Early Effects of Castration Therapy in Non-malignant and Malignant Prostate Tissue

by

Nina Ohlson

Department of Medical Biosciences, Pathology
Department of Surgical and Perioperative Sciences, Urology and Andrology
Umeå University
Umeå 2005
CONTENTS

ABBREVIATIONS - 1 -
ABSTRACT - 2 -
ORIGINAL PAPERS - 3 -
INTRODUCTION - 4 -
  PROSTATE CANCER - 4 -
  THE PROSTATE GLAND - 5 -
  REGULATION OF PROSTATE GROWTH - 5 -
    Hormonal regulation - 5 -
    Epithelial and stromal interactions - 7 -
    Growth factors - 8 -
  THE INSULIN-LIKE GROWTH FACTOR SYSTEM - 9 -
    Insulin-like growth factor-1 signaling - 10 -
    Insulin-like growth factor 1 and its receptor in prostate cancer - 12 -
AIMS - 14 -
MATERIALS AND METHODS - 15 -
  PATIENTS - 15 -
    Patients treated with castration (paper I, II, IV) - 15 -
    Tissue processing (paper I, II, IV) - 15 -
  ANIMALS - 15 -
    Castration treatment (paper III) - 15 -
    Injection analysis (paper III) - 15 -
    Tissue processing (paper III) - 15 -
  MORPHOLOGY - 16 -
    Apoptosis (paper I - IV) - 16 -
    Cell Proliferation (paper I - IV) - 16 -
    Stereology (paper III) - 16 -
  MICRO-DISSECTION - 17 -
    Laser capture micro-dissection (paper II, IV) - 17 -
    Laser micro-dissection and pressure catapulting (paper III, IV) - 17 -
  RNA ANALYSIS - 18 -
    RNA preparation (paper II, III, IV) - 18 -
    cDNA array hybridization (paper III) - 18 -
    cDNA preparation (paper II, III, IV) - 18 -
    Real-time quantitative PCR (paper II, III, IV) - 18 -
  PROTEIN ANALYSIS - 19 -
    Protein extraction (paper III) - 19 -
    Western blot analysis (paper III) - 19 -
    Immunohistochemistry (paper II, III, IV) - 19 -
STATISTICS - 20 -
RESULTS AND COMMENTS - 21 -
  Paper I - 21 -
  Paper II - 22 -
  Paper III - 24 -
  Paper IV - 26 -
GENERAL DISCUSSION - 28 -
CONCLUSIONS - 31 -
ACKNOWLEDGEMENTS - 32 -
REFERENCES - 34 -
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALS</td>
<td>Acid-labile subunit</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>BAD</td>
<td>Bcl-2/Bcl-X₁-associated death promoter</td>
</tr>
<tr>
<td>BPH</td>
<td>Benign prostatic hyperplasia</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>DHT</td>
<td>Dihydrotestosterone</td>
</tr>
<tr>
<td>EGF</td>
<td>Epithelial growth factor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FKHR/FOXO1</td>
<td>Forkhead transcription factor</td>
</tr>
<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>GRB-2</td>
<td>Growth factor receptor binding-2 protein</td>
</tr>
<tr>
<td>GS</td>
<td>Gleason score</td>
</tr>
<tr>
<td>HE</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IGFBP</td>
<td>Insulin-like growth factor binding protein</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin receptor</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>KGF</td>
<td>Keratinocyte growth factor</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate specific antigen</td>
</tr>
<tr>
<td>PVDF</td>
<td>Hydrophobic polyvinylidene difluoride</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SHC</td>
<td>Src homology domain-containing protein</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TRAMP</td>
<td>Transgenic adenocarcinoma of the mouse prostate</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VP</td>
<td>Ventral prostate</td>
</tr>
</tbody>
</table>
ABSTRACT

BACKGROUND. Androgen ablation, the standard treatment for advanced prostate cancer, results in increased apoptosis, decreased cell proliferation and subsequent involution of the prostate gland. The mechanisms behind these responses are largely unknown, but effects in the prostatic epithelium are believed to be mediated by primary changes in the stroma. The purpose of this thesis was to investigate short-term cellular effects of castration-induced prostate tissue involution in mice and humans.

METHODS. Prostate tissue factors affected by castration were investigated using cDNA-arrays, micro-dissection, RT-PCR, immunohistochemistry and Western blot analysis. The effects of local insulin-like growth factor-1 (IGF-1) administration were investigated in intact and castrated mice. Non-malignant and malignant epithelial and stromal cells were micro-dissected from human prostate biopsies taken before and within two weeks after castration treatment from patients with advanced prostate cancer. These tissue compartments were analyzed by RT-PCR and/or immunohistochemistry for IGF-1, IGF-1 receptor, androgen receptor (AR) and prostate specific antigen (PSA) expression. Treatment-induced changes in these factors were related to apoptosis and proliferation as well as to clinical data and cancer specific survival.

RESULTS. Similar to our observations in mouse ventral prostate (VP), non-malignant and malignant human prostate tissues responded with increased epithelial cell apoptosis and decreased proliferation after androgen withdrawal. Also, the PSA mRNA levels were reduced within the first days after therapy both in non-malignant and malignant human prostate epithelial cells. However, neither of these changes was related to subsequent nadir serum PSA or to survival. Locally injected IGF-1 increased epithelial cell proliferation and vascular volume in intact but not in castrated mice. IGF-1 was found to be mostly, but not exclusively, expressed in the stroma, and it decreased rapidly after castration in both humans and mice. This decrease was, however, largely absent in prostate tumor stroma, and tumor stroma cells showed lower pre-treatment levels of AR than stroma surrounding normal epithelial glands. Furthermore, decreased levels of IGF-1 mRNA in the non-malignant and tumor stroma cells, and in tumor epithelial cells in response to castration, were associated with high levels of apoptosis in epithelial cells after therapy.

CONCLUSIONS. In the prostate, IGF-1 may be an important mediator of stroma-epithelial cell interaction that is involved in castration-induced epithelial and vascular involution. Moreover, reduced AR in the tumor stroma may play an important role in prostate cancer progression towards androgen-independency, resulting in inadequate IGF-1 reduction and apoptosis induction in response to castration. Most primary tumors initially respond to castration with markedly decreased PSA synthesis and cell proliferation, and moderately increased apoptosis. Death due to metastatic disease is, however, still common, despite primary tumor regression. This may suggest that tumor cells in metastases respond differently to treatment than primary tumor cells, probably influenced by a different and possibly androgen-independent stroma. Further studies should test the hypothesis that the effect of castration therapy can be enhanced by simultaneous blocking of IGF-1 signaling.

KEY WORDS. Prostate cancer, human biopsies, androgen ablation, stroma, epithelium, micro-dissection, PSA, IGF-1, IGF-R1, AR
This thesis is based on the following papers, which are referred to in the text by their roman numerals:


III. Nina Ohlson, Anders Bergh, Malin Lindhagen Persson, and Pernilla Wikström. Castration rapidly decreases local insulin-like growth factor-1 levels and inhibits its effects in the ventral prostate in mice. Accepted in Prostate. 2005.


The original articles were reprinted with permission from the publishers.
INTRODUCTION

PROSTATE CANCER

Prostate cancer is the most common disease among men in the westernized countries [Parkin et al, 2005], and a recent survey reported approximately 9000 new cases in Sweden in 2003 [The National Board of Health and Welfare, 2003]. The incidence in different regions around the world shows large variation, implying that the environment and lifestyle are important factors for development of the disease. The genetic background is also believed to be of importance, but no major genes responsible for prostate cancer have yet been identified. It is hypothesized that most hereditary prostate cancers are due to multiple genes with small to moderate effects on disease risk [Konishi et al, 2005].

Serum levels of prostate specific antigen (PSA), an androgen-regulated serine protease secreted by prostatic glandular epithelial cells [Yousef & Diamandis, 2001, Wang et al, 1979], is used for monitoring treatment response and, to some extent, for diagnosis of prostate cancer. Although PSA production is decreased in prostate tumor cells, compared to non-malignant tissue, circulating PSA levels are largely elevated in prostate cancer patients due to increased leakage into the blood stream. Benign prostatic hyperplasia (BPH) or prostatitis can also result in elevated serum PSA levels. Thus, a PSA test alone can not confirm prostate cancer. Tumor staging [Hermanek & Sobin, 1992] and histological grading according to Gleason [Gleason, 1992], give additional information about the presence and severity of the disease. There is, however, a limited ability to predict the aggressiveness of a prostate tumor. The outcome differs largely between prostate cancer patients, even among the majority of patients that display intermediate levels of serum PSA, Gleason score (GS) and stage at diagnosis [Blute et al, 2001].

Prostate cancer is diagnosed as local or advanced disease, the latter definition describing an adenocarcinoma that has spread beyond the prostate capsule. Prostate cancer is mainly a disease of elderly men, as the median age at diagnosis is approximately 75 years [Aus et al, 2005]. Treatment recommendations vary depending on age, prognostic grouping, and the presence of co-morbidity. Treatments for malignancies confined to the prostate range from radical surgery, irradiation and anti-androgens to, so called, “watchful waiting” [Sharifi et al, 2005, Bhatnagar & Kaplan, 2005]. In advanced prostate cancer the most common treatment is chemical or surgical castration (orchietomy). This form of therapy lowers androgen levels, thus reducing tumor growth and giving symptomatic relief. Androgen deprivation therapy is sometimes also used as an adjuvant to local treatment of high-risk disease. Serum PSA levels are used to monitor the response to treatment and the lowest value (nadir serum PSA) is often reached after approximately six months or later [Benaim et al, 2002, Kwak et al, 2002, Fowler et al, 1995]. However, castration therapy is only a
palliative treatment, as the tumor will relapse within a few years, i.e. a subpopulation of tumor epithelial cells will begin to grow independently of androgen [Feldman & Feldman, 2001], leading to death of the patient. Development and evaluation of new therapeutic markers and strategies to defeat prostate cancer is clearly an important challenge in prostate cancer research today.

THE PROSTATE GLAND

The prostate in man is an exocrine gland that lies just below the bladder, wrapped around the urethra. It is enclosed in a thin fibrous capsule and its parenchyma consists of numerous branching tubuloalveolar glands terminating in ducts that finally empty into the urethra. The secretion is related to male fertility as it is believed to enhance sperm motility and survival [Arienti et al, 2004]. The human prostate is composed of four different regions based on morphology, and named using the urethra as reference point. Most prostate tumors are associated with the peripheral zone that together with the central and the transition zones constitute the glandular prostate. The fourth, non glandular, region is a fibromuscular stroma compartment [McNeal, 1981]. The mouse prostate differs in anatomy since it is subdivided into anatomically distinct lobes, named the ventral (VP), dorsal, lateral, and anterior lobes [Roy-Burman et al, 2004].

The prostate is comprised of two cellular compartments. The human epithelial compartment consists of a basal cell layer and a layer of differentiated secretory luminal cells. A basement membrane separates the glands from the dense fibromuscular stroma tissue, mainly composed of smooth muscle cells (SMCs) but also comprising fibroblasts, neuronal, lymphatic and vascular components. The dense fibromuscular stroma in human prostate is largely absent in mouse prostate, where a thin border of fibromuscular stroma surrounds the epithelial gland and loose connective tissue extends between individual ducts [Roy-Burman et al, 2004, Shappell et al, 2004].

REGULATION OF PROSTATE GROWTH

Hormonal regulation

The prostate is hormonally regulated and dependent on androgens for development, growth and maintenance of size and function. In the developing prostate, low levels of androgens act upon mesenchymal cells to induce SMC differentiation [Hayward et al, 1996], epithelial cell proliferation, and differentiation [Hayward & Cunha, 2000]. In the adult prostate, the smooth muscle cells are fully differentiated in the presence of high levels of androgen, and the epithelial proliferation and apoptotic levels are low and balanced.
The major circulating androgen in man is testosterone, mainly produced by the testes under hypothalamic control. The hypothalamus releases gonadotropin-releasing hormone (GnRH) that stimulates the production and release of luteinizing hormone (LH) from the anterior pituitary gland. LH then promotes the Leydig cells in the testis to produce and secrete testosterone. Most of the circulating testosterone is bound by different plasma proteins and is therefore not accessible in target tissue. In the prostate, testosterone is converted to the more potent androgen, dihydrotestosterone (DHT), by 5-alpha-reductase [Krieg et al, 1995]. Testosterone and DHT can both bind to the androgen receptor (AR), although DHT has a higher binding affinity. Ligand-free AR is found in the cytoplasm bound to heat-shock proteins (HSP-70 and -90) that stabilize the AR during folding, and protect it from degradation. Ligand binding to the AR induces a conformational change and dissociation of HSP-70 and -90. The AR then forms a homodimer and subsequent phosphorylations at several sites stabilize the complex. Activated AR is translocated to the nucleus where it initiates gene transcription by binding to specific androgen-responsive elements in the promoter regions of targeted genes.

Hormonal regulation of the prostate has mainly been studied in rodents where the VP strongly responds to androgen deprivation [Banerjee et al, 1995]. When androgens are removed from the normal prostate, apoptosis is induced in the epithelium [Kurita et al, 2001, Banerjee et al, 1995, Westin et al, 1993], probably by altered paracrine signaling from the stroma [Kurita et al, 2001] and decreased blood flow [Lekås et al, 1997]. The castration-induced epithelial apoptotic induction in rodents peaks around three days after castration and is preceded by a major decrease in blood flow as early as one day after androgen withdrawal [Shabsigh et al, 1998, Lekås et al, 1997]. Castration also increases endothelial cell apoptosis [Shabsigh et al, 1998], decreases endothelial and epithelial cell proliferation, and induces loss of secretory function. Taken together this causes involution of the gland [Kerr & Searle 1973, English et al, 1987]. The AR is present in glandular epithelium, stroma, and vascular SMCs [Prins et al, 1991, Johansson et al, 2005] (Figure I), but not in endothelial cells [Lissbrant et al, 2004, Buttyan et al, 2000, Prins et al, 1991]. This suggests that the castration-induced changes in the endothelial cells must be indirectly caused by altered signaling in surrounding AR-positive cells. Following castration, the stroma SMCs also fall into atrophy and apoptosis processes, but these morphological changes are less marked and more prolonged than in the epithelium [Niu et al, 2001].

As described above, patients with advanced and metastatic prostate cancer are mostly treated with androgen-deprivation therapy. The mechanisms behind the initial response to castration are largely unknown but the presence of functional AR is essential. The initial response to this therapy is usually high but the tumor generally begins to grow independently of androgen within a few years [Eisenberger et al, 1998]. There are several possible mechanisms by which prostate cancer may circumvent the loss of androgen [Feldman & Feldman, 2001]. The prostate tumor cells may have increased sensitivity to...
Introduction

androgens and other steroids due to AR gene amplification and increased AR protein expression. AR mutations may allow an increased number of possible AR activating ligands, thus compensating for low levels of androgen. Another possibility is that although circulating testosterone levels are decreased, the local androgen production is largely maintained. Furthermore, certain growth factors have been shown to activate the AR in the absence of androgen. Thus, even if androgen is withdrawn the presence of AR in prostate tumor tissue is an important factor for subsequent androgen-independent regrowth of the tumor [Chen et al., 2004].

Epithelial and stromal interactions

It appears that prostate tumorigenesis involves the acquisition of genetic alterations in the epithelium [Konishi et al., 2005], resulting in aberrant signaling to the surrounding stroma. Moreover, genetic alterations in stromal cells may precede and induce genotypic changes in the epithelial cells [Moinfar et al., 2000, Barcellos-Hoff & Ravani, 2000, Olumi et al., 1999]. In the normal prostate, epithelial cell differentiation, proliferation, and apoptosis are controlled by androgens. When stromal-epithelial interactions are interrupted, however, the stroma may lose its AR expression, thus influencing factors that normally regulate homeostasis of epithelial cells. Loss of AR expression in stromal cells surrounding human prostate cancer cells may be an early event in prostate cancer progression [Olapade-Olaopa et al., 1999]. Altered AR expression in tumor associated stroma (Figure I), along with genetic alterations within the epithelium, may thus promote the development of androgen-independent epithelial cell proliferation [Chung et al., 2005, Hayward et al., 2001].

Stromal-epithelial interactions are not only important for primary tumor growth but are also central to the understanding of metastatic disease. Cancer cells can alter their adjacent stroma to form a permissive and supportive environment for tumor progression [Mueller & Fusenig, 2004]. Thus, the formation of a secondary tumor probably depends on the ability of the metastatic cells to interact with and make use of the resources, including other cells and extra cellular matrix, in the new microenvironment [Condon, 2005]. Angiogenesis is the best described host-mediated response to cancer that is crucial for cancer progression. In the prostate, pro-angiogenic factors are produced both by cancer cells and stroma cells [Nicholson & Theodorescu, 2004]. Therapeutic targeting of the stroma is therefore probably advantageous as a complementary prostate cancer treatment.
Introduction

Figure I. Schematic representation of epithelial and stromal interactions, exemplified by IGF-1 signaling in normal (A) and malignant (B) cells. Growth factor signaling in tumors is often associated with a switch of paracrine to autocrine mode. Tumor stromal cells express less AR than their normal counterpart and tumor vasculature partly lacks mural cells (vascular SMCs and pericytes).

Growth factors

AR-stimulated growth is mediated via locally produced growth factors. Both epithelial and stromal cells are known to produce growth factors that can either promote or inhibit prostatic growth through autocrine or paracrine signaling, and several growth factor interactions are altered in tumors [Wong et al, 2003]. These growth factors include insulin-like growth factor (IGF) family, fibroblast growth factor (FGF) family, transforming growth factor-β (TGF-β) family, epidermal growth factor (EGF) receptor family, hepatocyte growth factor (HGF), and vascular endothelial growth factor (VEGF) family. Tumor progression often involves up- or down-regulation of growth factors and their receptors, as well as changes from paracrine to autocrine mediation of growth (Figure I).

Briefly, VEGF has an important role in prostate cancer by stimulating angiogenesis, inducing endothelial cell proliferation, and increasing vascular permeability [Lissbrant et al, 2004, Ferrara et al, 1996]. Secretion of VEGF has been observed in epithelial as well as surrounding stromal cells, and an androgen-deprivation-induced decrease in VEGF expression in prostate cancer is thought to be a mechanism for castration-induced apoptosis [Häggström et al, 1998]. Another major growth factor with angiogenesis promoting activity is basic fibroblast growth factor (bFGF), or FGF-2, which is synthesized mainly by stromal fibroblasts [Dow & deVere White, 2000]. bFGF stimulates prostate fibroblasts in an autocrine manner and has paracrine effects on prostate epithelial cell growth. Due to its angiogenic properties, bFGF is believed to be involved in progression from primary to
metastatic prostate cancer. Keratinocyte growth factor (KGF), or FGF-7, is also a member of the FGF family that normally is expressed in the prostatic stroma, and it is postulated that KGF acts with the aid of androgens in a paracrine fashion. In the absence of androgen, KGF may also directly activate the androgen and KGF receptors promoting epithelial cell survival and growth [Culig et al., 1994]. Another stroma-derived growth factor is HGF, whose receptor is mostly expressed on epithelial cells. Activation of the receptor affects a wide range of cellular processes, including growth and differentiation, morphogenesis and invasiveness [Nakamura et al., 1997]. The expression of EGF in the prostate is androgen dependent and will decrease following androgen withdrawal [Nishi et al., 1996] Stimulation of the EGF receptor pathways in tumor cells enhances cellular proliferation, prevents apoptosis, and promotes tumor-cell mobility, adhesion, and invasion, as reviewed by Woodburn, 1999. TGF-β can act both as a positive and a negative regulator of prostatic growth depending on the cell type and state of cell differentiation [Zhu & Kyprianou, 2005]. In the normal prostate, TGF-β primarily acts as a growth inhibitor for epithelial cells by inhibiting proliferation and inducing apoptosis. However, prostate cancer cells frequently lose their sensitivity to TGF-β, and are also able to over express TGF-β, leading to more aggressive phenotypes [Chang et al., 1993].

THE INSULIN-LIKE GROWTH FACTOR SYSTEM

The insulin-like growth factor (IGF) system has two major ligands, IGF-1 and IGF-2. IGF-1 and IGF-2 are single-chain peptides of 70 and 67 residues, respectively. These are predominantly produced in liver in response to growth hormone (GH) [Roberts et al., 1986] but are also synthesized in other adult tissues, including brain, muscle, and bone. The IGFs are involved in cell growth and development as well as in tumorigenesis [Yakar et al., 2005, Khandwala et al., 2000].

IGF-1 is expressed at low levels embryonically in mice, and is thought to be principally important for post-natal growth and development. However, IGF-1 also appears to be essential for correct embryonic development and fetal growth in mice and possibly also in humans [Liu et al., 1998, Woods et al., 1996, Powell-Braxton et al., 1993]. IGF-1 knockout mice generally die before birth, but those that survive are born smaller than wild-type mice and continue to grow with a retarded rate [Powell-Braxton et al., 1993, Baker et al., 1993]. Liver-specific deletion of the IGF-1 gene caused a dramatic reduction in circulating IGF-1 levels, but had no significant effect on the overall growth [Yakar et al., 1999], indicating that locally produced tissue IGF-1 is of importance for post-natal growth and development.

In association with the major ligands are at least six multi-functional IGF binding proteins (IGFBP-1 to 6). The majority of circulating IGF-1 is bound in a ternary complex that includes IGF-1, IGFBP-3, and ALS (acid-labile subunit) [Baxter & Martin, 1989]. IGFBP-3 is thus the major IGF transporting IGFBP and the most abundant IGFBP in the
circulation. Ternary complexes appear to be confined to the vascular compartment, prolonging the half-life of IGFs in the blood [Guler et al., 1989]. When the ternary complex dissociates, IGFBP and IGF, in free form or as binary complexes, can cross the vascular endothelium to interact with cell surface receptors in the target tissues [Rajaram et al., 1997]. In the tissue, IGFBPs can either enhance or inhibit IGF action, depending on various conditions, as reviewed by Firth & Baxter, 2002. The IGFBPs can prevent receptor binding and subsequent signaling when possessing a higher affinity for the IGFs than the receptors [Moore et al., 2003, Miyakoshi et al., 1999, Mohan et al., 1995]. The IGFBP-affinity for the IGFs may be influenced by post-translational modifications such as binding to extracellular matrix or cell surfaces, phosphorylation, and proteolysis. For example, IGFBPs associated with extracellular matrix or cell surfaces generally exhibit less affinity for IGFs. By recruiting and deliver IGFs to the IGF receptors, where the IGFs can be slowly released, they are thus able to stimulate IGF actions [Miyake et al., 2000, Mohan et al., 1995]. In addition, some IGFBPs have IGF-independent effects [Firth & Baxter, 2002, Mohan & Baylink, 2002].

IGF-R1 is a member of the family of tyrosine kinase growth factor receptors, and is highly homologous to the insulin receptor (IR). IGF-1 and insulin are capable of binding to each other’s receptors, but with considerable lower affinity than to their own receptors. IGF-2 availability is modulated by the type 2 IGF receptor (IGF-R2), also known as the mannose 6-phosphate receptor. IGF-R2 removes IGF-2 from the circulation by internalizing and targeting it for lysosomal degradation [Braulke, 1999]. IGF-R1 knockouts exhibit retarded growth and die at birth, due to poor organ development [Liu et al., 1993]. If both the IGF-1 and the IGF-R1 genes in mice are disrupted, a phenotype similar to that of the IGF-R1 knockout results, suggesting that the actions of IGF-1 are mediated primarily via the IGF-R1 [Liu et al., 1993].

**Insulin-like growth factor-1 signaling**

IGF-R1 consists of two extracellular α-chains and two intracellular β-chains, where the α-subunit bind the IGF-1 ligand and the β-subunit include a tyrosine kinase domain that is essential for most of the receptor’s actions. Ligand binding to the α-subunits leads to a signal transmission through the transmembrane domain to the β-subunits. The β-subunits respond by undergoing a conformational change followed by auto-phosphorylation of tyrosine residues 1131, 1135, and 1136 of the receptor complex [Kato et al., 1994]. The subsequent phosphorylations of other tyrosine residues within the cytoplasmic domain of the receptor provide docking sites for IRS (insulin receptor substrate) and SHC (src homology domain containing protein). These are the major substrates for IGF-R1-activated intracellular signaling pathways including the RAS/RAF/MEK pathway and the phosphatidylinositol 3-kinase (PI3K)/AKT pathway (Figure II).
Activated IGF-R1 initiates signaling through two primary cascades, the MAP kinase and the PI3 kinase pathways. IRS-1 connects the receptor to the PI3K pathway, while the MAP kinase pathway is activated through SHC. IRS-1 also connects to the MAP kinase pathway through its association with GRB-2. Overall, activation of the IGF-R1 signaling pathways leads to cell survival, through inhibition of pro-apoptotic signals, and cell proliferation, through transcriptional activations of genes involved in cell cycle progression.

The IRS protein family includes at least four members that are responsible for connecting IGF-R1 activation to several cell responses. Mice that lack the IRS-1 gene have fetal and post-natal growth retardation [Tamemoto et al, 1994], and cell lines derived from IRS-1 negative embryonic mice exhibit a major reduction in IGF-1-stimulated cell growth [Brüning et al, 1997]. IRS-1 mediated signaling is regulated by mechanisms including
regulation of the basal levels of IRS-1 [Zhang et al., 2000] and the phosphorylation status of the protein [Rui et al., 2001]. Serine/threonine phosphorylation of IRS-1 has been shown to inhibit IRS-1 signaling and thus counteract IGF-1 signaling. For example, IGF-1/insulin–induced phosphorylation of Ser\textsuperscript{307} (corresponding to Ser\textsuperscript{312} in humans) inhibits subsequent tyrosine phosphorylation of IRS-1 and activation of IRS-1–mediated signaling pathways [Rui et al., 2001].

Tyrosine phosphorylation of IRS-1 creates binding sites for numerous SH2 domain-containing proteins, including SHC and the p85 regulatory subunit of PI3K. One of the major effects of IGF-1 is to promote cell survival by influencing proliferation and apoptosis. The main anti-apoptotic signaling pathway is mediated by PI3K which activates AKT, also known as protein kinase B [Peruzzi et al., 1999]. Activated AKT can phosphorylate several proteins involved in programmed cell death, including BAD, a member of the BCL-2 family of proteins, and FKHR (FOXO1), a member of the forkhead transcription factor family (Figure II). Phosphorylation of these proteins by AKT suppresses their proapoptotic function [Brunet et al., 1999, Bai et al., 1999, Adams & Cory, 1998].

The RAS/RAF/MEK pathway is activated when GRB-2 (growth factor receptor binding-2 protein) associates with either IGF-1 activated SHC or IRS-1 (Figure II). This pathway is thought to primarily mediate a cell proliferative response to growth factors, such as IGF-1.

**Insulin-like growth factor 1 and its receptor in prostate cancer**

One of the mechanisms proposed to explain uncontrolled prostate cancer cell growth and tumor progression involves the local production of growth factors by tumor and stroma cells [Steiner, 1995]. One of these growth factors is IGF-1, which has been shown to be an important factor in prostate cancer by stimulating cancer cell growth and exerting anti-apoptotic effects [O’Brien et al., 2001, Djavan et al., 2001].

IGF-1 can activate the AR in the absence of androgens [Culig et al., 1994] and synergistically potentiate the effects of low androgen concentrations [Kollara et al., 2003]. Overexpression of IGF-1 in prostate epithelial cells induced tumorigenesis in a transgenic model [DiGiovanni et al., 2000]. Furthermore, most epithelial prostate cancer cells express high levels of IGF-1 together with its receptor [Cardillo et al., 2003, Nickerson et al., 2001, Kaplan et al., 1999] and thus probably autocrine signaling [Cardillo et al., 2003, Wang & Wong, 1998] (Figure I). Prostate cancer cells expressing IGF-1 might have a growth advantage, and the observed increase in serum IGF-1 might reflect the increased number of these cells [Kaplan et al., 1999]. However, circulating IGF-1 levels are regulated by multiple factors such as nutrition [Larsson et al., 2005, Underwood et al., 1986] and age [Juul et al., 1994], and studies on the association between serum IGF-1 levels and the risk of
Introduction

developing prostate cancer have yielded inconsistent results. Some studies have related high circulating IGF-1 levels to an increased prostate cancer risk [Stattin et al, 2004 and 2000, Djavan et al, 1999, Chan et al, 1998], while others have reported no association [Woodson et al, 2003, Kurek et al, 2000].

There is no consensus regarding IGF-R1 expression levels in non-malignant and malignant prostate and the role of IGF-R1 in metastases. IGF-R1 expression is generally thought to be increased in advanced and metastatic prostate cancer [Krueckl et al, 2004, Cardillo et al, 2003, Hellawell et al, 2002, Nickerson et al, 2001], but there are also studies reporting lower or unchanged levels of IGF-R1 levels in malignant prostate [Kaplan et al, 1999, Chott et al, 1999, Tennant et al, 1996]. However, if IGF-R1 signaling is inhibited or down-regulated, this sometimes leads to decreased tumor growth and metastasis [Wu et al, 2005, Grzmił et al, 2004, Burfeind et al, 1996], as well as reduced prostate cancer growth in bone [Goya et al, 2004]. These results suggest that inhibition of IGF-R1 signaling could be a target for prostate cancer therapy.
Aims

AIMS

Although Charles Huggins received the Nobel Prize in 1966 "for his discoveries concerning hormonal treatment of prostatic cancer," the mechanism behind the short- and long-term treatment response is, surprisingly, largely unknown. The overall purpose of this thesis was to elucidate short-term cellular responses to castration treatment in human and mouse prostate.

Specific aims:

- To study the time-frame and magnitude of early castration-induced responses in mouse ventral prostate as well as in non-malignant and malignant human prostate tissue
- To investigate if short-term therapy-induced changes in apoptosis, proliferation, and tissue PSA mRNA levels in primary tumor cells were related to disease progression and survival in prostate cancer patients
- To identify important factors and/or pathways mediating castration-induced prostate involution using cDNA array studies in mice, and consequently
  - To analyze the role of the IGF-system in castration-induced prostate involution in non-malignant human and mouse prostate tissue, as well as in human prostate cancer
MATERIALS AND METHODS

PATIENTS

Informed consent was obtained from all patients, and the studies were approved by the Research Ethics Committee of Umeå University Hospital, Umeå, Sweden.

Patients treated with castration (paper I, II, IV)

Ultrasound-guided core biopsies were taken shortly before and within 15 days after castration therapy in men diagnosed with prostate cancer at the Umeå University Hospital. 77, 83 and 55 patients were included in paper I, II and IV, respectively. Local tumor stage was evaluated by rectal digital examination, according to the 1992 UICC classification [Hermanek & Sobin, 1992], and radionuclide bone scan was performed for metastasis staging at the time of diagnosis. Nadir serum PSA level was defined as the lowest serum PSA level after therapy and cause of death was determined by evaluation of patient records and by linkage to the regional Cause of Death Registry. A strong response to castration was defined as a nadir serum PSA value equal to or less than 1 ng/ml.

Tissue processing (paper I, II, IV)

Biopsies were fixed in phosphate buffered formalin for 24 hours and embedded in paraffin. The biopsies were sectioned into four μm thick sections and stained with hematoxylin and eosin (HE). Tumor histology was graded according to Gleason [Gleason, 1992]. HE-stained sections were used to identify apoptotic cells and for micro-dissection of non-malignant and malignant epithelial and stromal cells.

ANIMALS

Adult C57 black mice (Taconic, Denmark) were housed in a controlled environment with free access to pelleted food and water. According to Swedish legislation on animal care, experiments were approved by the Animal Research Ethics Committee of Umeå, Sweden.

Castration treatment (paper III)

Animals were anaesthetized and surgically castrated, as previously described [Lissbrant et al, 2004]. Seven to nine days after castration, the animals were given a daily subcutaneous injection of testosterone esters (Sustanon, Organon, Oss, The Netherlands, 10 mg/kg). One, three, and seven days after castration treatment and one, two, and three days
after androgen administration (day eight to ten) the animals were anaesthetized and the VP were surgically removed. Intact animals were used as controls. To prevent RNA degradation, tissues were rapidly removed and snap frozen in liquid nitrogen.

Injection analysis (paper III)

Murine recombinant IGF-1 (0.5 μg, R&D Systems, UK, dissolved in 5 μl sterile saline) or saline only was injected into the left VP lobe in intact mice or simultaneously with castration. After 23 hours, the animals were given bromodeoxyuridine (BrdU, 50 mg/kg) intraperitoneally in order to label proliferating cells. BrdU is a thymidine analogue that is incorporated into the DNA during the S-phase of the cell cycle. One hour later the animals were fixed by vascular perfusion with Bouins solution. The left and right VP lobes were dissected out, post-fixed and embedded in paraffin for morphological analyses.

Tissue processing (paper III)

For subsequent use in cDNA arrays, micro-dissection, RT-PCR, or Western blot analyses, VP lobes were removed and frozen in liquid nitrogen. For morphological analyses the tissues were fixed by vascular perfusion using Bouins solution, the VP lobes were dissected out and post-fixed in the same fixative, dehydrated, and embedded in paraffin [Lissbrant et al, 2004].

MORPHOLOGY

Apoptosis (paper I - IV)

Apoptotic epithelial cells were identified in HE-stained sections, as single rounded cells or cells with apoptotic bodies (paper I, II, IV) or by TUNEL stained sections (In situ cell detection kit POD, Roche Diagnostics, Mannheim, Germany, paper III). Approximately 1000 malignant and/or 1000 non-malignant epithelial cells were assessed in each tissue sample. The percentage of TUNEL-labeled endothelial cells per blood vessel profile was also measured in paper III, where 200 blood vessels per prostate were examined.

Cell Proliferation (paper I - IV)

Biopsies were immunostained with an antibody against the Ki-67 antigen (Mib1, Dako, Denmark, paper I, II, IV). Mouse VP sections were immunostained for Ki-67 with TEC-3 antibody or for BrdU (DakoCytomation, Glostrup, Denmark, paper III), as previously described [Lissbrant et al, 2003]. The Ki-67 antigen is expressed during all stages of the cell cycle except in G0 and is commonly used to quantify cell proliferation [Scholzen & Gerdes, 2000]. The percentage of Ki-67 or BrdU positive epithelial cells was evaluated in 1000 malignant and/or adjacent non-malignant prostate cells in each tissue sample.
Stereology (paper III)

The densities of glandular epithelial cells and blood vessel lumina were determined in HE-stained paraffin sections from mouse VP using point counting morphometry [Lissbrant et al, 2004]. Briefly, a square lattice in the eye-piece of a light microscope was used to count the number of grid intersections for the measured tissue compartment and the reference space. Vessel volume density was assessed as the percentage of prostate volume composed of blood vessels positively immunostained for von Willebrand Factor (A0082, DakoCytomation) [Johansson et al, 2005].

MICRO-DISSECTION

Laser capture micro-dissection (paper II, IV)

Malignant and non-malignant epithelial cells from archived human prostate biopsies were retrieved by the Arcturus PixCell II LCM system (Arcturus, Mountain View, CA, USA). In brief, paraffin-embedded tissue samples were sectioned and mounted on HistoGene laser capture micro-dissection slides (Arcturus). Sections were deparaffinized twice in xylene for 5 minutes, rehydrated in 5 minutes washes in 100 %, 95 % and 70 % ethanol, respectively, and stained with Arcturus staining solution for 20 seconds. Sections were then rinsed twice in RNase-free water and dehydrated with 70 %, 95 % and 100 % ethanol, 30 seconds each, followed by two changes of xylene for 5 minutes. Sections were air dried before stored in an exicator and micro-dissected. Five hundred shots (diameter:15 µm) were captured in each fraction.

Laser micro-dissection and pressure catapulting (paper III, IV)

Frozen mouse VP tissue samples (paper III) were sectioned and mounted on PALM MembraneSlides (P.A.L.M Microlaser Technologies AG, Bernried, Germany). Sections were fixed in ice-cold 70 % ethanol for one minute and rinsed in RNase-free water for another minute. Sections were stained with HE and dehydrated by 70 % and 95 % ethanol incubations for 30 seconds, each followed by two changes of 99.5 % ethanol for 30 seconds.

Sections from paraffin-embedded human biopsies (paper IV) were deparaffinized in two changes of xylene for 2 minutes and rehydrated in changes of 99.5 % ethanol, 95 % ethanol, 70 % ethanol, and RNase-free water for 1 minute each, before being stained with Arcturus staining solution and dehydrated in 75 % ethanol, 95 % ethanol, and two changes of 99.5 % ethanol for 30 seconds each.
Materials and Methods

Sections were air-dried and stored in an exicator before micro-dissection. Approximately \(1 \times 10^6 \mu m^2\) (VP) or \(4 \times 10^5 \mu m^2\) (human prostate) of each cell compartment was retrieved separately by the PALM MicroLaser system in PALM AdhesiveCaps, according to the manufacturer’s description.

RNA ANALYSIS

RNA preparation (paper II, III, IV)

In paper II and IV, total RNA from fixed human prostate biopsies was obtained by extracting micro-dissected cells in 200 µl RNA lysis buffer containing 10 mM Tris/HCl (pH 8), 0.1 mM EDTA (pH 8), 2 % sodium dodecyl sulfate (SDS, pH 7.3) and 500 µg/ml proteinase K in 60°C over night, followed by proteinase K heat inactivation at 95°C for 5 minutes, extraction with phenol and chlorophorm and precipitation with an equal volume of isopropanol in the presence of 0.1 volume of 2 M sodium acetate (pH 4). The RNA pellet was washed with 70 % ethanol, then dried and dissolved in RNase-free water.

In paper III, total RNA was prepared from the frozen mice VP by the TRIzol extraction method (Invitrogen, Stockholm, Sweden), according to the manufacturer’s description. The RNA concentrations were determined spectrophotometrically and the RNA integrity was verified by ethidium bromide staining of 28 S and 18 S ribosomal RNA after agarose gel electrophoresis. RNA from micro-dissected cells was extracted using the PicoPure RNA Isolation Kit (Arcturus) and the RNA integrity was assured using the RNA 6000 Pico LabChip kit and the Agilent 2100 Bioanalyzer (Agilent Technologies, Kista, Sweden).

cDNA array hybridization (paper III)

Total RNA from mouse VP was pooled into groups of intact, one, and three days castrated animals, respectively. Five µg of total RNA was converted into 32P-labelled probes that were hybridized to Atlas Mouse cDNA Expression Arrays (Clontech, CA, USA) over night. Comparisons were made for intact animals with one and three days castrated animals, respectively. cDNA filters were exposed 7 to 14 days and scanned by a Typhoon 9400. The arrays were manually aligned and compared using AtlasImage™ 2.01.

cDNA preparation (paper II, III, IV)

Total RNA from micro-dissected human prostate biopsies (paper II) or from whole VP RNA extracts and micro-dissected mouse VP samples (paper III) were reverse transcribed in a total volume of 10 µl using random hexamers (Applied Biosystems, Sundbyberg, Sweden) and Superscript II reverse transcriptase (Invitrogen Life Technologies, Paisley, UK). All cDNA-reactions were run in duplicate and post-diluted in nuclease free water.
Materials and Methods

Real-time quantitative PCR (paper II, III, IV)

In paper II, mRNA expression levels of PSA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in human prostate cancer were analyzed by real-time PCR using the LightCycler SYBR Green I technology (Roche Diagnostics, Bromma, Sweden). In paper III, mRNA expression levels of IGF-1, IGF-R1, IGFBP-2, -3, -5, -6, and GAPDH were assessed in mouse VP and in paper IV IGF-1 and GAPDH mRNA levels in human biopsy tissues were analyzed using the same methodology. The reactions were performed with 2 µl (whole VP samples) or 3 µl (LMPC and LCM samples) of diluted cDNA, 0.5 µM of each specific primer and 2 to 4 mM MgCl2 (see indicated papers for specific primer pair condition and design) in a total volume of 20 µl. Negative controls were always run in parallel. The data were analyzed using the LightCycler analysis Software 3.5.3. Relative mRNA values were calculated from standard curves obtained by amplification of serial dilutions of cDNA corresponding to pooled human prostate tissue RNA, or RNA from intact mouse ventral prostate. In order to adjust for dissection and RNA extraction efficiency as well as for differences in RNA degradation, specific mRNA levels in micro-dissected cell areas were corrected for their corresponding GAPDH levels.

PROTEIN ANALYSIS

Protein extraction (paper III)

VP tissues were homogenized using a Micro Dismembrator (B. Braun Biotech International GmbH, Melsungen, Germany) at 2000 rpm for 45 seconds. Proteins were extracted using 0.5 % NP-40, 0.5 % NaDOC, 0.1 % SDS, 50 mM Tris-HCL (pH = 7.5), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1 mM NaF, Complete Protease Inhibitor Cocktail (Boehringer Mannheim, Germany), and 1 mM Na3VO4. Samples were mixed and incubated on ice for 30 minutes. The supernatants were isolated following centrifugation (20000 x g, 4°C, 30 min) and the protein concentration was determined using BCA Protein assay reagent Kit (Pierce Chemical Co., IL, USA).

Western blot analysis (paper III)

Proteins were separated by 15 % (IGF-1) or 7.5 % (IRS-1) SDS-polyacrylamide gel electrophoresis under reducing conditions and subsequently electro-blotted to a PVDF membrane. The membrane was blocked in 5 % milk followed by primary (IGF-1, 0.4 µg/ml, AF791, R&D Systems, Abingdon, UK; IRS-1, 0.05 µg/ml, 06-248, Upstate cell signaling solutions, Lace Placid, NY; IRS-2, 1 µg/ml, 06-506, Upstate; anti Ser 307 IRS-1, diluted 1:1000, Upstate; IRS-1 [pY612], 0.05 µg/ml, 44-816, BioSource, Camarillo, CA, and actin, 0.1 µg/ml A2066, Sigma, Saint Louis, MO) and secondary (anti-goat IgG, Jackson Laboratory, Pierce Biotechnology, Rockford IL, US or anti-rabbit IgG, Amersham
Biosciences, Buckinghamshire, UK) antibody incubations. Protein expression was visualized using Enhanced Chemiluminescence Advanced or Plus (Amersham Biosciences) and quantified using Fluor-S MultiImager scanning and Quantity One (4.5.0) software analysis (Bio-Rad laboratories, Hercules, CA, USA).

**Immunohistochemistry (paper II, III, IV)**

Human paraffin-embedded biopsies were stained for PSA (paper II) and IGF-1, IGF-R1 and AR (paper IV). Briefly, sections were incubated with primary antibodies against PSA (A 0562, DakoCytomation, Denmark), IGF-1 (C-20: sc-7144, Santa Cruz Biotechnology, Santa Cruz, CA, USA), IGF-R1 (Ab-1, 24-31, Neomarkers, Fremont, CA, USA), and AR (PG-21, Upstate, Lake Placid, NY, USA). After washing and secondary antibody incubation, the sections were processed using Ventana Basic AEC kit (Ventana Medical systems, Tuscon, AZ, USA), SuperPicTure polymer detection kit (Zymed laboratories, San Fransisco, CA, USA), Catalyzed signal amplification systems II (DakoCytomation, Glostrup, Denmark), and Envision plus kit (DakoCytomation), respectively.

In paper II, IGF-R1 was localized in paraffin-embedded sections of mouse VP using primary antibodies (IGF-IRα (N-20) and IGF-IRβ (C-20), Santa Cruz Biotechnology) in the Ventana automatic immunostaining system and visualized using the Ventana DAB iVIEW (Ventana Medical Systems).

**STATISTICS**

Groups were compared by the Kruskal Wallis H test followed by the Mann-Whitney U-test, and paired observations were compared with the Wilcoxon signed rank test. Correlations between continuous variables were analyzed using the Spearman rank test. Associations between categorical variables were analyzed by Pearson’s Chi-Square test. Kaplan-Meier analysis of cancer specific survival was performed with death of prostate cancer as event, and analysis of time to progression with death of prostate cancer or PSA relapse as events. Groups were compared with the log rank test. Statistical analysis was performed using the SPSS 12.0.1 software (SPSS Inc., Chicago, IL, USA). All tests were two-sided, and p-values less than 0.05 were considered statistically significant.
RESULTS AND COMMENTS

The following section is a summary of papers I to IV. Figures and tables are referred to by their numbers given in the original paper.

Cell proliferation and apoptosis in prostate tumors and adjacent non-malignant prostate tissue in patients at different time-points after castration treatment (paper I)

Although androgen ablation is the standard therapy for advanced prostate cancer, the short-term effects of this treatment are largely unknown. Early changes in apoptotic and proliferation index in non-malignant and malignant prostate tissue were analyzed in 77 patients before and within ten days after castration treatment (Fig. 2, paper I). We demonstrated that castration induced a decrease in epithelial Ki-67 labeling both in non-malignant and malignant prostate tissue within one day, and that this level remained low until seven days after treatment. However, the proliferation level tended to increase in the non-malignant epithelial cells about one week after treatment, probably because the remaining epithelium, enriched for basal epithelial cells, still had some proliferating activity. The proliferation index was generally decreased in the malignant prostate epithelial cells within the first days after castration while it was highly variable seven days after treatment, suggesting that the sustained anti-proliferative effect of castration may vary between tumors. The apoptotic index increased already within one day after castration treatment both in non-malignant and malignant prostate epithelial cells and the apoptotic levels remained upregulated for about a week. It has previously been suggested that impaired apoptotic response in the primary tumor can be related to a metastatic phenotype [Glinsky et al, 1997, McConkey et al, 1996] and in the present study patients without metastatic disease at diagnosis did show a more sustained apoptotic response than patients with metastases (Table II, paper I). We also observed that both pre- and post-treatment biopsies showed areas with variable levels of proliferating and apoptotic cells. Thus, it is possible that the treatment response may vary within the same tumor.

Human prostate tumors are extremely heterogeneous with mixed populations of cells with different characteristics. Biopsies taken from different time-points after treatment probably are sampled from a variety of subpopulations, which can make comparative analysis difficult. A large number of patients and several biopsies per patient must be studied in order to demonstrate statistically significant differences. However, if significant statistical differences are found between pre- and post-treatment groups in these heterogeneous biopsies, they will probably be underestimated rather than observed by chance.
The castration-induced response in the human prostate was similar to the response seen in the VP of mouse and rat [Banerjee et al, 1995, Westin et al, 1993, Kurita et al, 2001, paper III]. The VP is highly sensitive to androgen regulation and diminishes drastically in size when androgen is withdrawn [Banerjee et al, 1995]. However, this study indicates that experimental findings made in rodents may be relevant also for human prostate biology. The reason for less and slower reduction in organ volume in man compared to rodents [Kucway et al, 2002] after castration is therefore apparently not the lack of epithelial involution but may instead be explained by the fact that human prostate consists of more fibro-muscular stroma than rodent prostate, as described in the introduction, and that there are only limited changes in proliferation and apoptosis in the stroma by castration [Niu et al, 2001]. Moreover, the rapid apoptotic and anti-proliferative castration-induced responses indicate that serum PSA, with its long half-life, is not an ideal marker for monitoring short-term, and thereby possibly not even long-term, responses to treatment.

The magnitude of early castration-induced primary tumor regression in prostate cancer does not predict clinical outcome (paper II)

Currently, methods of monitoring response to treatment are limited and mainly based on nadir serum PSA levels obtained several months after treatment. It would be desirable and probably also beneficial to the patient and the health care sector to be able to predict which patients would be suitable for additional early adjuvant therapy that now is available [de Wit, 2005, Pienta & Smith, 2005]. The study presented in paper II was therefore performed to pursue and relate the findings regarding apoptosis and proliferation in paper I to clinical outcome, using partly the same patient material (n = 83). In addition to clinical data, we added measurements of tissue PSA mRNA and serum PSA levels. The time-points evaluated were also extended to fifteen days after treatment.

Patients were divided into two groups according to their nadir PSA value. Patients with a nadir PSA less or equal to 1 ng/ml, called the strong responders (including 43 % of the patient material), had significantly longer cancer-specific survival than patients with a weak response (Fig. 1A, paper II) as earlier described [Kwak et al, 2002]; 7.2 years vs. 3.3 years, respectively. The strong responders had a lower share of detectable metastases, lower GS, and less advanced tumor stage at diagnosis compared to the weak responders (Table 1, paper II). As assumed, metastasis and GS were also found to be related to survival. All patients with local relapse after therapy, had stage T3-T4 tumors and GS 8-10 at diagnosis.

In our study, the median time to nadir PSA after castration treatment was approximately four months, but longer times have also been reported [Fowler et al, 1995, Benaim et al, 2002, Kwak et al, 2002]. However, in line with results found in non-malignant epithelium,
castration induced a decrease in median primary tumor cell PSA mRNA levels within a few days in patients of all tumor grades and stages, and in patients with and without metastases at diagnosis (Fig. 4A, paper II). Similar results were observed for proliferation, which decreased and for the apoptotic index, which increased (Fig. 4B,C, paper II). The relative changes of these responses were not correlated (Table 2, paper II), indicating that they mirror different cell responses. Similar dissociation between tumor cell growth and PSA expression has previously been described [Denmeade et al, 2003]. The early changes in primary tumor PSA mRNA levels were not related to nadir serum PSA, time to PSA relapse, or to cancer specific survival. Moreover, neither the pre-treatment values nor the relative changes by castration therapy of tumor cell apoptosis or proliferation could predict PSA nadir value, cancer-specific survival, or time to disease progression. Patients with marked reductions in primary tumor cell proliferation and increased tumor cell death could still have a week serum PSA response and short cancer-specific survival. The decrease in tissue PSA mRNA expression level was followed by a decrease also in tissue PSA protein levels. The PSA response in tumor epithelial cells were, however, more heterogeneous than in non-malignant areas, as intense tumor epithelial PSA immunostaining was occasionally found adjacent to negatively stained tumor cells.

In this study most of the patients had metastatic disease (61 %). This was probably the reason for the lack of correlation found between primary tumor epithelial cell PSA mRNA levels and serum PSA levels, since metastases likely contributed substantially to the serum PSA levels. However, in patients without detectable metastases, the pre-treatment tumor epithelial cell PSA mRNA level seemed to be related to the pre-treatment serum PSA level ($r_s = 0.56, p = 0.07, n = 11$), and the relative change of tumor epithelial cell PSA mRNA level by therapy seemed to be related to the nadir PSA level ($r_s = 0.56, p = 0.08, n = 11$). Although tissue PSA levels may not be the ideal marker for monitoring the overall response to castration therapy, tissue PSA measurements may be of some value. We observed a limited and variable reduction of the tissue PSA mRNA levels nine to fifteen days after castration, suggesting active PSA synthesis in some remaining tumor epithelial cells not affected by castration. Tissue PSA may also be related to local tumor relapse since patients with a clear-cut local relapse after therapy had lower pre-treatment tumor epithelial cell PSA mRNA levels than patients without clinical signs of local relapse after therapy ($p = 0.043$).

In this study, tumor epithelial cells from human prostate biopsies were micro-dissected. Micro-dissected cells from small areas may not describe the whole tumor and to avoid this, several biopsies and several areas per biopsy were micro-dissected whenever possible. Since the amount of cell material in different cell compartments and the percentage of successfully transferred micro-dissected cells to the cap may differ, GAPDH was used as a control in the mRNA analysis. GAPDH, a catalytic enzyme involved in glycolysis [Kim & Dang, 2005] is believed to be constitutively expressed in almost all tissues and was
therefore used to normalize samples. However, some physiological factors, such as hypoxia [Graven et al., 1994] and prostate cancer progression [Altenberg & Greulich, 2004] increase GAPDH expression in certain cell types and GAPDH may also be involved in apoptosis [Kim & Dang, 2005]. This does not make GAPDH an ideal housekeeping gene, and in all our studies we have analyzed GAPDH expression separately to ensure that this is not a factor that influences our conclusions.

The important conclusions from this study were that most primary tumors seemed to respond to castration with moderately increased apoptosis as well as decreased cell proliferation and PSA mRNA levels, and that these responses were not related to changes in serum PSA levels or to clinical outcome in patients with advanced prostate cancer.

**Castration rapidly decreases local insulin-like growth factor-1 levels and inhibits its effects in the ventral prostate in mice (paper III)**

Castration-induced prostate involution is probably partly regulated through paracrine mechanisms mediated by hormone receptors in the prostatic stroma [Kurita et al., 2001]. In this study, we used micro-array and micro-dissection techniques to search for stroma-derived factors involved in this signaling.

Our previous studies had shown early castration-induced changes in proliferation and apoptosis in human prostate tissue. In paper III, this was verified in the mouse VP. Androgen ablation decreases blood flow [Lekås et al., 1997] and increases endothelial cell apoptosis [Shabsig et al., 1998] in rat VP, and similar castration-induced effects were observed in this study in mice. Increased endothelial cell apoptosis and decreased endothelial cell proliferation preceded decreased epithelial cell proliferation and increased epithelial cell apoptosis and a reduction in wet weight (Table II, paper III).

Studies using cDNA arrays were used to analyze changes in gene expression in mouse VP at early time-points, i.e. one and three days after castration, when tissue composition was essentially unaffected. The cDNA arrays were used for screening purposes, and the results obtained must therefore be verified by other methods. In order to decrease the number of false positive and false negative results, experiments should be repeated. In this study, quantitative RT-PCR was used to verify interesting cDNA data. Changes in IGF-1, IGFBP-2, -3, -5, and -6 mRNA levels were observed and further evaluated using quantitative RT-PCR. IGF-1 mRNA was significantly decreased already one day after castration treatment and returned to basal levels when androgen was administrated to castrated animals (Fig. 1A, paper III). The IGFBP-2 mRNA level was also significantly decreased after castration but remained unchanged in response to androgen (Fig. 1B, paper III). IGFBP-3 expression increased significantly three days after treatment, and levels
Results and Comments

returned to normal when androgen was administrated (Fig. 1C, paper III). We could not confirm any androgen dependent regulation of IGFBP-5 or IGFBP-6, and these molecules were therefore not further examined. Since IGF-1 primarily signals through its receptor IGF-R1, we also wanted to analyze IGF-R1 mRNA levels in response to androgen withdrawal and substitution. IGF-R1 mRNA levels increased one to seven days after castration and the levels were normalized after androgen replacement (Fig. 1D, paper III).

The decrease in IGF-1 mRNA levels after castration was accompanied by a decrease in IGF-1 protein levels within one day, and after seven days no IGF-1 expression was detectable with Western blot analysis (Fig. 2, paper III). The effect of this decreased IGF-1 protein level was further evaluated by comparing intact levels of IRS-1 and IRS-2 with their corresponding levels one and three days after treatment. To monitor down-stream events, the phosphorylation level of IRS-1 at tyrosine 612 (pY612IRS-1) and serine 307 (pSer307IRS-1) was analyzed. Both basal IRS-1 and pY612IRS-1 levels were reduced at day three after castration while the pSer307IRS-1 level was increased within one day of therapy and reduced thereafter (Fig. 3, paper III). The IRS-2 level was not changed. Two intracellular signaling pathways important for cell survival, the PI3K/AKT and the RAS/RAF/MEK pathways, are activated through IRS-1 (Figure II). Phosphorylation of serine residues, as pSer307IRS-1, is known to counteract activating tyrosine phosphorylations, as pY612IRS-1 [Rui et al., 2001]. Thus, our results indicated reduced IGF-R1 signaling in the VP one to three days after castration.

As described in the introduction, prostate tissue involution in response to castration is apparently caused by changes in prostate stroma [Kurita et al., 2001]. Thus, we wanted to examine in which cellular compartment IGF-1 and its receptor were produced. LMPC was used to collect epithelial calls and adjacent stroma from frozen mouse VP tissue before and after castration. The median IGF-1 mRNA level, measured using real time RT-PCR, was considerably higher in the stroma (more than 20-times) than in the epithelium before castration. The IGF-1 mRNA levels were decreased by castration both in the stroma (p = 0.03) and in the epithelium (p = 0.05). In four out of five animals, the IGF-R1 mRNA expression levels were higher in the epithelium than in the stroma, but this difference was not statistically significant.

In order to investigate if IGF-1 could affect growth of prostate glandular and vascular compartments, IGF-1 was injected locally into the left lobe of the mouse VP, and tissue was analyzed 24 hours later. Epithelial cell proliferation and volume density of von Willebrand factor stained blood vessels increased after IGF-1 administration (Table IV, paper III). Interestingly, when IGF-1 was administrated to castrated animals neither epithelial cell proliferation nor vascular density were affected (Table IV, paper III). Reduced IGF-R1 cell signaling in vitro has previously been shown in the absence of androgen [Pandini et al., 2005] or EGF [Zhang et al., 2000], but the mechanism behind this
Results and Comments

effect is unknown. However, the block is not related to receptor down-regulation since IGF-R1 levels are maintained and even increased after castration. There is also probably no general lack of growth factor responsiveness since EGF has been shown to be able to delay castration-induced VP involution in rats [Torrинг et al., 2000].

Our results indicate that reduced stroma-derived IGF-1 and reduced IGF-1 responsiveness may be an important factor in castration-induced prostate involution.

**Reduction of local IGF-1 synthesis is of importance for castration-induced regression of normal and malignant human prostate tissue (paper IV)**

Previous observations that castration rapidly reduced stroma IGF-1 level and action in the prostate epithelium in mice (paper III) was the base for this study. We wanted to see whether changes in the IGF-system are of importance for castration-induced prostate and prostate cancer regression in humans.

As in the mouse VP, IGF-1 mRNA was primarily expressed in the stroma in non-malignant human prostate tissue, and IGF-1 receptor was expressed in epithelial cells, as previously suggested [Cardillo et al., 2003]. This indicates that epithelial cells are the main target for IGF-1 action in the human prostate. Also, in malignant GS 6 to 7 prostate tissues IGF-1 mRNA was primarily, but not exclusively, found in the stroma. In line with the results found in mice (paper III), castration resulted in rapidly decreased IGF-1 mRNA levels in the stroma cells surrounding non-malignant epithelial glands (Fig. 2, paper IV). Significantly decreased levels of IGF-1 mRNA were seen one to seven days (p = 0.006) but not eight to fourteen days (p = 0.159) following castration. Epithelial IGF-1 mRNA levels did not change by castration. Interestingly, castration failed to significantly decrease IGF-1 levels in stroma derived from GS 6 (n = 1) and 7 (n = 6) tumors. Reduced IGF-1 mRNA levels were, however, sometimes observed in tumor stroma as well as in tumor epithelial cells, which seemed to be related to an apoptotic response after castration. The relative apoptosis induction was higher in tumors with stroma IGF-1 reduction (p = 0.077) and the relative change of IGF-1 mRNA levels in the epithelium was inversely correlated to the post-therapy apoptosis levels (p = 0.020). This was in line with the observations in the non-malignant prostate stroma, in which IGF-1 mRNA levels after treatment were inversely correlated to relative change in apoptosis (p = 0.002). This indicates a high magnitude of apoptotic induction after castration treatment in normal and malignant epithelial cells surrounded by stroma cells with markedly reduced IGF-levels after therapy as well as in tumor epithelial cells with reduced intrinsic levels of IGF-1. Thus, lack of IGF-1 reduction in response to castration therapy may contribute to inadequate apoptosis induction in prostate cancer after therapy.
Since castration failed to suppress stroma IGF-1 synthesis it was of interest to investigate possible reasons for this lack of response. Pre-treatment biopsies were therefore stained and scored for the presence of nuclear AR in the stroma tissue adjacent to non-malignant and malignant prostate glands in the same biopsies. In non-malignant prostate tissue, 56 % of the stroma cells were nuclear AR receptor positive (Fig. 3A, paper IV). In contrast, tumor stroma contained significantly less cells that expressed nuclear AR (10 %, p = 0.001, Table 1, Fig. 3C). The AR level in the tumor stroma was correlated to the stroma IGF-1 mRNA level before therapy (p = 0.006) and inversely correlated to the relative IGF-1 mRNA reduction in the tumor stroma after castration (p = 0.033). These results indicate a higher magnitude of therapy response with increasing AR level in the tumor stroma. Almost all tumor epithelial cells initially showed nuclear AR staining, and castration reduced the percentage of epithelial cells with strong nuclear staining in most tumors, from a median staining level of 68 % to 28 % after castration (p = 0.008, Table 1, paper IV). The magnitude of this response was, however, not correlated to the castration-induced changes in epithelial cell IGF-1 mRNA levels or apoptosis. Thus, androgen stimulated IGF-1 synthesis in the stroma apparently acts as an anti-apoptotic factor in the human prostate epithelium. This does not exclude the possibility that IGF-1 may also act on the relatively few IGF-R1 bearing cells in the prostate stroma [Cardillo et al, 2003] or vasculature (paper III), but this possibility was not examined further.

In this study we demonstrated that low stromal AR expression in prostate cancer may mean that the tumor stroma, in contrast to normal prostate stroma, is largely androgen-independent and that this is of importance for the overall tumor response to castration. This is in line with previous studies showing that the number of AR positive stroma cells is reduced in prostate cancer compared to non-malignant prostate tissue [Ricciardelli et al, 2005, Henshall et al, 2001, Olapade-Olaopa et al, 1999]. In prostate tumors, castration may thus fail to reduce the secretion of stroma-derived factors, like IGF-1, that normally regulates epithelial cell proliferation and death. In non-malignant as well as in malignant human prostate tissue, castration did however result in a rapid and major decrease in cell proliferation (paper I), suggesting that factors apart from decreased IGF-1 level mediate the castration-induced decrease in tumor cell proliferation.

Our observations, suggest that the local reduction in IGF-1 level is of importance for therapy-induced induction of tumor cell apoptosis, although other factors must be involved as well. The question of whether castration therapy also decreases IGF-1 responsiveness in human tumor epithelial cells as in normal epithelial cells in the mouse prostate (paper III) remains to be examined. Most primary prostate tumors stained positively for IGF-R1 before [Krueckl et al, 2004, Hellawell et al, 2002] and after castration, indicating that tumor epithelial cells are likely targets for local IGF-1 action. This knowledge could possible be used to improve therapy.
GENERAL DISCUSSION

Early castration-induced responses in mouse and human prostate

Androgen ablation treatment is the standard therapy for metastatic prostate cancer. The basis of this therapeutic approach is that androgen stimulation is essential for growth and resistance to apoptosis in most prostate carcinomas. The mechanism behind castration-induced prostate tissue involution is incompletely understood and studies of non-malignant prostate tissue elucidating the normal response to castration can thus be useful when trying to understand the response, or lack of response, in malignant tissue. This thesis shows that early castration-induced effects in human prostate tissue resemble those of the mouse VP. This indicates that the VP in mice can be used as a model system to study androgen ablation treatment. The studies included in this thesis also indicate, for the first time, that both non-malignant and primary tumor epithelial cells in patients with advanced disease, respond in a similar manner with regard to apoptosis and proliferation after castration, although the magnitude of the apoptotic response was more pronounced in the normal epithelial compartment than in the malignant epithelial cells. Changes neither in tumor cell apoptosis nor in proliferation could predict serum PSA levels or clinical outcome.

As mentioned in the results above, serum PSA may not be the ideal marker for monitoring the effect of treatment, since its half-life in serum and thus the time to reach nadir serum PSA levels is very long. In addition, changes in PSA levels are apparently not related to changes in tumor cell proliferation and death. Additional therapies for metastatic prostate cancer are available [de Wit, 2005, Pienta & Smith, 2005], and an early prediction of patients suitable for this early adjuvant treatment is thus desirable. The results presented in this thesis also suggest that the time-frame is of importance when analyzing different responses to treatment and possible markers for prostate cancer. For example, detectable prostate tissue PSA levels 6 to 12 months after hormone therapy in biopsies indicated a poor prognosis [Grande et al, 2000], while we, in paper II, did not find an association between early (within two weeks) tissue PSA response and outcome. However, our results suggests that additional studies of tissue PSA mRNA levels approximately one to two weeks after treatment could give further prognostic information, since biopsies from the group of patients with weak serum PSA response showed a limited tissue PSA mRNA response at that time-point.

Due to heterogeneity within the primary tumors, our results may not mirror the overall response of the tumor. Furthermore, if metastases are present, it is likely that their response to treatment is not reflected in the principal primary tumor response.
Aberrant signaling between stroma and epithelium in prostate cancer

Understanding local cellular interactions within normal tissue can facilitate an understanding of corresponding aberrant communication in tumors and yield novel therapeutic targets for prostate cancer.

As previously reported we found the number of AR positive stroma cells to be reduced in prostate cancer compared to non-malignant prostate tissue [Ricciardelli et al, 2005, Henshall et al, 2001, Olapade-Olaopa et al, 1999]. Low stroma AR expression in tumors has been related to early PSA relapse after radical prostatectomy [Ricciardelli et al, 2005, Henshall et al, 2001]. We now suggest that this low AR expression in prostate cancer might also mean that the tumor stroma, in contrast to normal prostate stroma, is largely androgen independent and that this is of importance for the overall tumor response to castration. Furthermore, in contrast to normal prostate, prostate tumors failed to reduce stroma IGF-1 mRNA levels that normally regulate epithelial cell proliferation and death. However, in both non-malignant and malignant human prostate tissue, castration did result in a rapid and major decrease in cell proliferation suggesting that factors other than decreased IGF-1 levels mediate the castration-induced decrease in tumor cell proliferation. In human prostate cancer, the epithelial apoptotic response in tumors peak at a 2-3 fold increase whereas in non-malignant tissue, in which stroma AR is abundant and stroma IGF-1 is reduced, apoptosis is increased by 7-fold. Thus, reduced IGF-1 level in the stroma seems to be important for the magnitude of castration-induced epithelial cell apoptosis, although other factors must be involved as well. Taken together, our results indicate inadequate apoptosis induction in tumor cells due to reduced androgen sensitivity and thus lack of IGF-1 reduction in the tumor stroma in response to castration. Accordingly, one can assume that metastatic tumor cells have an even less ability than primary tumor cells to respond to treatment, due to their different environment with often high levels of stimulatory growth factors such as IGF-1 and probably androgen-independent stroma.

In line with this hypothesis, castration controls growth of the primary tumor in a transgenic mice model of prostate cancer (TRAMP), but fails to control growth of its metastases [Wikström et al, 2005]. Moreover, metastases in hormonally treated prostate cancer patients demonstrate a large heterogeneity, both between patients and within the same patient [Shah et al, 2004] indicating that the response to treatment may vary. Consequently, interactions between metastatic tumor cells and their surrounding stroma need to be further investigated.
Insulin-like growth factor-1 signaling in prostate cancer and possible future targets for therapy

Whether high serum IGF-1 level is a risk factor for prostate cancer is debated, and observed associations between circulating levels of IGF-1 and risk have been inconsistent (as described in the introduction of this thesis). An increase in serum IGF-1 levels with increasing tumor burden in TRAMP mice has been reported [Kaplan et al., 1999], but there are also contradicting reports that there are no correlation between preoperative plasma IGF-1 levels and prostate cancer pathologic features in patients [Shariat et al., 2000].

Prostate cancer preferentially metastasizes to bone, which is the largest store of IGFs in the body [Goya et al., 2004, Hauschka et al., 1986]. IGF-R1 is generally up-regulated in advanced and metastatic prostate cancer [Krueckl et al., 2004, Hellawell et al., 2002, Nickerson et al., 2001] and antibody-inhibition of IGF-1 and IGF-2 reduced prostate cancer growth within the bone [Goya et al., 2004]. Down-regulation of IGF-1 synthesis in the bone micro-environment thus appears to be particularly important for therapy outcome. One important question that should be examined further is whether the high IGF-1 levels in bone are influenced by castration therapy.

Since the IGF axis seems to play an important role in prostate cancer biology, the IGF-R1 may be a novel target for prostate cancer treatment to be combined with androgen ablation for increased efficiency. Methods to inhibit IGF-R1 signaling such as antibodies, anti-sense strategies, dominant negative IGF-R1 mutants, or small-molecules targeting the tyrosine kinase domain [Garber, 2005, Bähr & Groner 2004, LeRoith & Helman 2004] are under development. However, some of these approaches have problems, such as lack of specificity and difficulty in drug deliverance. Targeting IGF-R1 could impair IR expression due to their extensive homology, and the side effects could thus be insulin resistance and diabetes. Since IGF-R1 is present throughout the body, normal tissue will probably also be affected by inhibited IGF-R1 signaling. Further studies will be needed in order to elucidate the effects of treatment, particularly in tissues with a high level of cellular turnover, such as the gastrointestinal tract and the bone marrow.
CONCLUSIONS

- The time-frame and nature of the early response to castration is apparently similar in the mouse prostate and in non-malignant human prostate. Information derived in mice models may therefore be relevant for human prostate.

- Castration therapy rapidly decreases tissue PSA mRNA and cell proliferation and increases apoptosis in most primary tumors. These responses are, however, not related to subsequent changes in serum PSA levels or to clinical outcome, possibly because metastases do not respond as favorably as the primary tumors to therapy.

- Changes in tissue PSA mRNA levels are not correlated with therapy-induced changes in cell proliferation and apoptosis. Therefore, measurements of therapy-induced changes in PSA are not necessarily representative of changes in tumor growth characteristics.

- IGF-1 is apparently an important mediator of stroma-epithelial cell interaction in the mouse and human prostate. Androgen ablation rapidly reduces stroma IGF-1 levels and inhibits its signaling via IGF-R1 in the epithelium. Castration-induced prostate tissue involution may be caused by locally decreased IGF-1 synthesis and action.

- A castration-induced decrease in human prostate cancer IGF-1 levels is of importance for therapy-induced tumor cell apoptosis. The limited therapy-induced decrease in IGF-1 in some patients could in part be related to the development of an androgen-independent tumor stroma. If so, it is possible that inhibition of IGF-1 signaling could enhance the effect of castration therapy.
ACKNOWLEDGEMENTS

Här är nu sidorna, som alla alltid hittar fram till (!), där jag får möjlighet att skriftligen tacka de personer som på alla möjliga sätt varit till hjälp och stöd under min tid som doktorand.

Jag vill rika ett stort TACK till:

Pernilla och Anders, som på ett föredömligt sätt handlett i ”forskten”. Bättre än er blir det inte!

Pär, som samlat massvis av data och material och som med kloka inlägg och språkliga rättelser gjort manuskripten förståeliga även för utomstående.

mina medförfattare Katarina och Malin för det jobb ni lagt ner.

Birgitta, Elisabeth och Pernilla, som med en positiv inställning gång på gång bevisat att ingenting är omöjligt. Siggan och Ulla-Stina, förutom att ni envisats med att servera oliver på gruppens gemensamma middagar är ni toppen bra! Åsa, du är en mycket klok kvinna med ett till synes oändligt tålamod. Ni är alla klippor, glöm aldrig det!

Sofia, Anna, Peter, Lotta och Andreas, det har varit väldigt skoj att vara doktorand tillsammans med er!

”Prostatagruppen”, för fredagslunchmöten, goda råd och Hemavan-turer (jag förstår dock fortfarande inte vad det är som är så lockande med fjällbranter!).

Karin, Anette och Solveig, för ovärderlig hjälp när det behövts ordning och reda.

Ulrica, för mikro-dissektions- och bioanalyzerassitans.

Åse, Andreas, Ylva och Stina, för att ni så snällt hjälp mig med mina disputationss- och avhandlingsrelaterade funderingar.

Bethany, för att du uppmärksammat och rättat till mina nedskrivna svagheter i det engelska språket.
Acknowledgements

nuvarande och dåvarande arbetskamrater i 6M, ”gamla 3B korridoren” och ”baracken”. Ni är sååå många, så jag räknar inte upp er vid namn, men det har verkligen varit roligt att jobba, surra bort fikatid och redovisa uppdrag tillsammans med er!

alla mina vänner; tisdagsmiddagsgänget, innebandyfolket och alla andra. Vad vore livet utan trevliga människor som förgyller ens fritiden?

mamma och pappa, för att ni ställer upp i alla lägen och är de bästa föräldrar man kan önska sig! Niklas och Thomas, mina fantastiska bröder i söder.

David, du är allt för mig!
REFERENCES


References


References


• English HF, Santen RJ, Isaacs JT. Response of glandular versus basal rat ventral prostatic epithelial cells to androgen withdrawal and replacement. Prostate. 1987;11(3):229-42.


- Hellawell GO, Turner GD, Davies DR, Poulson R, Brewster SF, Macaulay VM. Expression of the type 1 insulin-like growth factor receptor is up-regulated
References


References


References


• Liu JP, Baker J, Perkins AS, Robertson EJ, Efstratiadis A. Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). Cell. 1993 Oct 8;75(1):59-72.


• Moinfar F, Man YG, Arnaould L, Brathhauer GL, Ratschek M, Tavassoli FA. Concurrent and independent genetic alterations in the stromal and epithelial...
References


- Nickerson T, Chang F, Lorimer D, Smeekens SP, Sawyers CL, Pollak M. In vivo progression of LAPC-9 and LNCaP prostate cancer models to androgen independence is associated with increased expression of insulin-like growth factor I (IGF-I) and IGF-I receptor (IGF-IR). Cancer Res. 2001 Aug 15;61(16):6276-80.


References


References

- Tennant MK, Thrasher JB, Twomey PA, Drivdahl RH, Birnbaum RS, Plymate SR. Protein and messenger ribonucleic acid (mRNA) for the type 1 insulin-like growth factor (IGF) receptor is decreased and IGF-II mRNA is increased in human prostate carcinoma compared to benign prostate epithelium. J Clin Endocrinol Metab. 1996 Oct;81(10):3774-82.


