonal therapy. After initial hormonal deprivation, the hormone-independent cells continue to proliferate and gradually, repopulate the tumor. Consequently, hormonal therapy is nearly always followed by recurrence of symptoms and progression of the disease. At the time of the patient's death, hormone-independent cells comprise the majority of tumor cells.

Many molecular mechanisms regulate prostate carcinoma pathogenesis, proliferation, and progression to bone metastases. The basic molecular mechanisms are endocrine, paracrine, autocrine, and intracrine. These mechanisms can be mediated by a variety of agents, including gonadal and adrenal steroids, retinoic acid and vitamin D derivatives, neuroendocrine factors, growth factors, cytokines, and lymphokines. Prominent among these factors is PTHRP. The expression of this oncoprotein by prostate carcinoma has been demonstrated (Dougherty et al., 1999; Iwamura et al., 1993; Wu et al., 1998). PTHRP is also expressed by normal and hyperplastic prostate cells, and there is a gradient of expression that peaks in malignant prostate cells (Deftos, 2000). PTHRP

overexpression by prostate carcinoma cells has been implicated in tumor progression. In prostate carcinoma, it was observed that novel intracrine pathways mediated the effects of PTHRP and its various processed fragments (Deftos, 2000). These mechanisms influence cytokine expression, transduction of growth regulatory signaling pathways, proliferation, immunoregulation, and angiogenesis.

PTHRP expression was studied in the regulation of prostate carcinoma growth using human and animal models. During cellular proliferation, an increased expression of PTHRP was observed in prostate cancer cell *in vitro* (Dougherty et al., 1999). The effects of PTHRP overexpression on prostate tumor growth was determined using the MatLyLu rat prostate carcinoma model. PTHRP- overexpressing cells injected into rat hind limbs, significantly enhanced primary tumor growth and tumor size as compared with control cells. Immunohistochemical analysis performed on metastatic bone lesions of prostate carcinoma patients revealed that PTHRP protein was found in the cytoplasm and nucleus of cancer cells in the bone microenvironment. Nuclear localization of PTHRP has been associated with an inhibition of apoptosis; therefore, the ability of PTHRP to protect prostate cancer cells from apoptotic stimuli was examined. PTHRP transfected cells showed significantly increased cell survival after exposure to apoptotic agents as compared with cells producing no PTHRP or cells transfected with PTHRP lacking its nuclear localization signal (Dougherty et al., 1999).

Demonstrated by immunostaining, expression of PTHRP in the normal prostate, seems to be confined to a subpopulation of neuroendocrine cells that are dispersed throughout the prostatic epithelium (Wu et al., 1998). On the other hand, a strong diffuse staining is apparent in prostate cancer tissues. Differential expression of PTHRP isoforms

might exist in neuroendocrine cells and in cancer cells of the prostate. Overexpression of PTHRP may be involved in the early phase of malignant transformation and development of prostate cancer (Wu et al., 1998).

Prostate cancer frequently metastasizes to bone and it is characteristically associated with a high incidence of osteoblastic and osteolytic skeletal lesions. Presently, there is little known about the biological interaction between human prostate cancer cells and bone. The development of an animal model using adult human bone enhanced the ability to study the biology of prostate cancer metastasis to bone (Tsingotjidou et al., 2001). Bone, harvested from patients was implanted in mice. After implantation, prostate cancer cells were injected near the bone implantation site. Histological analysis revealed that prostate cancer cells homed to the reconstituted human bone marrow cavity. Analysis of the bone-tumor interaction after injection of prostate cancer cells revealed strong labeling for PTHRP, TNF alpha and IL-6, and showed evidence of osteoclast recruitment and osteoclast activity. This data suggest that the tumor cells may induce an osteolytic response to enhance their ability to metastasize to bone. This animal model allows the study of the biologic interaction between prostate cancer cells and human bone and it enhanced the understanding of the events associated with prostate cancer metastasis to bone.

Using a rat prostate cancer model, the role of PTHRP was assessed in contributing to bone breakdown and prostatic skeletal metastasis. Results demonstrate that PTHRP can increase osteoclastic osteolysis in the presence of focal osseous prostate cancer metastases and may contribute to the lytic lesions (Rabbani et al., 1999). PTHRP was identified among the bioactive prostate factors that appear to participate in

prostate carcinoma pathogenesis and progression. Understanding these regulatory interactions among prostate carcinoma, its cell products, and bone should therefore provide new insights into the pathogenesis of this disease.

Objectives of Thesis

Understanding the molecular and cellular basis for PTHRP over expression in malignancy is important in determining future avenues for therapy. The objectives of this thesis are to confirm previous findings and to examine the regulation of PTHRP in prostate cancer.

1) To characterize PTHRP gene expression in late stage prostate cancer tumor cells, PC-3 and PC-3T, *in vivo* and *in vitro* and understand its role in the progression of prostate cancer.
2) To directly examine and establish the role of androgens (DHT) in the molecular regulation of PTHRP production in prostate cancer.
3) To study the effects of the interaction of androgens and PTHRP on prostate cancer growth *in vivo* and *in vitro*.

Chapter 2

Androgen Regulation of Parathyroid Hormone Related Peptide (PTHRP) Production in Human Prostate Cancer Cells

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The study in this chapter is aimed at investigating the role of androgens in the regulation of PTHRP *in vitro* and *in vivo* using a prostate cancer cell model system. The study is presented in the form of a paper to be submitted for publication. I was responsible for all the experimental work present in this chapter. I greatly appreciate the advice of Dr. Rabbani and Dr. Goltzman in the design of the experiments and the expertise of Dr. Miao in immunohistochemical analysis.

Abstract

Androgen insensitive prostate cancer is known to be more aggressive than androgen responsive cancer. Parathyroid hormone related peptide (PTHRP) has been described in prostate tissues and higher concentrations of PTHRP have been found in neoplastic versus hyperplastic prostatic lesions. We therefore assessed the ability of androgens to regulate PTHRP in prostate cancer by using androgen insensitive human PC-3 prostate cancer cells and cells transfected with a functional human androgen receptor (PC-3T). PC-3T cells grew more slowly *in vitro* in whole serum or in charcoal stripped serum supplemented with dihydrotestosterone (DHT) than in stripped serum alone. Additionally, treatment of PC-3T cells with DHT or in the presence of whole serum resulted in the inhibition of PTHRP mRNA expression and its release into cell culture media as determined by immunoradiometric assay. These effects of DHT in decreasing PTHRP production were dose-dependent in nature and were readily blocked by co-incubation of PC-3T cells with DHT and with the androgen receptor antagonist flutamide.

In order to determine the effect of sex steroids on tumor growth and PTHRP expression *in vivo*, PC-3 and PC-3T cells were injected subcutaneously into male Balb/c nu/nu mice. Animals inoculated with PC-3T cells developed palpable tumors at a much later time (4 weeks) than did animals receiving wild type PC-3 cells (2 weeks). Inoculation of PC-3T cells in castrated host animals resulted in rapid tumor growth which closely resembled that of PC-3 tumor bearing animals. Determination of PTHRP mRNA expression and protein production in primary tumors of control and experimental animals

showed a decrease in PTHRP levels in PC-3T tumors, effects which were seen to be reversed in PC-3T tumors from castrated hosts.

The well characterized rat H-500 Leydig tumor cell model is known to express high levels of PTHRP. To determine the effects of androgens on PTHRP promoter activity, H-500 cells were transiently transfected with a PTHRP promoter- luciferase reporter construct. Cells treated with DHT or with whole serum resulted in a 30% decrease in luciferase activity. These results indicate that prostate tumor cell growth correlates inversely with androgen sensitivity and directly with PTHRP production *in vitro* and *in vivo* in this model, that androgens can directly regulate PTHRP production, and that the androgen effect is mediated by transcriptional regulation via the androgen receptor.

Introduction

Prostate carcinoma is the leading hormone- dependent malignancy affecting males in North America and results in a high incidence of cancer- related deaths (Deftos, 2000; Zaccagnini, 1999). This hormone- dependent malignancy is characteristically associated with a high incidence of osteoblastic as well as osteolytic skeletal lesions (Chackal-Roy et al., 1989; Haq et al., 1992; Rabbani et al., 1999). Malignant transformation of prostatic epithelial cells may be the result of aberrant expression of growth factors or of their receptors (Damon et al., 2001; Kim et al., 1999; Tang et al., 1999). Androgens play an important role in the proper development and physiological function of the prostate (Brinkmann, 2001; Slater and Oliver, 2000). The actions of androgens on target cells are initiated by the binding of the steroid to a stereospecific high affinity intracellular receptor. Due to a conformational change, the androgen- bound receptor complex is then able to interact with specific androgen response elements (ARE) in the promoter region of target genes that modulate cellular growth and differentiation. Testosterone, synthesised in the testes and to a lesser extent in the adrenals, is converted to DHT by the enzyme 5α- reductase, which is localized mainly in the prostate (Lobaccaro et al., 1997; Shiina and Igawa, 1997). Although testosterone and DHT both stimulate the growth of normal and malignant prostate tissue, DHT is believed to be the more potent androgen (Klus et al., 1996). Total androgen ablation therapy in combination with anti- androgens has been shown to produce the most beneficial responses in patients with hormone-responsive prostatic tumors (Denis, 1994). However, most patients experience disease progression due to the development of an androgen- independent tumor associated with a lack of or mutation of the androgen receptor (Veldscholte et al., 1992).

Originally discovered as a product of tumors that produce hypercalcemia, parathyroid hormone related peptide (PTHRP) has been demonstrated to be a product of many malignant tissues, including prostate carcinoma. PTHRP is also widely distributed in many normal adult and fetal tissues where it exerts an autocrine or paracrine effect in modulating cellular growth and differentiation (Juppner et al., 1991). Suggested physiological roles of PTHRP include regulation of ion transport (Kovacs et al., 1996; Orloff et al., 1996), smooth muscle relaxation (Martin et al., 1997) and modulation of cell growth and differentiation of a variety of tissues. The biological effects of PTHRP are mediated at least in part, through a cell surface seven transmembrane spanning G-protein coupled receptor that it shares with parathyroid hormone (PTH), the type I PTH/PTHRP receptor (PTHR) (Juppner et al., 1991). Several growth factors and steroid hormones have been shown to regulate PTHRP production in tumor cells (Falzon, 1996b; Holt et al., 1994; Sebag et al., 1994). Compounds which induce PTHRP production include fetal bovine serum (FBS) and a variety of growth factors whereas, dexamethasone, vitamin D and androgens suppress PTHRP in a number of cell types (Glatz et al., 1994; Liu et al., 1993). Normal prostate epithelial cells produce PTHRP; however, PTHRP is found in higher levels in prostate carcinoma than in benign prostatic hyperplasia or normal cells.

In the current study we examined the regulation of PTHRP production by androgens in the androgen- insensitive human prostate cancer cell line PC-3, which lacks a functional androgen receptor, and in androgen sensitive PC-3 cells transfected with the full length human AR complementary DNA (PC-3T) (Yuan et al., 1993). The effects of androgen on tumor cell growth and PTHRP production were evaluated both *in vitro* and *in vivo*.

Materials and Methods

Cell Culture.

Human prostate cancer cell line PC-3 was obtained from the American Type Culture Collection (Rockville, MD). PC- 3 cells transfected with a full- length, functional human androgen receptor cDNA (PC-3T) were kindly provided by Dr. T. J. Brown (Toronto Hospital Research Institute, Toronto, Canada). Rat Leydig tumor cells H-500 were obtained from the E. G. and G. Mason Research Institute (Worcester, MA). All cell types were maintained *in vitro* in RPMI- 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10 % fetal bovine serum (FBS), 100 U/ml of penicillin-streptomycin sulfate (GIBCO- BRL). PC-3T cells were maintained under selection by supplementing the media with 100 μg/ml hygromycin B (Sigma Chemical Co., St. Louis, MO). Cells were incubated at 37°C in 5% $C0_2$.

Cell Proliferation Assays.

PC-3 and PC-3T cells were plated in triplicate at a density of 10, 000 cells per well, in 2 ml of media, in 6- well plates. Cells were grown in FBS or in charcoal-stripped FBS, depleted of androgens. Where indicated, PC-3T cells in stripped serum were treated with either 100 nM DHT alone or with DHT and 1 μM flutamide. Cells were trypsinized and counted, at the times indicated, using a Coulter Counter (model ZF, Coulter Electronics, Harpenden, Herts, UK). Cell culture medium was replenished every third day.

Reverse Transcriptase-PCR.

Total RNA was extracted from PC-3 and PC-3T cells by a single-step method using Trizol reagent. The reaction template, denatured for 10 minutes at 70°C, consisted of 2 μg of total RNA in 10μL of DEPC H_2O. To the reaction mixture, 1X PCR buffer (MBI Fermentas), dNTP mix 0.2 mM each (MBI Fermentas), 10 units of RNasin inhibitor (Amersham Pharmacia Biotech Inc.) and of M-MuLV Rtase were added. The RNA was reversed transcribed for 1 hour at 42°C. Using 2.5 units of Taq DNA polymerase (Gibco BRL), the product was amplified by PCR, for 25 cycles (melting 94°C, annealing 55°C, extension 72°C, each for 1 minute followed by an extension cycle at 72°C for 10 minutes). The following primers (0.5 μM) were used to amplify PTHRP: forward 5'-GAGCACCAGCTACTGCATGACAAG-3' and reverse 5'-CTGAGTTAGGTATCTGCCCTCGT-3'. The amplified PCR product was fractionated on a 1.3 % agarose gel and visualized by ethidium bromide staining. Band intensities for PTHRP were quantitized using a densitometric software, Quantity One (BioRad).

Northern blot analysis.

Total cellular RNA was isolated by Trizol extraction from control and experimental PC-3 and PC-3T cells and tumors after treatment in the presence of absence or androgens. Briefly, 20 μg of total cellular RNA was electrophoresed on a 1.1% agarose-formaldehyde gel, transferred to a nylon membrane (Nytran; S&S, Keene, NH) by capillary blotting, and then fixed by drying and ultraviolet cross- linking for 10 min. The integrity of the RNA was assessed by ethidium bromide staining. Hybridization was carried out with [^{32}P]dCTP-labeled human PTHRP and 18S cDNA. After 24 hour

incubation at 65°C, filters were washed twice under low- stringency conditions [1x standard sodium citrate (SSC) and 1% SDS at 60°C for 2x 20 min] and under high stringency conditions [0.1x SSC and 0.1% SDS at 60°C for 2 x 20 min]. Autoradiography of filters was carried out at -70°C using Biomax film (Eastman Kodak Co., Rochester, NY) with two intensifying screens. The level of PTHRP and of 18S messenger RNA (mRNA) expression were quantified using the densitometric software, Quantity One (BioRad, Mississauga, Can.).

Immunocytochemistry and *immunohistochemistry.*

PC-3 and PC-3T cells, cultured in 35 mm plates, and paraffin tumor sections were stained immunocyto/histochemically for PTHRP as previously described (He et al., 2001) using the avidin-biotin-peroxidase complex (ABC) technique. Rabbit antiserum against PTHRP (1–34) peptide was applied to cells and tumor sections overnight at room temperature. As the negative control, the primary antibody was omitted from the reaction mixture. After washing with high TBS salt buffer (50 mM Tris-HCl, 2.5% NaCl, 0.05% Tween 20, pH 7.6) for 10 min at room temperature followed by two 10-min washes with TBS, the samples were incubated with secondary antibody (biotinylated rabbit antigoat IgG; Sigma), washed as before and processed using the Vectastain ABC-AP kit (Vector Laboratories, Inc.). Red pigmentation to demarcate regions of immunostaining was produced by a 10- to 15-min treatment with Fast Red TR/Naphthol AS-MX phosphate (Sigma, containing 1 mM levamisole as endogenous alkaline phosphatase inhibitor). The sections were then washed with distilled water, counter-stained with methyl green, and mounted with Kaiser's glycerol jelly.

Computer-assisted image analysis.

After immunostaining of culture cells and tumor sections, computer-assisted image analysis was performed as described previously (Miao et al., 2001a). Briefly, images of stained culture dishes and tumor sections were photographed with transmitted light over a light box. All images were taken with a Sony digital camera and processed using Northern Eclipse image analysis software, version 5.0 (Empix Imaging Inc., Mississauga, ON, Canada). For determining the area of positive cells, thresholds were set using green and red channels. The thresholds were determined interactively and empirically on the basis of three different images. Subsequently, this set threshold was used to automatically analyze all recorded images of all samples that were stained in the same staining session under identical conditions. The area of immunocytochemical or immunohistochemical stained regions was calculated automatically by the software in each microscopic field. Pixel counts (summary total gray) of immunoreaction product was calculated from thresholding of each microscopic field area of 0.8 mm^2. These data reflect the relative amount of PTHRP as measured by immunostaining intensity. The software therefore determines summary total gray as the intergrated immunostaining intensity over a given area. Data from image analysis of stained cell cultures and tumor sections are presented as means ± SEM of four different samples.

Immunoradiometric assay.

PTHRP secreted in the conditioned media was measured using a non-competitive, two- sided immunoradiometric technique IRMA (Diagnostic Systems Laboratories, Webster, Texas). PTHRP is recognized by an NH_2-terminal reactive

antibody (raised against PTHRP (1-34)) immobilized on a solid phase (capture antibody), and with a second, I^{125} radiolabeled COOH- terminal reactive antibody (raised against PTHRP (47- 86)), for detection (signal antibody). PC-3 and PC-3T cells were plated at a density of 10^6 cells in 10 ml in 60 mm plates. At 70% confluence, cells were deprived of serum for 24 hours and then treated in the presence or absence of androgens for 12 hours. The conditioned media was collected and the final cell number was determined using a coulter counter. The assay was carried out per the manufacturer's specifications. The reaction mixture was incubated at room temperature for 20 hours, washed with deionized water and the radioactivity from each sample was counted on a gamma counter for 1 minute. The detection limit of the assay is 0.3 pmol/L (3.0 pg/mL).

Luciferase and ß-Galactosidase (ß-gal) Assays.

Transient transfection for promoter assays was performed by the lipofectamine method (Gibco BRL, Burlington, Can.). In brief, 0.8 x 10^6 H-500 cells were plated in 35-mm dishes a day before transfection. The cells were co- transfected with 1 μg of pGL3 vector, containing the PTHRP rat promoter upstream of the luciferase reporter gene, and 1 μg pCMV-ß- galactosidase reporter plasmid to determine transfection efficiency. The transfected cells were washed the next day and allowed to grow for 24 hours in medium containing 10% FBS. After cells were serum deprived for 24 hours, they were treated with media containing FBS, SFBS or SFBS plus 100 nM DHT. The cells were harvested by scraping and used for luciferase assays as described. Total light emission during the initial 10 seconds was measured in a luminometer. The β-galactosidase assay was performed with the same samples as described. All luciferase assay readings were

performed in triplicate and were corrected for ß- galactosidase expression levels in each cell population.

Animal protocols.

Six-week-old male BALB/c nu/nu male normal and castrated mice were obtained from Charles River Laboratories, Inc. (St. Constant, Québec, Canada). Before inoculation, PC-3 and PC-3T tumor cells, grown in serum-containing medium, were washed with Hanks' buffer and were trypsinized for 5 min. Cells were then collected in Hanks' buffer and centrifuged at 1500 rpm for 5 min. Cell pellets (3×10^6 cells) were resuspended in 200 µl of a matrigel and saline mixture (20% matrigel) and injected subcutaneously into the flank region of the hind leg of the mice. All animals were numbered, kept separately and examined for the development of tumors weekly. The tumor mass was measured in two dimensions by calipers, and the tumor volume was calculated according to the equation $(l \times w^2)/2$. Animals were sacrificed and the tumors were isolated.

Statistical analysis.

Results are expressed as the mean ± SEM of at least triplicate determinations. Statistical comparisons were made using the Student's *t* test or ANOVA, with a probability of less than 0.05 being considered significant.

Results

Effect of androgens on prostate cancer cell growth in vitro.

A cell proliferation assay was performed to study the effects of restoration of androgen responsiveness on the growth rate of PC-3T cells in comparison to the wild type PC-3 cells. PC-3T cells showed a 45% reduction in proliferation rate throughout the course of the study. No significant difference in cell growth was seen between PC-3 cells and cells mock transfected with an empty vector (data not shown). PC-3T cells showed a 30% increase in proliferation rate when grown in media containing charcoal- stripped FBS, depleted of androgens (Fig. 1). Media containing SFBS supplemented with 100 nM DHT resulted in a decrease in PC-3T cell growth to a rate comparable to PC-3T cells incubated in media containing FBS. The specificity of the androgen receptor in mediating this androgen growth inhibitory effect was confirmed by co-incubation of PC- 3T cells with 100 nM DHT and 1.0 μM AR antagonist, Flutamide (FLU), where the DHT growth inhibition was abolished (Fig. 1).

Effect of androgens on PTHRP gene transcription.

To examine the effects of the restoration of androgen responsiveness on PTHRP mRNA expression, reverse transcriptase PCR and Northern blot analysis were performed on PC-3 and PC-3T cultured *in vitro* in the presence or absence of androgens. Cells in serum free conditions for 24 hours exhibited a maximal induction in PTHRP mRNA levels after a 12 hours treatment (data not shown). PC-3T cells, incubated in FBS, showed a 35% reduction in PTHRP mRNA levels compared to the parental cell line, PC-3 also incubated in FBS (Fig. 2). PC-3T cells cultured in charcoal-stripped FBS

increased PTHRP mRNA levels by 24%; subsequently, the addition of 100 nM DHT reduced mRNA production to a level comparable to FBS treated PC-3T cells (Fig. 2A). Similar results were observed when Northern blot analysis was performed on the experimental groups. PTHRP mRNA levels were higher in PC-3 cells by 40% than in androgen- responsive, PC-3T cells. Removal of androgens from the culture media of PC-3T cells caused mRNA levels to increase by 30%; furthermore, cells treated with DHT resulted in a substantial reduction in PTHRP mRNA levels (Fig. 2B).

The effect of androgens on PTHRP production.

An immunocytochemical assay specific for PTHRP (1- 34) showed strong staining in the cellular nucleus and cytoplasm of PC-3 cells. The PTHRP specific color staining was quantitated and results clearly indicated that PTHRP protein levels were 45% greater in PC-3 cells than in PC-3T cells (Fig. 3). PTHRP protein levels produced by control and experimental cells were determined by an immunoradiometric assay (IRMA) that measures PTHRP (1- 86) secreted into the conditioned media. PC-3 cells produced 60% more PTHRP than the protein levels produced by androgen- sensitive PC-3T cells. As observed for the mRNA levels, protein levels produced were increased by 35% by incubating the cells in media containing stripped- FBS. This increase in PTHRP production could be inhibited by treating PC-3T cells with 100 nM DHT for 12 hours (Fig. 4). The specificity of the effect of androgen on PTHRP production was further evaluated by treating PC-3T cells with different concentrations of DHT. IRMA measuring PTHRP (1-86) secreted into conditioned media indicated that DHT, at

increasing concentrations from 1 to 100 nM, inhibited PTHRP production in a dose-dependent manner (Fig. 5).

Androgen regulation of PTHRP promoter activity.

The effect of androgens on PTHRP promoter activity was studied using the rat Leydig tumor cell line, H-500, that produce high levels of PTHRP and contain endogenous androgen receptor. The transient transfection of pGL3 vector containing the rat PTHRP promoter upstream of the luciferase reporter gene allowed measurement of promoter activity in response to androgen treatment. The pGL3 vector not containing the PTHRP promoter was used as a control and the relative luciferase activity measured was calculated as fold increases from the empty vector. The highest PTHRP promoter activity was observed for experimental cells incubated in SFBS, in the absence of androgens. H-500 cells treated with FBS or treated with SFBS + 100 nM DHT resulted in a 35% reduction in PTHRP promoter activity (Fig. 6).

Effect of androgens on tumor growth in vivo and tumoral PTHRP mRNA and protein levels.

Subcutaneous inoculation of the wild- type PC-3 cells into the right flank of 6-weeks old male BALB/c nu/nu mice resulted in the development of palpable tumors by week 2 post tumor cell inoculation. In contrast, development of primary tumors was significantly delayed when the animals were inoculated with PC-3T cells, where palpable

tumors were only detected at week 5, post tumor inoculation. Furthermore, the tumor volumes recorded, on week 9, was 50% less than that for PC-3 tumors. To evaluate the effects of androgen ablation on PC-3T tumor development, PC-3T cells were injected into castrated animals. Castrated nude mice developed palpable tumors by week 4 but more importantly the tumor volumes were significantly larger than the PC-3T tumor volumes recorded for non- castrated animals (Fig. 7).

The primary tumors were removed and RT-PCR was performed on the RNA isolated to measure the relative PTHRP mRNA levels. PC-3T tumors showed lower levels of PTHRP mRNA expression compared with PC-3 tumors. Moreover, the tumoral level of PTHRP mRNA was significantly higher in castrated animals bearing PC-3T tumors than compared to non-castrated animals (Fig. 8).

An immunohistochemical reaction specific for PTHRP (1-34) was performed on tumor sections obtained from each of the 3 animal groups. Intense color staining, of PC-3 tumor cells was observed and quantitated. PC-3T tumor sections exhibited a greater than 60% reduction in PTHRP immuno- staining when compared to PC-3 tumor cells. PC-3T tumor sections isolated from castrated animals showed a 45% increase in staining specific for PTHRP when compared to non- castrated animals (Fig. 9).

Collectively, our results demonstrate that restoration of hormone responsiveness in PC-3 cells resulted in a decrease in cellular proliferation *in vitro* and a decrease in tumor growth *in vivo*. These results also demonstrate that androgens regulate PTHRP production, which may play a key role in prostate tumor growth.

Figure 1. Effect of androgens on the proliferation of PC-3T cells.

PC-3 and PC-3T cells (10^4) were grown in RPMI- 1640 media containing FBS or stripped FBS in 6- well plates. PC-3T cells incubated in stripped serum were treated with 100nM DHT alone or in combination with 1μM flutamide. At various time points, cells in groups of triplicates, were trypsinized and counted using a coulter counter. Bars represent the mean ± SEM of three experiments. *Significant differences in the growth from control PC-3 cells ($P < 0.05$); ** Significant differences in the growth from PC-3T cells in FBS ($P < 0.05$).

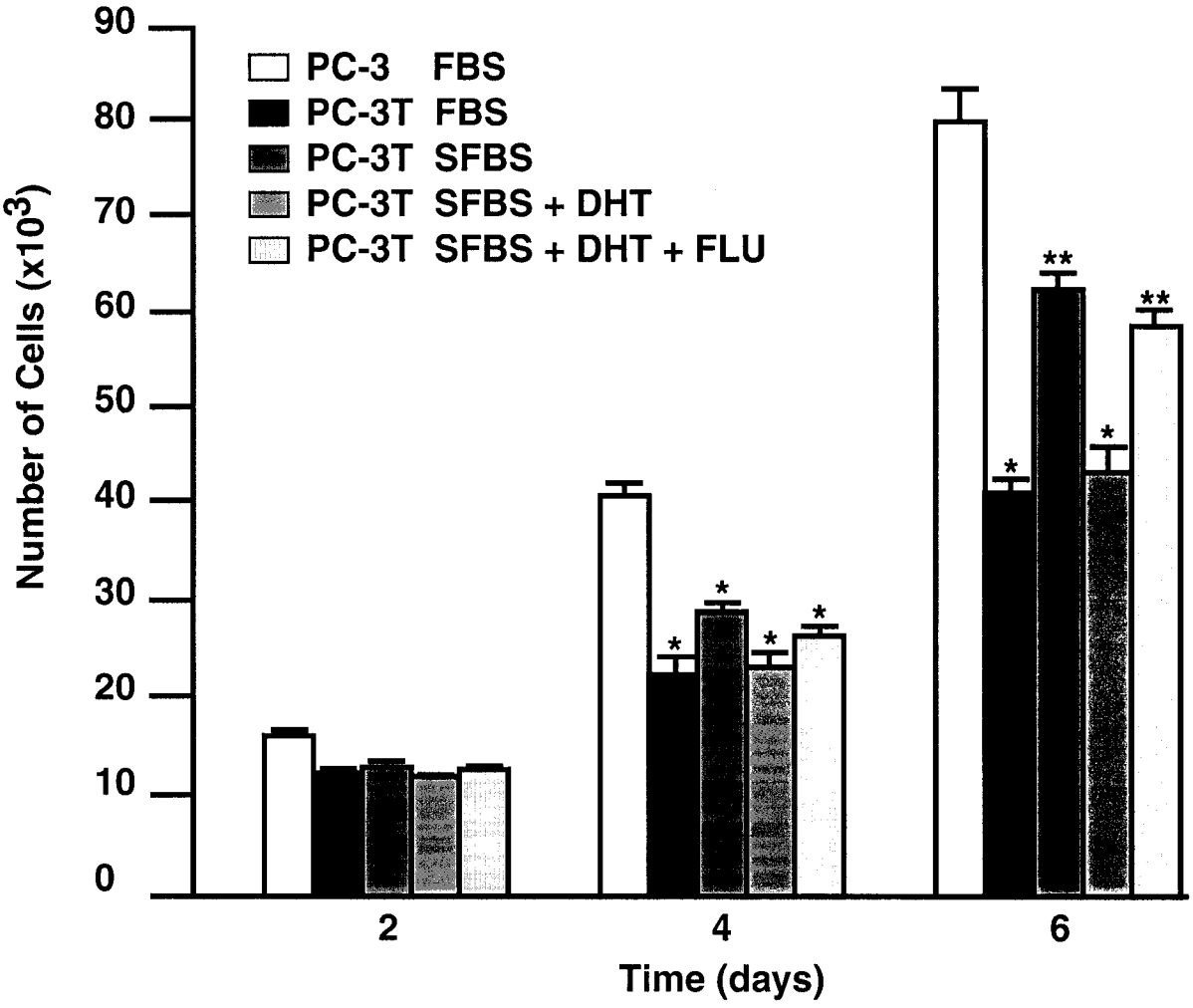

90
80
70
60
50
40
30
20
10
0
Number of Cells (x10^3)
PC-3 FBS
PC-3T FBS
PC-3T SFBS
PC-3T SFBS + DHT
PC-3T SFBS + DHT + FLU
*
**
2
4
6
Time (days)

Figure 2. Effect of androgens on PTHRP gene expression in PC-3T cells.

PC-3 and PC-3T cells were grown to 70% confluence in RPMI media containing FBS and incubated overnight in serum- free conditions. Cells were then treated for 12 hours in media containing FBS, stripped FBS or in media containing stripped FBS with 100 nM DHT. A: Level of PTHRP and GAPDH mRNA determined by RT- PCR as described in Materials and Methods. Results are expressed as the ratio of PTHRP to GAPDH mRNA. B: Level of PTHRP and 18S mRNA determined by Northern blot analysis as described in Materials and Methods. Results represent the ratio of PTHRP to 18S mRNA. Bars represent the means ± SEM of 3 different experiments. *Significant differences in the ratios from control PC-3 cells ($p< 0.05$); ** Significant differences in the ratios from PC-3T cells in FBS ($P < 0.05$).

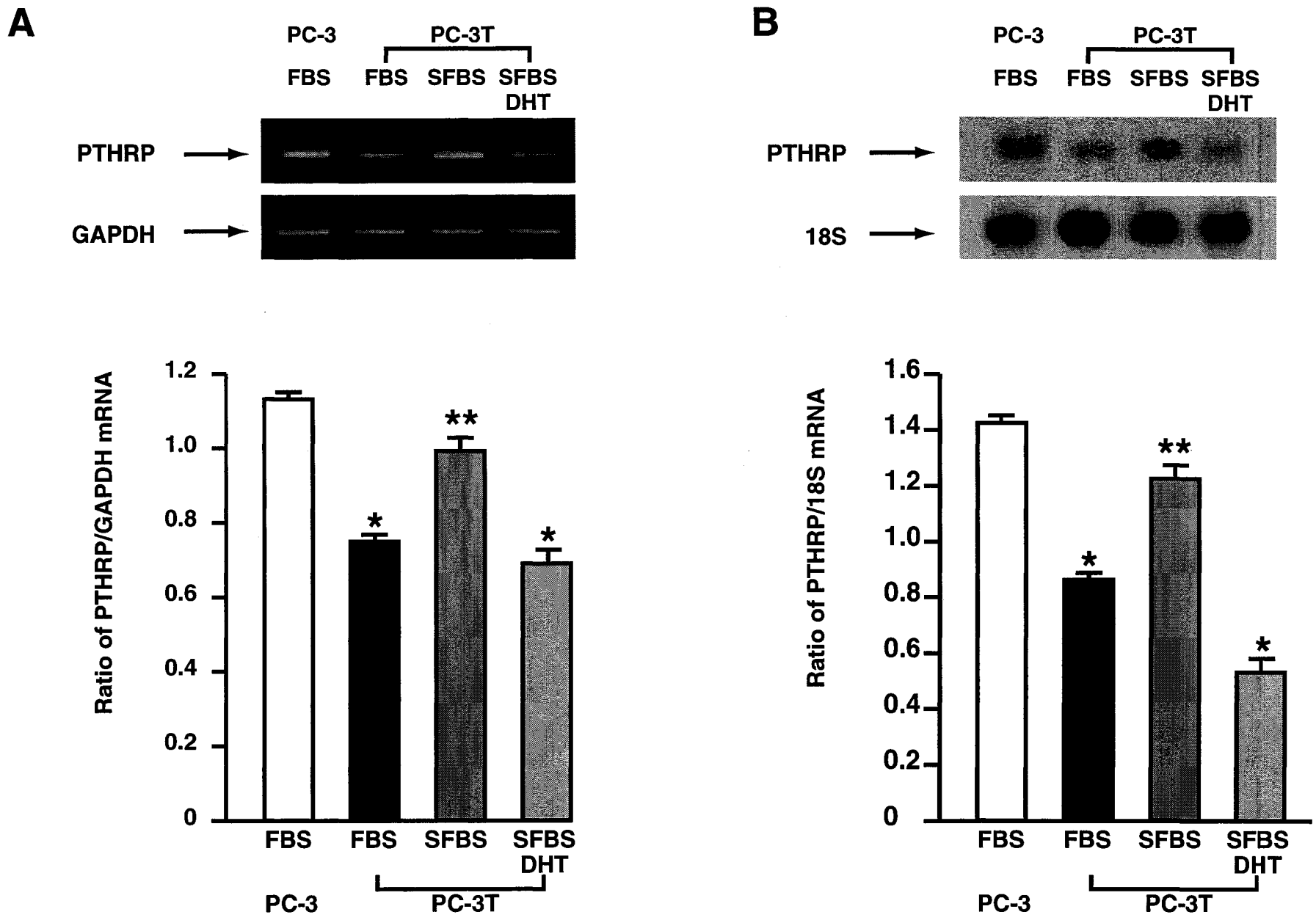
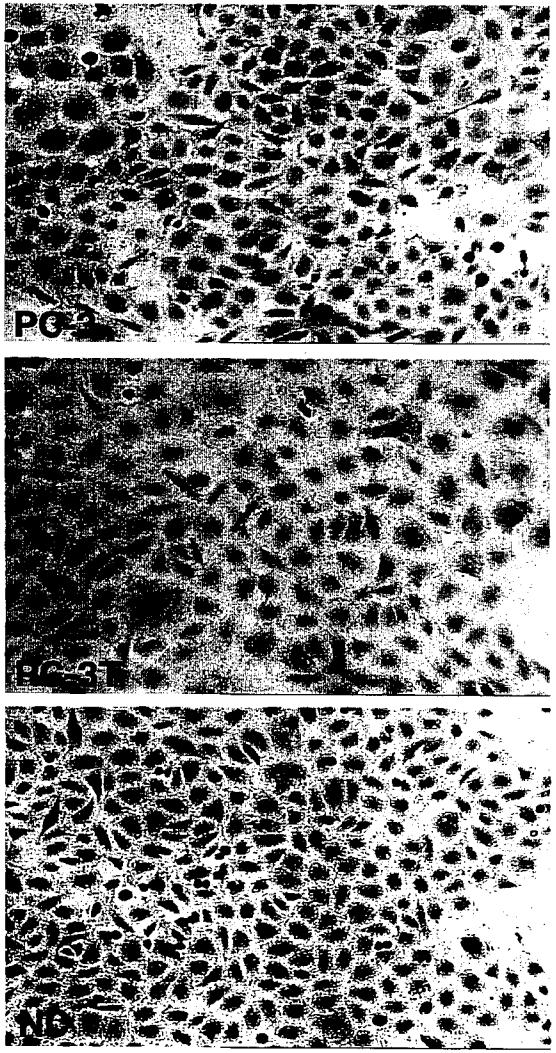

A
PC-3
PC-3T
FBS
FBS
SFBS
SFBS DHT
PTHRP
GAPDH
Ratio of PTHRP/GAPDH mRNA
1.2
1.0
0.8
0.6
0.4
0.2
0
*
**
*
FBS
FBS
SFBS
SFBS DHT
PC-3
PC-3T
B
PC-3
PC-3T
FBS
FBS
SFBS
SFBS DHT
PTHRP
18S
Ratio of PTHRP/18S mRNA
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
**
*
FBS
FBS
SFBS
SFBS DHT
PC-3
PC-3T

Figure 3. PTHRP protein expression in PC-3 and PC-3T cells.

PC-3 and PC-3T cells were grown to 80% confluence in RPMI media containing FBS. Cells were fixed and examined microscopically. A: PTHRP protein expression was determined by an immunocytochemical reaction as described in Materials and Methods. The negative control (NC) resulted from the omission of the primary antibody specific to PTHRP (1-34). B: The immunocytochemical reaction was quantified as described in Materials and Methods. Quantification is presented as summary total gray demonstrating a positive reaction. Results represent the means ± SEM of 3 different experiments. *Significant differences in immunostaining value from control PC-3 ($P < 0.05$).

A



B

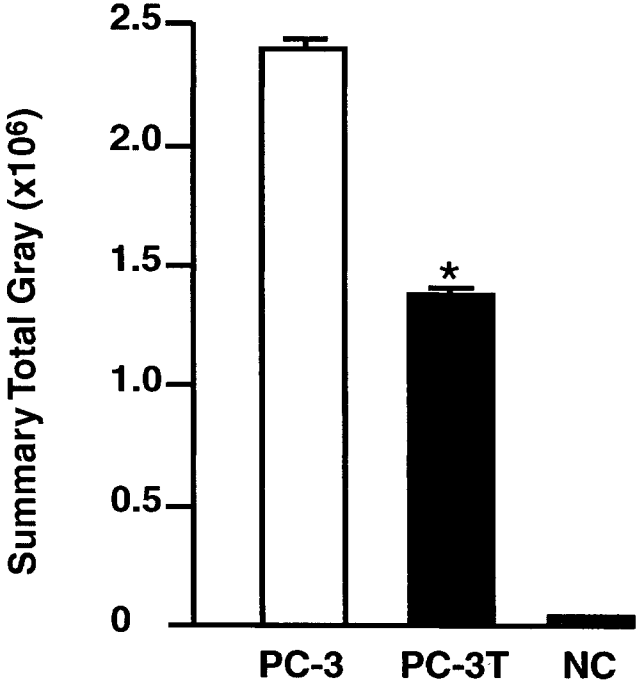


Figure 4. Effects of androgens on the production of immunoreactive PTHRP in PC-3 and PC-3T cells.

Cells were grown to 70% confluence in RPMI media containing FBS and incubated overnight in serum- free conditions. Cells were then treated for 12 hours in media containing FBS, stripped FBS or in stripped FBS with 100nM DHT. PTHRP (1-86) secreted in conditioned media was determined using IRMA as described in Materials and Methods. Results are expressed as picograms of PTHRP (1-86) per 10^6 cells per 12 hours. Bars represent the means ± SEM of 3 different experiments. *Significant differences in PTHRP values from control PC-3 cells ($P< 0.05$); ** Significant differences in PTHRP values from control PC-3T cells in FBS ($P< 0.05$).

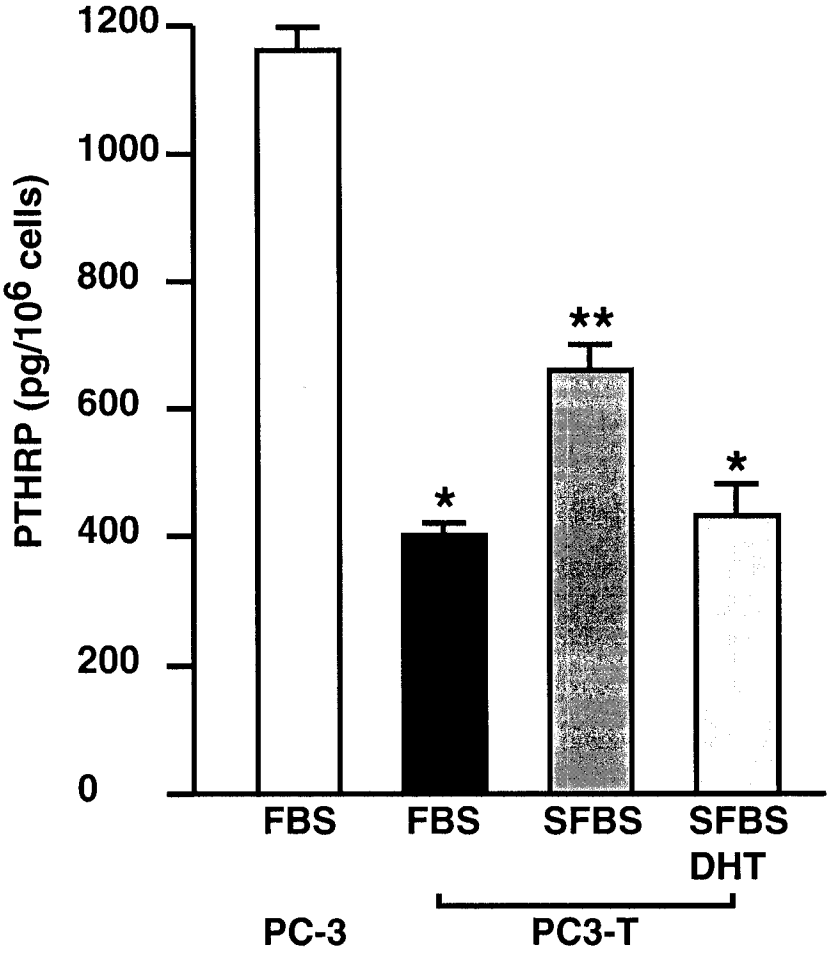

1200
1000
800
600
400
200
0
PTHRP (pg/10^6 cells)
*
**
*
FBS
FBS
SFBS
SFBS
DHT
PC-3
PC3-T

Figure 5. Effects of DHT on PTHRP production in PC-3T cells.

PC-3T cells were grown to 70% confluence in RPMI media containing FBS and incubated overnight in serum- free conditions. Cells were then treated for 12 hours in media containing stripped FBS with different concentrations of DHT (1, 10, 100nM). PTHRP (1-86) secreted in conditioned media was determined using IRMA as described in Materials and Methods. Results are expressed as picograms of PTHRP (1-86) per 10^6 cells per 12 hours. Bars represent the means ± SEM of 3 different experiments. *Significant differences in PTHRP values from control PC-3T cells in SFBS alone ($P < 0.05$).

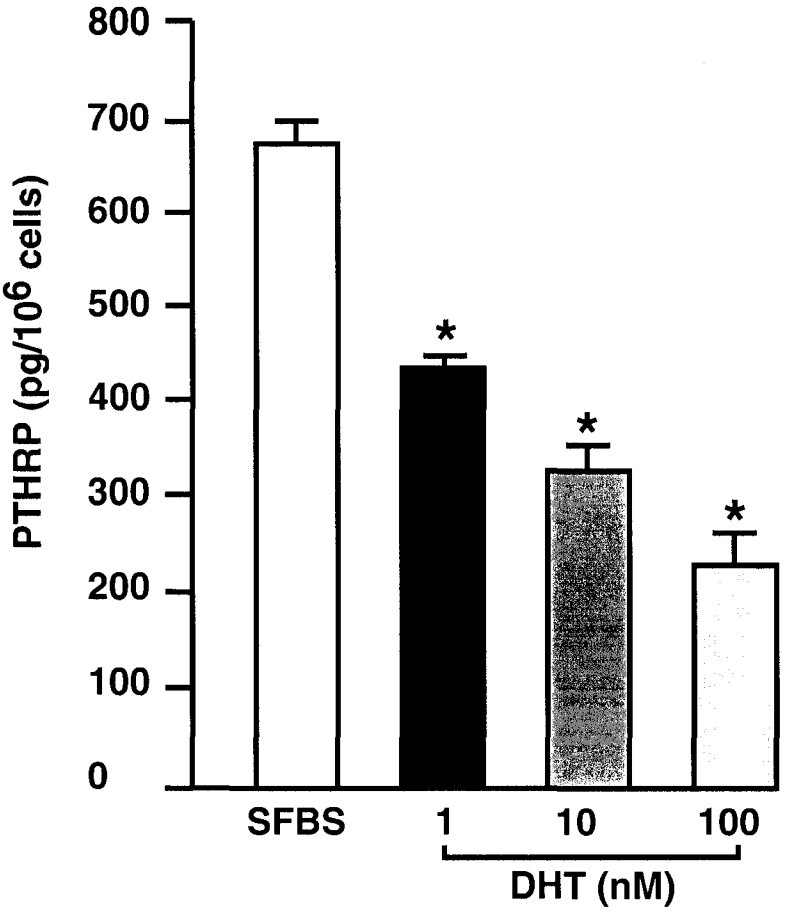

800
700
600
500
400
300
200
100
0
PTHRP (pg/10^6 cells)
*
*
*
SFBS
1
10
100
DHT (nM)

Figure 6. Effects of androgens on PTHRP promoter activity.

Rat Leydig tumor cells H-500 were grown to 50- 70% confluency. Cells were transiently co- transfected with pGL3 vector containing the rat PTHRP promoter upstream a luciferase reporter gene and with basic β- galactosidase vector. Cells were incubated in FBS for 24 hours and then incubated in serum free conditions for 24 hours. Subsequently cells were treated for 1 hour with media containing FBS, SFBS or SFBS and DHT (100nM). Luciferase activity was quantified using a luminometer and results were normalized for transfection efficiency as measured by β- galactosidase reaction. Values represent fold increase in luciferase activity as compared to control pGL3 vector containing no PTHRP promoter. Results represent means ± SEM of 3 different experiments. * Significant differences from control H-500 cells in FBS ($P < 0.05$).

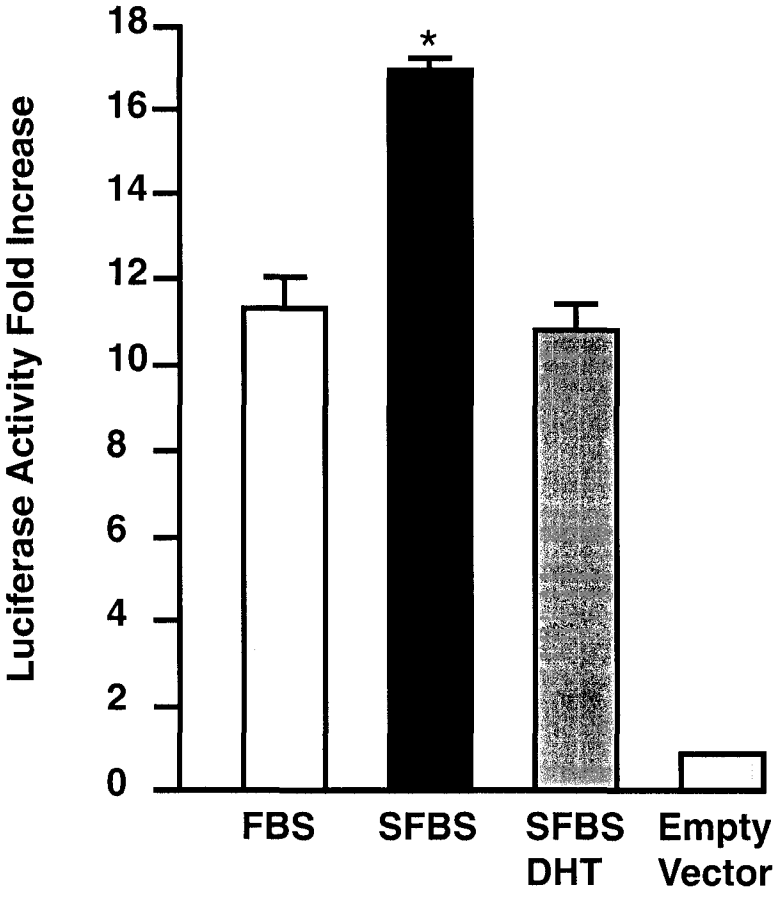

Luciferase Activity Fold Increase
18
16
14
12
10
8
6
4
2
0
*
FBS
SFBS
SFBS DHT
Empty Vector

Figure 7. Effect of androgens on the growth of human prostate tumor cells in nude mice.

PC-3 and PC-3T cells (1 x 10^6 cells in 20% matrigel) were injected subcutaneously in the right flank of nu/nu BALB/c male mice. Additionally, PC-3T cells were similarly injected into castrated male animals. Tumors were measured weekly using caliper and tumor volumes were calculated according to the equation $(l \times w^2)/2$. Results represent means ± SEM of 3 different experiments with 6 animals in each group. * Significant differences in tumor growth from control animals inoculated with PC-3 cells; ** Significant differences in tumor growth from non- castrated animals inoculated with PC-3T cells ($P < 0.05$).

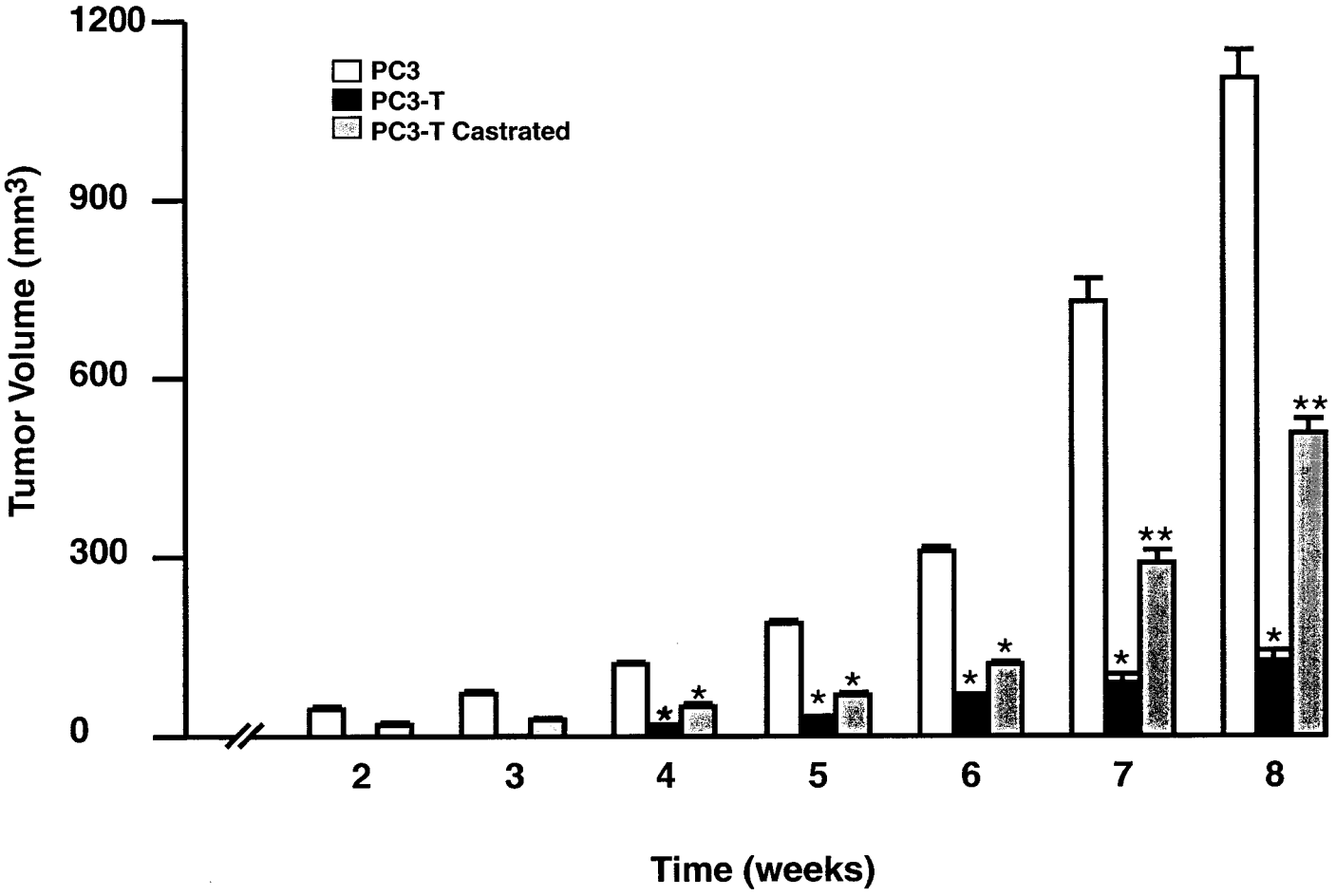
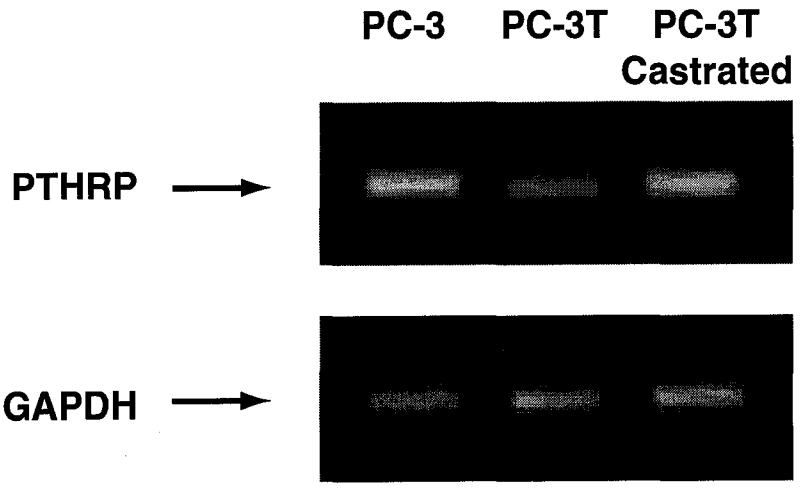

PC3
PC3-T
PC3-T Castrated
Tumor Volume (mm3)
1200
900
600
300
0
2
3
4
5
6
7
8
Time (weeks)
*
**

Figure 8. Effect of androgens on PTHRP gene expression in tumors.

Total cellular RNA extracted from tumors of normal male animals inoculated with PC-3 and PC-3T cells and from tumors of castrated animals inoculated with PC-3T cells. Top: Level of PTHRP and GAPDH mRNA determined by RT- PCR as described in Materials and Methods. Bottom: Results are expressed as the ratio of PTHRP to GAPDH mRNA. Bars represent the means ± SEM of 3 different experiments. *Significant differences in the ratios from control PC-3 tumors ($P < 0.05$).


PC-3
PC-3T
PC-3T
Castrated
PTHRP
GAPDH

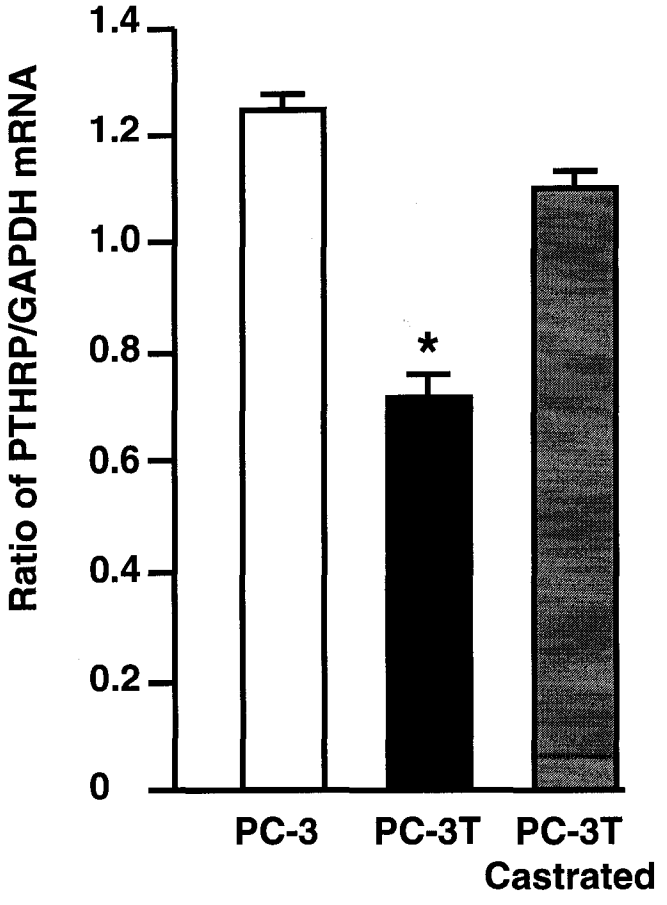
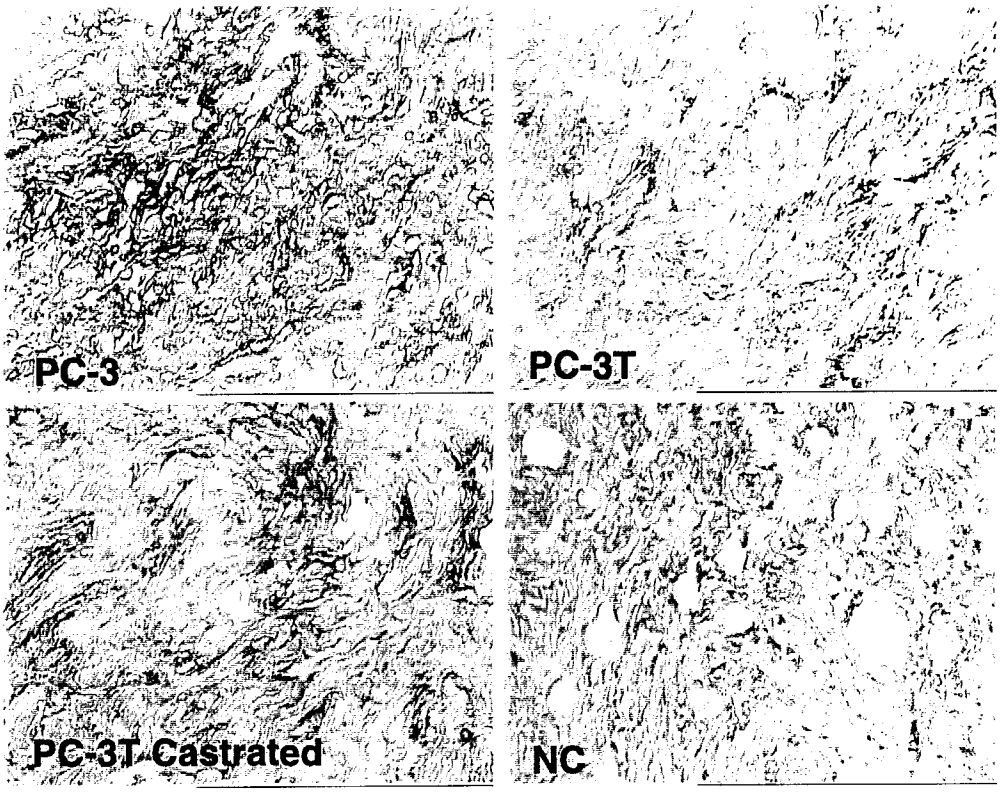

Ratio of PTHRP/GAPDH mRNA
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
PC-3
PC-3T
PC-3T
Castrated

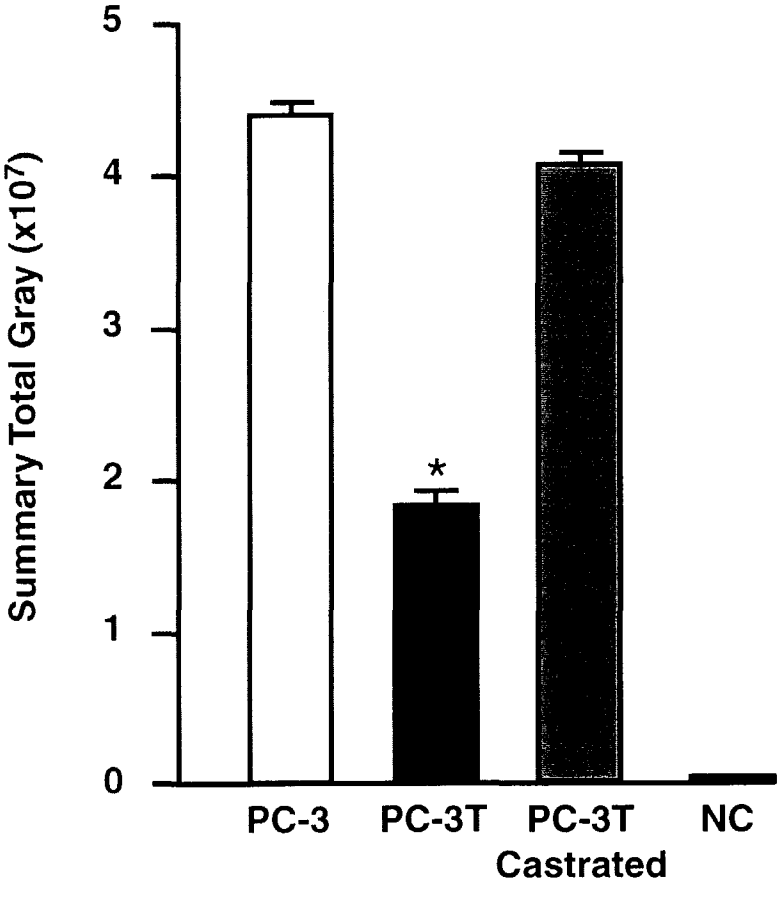
Figure 9. Effect of androgens on PTHRP production by tumors.

A: Immunohistochemistry for PTHRP was performed, as described in Materials and Methods, in tumors sections of animals inoculated with PC-3 or PC-3T cells and in tumor sections of castrated animals inoculated with PC-3T cells. Four animals were present in each group and three tumor sections were analysed for each animal. The negative control (NC) was performed by the omission of the primary antibody specific to PTHRP (1-34). B: Immunohistochemical activity was quantified as described in Materials and Methods. Quantification is presented as the summary total gray demonstrating a positive immunohistochemical reaction. Results represent means ± SEM of 3 different experiments. * Significant differences in PTHRP values from control PC-3 tumors ($P < 0.05$).

A


PC-3
PC-3T
PC-3T Castrated
NC

B


Summary Total Gray (x10^7)
5
4
3
2
1
0
*
PC-3
PC-3T
PC-3T
Castrated
NC

Discussion

Normal and hyperplastic prostate cells require androgens for their survival and proliferation whereas androgen insensitive malignant prostate carcinoma cells exhibit a reduced androgen requirement for survival (Lobaccaro et al., 1997; Lu and Danielsen, 1998; McKeehan et al., 1987). Androgen ablation and anti- androgen hormonal therapies initially benefit early stage prostate cancer patients (Catalona, 1994; Denis and Griffiths, 2000). However, the success of these therapies is largely dependent on the hormone-sensitive status of the malignant cells. The prostate tumor is composed of non- uniform sub-populations of cells possessing different hormonal responsive abilities (Knudson, 1993). The removal of endogenous circulating androgens and the blocking of androgen receptor with AR antagonist prevents the growth and survival of hormone-dependent prostate cancer cells resulting in an initial tumor mass reduction. However, the proliferative capacity of androgen insensitive tumoral cellular subpopulations will not be inhibited. The onset of cancer progression, results in the primary tumor being composed predominately of androgen insensitive malignant cells that are no longer affected by the androgen deprivation (Catalona, 1994). In the later stages of prostate cancer, hormone therapy becomes ineffective and other forms of treatment must be used.

PC-3 cells representing late stage prostate carcinoma, exhibit a high proliferation rate *in vitro* and *in vivo.* The stable transfection of a functional, human androgen receptor caused cellular proliferation to be substantially reduced in PC-3T cells (Yuan et al., 1993). When tested *in vivo*, athymic nude mice inoculated with hormone responsive PC-3T cells, developed significantly smaller tumors and exhibited a later onset of tumor development, as compared with animals receiving wild- type hormone- insensitive PC-3

cells. In this system, androgens were found to inhibit cellular growth as opposed to androgens promoting the growth of prostatic malignant cells. This paradox has also been observed in a breast cancer cell system where contrary to what was expected, estrogens were found to inhibit cellular proliferation of late stage human breast cancer. S30 cells, transfected with a functional estrogen receptor, grow more slowly than the parental cells that are estrogen non-responsive, MDA- MB 231 cells (Jeng et al., 1994). However these studies, while demonstrating a different growth pattern of hormone responsive tumor cells, did not examine the effect of hormonal status of tumor cells on the expression of growth factors and proteases which in turn can lead to altered tumor growth. This inhibition in cellular growth could be mediated by the suppression of PTHRP. In other reports, PTHRP has been found to stimulate growth of chondrocytes and keratinocytes (Henderson et al., 1996; Lam et al., 1997).

Higher PTHRP expression levels have been associated with late stage prostate cancer cells than with normal prostate cells (Deftos, 2000) and in our studies PC-3T cells were found to express on average 40% less PTHRP when compared to the parental cells PC-3. The effect of androgens on PTHRP production have been further confirmed by treating cells in the absence of androgens and then with the addition of the active androgen metabolite dihydrotestosterone. PTHRP production was dose- dependently inhibited by increasing concentrations of DHT in this human prostate cancer cell system. Studies have been performed on the full-length rat PTHRP promoter. The removal of androgens was found to enhance PTHRP promoter activity. As well, and of importance, DHT inhibited promoter activity. Future in depth promoter analysis studies are needed to identify a putative negative androgen response element. These studies would help clarify

the mechanism of the inhibitory effect of DHT on PTHRP promoter regulation and provide new strategies to treat PTHRP overproduction. Our findings therefore could have important implications for the role of PTHRP in prostate cancer progression and new directions in the future treatment of both androgen- dependent and androgen- independent prostate cancer. Hormonal therapies in combination with other anti- cancer therapies may prove to provide the best strategy (Chao and Harland, 1997) for the prevention of prostate cancer progression and metastasis and reduction of the morbidity and mortality of this prevalent disease.

Chapter 3

General Discussion

PTHRP was discovered as the main factor responsible for hypercalcemia of malignancy (Nissenson et al., 1981; Rodan et al., 1983). Binding of PTHRP to the PTH/PTHRP cell surface receptor mediates a variety of physiological functions responsible for the regulation of calcium homeostasis (Kovacs et al., 1996; MacIsaac et al., 1991) and endochondrial bone formation (Amizuka et al., 1994; Lee et al., 1996). Several studies have demonstrated that increased expression of PTHRP in cancer is associated with accelerated tumor growth and a more malignant phenotype, suggesting that PTHRP may play a role in promoting tumor progression (Alipov et al., 1997; Bouvet et al., 2001; Dougherty et al., 1999; Hidaka et al., 1998). PTHRP was found to have a mitogenic effect by increasing the rate of proliferation in normal and malignant cells (Henderson et al., 1996). The relative level of PTHRP expression is highest in transformed malignant cells than in normal cells (Asadi et al., 1996; Dougherty et al., 1999).

Prostate cancer is the highest diagnosed cancer among men in North America (Coffey, 1993; Wingo et al., 1995). PTHRP expression has been identified in prostate tissue but more importantly, an increase in PTHRP expression has been observed in benign hyperplasia of the prostate gland and in advanced prostate cancer (Asadi et al., 1996; Iwamura et al., 1993). The most common site of prostate cancer metastasis is to bones, lungs and lymph nodes (Kohno et al., 1994; Rabbani et al., 1999; Tsingotjidou et

al., 2001). PTHRP is implicated in the pathogenesis of metastasis to bone by several cancers including breast cancer (Kohno et al., 1994; Yoshida et al., 2000) and prostate cancer (Blomme et al., 1999; Sugihara et al., 1998). The prostate gland is largely dependent on androgens for its normal development. Prostate cells are normally androgen sensitive however through malignant transformation the requirement of androgens for cellular survival is greatly reduced (Brinkmann, 2001). Prostate tumors are composed of various sub populations of cells that have different androgen requirements. Androgen ablation therapy is currently used to reduce prostate tumor growth. This treatment results in reduction of tumor volume by inhibiting the growth of androgen sensitive cells but unfortunately, the androgen independent cells present in the tumor continue to proliferate (Catalona, 1994).

PTHRP gene expression has been found to be regulated by several steroidal compounds including estrogens, dexamethasone, vitamin D and androgens (Liu et al., 1993; Paspaliaris et al., 1995; Sebag et al., 1994). Androgens were found to inhibit PTHRP expression in rat Leydig tumor cells *in vitro* (Liu et al., 1993). This androgen regulation of PTHRP was now studied in a human prostate cancer model *in vitro* and *in vivo*. Human prostate cancer cells PC-3, lack a functional androgen receptor and therefore are non-responsive to androgen treatment. PC-3T cells, transfected with a functional androgen receptor, are responsive to androgen treatment. In this cell model system, it was demonstrated than androgens down regulate PTHRP expression *in vitro* and *in vivo*. Reduced PTHRP production correlated with a later onset of prostate tumor development in experimental nude mice. Furthermore, compared to control animals, smaller tumor volumes were measured in animals inoculated with androgen sensitive tumor cells.

Androgens negatively regulate PTHRP expression in prostate cancer cells consequently, this inhibition of PTHRP production resulted in reduced prostate tumor growth *in vivo*.

Studies suggest that PTHRP may play a significant role in the growth of human prostate cancer by acting locally in an autocrine fashion (Iwamura et al., 1994). In this study, androgens were shown to regulate the expression of PTHRP and to regulate the growth of prostate tumor cells; however a more in depth study is needed to demonstrate a PTHRP- mediated cell growth *in vitro* and *in vitro*. As described for the breast cancer cell line MCF-7 cells (Falzon and Du, 2000), proliferation assays involving cells stably transfected with PTHRP in the sense and the antisense orientation are required to demonstrate PTHRP mediated cell growth in the PC-3 and PC-3T human prostate cancer cells. Moreover, further experiments are required to demonstrate the effects of androgens (DHT), in the absence of serum, on prostate cancer cell growth and tumor progression.

PTHRP is expressed in many prostate cancer cell lines *in vitro* and in metastatic bone lesions *in vitro* (Rabbani et al., 1999). PTHRP expression positively influences primary tumor size *in vivo* and protects cells from apoptotic stimuli (Dougherty et al., 1999). PTHRP therefore plays an important role in the promotion of prostate tumor establishment and/or progression and should be an important target for modifying the cause of this prevalent tumor.

Chapter 4

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