ANDROGEN-INDEPENDENT PROSTATE CANCER

– STUDIES ON ANGIOGENESIS AND ADAMTS1

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Front cover: Immunohistochemical staining of blood vessels in a tumor xenograft.

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To my family

Androgen-Independent Prostate Cancer – studies on angiogenesis and ADAMTS1

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ABSTRACT

Androgen deprivation therapy (ADT) is the standard treatment for advanced prostate cancer since prostate tumors initially are dependent on androgens for growth. However, most tumors will eventually relapse and grow in a highly aggressive and androgen-independent (AI) manner. AI prostate cancer is associated with poor prognosis and new treatment modalities are therefore urgently needed. Anti-angiogenic therapy could be one strategy to suppress tumor growth, but this requires an increased understanding about the regulation of angiogenesis in AI prostate cancer. The aim of this thesis was therefore to increase the knowledge about AI prostate cancer, with special focus on angiogenesis. First, an experimental model system that allows comparative studies of androgen-dependent (AD) and AI prostate cancer was established. An AD human prostate cancer cell line was cultured under selective pressure in androgen depleted medium, which resulted in an AI subline. Characterization of the newly established AI cell line revealed that transition into androgen-independency was associated with a more rapid tumor take, decreased PSA levels, increased microvessel density (MVD) and altered blood vessel morphology. To identify factors that could be of importance for the increased angiogenesis observed in AI tumors, a gene expression analysis was performed. The results demonstrated that transition into androgen-independency was accompanied with altered expression of a number of genes associated with angiogenesis, including ADAM metallopeptidase with thrombospondin type 1 motif, 1 (ADAMTS1). ADAMTS1 is a potent anti-angiogenic factor that was found to be significantly downregulated in AI cancer cells and its expression correlated negatively with MVD in the tumor xenografts. Furthermore, immunohistochemical studies of tumor tissue from prostate cancer patients demonstrated significantly lower levels of ADAMTS1 in cancer areas than in benign glands. In addition, low levels of ADAMTS1 were associated with metastatic disease and higher MVD in AI tumors. In order to further elucidate the role of ADAMTS1 in prostate cancer progression and tumor angiogenesis, the expression of ADAMTS1 was modified by transfection in the experimental model system. The results revealed that altered expression of ADAMTS1 markedly affected the blood vessel morphology but not the number of blood vessels in the tumor xenografts. Modified expression of ADAMTS1 also affected the levels of the anti-angiogenic protein TSP1, whose expression was inversely related to ADAMTS1. Moreover, upregulation of ADAMTS1 resulted in a markedly delayed growth of AI tumors, while the opposite was observed in AD tumors. In summary, the results show that transition into androgen-independency is associated with increased angiogenesis and altogether the data from this thesis suggest that ADAMTS1 is an important factor in prostate cancer biology that is lost during disease progression and that is associated with decreased angiogenesis, tumor growth and metastasis in AI prostate cancer.

Key words: Prostate cancer; Androgen-independent, Castration resistant; Hormone refractory; Angiogenesis, Microvessel density; ADAMTS1; LNCaP, VEGF; TSP1

LIST OF PUBLICATIONS

This thesis is based on the following papers, referred to in the text by their Roman numerals:

I. **Gustavsson H.**, Welén K. and Damber JE.
Transition of an androgen-dependent human prostate cancer cell line into an androgen-independent subline is associated with increased angiogenesis
The Prostate 2005 Mar 1;62(4):364-373

II. **Gustavsson H.**, Jennbacken K., Welén K. and Damber JE.
Altered expression of genes regulating angiogenesis in experimental androgen-independent prostate cancer
The Prostate 2008 Feb 1;68(2):161-170

III. **Gustavsson H.**, Wang W., Jennbacken K., Welén K. and Damber JE.
ADAMTS1, a putative anti-angiogenic factor, is decreased in human prostate cancer
BJU International 2009, In press

IV. **Gustavsson H.**, Tešan T., Jennbacken K., Kuno K., Damber JE. and Welén K.
ADAMTS1 is involved in the regulation of blood vessel morphology, TSP1 levels and tumor growth in experimental prostate cancer
In manuscript

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ABBREVIATIONS

ADAM	A disintegrin and metalloproteinase
ADAMTS	ADAM metallopeptidase with thrombospondin type 1 motifs
AD	Androgen-dependent
ADT	Androgen deprivation therapy
Ang	Angiopoietin
AI	Androgen-independent
AR	Androgen receptor
ARE	Androgen response element
α-SMA	Alpha-smooth muscle actin
bFGF	Basic fibroblast growth factor
DCC-FBS	Dextran charcoal treated fetal bovine serum
DELFIA	Dissociation-enhanced lanthanide fluorescent immunoassay
DHT	Dihydrotestosterone
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
FBS	Fetal bovine serum
GnRH	Gonadotropin releasing hormone
HB-EGF	Heparin-binding EGF-like growth factor
HIF-1	Hypoxia inducible factor 1
LH	Luteinizing hormone
LNCaP	Lymph node carcinoma of the prostate
MMP	Matrix metalloproteinase
MVD	Microvessel density
PDGF	Platelet-derived growth factor
PIN	Prostatic intraepithelial neoplasia
PSA	Prostate specific antigen
RT-PCR	Reverse transcriptase polymerase chain reaction
SEM	Standard error of the mean
SRC1	Steroid receptor coactivator 1
TIF2	Transcriptional intermediary factor 2
TGF-β	Transforming growth factor-β
TIMP	Tissue inhibitor of matrix metalloproteinases
TRAMP	Transgenic adenocarcinoma of mouse prostate
TSP	Thrombospondin
TURP	Transurethral resection of the prostate
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

INTRODUCTION

THE PROSTATE GLAND

Anatomy, morphology and physiology

The prostate is an exocrine gland that is part of the male reproductive system. It is located just below the urinary bladder and surrounds the urethra. The adult human prostate is composed of three distinct zones; the peripheral zone, the transitional zone and the central zone.[1] The peripheral zone is the largest, which constitutes approximately 70% of the prostate, and the most common origin of prostate tumors.[2]

The prostate is composed of epithelial lined acini and ducts that are surrounded by a dense fibromuscular stroma and enclosed by a fibrous capsule. The prostate epithelium consists of three different cell types; the luminal epithelial cells, the basal epithelial cells and the neuroendocrine cells (Fig. 1). The predominant cell type is the luminal epithelial cells that produce and secrete the components of the prostatic fluid. They are terminally differentiated cells that express the androgen receptor (AR)[3-5] and are dependent on androgens for survival.[6,7] The basal cells are localized between the luminal cells and the underlying basement membrane. The basal cells also express the AR,[3,4,8] but at lower levels and do not require androgens for survival.[6,7] A subset of the basal cells are believed to function as renewing stem cells that are stimulated by androgens to proliferate and differentiate into the secretory luminal phenotype via transit amplifying cells.[9,10] The third cell type, the neuroendocrine cells, are terminally differentiated cells that are dispersed throughout the basal cell layer. They are believed to support the luminal cells in a paracrine fashion and are independent of androgens.[11,12] An underlying basement membrane separates the epithelium from the surrounding stroma. The stroma consists of different cell types, including smooth muscle cells, fibroblasts, mast cells and macrophages that are embedded in a collagenous matrix together with blood vessels, lymphatic vessels and nerves.

The primary function of the prostate is to produce a fluid that drains into the urethra and together with the spermatozoa and secretion from the seminal vesicles constitutes the majority of the semen. The prostate secretion is believed to enhance sperm motility and survival, but is not required for fertility. The fluid is weakly alkaline and protects the sperm from the acidic milieu in the vagina. In addition,

several proteolytic enzymes are produced and secreted by the prostate. One of the major proteins in prostate secretions is prostate specific antigen (PSA) that belongs to the family of human glandular kallikreins.[13] PSA is a serine protease that acts as a liquefying agent and facilitate sperm motility by cleaving the gel forming proteins semenogelin I and II in semen.[14] Under normal conditions PSA is secreted into the ducts and ejected into the urethra, but during pathological conditions the basal cell layer and the basement membrane can be disrupted, causing PSA leakage into the surrounding stroma and vasculature.

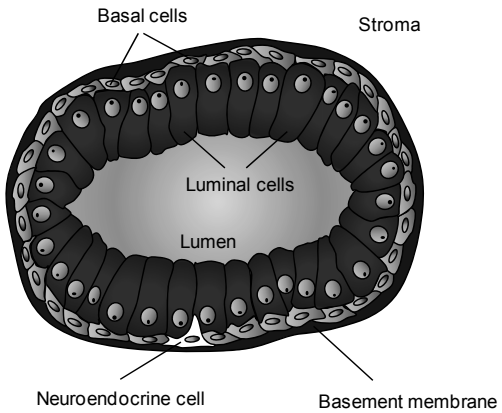

Figure 1: Schematic illustration of a human prostate gland. The epithelium consists of three different cell types; luminal cells, basal cells and neuroendocrine cells. The basement membrane separates the epithelium from the surrounding stroma.

Androgen regulation of the prostate

The prostate gland is dependent on a continuous supply of androgens for development, growth, differentiation, maintenance and function. Testosterone is the most abundant circulating androgen in men, with the testes producing more than 95%. The remaining 5% is derived from the adrenal glands.[15] The production and secretion of androgens is regulated by the hypothalamus and the pituitary gland (Fig. 2). The release of gonadotropin releasing hormone (GnRH) from the hypothalamus results in secretion of luteinizing hormone (LH) from the pituitary. Circulating LH then stimulates the Leydig cells of the testes to produce and secrete testosterone. The hypothalamus also releases corticotrophin releasing hormone (CRH) that stimulates the pituitary to secrete adrenocorticotropic hormone (ACTH). ACTH in turn, induces the adrenal production of testosterone and other weak androgens, like androstenedione and dihydroepiandrosteron. These androgens can subsequently be converted to testosterone in peripheral tissues. The majority of circulating testosterone is bound to plasma proteins and only a small portion remains free. In the prostate, free testosterone diffuses into the cells and is converted into dihydrotestosterone (DHT) by the enzyme 5α-reductase.[15] DHT

binds the AR, causing dissociation of the receptor from heat shock proteins followed by receptor dimerization and phosphorylation. Testosterone can also bind and activate the AR, but DHT has a stronger binding affinity for the AR and is more potent than testosterone.[16] The activated receptor complex is then translocated to the nucleus. Inside the nucleus it binds to androgen response elements (AREs) in promoter regions of different target genes and regulates transcription.

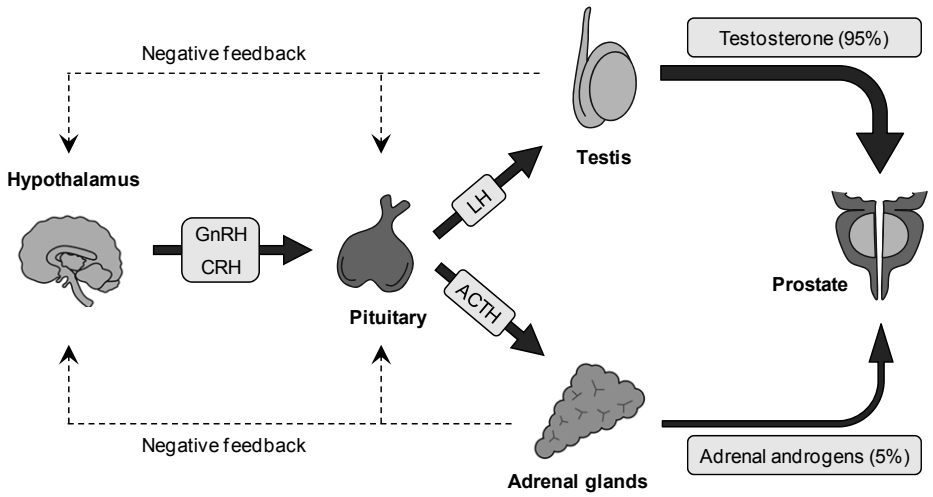

Figure 2: Regulation of testosterone production. The hypothalamus secretes GnRH and CRH that stimulate the pituitary to release LH and ACTH, respectively. LH stimulates testosterone production in the testis and ACTH stimulates production of testosterone and other weak androgens in the adrenal glands. Testicular testosterone accounts for approximately 95% of the circulating testosterone. GnRH = gonadotropin releasing hormone; CRH = corticotropin releasing hormone; LH = luteinizing hormone; ACTH = adrenocorticotropic hormone.

Removal of androgens results in loss of secretory function, decreased cell proliferation, increased apoptosis and thereby to involution of the gland.[17-20] Initially this was believed to be a direct effect on the epithelium due to lack of AR activation. However, more recent studies have shown that the main function of androgens on luminal epithelial cells is to stimulate the secretory function and keep the cells in a differentiated and growth quiescent state, while androgen regulation of epithelial growth and regression mainly is mediated by the stroma.[21,22] In response to androgen stimulation, stromal cells that also express AR[3-5] produce several growth factors that act in a paracrine fashion to sustain the vitality of the epithelium.[23] Thus, the widespread apoptosis and loss of epithelium induced by castration is primarily due to inactivity of the AR in stromal cells rather than in epithelial cells[24,25] In addition, androgen regulation of prostate growth and regression has been suggested to be mediated by the vasculature.[26,27] In the normal

prostate and in androgen-dependent (AD) tumors, castration induces endothelial cell apoptosis, vascular regression and decreased blood flow.[28-33] These vascular changes precede the castration induced effects on the epithelium, suggesting that the involution of the prostate gland partly is due to insufficient blood supply.[28-30] Furthermore, testosterone induced regrowth of the prostate is preceded by increased angiogenesis and thereby increased blood flow.[31] Since murine prostate endothelial cells have been reported to lack expression of AR,[5] it was earlier believed that the androgen regulation of the vasculature primarily was an indirect effect mediated by epithelial and stromal cells in a paracrine fashion.[33-35] However, more recent studies have demonstrated that the AR indeed is expressed in human prostate endothelial cells and can mediate androgen regulated gene transcription, ultimately leading to increased proliferation of these cells.[36]

PROSTATE CANCER

General background

Prostate cancer is the most common cancer form among men in the Western countries.[37,38] In Sweden approximately 9 000 men are diagnosed with the disease every year and approximately 2300 will die from their cancer (Swedish Cancer Registry 2007). The incidence rate has increased during the past few decades, primarily due to the introduction of PSA testing, while the mortality rate almost has remained unchanged. Ageing is the single most significant risk factor for developing prostate cancer, and the disease is most common among men over 60 years of age.[39] In fact, small foci of prostate cancer can be found in approximately 50% of elderly men,[40] but only a small fraction of these will develop into a clinically significant disease. Epidemiological studies have shown large variations in the incidence and prostate cancer is more common in Europe and USA than in Asia. However, the presence of small latent carcinomas seems to be equally frequent throughout the world[41] and the risk of developing prostate cancer increases markedly in native Asians that migrate to USA,[42] indicating that dietary and environmental factors are of importance. There is also a hereditary form of prostate cancer, and a number of prostate cancer susceptibility genes have been identified.[43]

Early prostate cancer demonstrates few symptoms, and most symptoms like voiding problems, hematuria and bone pain are signs of advanced prostate cancer that have spread outside the prostate. Most prostate cancer is today diagnosed on the basis of PSA testing, followed by rectal palpation or transrectal ultrasound together with sampling of biopsies that are examined histologically. The Gleason score system is used to assess the differentiation grade of the tumors and is one of

the most important prognostic factors for disease progression.[44,45] The tumor tissue is graded from 1 to 5, where 5 represent the most aggressive tumor pattern with the lowest differentiation grade. The sum of the two most common grades is then giving the Gleason score of the tumor. In a recent modification of the Gleason score system, the highest grade should also be included.

Prostate cancer can be diagnosed as local, locally advanced or metastatic disease. Treatment options of organ confined disease include radical prostatectomy and radiation therapy that aims to remove the tumor.[43] Another option for men with a short life expectancy and with clinically low-risk prostate cancer is active surveillance with regular controls followed by treatment in the case of disease progression.[43] Advanced prostate cancer is characterized by spreading outside the prostate capsule and the most common site for prostate cancer metastasis is to lymph nodes and bone. Like the normal prostate, prostate cancer growth is initially dependent on androgens and androgen deprivation therapy (ADT) is therefore the standard treatment for locally advanced and metastatic disease.[20,46-50] The beneficial effects of ADT were first described by Huggins in 1941 and he was later awarded the Nobel Prize for his pioneering work.[51,52] Deprivation of testicular androgens can be achieved by surgical castration, or more often, by chemical castration. Production of testosterone can be inhibited by GnRH agonists or antagonists that downregulate or inhibit GnRH receptors and thereby result in suppressed pituitary LH release.[43,53] The effects of androgens can be further blocked by AR antagonists, i.e. anti-androgens that exert their effect peripherally by blocking the AR in the prostate cancer cells.[43] The majority of patients respond to ADT with decreased tumor burden, symptomatic relief and decreased PSA. However, ADT is not curative and even if the growth arrest can last for several years, the tumors will eventually relapse and continue to grow in an androgen-independent (AI) manner. This stage of the disease is referred to as castration resistant or hormone refractory prostate cancer. Recurrent castration resistant prostate cancer is usually highly aggressive and metastatic and is the terminal stage of prostate cancer that ultimately leads to the death of the patients. Castration resistant prostate cancer is today an incurable disease and the treatment options, including radiation and cytotoxic treatment are only palliative. Treatment with the cytotoxic drug docetaxel in combination with prednisone has been shown to improve survival, but only with a few months.[54,55] The need for new treatment options is urgent and several promising drugs, including cancer vaccines,[56] angiogenesis inhibitors[57] and inhibitors of androgen biosynthesis[58] are at present evaluated in clinical trials.

Development and progression of prostate cancer

Prostate cancer origins in the glandular epithelium and the tumor cells have generally been believed to originate from the luminal cells, since they are dependent on androgens and express luminal cell markers. However, there are increasing evidence indicating that the cancer cells are derived from less differentiated stem cells or transit amplifying cells.[59,60] Most prostate tumors are heterogeneous and multifocal, suggesting that multiple neoplastic foci have emerged and evolved independently.[61]

Prostate cancer development and progression is a multistep process (Fig. 3). Through genetic and epigenetic alterations normal epithelial cells develop into malignant cells escaping from normal regulatory control. The genetic alterations causing prostate cancer are not well understood, but premalignant lesions, so called prostatic intraepithelial neoplasia (PIN), are often found within the prostate. PIN is considered to be a precursor stadium to prostate cancer that histologically is very similar to prostate cancer with the exception that the basal layer is still present.[62] Through additional alterations malignant tumors develop that initially are restricted to the prostate, but eventually penetrate the prostate capsule, invade surrounding tissues and ultimately form metastases. As mentioned earlier, prostate cancer is initially dependent on androgens, but the transformation into a malignant phenotype is associated with a shift from paracrine to a more autocrine androgen stimulation.[63] Since ADT is used to treat advanced prostate cancer, further tumor progression is associated with the loss of androgen-dependency and the development of highly aggressive castration resistant disease.

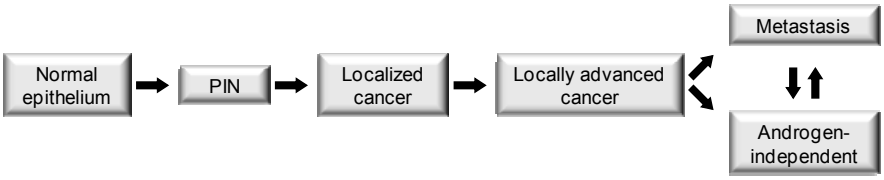

Figure 3: Prostate cancer development and progression is a multistep process. Prostatic intraepithelial neoplasia (PIN) is considered to be a precursor stadium to prostate cancer. Early stages of prostate cancer are generally dependent on androgens and can be treated with androgen deprivation therapy, while late stages of prostate cancer are associated with androgen-independent and metastatic disease.

Development of AI prostate cancer

Androgen withdrawal causes growth arrest and massive apoptosis of the vast majority of prostate cancer cells, but does not eliminate all cancer cells. Through cellular adaptation or selection of preexisting cancer cells that have the capacity to

grow in the absence of androgens, AI tumors will eventually relapse. The progression into androgen-independency is not fully understood, but a number of potential mechanisms have been proposed (Fig. 4).[64,65] Several of them involve alterations that result in retained AR activation in spite of low serum concentrations of testosterone.

AR-dependent mechanisms

One possibility is that increased levels of AR make the cancer cell hypersensitive to low concentrations of androgens. This has been demonstrated in experimental androgen-independent cancer, where an increase in expression, stability and nuclear localization of the AR was associated with growth stimulation at lower androgen concentrations.[66] Increased expression of the AR is commonly observed in castration resistant tumors and AR gene amplification is found in approximately 30% of relapsed tumors after ADT, but not in hormonally untreated tumors.[67-69]

There are several studies reporting that AR mutations are frequently found in metastatic and AI prostate cancer.[70-73] Mutations in the ligand binding domain of AR can result in altered specificity and thereby activation by other ligands than DHT and testosterone. The most studied AR mutation is the T877A mutation, which results in the substitution of threonine to alanine at position 877 in the ligand binding domain. It was first described in the LNCaP cell line,[74] but has also been observed in clinical samples.[75] Molecular studies have demonstrated that estrogens, progestagens and anti-androgens can bind and activate ARs with this mutation and thereby function as agonists.[74] In addition, there are studies demonstrating that glucocorticoids can function as agonists in other forms of mutated AR.[76] The fact that anti-androgens can function as agonists may explain the beneficial effects of anti-androgen withdrawal on some patients with progressive prostate cancer after combined androgen blockade. This is further supported by the fact that mutations in codon 877 are frequently found in metastases from patients treated with flutamide.[77]

When the AR is activated it binds to AREs in the promoter regions and regulates transcription of different target genes. However, the transcription is also regulated by several co-activators and co-repressors that bind the AR complex and facilitates or prevent transcription. Modulation of these co-regulatory proteins have been suggested to be another potential mechanism responsible for development into androgen-independency and overexpression of co-activators like steroid receptor coactivator 1 (SRC1) and transcriptional intermediary factor 2 (TIF2) have been described in recurrent prostate cancer.[78]

Another mechanism whereby the cancer cells can circumvent androgen deprivation is by an increased local production of androgens. In fact, there are studies showing that the intraprostatic concentrations of androgens remain moderately high after ADT.[79,80] Furthermore, the levels of testosterone is higher in metastases from castration resistant prostate cancer than in untreated primary tumors.[81] Elevated intratumoral levels of androgens have also been associated with progression of LNCaP tumors after castration.[82] It has been suggested that the maintained levels of testosterone is due to intracrine production of testosterone or by an upregulation of genes converting adrenal androgens to testosterone.[81-83]

All of the potential mechanisms described so far require a ligand for activation of the AR, but another hypothesis is that the AR can be activated in a ligand-independent manner. In fact, constitutively active variants of AR that lack the ligand-binding domain have been identified in castration resistant prostate tumors.[84] Moreover, several growth factors and their downstream tyrosine kinases, including insulin-like growth factor 1 (IGF-1), keratinocyte growth factor (KGF), epidermal growth factor (EGF) and HER-2/neu, can induce phosphorylation of the AR in the absence of androgens.[85,86]

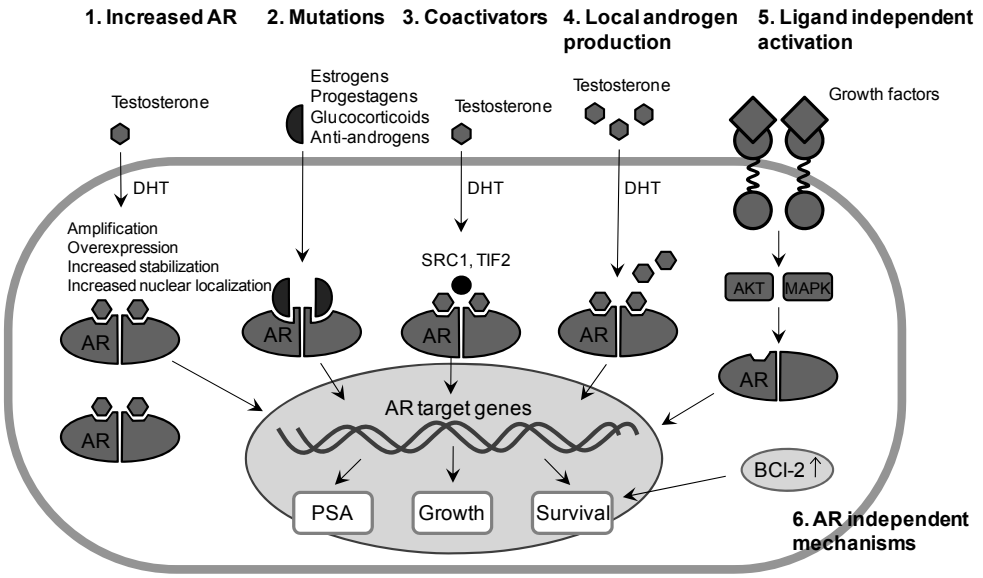

Figure 4: Several mechanisms have been proposed to be responsible for the development of AI prostate cancer. Many of them result in activation of the androgen receptor (AR) in spite of low serum concentrations of testosterone (1-5), but there are also AR independent mechanisms (6). DHT = dihydrotestosterone; PSA = prostate specific antigen; SRC1 = steroid receptor coactivator 1; TIF2 = transcriptional intermediary factor 2.

AR-independent mechanisms

Even if the AR is believed to be of importance in the majority of castration resistant tumors, the variability in AR levels increases with progression and there are also tumors that are AR negative.[87] In addition, there are a number of AI cell lines lacking the AR.[88] This indicates that there are other pathways not involving the AR. Isaacs postulated that prostate tumors contain a mixture of AD and AI cells already from the initiation of cancer development.[89] According to this hypothesis, prostate cancer would originate from a population of epithelial stem cells that are independent of androgens. In the presence of androgens, the cancer stem cell progeny will differentiate into AD cancer cells that express AR and secrete PSA. Androgen deprivation will eliminate these cells, and results in the selective outgrowth of AI cancer stem cells.

Another mechanism that has been suggested is related to altered apoptosis regulation. Removal of androgens induces apoptosis in AD cancer cells, while AI cells are resistant. This could be explained by an upregulation of anti-apoptotic factors like Bcl-2. Bcl-2 is frequently overexpressed in castration resistant tumors and experimental studies have demonstrated that upregulation of Bcl-2 in AD cancer cells protects them from apoptosis when androgens are removed.[90,91]

ANGIOGENESIS

The vasculature provides tissues with oxygen, nutrients and removes metabolic waste products. The blood vessels also transport signal molecules like hormones and produce growth factors that can act in a paracrine manner on surrounding cells. Blood vessels are composed of endothelial cells and mural cells. The endothelial cells form a vessel lumen that is surrounded by a basement membrane and stabilized by a coat of vascular smooth muscle cells and/or pericytes (Fig. 5).

The formation of new blood vessels occurs via two processes; vasculogenesis and angiogenesis. Vasculogenesis is the process when blood vessels are created *de novo* from endothelial progenitor cells. This process mainly occurs during embryonic development, but also in adult tissue.[92,93] Vasculogenesis involves migration, differentiation and organization of endothelial progenitor cells into capillary-like tubes forming a primitive vascular network.[27,94,95] This network is then further developed into mature vasculature by the process of angiogenesis. Angiogenesis is the formation of new blood vessels from preexisting vasculature, and occurs both during embryonic development and in postnatal life. Angiogenesis in adult tissues is a tightly regulated process that mainly occurs during tissue repair and during the

female reproductive cycle. In addition, angiogenesis is observed in different pathological conditions such as cancer.

Angiogenesis is a complex process that is tightly regulated by several factors. The angiogenic process starts with enlargement of the existing vessel, which then sprouts or splits into new vessels.[27,94,95] Sprouting angiogenesis is a multistep process encompassing vessel destabilization, basement membrane degradation, endothelial cell proliferation and migration, lumen formation, sprout merging and stabilization (Fig 5).[94,95]

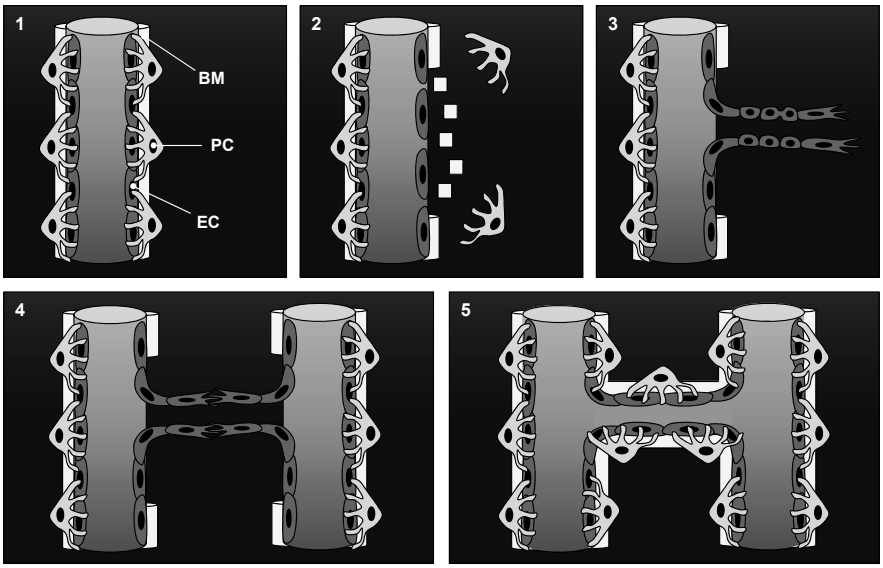

Figure 5: Non-angiogenic, mature blood vessels are composed of closely attached endothelial cells (EC) forming a vessel lumen that is stabilized by pericytes (PC) and is surrounded by a basement membrane (BM) (1). Angiogenesis is a complex process encompassing vessel destabilization through the removal of pericytes and degradation of the basement membrane (2), endothelial cell migration and proliferation (3), sprout merging (4) and vessel stabilization (5).

First, the blood vessel is dilated and in response to vascular endothelial growth factor (VEGF) the permeability increases.[96] This allows for extravasation of plasma proteins into the tissue, which subsequently facilitate the migration of endothelial cells. The vessel is then destabilized through the removal of pericytes from the endothelium. The angiopoietins (Ang) are believed to be of importance for this process, where a shift from Ang-1 to Ang-2 expression results in a more plastic and proliferative phenotype of the vessels.[97] Next, proteases like matrix metalloproteinases (MMPs) degrade the surrounding basement membrane and facilitate sprouting into the tissue through degradation of the extracellular matrix

(ECM).[98] This is followed by endothelial cell proliferation and migration into the tissue induced by different growth factors, including VEGF, basic fibroblast growth factor (bFGF), interleukin 8 (IL-8) and EGF.[27,94,95] The tip cell moves in the front of the proliferative cells and guides the vessel in the correct direction.[99] The endothelial cells then coassemble into capillary structures, where adhesion molecules like VE-cadherin are of importance for a close cell-cell contact.[95,100] The angiogenic process is ended by the stabilization and maturation of the newly formed vessels. Pericytes are recruited to the new vessels by release of platelet-derived growth factor B (PDGF-B)[101,102] and heparin-binding EGF-like growth factor (HB-EGF)[103] from the endothelial cells. Finally, a switch back to Ang-1 expression leads to the stabilization of vessels.

Tumor angiogenesis

Angiogenesis is a prerequisite for solid tumor growth and is also important for the dissemination of tumor cells. That tumor growth is angiogenesis dependent was first postulated by Judah Folkman in 1971.[104] He demonstrated that tumors cannot grow beyond a few millimeters without the induction of angiogenesis. In prostate cancer, increased microvessel density (MVD) is related to clinical stage, progression, metastasis and survival,[105-108] which highlights the importance of angiogenesis for disease progression. This makes angiogenesis an interesting therapeutic target in castration resistant prostate cancer.

Tumor development can be divided into two phases; a prevascular phase and a vascular phase.[109,110] When tumors develop they are initially not vascularized. During this prevascular phase tumors cannot grow beyond a certain size that allows adequate oxygen supply by passive diffusion from surrounding blood vessels. Approximately 0.2 mm represents the threshold distance that oxygen effectively can diffuse through tissues,[111] but in tissues with high metabolic activity the distance is less.[112] Tumor cells located more than 0.1-0.2 mm away from a blood vessel will suffer from hypoxia that eventually will cause necrosis or induce apoptosis of the cells.[112] Tumors can persist in this avascular and dormant stage for many years, in which cell death rate equals growth rate, before switching to the vascular phase where angiogenesis is induced and the formation of new blood vessels allows tumor expansion.[113,114] The progression of tumors into this angiogenic phenotype is called the “angiogenic switch” and results from an imbalance between pro-angiogenic and anti-angiogenic factors.[109,110] Important inducers of this angiogenic switch are genetic mutations and hypoxia. Hypoxia results in increased levels of the transcription factor hypoxia inducible factor-1 (HIF-1), that is rapidly degraded under normal conditions. Accumulation of HIF-1

drives the transcription of several genes important for angiogenesis, including VEGF.[115]

Tumor blood vessels are not only important for supplying the cancer cells with oxygen and nutrients. They are also an important route for dissemination of the cancer cells to distant organs where they can establish new metastases. In the process of intravasation the cancer cells invade the blood vessels and enter the circulation. Once in the circulation the cancer cells can be transported to other organs in the body. The cancer cells leave the circulation and penetrate into the surrounding tissue by the process of extravasation. The cancer cells can either extravasate through the vessel wall directly or first start to proliferate within the blood vessels, generating a small tumor that subsequently grows through the vessel wall.[116] All of these processes rely on complex interactions between the cancer cells and the blood vessels. Many cancer cells are seeded to the circulation but only a few will succeed to colonize and form new metastases at distant sites.[117] Like primary tumors, dormant micrometastases can be present in the body several years before some of them grow to clinically detectable tumors. Even if the cancer cells initially can get access to the circulation by growing around existing blood vessels, induction of angiogenesis is necessary for continuous expansion of the micrometastases. Tumor dormancy is therefore partly explained by the failure of tumor cells to induce angiogenesis.[118]

Tumor blood vessels can grow by several mechanisms, including angiogenesis, recruitment of circulating bone marrow-derived endothelial progenitor cells and by vasculogenic mimicry (the formation of blood vessels by tumor cells instead of endothelial cells) (Fig. 6).[119-121] In addition, tumors can get access to the vasculature by so called co-option of blood vessels,[97,119] which means that a tumor establishes and grows around an existing vessel (Fig. 6). As described above, this is probably an important mechanism in the establishment of distant metastases. Tumor angiogenesis is regulated by a dynamic balance of positive and negative regulators that are produced by the tumor cells themselves or by other tumor associated cells, like macrophages, mast cells or endothelial cells.[27,94,95] The angiogenic stimulation in tumors is not as well orchestrated as during normal vessel formation resulting in reduced functionality of the vessels. Tumor vessels therefore differ in many aspects from normal blood vessels. They are highly irregular and tortuous, and have several blind ends.[122,123] The vessels can be incompletely lined by endothelial cells and lack a complete basement membrane.[124,125] The endothelial cells are loosely attached to each other and a large proportion of the vessels are immature vessels that are devoid of closely attached pericytes.[126,127] The abnormal morphology of tumor vessels results in increased leakage, irregular and sluggish blood flow and thereby often in hypoxic regions.

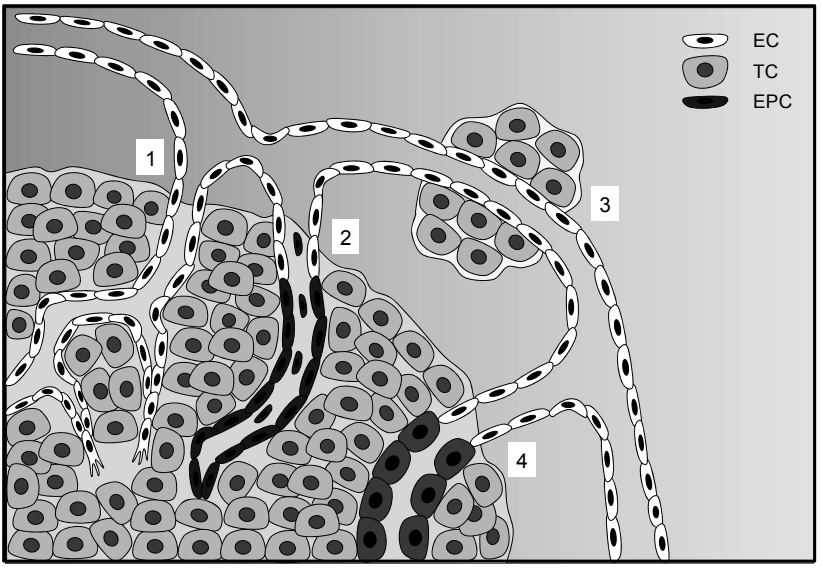

Figure 6: Different mechanisms of tumor vascularization, including angiogenesis (1), recruitment of endothelial progenitor cells (2), co-option (3) and vasculogenic mimicry (4). EC = endothelial cell; TC = tumor cell; EPC = endothelial progenitor cell.

Vascular endothelial growth factor

VEGF is one of the most important factors that stimulate normal and tumor angiogenesis.[128,129] It is a potent pro-angiogenic factor that stimulates endothelial cell proliferation, migration and tube formation.[130,131] It is also an important survival factor for endothelial cells and leads to vasodilation and increased vascular permeability. VEGF expression can be stimulated by several factors, including hypoxia, hormones, growth factors and cytokines.[130,131] Alternative splicing can generate seven different isoforms ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{165b}$, $VEGF_{183}$, $VEGF_{189}$ and $VEGF_{206}$). $VEGF_{121}$ and $VEGF_{165}$ are secreted forms, whereas $VEGF_{189}$ and $VEGF_{206}$ are bound to the cell surface and ECM due to the presence of heparin binding regions.[132] $VEGF_{165}$ is the predominant isoform and is overexpressed in a variety of tumors. VEGF is present as disulfide-linked homodimers and mediates its biological effects via the transmembrane tyrosine kinase receptors vascular endothelial growth factor receptor 1 (VEGFR-1/Flt-1)[133] and VEGFR-2 (Flk-1).[134] These receptors are primarily expressed on endothelial cells, but can also be found on other cell types, including prostate tumor cells.[135,136] In addition, VEGF can bind the cell surface receptor neuropilin 1 that acts as a co-receptor for VEGFR-2 by increasing the binding affinity to VEGF.[137,138] VEGFR-2

is the key receptor mediating the pro-angiogenic effects of VEGF in endothelial cells.[130,131] Upon binding of VEGF, receptor dimerization and autophosphorylation initiates an intracellular signal cascade ultimately leading to mitogenic, chemotactic and pro-survival signals in the endothelial cells.

VEGF has been described as one of the most important inducers of angiogenesis in prostate cancer. It is upregulated in prostate cancer compared to benign tissue and correlates with increased MVD, Gleason score and metastasis.[139-143] Furthermore, inhibition of VEGF suppresses angiogenesis, tumor growth and metastasis in experimental studies.[144-146] VEGF is known to be stimulated by androgens in the prostate and castration results in decreased levels of VEGF in the normal prostate and in prostate tumors.[32,34,147,148]

Thrombospondin 1

Tumor angiogenesis is not only due to an increased expression of pro-angiogenic factors, but also to the loss of naturally occurring anti-angiogenic factors that are present in normal tissues to keep the vasculature quiescent.[110] One of the first described and most studied endogenous angiogenesis inhibitor is thrombospondin 1 (TSP1),[149] a large glycoprotein present as trimers in the extracellular matrix. It is a major constituent of platelet α granules, but is also secreted by a wide variety of epithelial and mesenchymal cells. TSP1 is a multifunctional protein that interacts with cell surface receptors, growth factors and proteases.[150,151] TSP1 inhibits angiogenesis by suppressing endothelial cell proliferation, migration and by inducing apoptosis.[150,151] The anti-angiogenic effects of TSP1 involve both direct effects and indirect effects on endothelial cells. Binding to the cell surface receptor CD36 on endothelial cells is considered to be one of the major mechanisms whereby TSP1 inhibits angiogenesis and results in decreased migration and induction of apoptosis of the endothelial cells.[152,153] TSP1 also interacts with several proteases involved in the angiogenic process, including MMP-9 that promotes endothelial cell migration and the mobilization of ECM-stored growth factors. By inhibiting the activation of MMP-9, TSP1 inhibits the release of ECM bound VEGF.[154] In addition, TSP1 reduces the levels of bioavailable VEGF through direct binding and sequestration of this pro-angiogenic factor.[155] TSP1 can also act as an activator of transforming growth factor-β (TGF-β) that is an important regulator of tumor growth and progression.[150,151] TSP1 contains three TSP type I repeats and several of the anti-angiogenic functions have been mapped to this protein motif, including CD36 binding, activation of TGF-β and inhibition of VEGF-induced angiogenesis.[151,156]

TSP1 has been shown to be an important regulator of tumor growth in several studies. Inhibition of tumor angiogenesis by TSP1 results in decreased tumor

growth and metastasis in experimental models,[157,158] and downregulation of TSP1 has been associated with tumor progression in a variety of tumor types, including prostate cancer.[159-162] In addition to its anti-angiogenic effects TSP1 has also been described to directly inhibit the proliferation of prostate cancer cells.[163]

EXTRACELLULAR MATRIX REMODELING

Interactions between different cell types play an important role in normal tissue as well as in tumors. Many of these interactions take place in the ECM that is surrounding the cells. The ECM is a complex network of proteins and carbohydrates that helps to bind the cells in tissues together.[164] The ECM is composed of three major protein components; collagen fibers, proteoglycans and multi-adhesive matrix proteins.[164] The collagen fibers have an enormous tensile strength and protect tissues from stretching forces. The proteoglycans are highly hydrated, and forms a viscous gel that largely is responsible for the volume of the ECM and permits diffusion of small molecules between cells. In addition, the highly hydrated gel makes the tissue resistant to compression forces. Multi-adhesive matrix proteins link cells to ECM by binding to cell surface proteins and are important for cell adhesion and migration. A specialized form of ECM is the basement membrane, or basal lamina, that separates epithelial cells from the surrounding stroma in normal tissues.[165] A basement membrane is also underlying endothelial cells in capillaries.[165] A variety of molecules passes through the basement membrane in both directions and allows the interaction between epithelial/endothelial cells and the stromal compartment.

The ECM is not only important for protecting cells and linking them together into tissues, it is also an important route for cell migration and functions as a reservoir of regulatory proteins. Many growth factors are bound in the ECM to proteoglycans, especially those containing heparan sulfate.[166,167] Binding to ECM components protects growth factors from being degraded and also regulates their activity. By binding to growth factors, proteoglycans can either block their activity by sequestration, or conversely, present them to the cells.[166,167] In fact, the interaction between several growth factors and their cell-surface receptors is greatly facilitated by, or even requires, presentation by proteoglycans.[166,167]

Matrix remodeling is an important process involved in almost every step of cancer progression. Proteases, like plasminogen activators, plasmin, MMPs, a disintegrin and metalloproteinase (ADAMs) and ADAM metallopeptidases with thrombospondin type 1 motifs (ADAMTSs) are key effectors of ECM remodeling.[98] Proteolytic cleavage of ECM components creates space for proliferating cells and facilitates cell migration and invasion. The proteases can also

release and modulate ECM bound proteins, and thus regulate the availability and activity of growth regulating factors.[98] In addition to direct effects on cancer cell growth and spreading, all of these events are also involved in the process of tumor angiogenesis.

ADAMTS1

The ADAMTS protein family

ADAMTS is a group of secreted proteases that belong to the superfamily of Zn^{2+}-dependent metalloproteinases. They are characterized by the presence of a disintegrin-like domain and a varied number of TSP type I motifs.[168,169] ADAMTS1 was the first identified member of this protein family,[170] today comprising 19 members in the human genome.[168,169]

ADAMTSs are complex multi-domain proteases, whose functions mainly depend on interactions with ECM and cell surface components. Based on similarities in structure and function the ADAMTSs can be categorized into four subgroups;[168,169] (1) aggrecanases (ADAMTS1, 4, 5, 8, 9, 15 and 20) with the ability to cleave aggrecan and other proteoglycans, (2) procollagen N-peptidases (ADAMTS2, 3 and 14) that are involved in the processing of procollagens to collagen, (3) von Willebrand factor cleaving protease (ADAMTS13) and (4) orphan ADAMTSs (ADAMTS6, 7, 10, 12, 16, 17, 18 and 19) referring to those with no identified function or substrate.

ADAMTS1 structure and activity

The ADAMTS1 gene is organized in 9 large exons and is located on chromosome 21 in the human genome. It is expressed in a wide range of human tissues, including the prostate.[171,172] Expression of ADAMTS1 is affected by a diverse range of growth factors, cytokines and hormones in a tissue dependent fashion.[173-178] Common to all members of the ADAMTS protein family, ADAMTS1 comprises a signal peptide and prodomain in the N-terminal, followed by a metalloproteinase domain, a disintegrin-like domain, a TSP type I motif, a cysteine rich domain and a spacer region. In the C-terminal ADAMTS1 has two additional TSP type I motifs, which can vary from none to 14 in other ADAMTS (Fig. 7).[168,169]

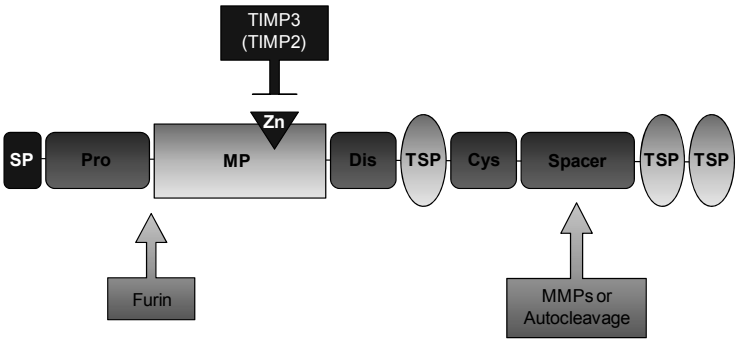

Figure 7: The multi-domain structure of the ADAMTS1 protein. SP = signal peptide; Pro = prodomain; MP = metalloproteinase domain; Dis = disintegrin-like domain; TSP = TSP type I motif; Cys = cysteine rich domain; Spacer = spacer region. Removal of the prodomain by furin yields the active form of ADAMTS1 that can be further processed in the C-terminal by matrix metalloproteinases (MMPs) or by autocleavage. The protease activity of ADAMTS1 can be inhibited by tissue inhibitors of matrix metalloproteinases (TIMP2 and 3).

ADAMTS1 is a catalytically active metalloproteinase with the ability to cleave several ECM components.[179] The substrates identified so far include aggrecan, versican, gelatin, nidogen 1 and 2, TSP1 and 2, tissue factor pathway inhibitor-2 (TFPI-2) and syndecan-4.[180-187] ADAMTS1 is produced as a latent pro-enzyme of approximately 110 kDa, and is processed in two consecutive steps to release active forms of 87 kDa and 65 kDa, respectively.[188] The active form of 87 kDa results from the removal of the prodomain that blocks the catalytic site and keeps the enzyme inactive. Removal of the prodomain is performed by pro-protein convertases such as furin in the secretory pathway (Fig. 7).[188,189] Once activated, the catalytic function of ADAMTS1 can be further regulated by tissue inhibitors of matrix metalloproteinases (TIMPs). TIMP3 is the major inhibitor of the proteolytic activity of ADAMTS1, but it can also be partially inhibited by TIMP2.[181]

The secreted, active form of ADAMTS1 (87 kDa) is mainly present in the ECM, where it is bound to heparin and heparan sulfates.[190] Deletion mutant analyses revealed that the C-terminal are responsible for anchoring to ECM.[190] This region is also important for substrate interaction.[186] Further processing of ADAMTS1 in the spacer region removes two TSP type I motifs in the C-terminal and thereby alter the affinity of the protein to ECM.[188] This proteolytic processing yields two soluble fragments; the 65 kDa active form plus a 22 kDa C-terminal fragment. This second cleavage can be mediated through autocleavage or by other MMPs, including MMP-2, -8 and -15 (Fig. 7).[188,191] By protecting the C-terminal region of ADAMTS1, heparin and heparan sulfate can inhibit the second proteolytic processing, indicating that the composition of the ECM is of importance for regulating the proteolytic status and solubility of ADAMTS1.[188,191]

Biological functions of ADAMTS1

ADAMTS1 is involved in several normal and pathological conditions, including angiogenesis, inflammation, ovulation, organ morphogenesis and cancer.[170,171,174,192,193] Knockout of the ADAMTS1 gene in mice revealed a number of defects, including post natal growth retardation, adipose tissue malformations and defects on kidneys and adrenal glands.[193] In addition, ADAMTS1 (-/-) mice displayed histological abnormalities of the uterus and ovaries with concomitant reduced fertility in females due to impaired ovulation.[193,194] During ovulation ADAMTS1 is temporally upregulated in the ovaries by progesterone and is involved in the release of the ovum through degradation of versican.[174]

ADAMTS1 and angiogenesis

The ADAMTS1 protein contains three TSP type I motifs that are conserved motifs also present in TSP1 and 2. In TSP1 and 2, several of their anti-angiogenic mechanisms have been mapped to these motifs, which led to the hypothesis that also ADAMTS1 may have anti-angiogenic activity. This idea was confirmed in a set of experimental studies showing that ADAMTS1 inhibits bFGF induced vascularization in the cornea pocket assay as well as VEGF induced angiogenesis in the chorioallantoic membrane (CAM) assay.[171] The suppressive effect of ADAMTS1 was even greater than that of TSP1 and endostatin. Both the full-length active form of 87 kDa, as well as the processed form of 65 kDa can reduce endothelial cell proliferation in a dose-dependent manner.[171,188] ADAMTS1 has also been identified as a downregulated gene in proliferating endothelial cells compared to tubular endothelial cells.[195] Furthermore, knockdown of ADAMTS1 in endothelial cells results in increased proliferation, and endothelial cell migration is inhibited by ADAMTS1.[177,196]

The anti-angiogenic properties of ADAMTS1 have been reported to involve different mechanisms. Through direct binding and sequestering of $VEGF_{165}$ by the TSP type 1 motifs, ADAMTS1 can inhibit activation of the VEGFR-2 receptor on endothelial cells and thereby proliferation.[197] ADAMTS1 has also been reported to bind bFGF.[196] Another mechanism involves the catalytic domain of ADAMTS1. By proteolytic cleavage of TSP1 and 2, ADAMTS1 releases soluble fragments with proven anti-angiogenic activity.[185] However, there are also studies describing ADAMTS1 as a pro-angiogenic factor that is upregulated by hypoxia in endothelial cells and promotes cell migration.[198] In addition, ADAMTS1 was shown to be upregulated in primary endothelial cells during growth factor induced angiogenic sprouting and knockdown of ADAMTS1 resulted in decreased invasive capacity.[199]

ADAMTS1 in cancer

ADAMTS1 has been reported to efficiently suppress tumor growth and metastasis in different experimental cancer models by inhibiting angiogenesis.[191,192,200] This anti-tumor effect of ADAMTS1 is mainly mediated by the C-terminal comprising the TSP type I motifs[191,192,200] and is believed to involve binding and thereby reduced bioavailability of different growth factors, including VEGF, HB-EGF and amphiregulin.[191,197] However, there are also contradictory data describing ADAMTS1 as a tumor promoting factor.[191,192,201] ADAMTS1 transfection of a bronchial epithelial tumor cell line accelerated subcutaneous tumor growth, together with a stromal reaction characterized by myofibroblast infiltration and excessive matrix deposition.[201] Furthermore, ADAMTS1 was upregulated in breast cancer cells with elevated metastatic activity.[202]

The contradictory results may partly be explained by the proteolytic status of ADAMTS1. In a study by Liu et al, full length ADAMTS1 (87 kDa) was found to promote subcutaneous tumor growth and pulmonary metastasis by enhancing invasion and angiogenesis, while overexpression of the two processed forms (65 kDa + 22 kDa) suppressed metastasis by inhibiting tumor cell extravasation, survival and angiogenesis. The tumor promoting effect was shown to involve shedding and activation of the growth factors HB-EGF and amphiregulin and thereby activation of the EGF receptor and Erb-B2.[191]

In human malignancies, decreased expression of ADAMTS1 has been reported in breast, pancreatic, hepatocellular and lung cancer compared to benign tissue.[203-206] In contrast, increased expression of ADAMTS1 has been observed in thyroid cancer,[207] while no clear difference was observed in cartilage tumors.[178] In a recently published paper, decreased expression of ADAMTS1 was associated with vascular invasion in hepatocellular cancer,[208] but there are also studies showing that elevated levels of ADAMTS1 is associated with metastasis and worse prognosis in pancreatic and breast cancer.[204,209]

AIMS

GENERAL AIM

Prostate tumors are initially dependent on androgens for growth and the standard treatment for advanced prostate cancer is therefore ADT. Removal of androgens results in decreased tumor burden and symptomatic relief in the majority of patients. Unfortunately, most tumors will eventually relapse and continue to grow and spread in an AI manner. Today, there are no effective treatment options for this stage of the disease that ultimately is fatal. The general aim of this thesis is to increase the knowledge of mechanisms that are altered during transition into androgen-independency. This is important in order to develop new treatment options for this stage of the disease. One possibility would be to target the tumor vasculature by anti-angiogenic treatment. The focus of this thesis is therefore on how tumor angiogenesis, and the regulation of this process, is altered in AI prostate cancer.

SPECIFIC AIMS

- To develop and characterize a model system for comparative studies of AD and AI prostate cancer *in vitro* and *in vivo*
- To compare the expression of angiogenesis related genes in AD and AI prostate cancer cells in order to identify factors that are of importance for the regulation of angiogenesis in AI tumors
- To verify the role of identified factors (ADAMTS1) for angiogenesis and tumor progression in tumor specimens from prostate cancer patients
- To further study the effects of ADAMTS1 on tumor growth and blood vessels in AD and AI tumor xenografts

MATERIALS AND METHODS

PATIENT MATERIAL

Human tumor material obtained by transurethral resection of the prostate (TURP) was used for analysis in paper III. Most prostate cancers relapse at distant sites after ADT and not locally in the prostate, making it difficult to achieve tumor material for studies on AI prostate cancer. TURP operations of patients with castration resistant prostate cancer are performed as palliative treatment due to obstructive voiding problems or bleeding, which ascertains that the material analyzed is from progressive AI prostate cancer. The castration resistant tumor material was compared with tumor material classified as T1b. T1b tumors are unapparent and not palpable tumors with incidental histological tumor finding in more than 5% of the resected tissue.

Formalin-fixed and paraffin-embedded tissue sections were obtained from patients with prostate cancer that underwent TURP at the Department of Urology, Sahlgrenska University Hospital, Gothenburg, Sweden. The study was approved by the local ethical committee. Of the analyzed tissue specimens, 30 were from patients with TURP-diagnosed untreated prostate cancer in stage T1b and 26 were from patients with recurrent castration resistant prostate cancer after ADT. Mean age in the untreated hormone naïve group was 77.0 years (range, 60-90 years) and mean Gleason score was 6.6 (range 5-9). Mean age in the castration resistant group was 78.8 years (range, 65-88 years) and mean Gleason score was 9.1 (range 7-10). The Gleason scores used in this study are the sum of the two most common Gleason grades according to the previous system. Among the castration resistant tumors, 8 were from patients without metastases, 16 were from patients with metastatic disease and 2 were from patients with unknown metastatic status. Adjacent benign areas from 35 tissue specimens (29 hormone naïve + 6 castration resistant) were also analyzed in the study.

CELL LINES AND CELL CULTURE

The human prostate cancer cell line LNCaP was used for experimental studies. The LNCaP cell line was originally established from a lymph node metastasis of a patient with castration resistant prostate cancer.[210] However, the LNCaP cell line is still AD and is the most commonly used AD cell line in prostate cancer research.

LNCaP cells were obtained from American Type Culture Collection (Manassas, VA, USA) and were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics according to the manufacturer´s recommendations.

In order to establish a model system for studying the transition into androgen-independency, LNCaP cells were cultured in medium containing 10% steroid depleted dextran charcoal treated serum (DCC-FBS) instead of regular FBS. LNCaP cells from different passages were subjected to the steroid reduced medium. An AI subline appeared from LNCaP cells in passage 19, where passage 1 represents the first passage in our laboratory, and was thereby named LNCaP-19. LNCaP-19 cells were routinely maintained in medium supplemented with 10% DCC-FBS instead of FBS. Cells were tested and found free of mycoplasma.

For analysis of androgen regulated gene expression in paper II, cells were cultured in the absence or presence of the synthetic androgen R1881 (NEN Life Science Products, Boston, MA, USA). After 3 days of starvation in medium containing 1% DCC-FBS, cells were treated with either 0 nM or 1 nM R1881. Four days later, cells were harvested for RNA and protein analyses.

Transfection of cells

In paper IV, ADAMTS1 expression was modified in LNCaP and LNCaP-19 cells by transfection. A shRNA plasmid for human ADAMTS1 with neomycin resistance (SABiosciences, Frederick, MD, USA) was used to downregulate the expression of ADAMTS1 by RNA interference in LNCaP cells. In addition, a plasmid expressing shRNA that does not match any human, mouse or rat gene was included as control. LNCaP-19 cells were transfected with a mammalian expression vector containing the full-length wild-type mouse ADAMTS1[192] or empty control vector (pcDNA3), kindly provided by Prof. Kouji Kuno. Cells were transfected over night by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Three days later, the cells were subjected to selective pressure by adding Geneticin (Invitrogen) that allows growth of cells that have been successfully transfected. Stably transfected clones could be isolated after 3 weeks. The isolated clones were expanded and screened for ADAMTS1 expression by real-time reverse transcriptase polymerase chain reaction (RT-PCR) and Western blotting.

Proliferation experiments

In paper I, androgen regulation of proliferation was analyzed by ^{3}H-thymidine incorporation. LNCaP and LNCaP-19 cells were cultured in various concentrations of FBS or DCC-FBS (1%, 2.5%, 5% or 10%). In addition, cells were treated with different concentrations of R1881 (0, 0.001, 0.01, 1 or 10 nM). The cells were incubated for 7 days and then pulsed for 4 hours with ^{3}H-thymidine (PerkinElmer Life Sciences, Boston, MA, USA). ^{3}H-thymidine was counted in a liquid scintillation counter after trichloroacetic precipitation and NaOH dissolution.

In paper IV, proliferation rate of transfected clones with modified ADAMTS1 expression was analyzed. Cells were seeded in their regular culture medium and incubated for 4 days. The cell density was analyzed by adding a fluorescent dye that binds to DNA (CyQuant Cell Proliferation Assay Kit, Invitrogen) and fluorescence was measured with a multilabel plate reader.

ANIMAL EXPERIMENTS

Male athymic BALB/c nude mice were used for *in vivo* analyses of tumor cells. These mice are T-cell deficient, which allows growth of human xenografts. In addition, their nude phenotype makes it easy to follow and monitor subcutaneous tumor growth. The mice were at least 8 weeks old when experiments were started, which ascertains sexual maturity. The use of animals was approved by the animal ethical committee in Gothenburg.

A total of 1×10^{7} cells + 0.5 μg/ml bFGF (paper I) or 2×10^{6} cells (paper II and IV) were inoculated subcutaneously in the flank of the mice. The cells were suspended in equal volumes of cell culture medium and matrigel (BD Biosciences, Bedford, MA, USA). Matrigel is a gelatinous protein mixture that resembles basement membrane matrix and supports the propagation of tumor cells in mice. Cells were grown in intact/sham-operated as well as in castrated mice. Castration was performed via a scrotal incision under anesthesia prior to cell inoculation (paper I and IV) or three days before sacrifice (paper II). Tumors were inspected and measured with a caliper weekly. Tumor volumes were calculated by the formula $(\text{length}\times\text{width}^{2})/2$. When tumors were about 1300 mm^{3}, or after a maximum of 16 weeks, mice were sacrificed and tumors were removed and weighed. One part of the tumors was fixed in formalin for paraffin embedding and one part was frozen in liquid nitrogen.

RNA ANALYSES

RNA preparation

Total RNA was isolated from cells and tumors with the RNeasy Mini kit and was further treated with RNase-free DNase I (Qiagen, Hilden, Germany). For the angiogenesis gene array used in paper II, RNA was extracted with TRIzol solution (Invitrogen) followed by further purification by the Clean Up step of RNeasy Mini kit. Since DNase treatment might generate small DNA fragments that can bind unspecifically to the microarray membrane this method allows the isolation and purification of RNA without DNase treatment. The concentration and purity of RNA were determined spectrophotometrically and the integrity of RNA was confirmed by gel electrophoresis.

Angiogenesis gene array

In paper II, the expression of angiogenic factors was compared in LNCaP and LNCaP-19 cells by performing a pathway specific microarray analysis. The cDNA array used (GEArray Q Series Human Angiogenesis Gene Array, SABiosciences) consisted of 96 angiogenesis related genes plus control genes. Biotin dUTP-labeled cDNA probes were generated from the isolated RNA by using the GEArray Ampo-Labeling LPR-kit (SABioscences). After prehybridization with sheared salmon sperm DNA, the array filters where hybridized with the denatured cDNA probes over night at 60 ºC. Alkaline phosphatase-conjugated streptavidin and CDP-star substrate were used for chemiluminescent detection. Signal intensities were quantified with the computer software ScanAlyze (Michael Eisen, Stanford University, USA) and normalized against the control genes included. Comparisons between LNCaP and LNCaP-19 were performed in cells grown in 0 nM and 1 nM R1881, respectively. A more than 2-fold difference in signal intensity between the cell lines was considered significant.

Real-time RT-PCR

Reverse transcription of total RNA into cDNA was performed by M-MLV Reverse Transcriptase in the presence of random primers, nucleotides and RNasin (Promega, Madison, WI, USA). Quantitative real-time RT-PCR was performed with the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Gene specific primers and probes for ADAMTS1, ephrin-A5, fibronectin 1, midkine, neuropilin 1, VEGF and 18S were purchased as TaqMan Gene Expression

Assays (Applied Biosystems). Data were analyzed according to the ΔΔCt method for relative quantification and were normalized to the expression of 18S rRNA.

PROTEIN ANALYSES

ELISA and DELFIA

In paper I, PSA and VEGF levels were analyzed in conditioned medium from LNCaP and LNCaP-19 cells treated with different concentration of R1881. Cells were seeded in medium containing 1% DCC-FBS and after 3 days of starvation, medium was changed and cells were cultured in various concentrations of R1881 (0, 0.1, 1 or 10 nM). Four days later, the conditioned medium was harvested, centrifuged and freezed. PSA was measured in the conditioned medium using a dissociation-enhanced lanthanide fluorescent immunoassay (DELFIA) for human total PSA (ProStatus, Wallac Oy, Turku, Finland) and VEGF was measured with an enzyme linked immunosorbent assay (ELISA) for human VEGF (Quantikine, R&D Systems, Minneapolis, MN, USA). Levels of secreted PSA and VEGF were normalized to the number of cells, which were trypsinized and counted in a Bürker chamber.

Western blotting

Total protein was extracted from cells and tumors by homogenization and sonication in the presence of protease inhibitors (Complete Mini, Roche Diagnostics, Mannheim, Germany). Protein concentrations were determined with the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Protein samples (50 µg) were size separated by SDS-polyacrylamide gel electrophoresis (NuPage, Invitrogen) and transferred to polyvinyldifluoride membranes. Membranes were blocked with 5% non-fat milk and incubated at 4 ºC over night with primary antibodies. Primary antibodies used were against human ADAMTS1 (Sigma-Aldrich, St Louis, MO, USA), mouse ADAMTS1 (kindly provided by Prof. Joanne S. Richards[174]), neoepitope of versican (Abcam, Cambridge, UK), TIMP2 (Sigma-Aldrich), TIMP3 (Chemicon, Temecula, CA, USA) and TSP1 (NeoMarkers, Fremont, CA, USA). As loading control an antibody against actin (Sigma-Aldrich) was used. Detection was performed with enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Buckinghamshire, UK). Semi-quantitative analysis of immunoreactive bands was performed with the free computer software Image J.

Immunohistochemistry

Immunohistochemistry was used to analyze blood vessels (paper I-IV) and to investigate the expression of ADAMTS1 (paper III). Tissue sections were deparaffinized, rehydrated through graded alcohol and heated in unmasking solution for antigen retrieval. After quenching of endogenous peroxidase activity and blocking of nonspecific antibody binding, tissue sections were incubated with primary antibodies over night at 4 ºC. For determination of MVD and blood vessel morphology, sections were stained with antibodies against CD34 (BD PharMingen, San Diego, CA, USA, paper I) (Abcam, paper II and IV) (NeoMarkers, paper III). ADAMTS1 expression (paper III) was analyzed with the same antibody that was used for Western blotting (Sigma-Aldrich). To evaluate pericyte coverage of blood vessels (paper IV), tissue sections were double stained for CD34 and alpha-smooth muscle actin (α-SMA) (Sigma-Aldrich). Immunoreaction of CD34 was detected with the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) and ADAMTS1 was detected with the Envision System (DAKO, Copenhagen, Denmark). Staining was visualized with DAB or Vector Red (α-SMA) and sections were counterstained with haematoxylin, dehydrated and mounted.

MVD was evaluated by counting the number of CD34-positive vessels in three (paper I and II) or five (paper III and IV) randomly chosen fields of representative areas in each section. A blood vessel was identified as any immunostained endothelial cell or endothelial cell cluster separated from adjacent microvessels, according to Weidner et al.[107] In paper IV, blood vessel morphology was evaluated by grouping the tumors into two groups according to the form and distribution of vessels. Tumors characterized by large, thick and singly distributed vessels, often with a visible lumen, were denoted as type 1. Tumors characterized small and thin vessels forming networks without visible lumen were assigned as type 2. To evaluate pericyte coverage of blood vessels (paper IV), the number of CD34-positive vessels associated with α-SMA positive cells were counted, and divided with the total number of CD34-positive vessels in five fields. In paper III, ADAMTS1 expression was analyzed in TURP specimens. A semi-quantitative scoring system, combining staining intensity (0-3) and proportion of positive tumor/epithelial cells (0-3) was used for evaluation of the ADAMTS1 staining. A total score in each field was obtained by multiplying the two scores. Mean tumor score was calculated from ten analyzed fields in randomly chosen areas. All analyses were performed unaware of origin, by two investigators at ×200 magnification.

STATISTICAL ANALYSES

Data are presented as mean ±SEM. The Mann Whitney *U*-test was used to compare differences between groups. Correlations were analyzed with Spearman´s rank correlation test. Fisher´s exact test was used to compare tumor take rate between groups in paper IV. A *P*-value ≤ 0.05 was considered statistically significant. All statistical analyses were performed using the SPSS software for Windows.

RESULTS AND COMMENTS

PAPER I

The progression into androgen-independency after ADT is a major clinical problem. In order to improve treatment for these patients it is of great importance to increase the knowledge of this stage of the disease. This requires good experimental model systems that are representative of the clinical situation.

In this paper an experimental model was established that allows comparative studies of AD and AI prostate cancer. We used the human prostate cancer cell line LNCaP that is the most studied AD cell line.[210] Through continuous culturing of LNCaP cells in their regular medium followed by selective pressure in androgen depleted medium, an AI subline was generated. In a first attempt, LNCaP cells from passage 12 were used, but these cells were unable to proliferate in the absence of androgens and a gradual decrease in cell number was observed until culture was terminated after approximately one year. The experiment was repeated with LNCaP cells from passage 19 and cells from this passage continued to proliferate in the absence of androgens and gave rise to the new AI subline, called LNCaP-19.

Characterization of the new cell line LNCaP-19 revealed that these cells still were androgen responsive even if they did not require androgens for growth. The proliferation rate in the presence of androgens did not differ between LNCaP and LNCaP-19 cells. However, in androgen depleted medium LNCaP cells did not grow at all while LNCaP-19 showed a moderately reduced growth rate. This is typical in the majority of castration resistant patients, where continued ADT still reduces tumor growth to some extent.[211,212] The PSA secretion was also stimulated by androgens in both LNCaP and LNCaP-19 cells. However, the concentration of PSA was much lower in LNCaP-19 cells compared to LNCaP cells, which is characteristic for more aggressive prostate tumor cells.[213,214] The high levels of PSA detected in serum from patients with castration resistant progressive disease, is primarily due to the large number of cancer cells and to increased leakage into the vasculature.[214]

LNCaP-19 cells displayed a more aggressive behavior compared to LNCaP when implanted subcutaneously in nude mice. They rapidly formed tumors both in sham-operated and castrated mice, indicating a true AI growth *in vivo*. In contrast, development of LNCaP tumors was much slower and was even more delayed in castrated animals. Analysis of the tumor xenografts revealed that LNCaP-19 tumors

displayed a more than 2-fold increase in MVD compared to LNCaP tumors, suggesting that increased angiogenesis could be involved in the rapid tumor growth. Interestingly, there was also a significant difference in blood vessel morphology. The blood vessels in LNCaP-19 tumors were mainly small and thin, while LNCaP tumors also contained large round vessels with a visible lumen. More recent data have also shown that blood vessels in LNCaP-19 tumors are less frequently covered by pericytes.[215]

VEGF is one of the most potent pro-angiogenic factors and upregulation of VEGF is associated with increased angiogenesis in many cancer forms, including prostate cancer. The role of VEGF in AI prostate cancer is not clarified and data are conflicting.[216-219] We therefore investigated whether the increased angiogenic capacity of LNCaP-19 cells was associated with elevated levels of VEGF. Measurements of secreted VEGF *in vitro* revealed androgen stimulation of VEGF both in LNCaP and LNCaP-19. However, the levels of VEGF were not higher in LNCaP-19 cells compared to LNCaP cells. This indicates that elevated VEGF levels are not responsible for the increased angiogenesis observed in LNCaP-19 tumors. Later studies have also verified this in tumor xenografts, where significantly lower levels of VEGF were found in LNCaP-19 tumors compared to LNCaP.[220]

In conclusion, the newly established AI cell line LNCaP-19 displays several properties that resemble the clinical situation. LNCaP-19 is independent of androgens for growth *in vitro* and *in vivo*, but is still androgen responsive to some degree and secretes lower levels of PSA. Furthermore, the transition into androgen-independency was associated with a more rapid tumor growth in nude mice and increased angiogenesis. However, the observed increase in angiogenesis does not seem to rely on increased VEGF production.

PAPER II

In prostate cancer, an increased MVD is observed in AI and metastatic disease and this is probably important for the rapid growth and spreading of the cancer cells.[107,221,222] According to data from paper I, the increased angiogenesis observed in AI tumor xenografts was not explained by elevated VEGF production. In this study an angiogenesis specific gene array was used in order to identify other factors that could be of importance for the increased angiogenesis. Gene expression of LNCaP and LNCaP-19 cells was compared both in the presence and absence of androgens *in vitro* and a number of genes were found to be differentially expressed. ADAMTS1, ephrin-A5, fibronectin 1 and neuropilin 1 were all identified as downregulated genes in LNCaP-19, while midkine and VEGF was upregulated. Even if higher VEGF mRNA was detected in LNCaP-19 compared to LNCaP in this study, previous data demonstrate that protein levels of VEGF are not elevated in LNCaP-19 cells (paper I) and are even lower in tumors.[220]

One of the most differentially expressed genes was ADAMTS1 that was significantly downregulated in LNCaP-19 cells both in the presence and absence of androgens. Analysis of tumor xenografts verified the downregulation of ADAMTS1 also *in vivo*. Furthermore, ADAMTS1 expression was not affected by androgens *in vitro* or in subcutaneous tumors. ADAMTS1 has been described as an effective inhibitor of angiogenesis, tumor growth and metastasis in other experimental models.[171,191,192,200] The loss of ADAMTS1 in LNCaP-19 indicates that ADAMTS1 may function as a tumor suppressor also in prostate cancer.

In paper I, we reported that the number of microvessels is about 2-fold higher in AI LNCaP-19 tumors compared to AD LNCaP tumors. Analysis of the tumor xenografts revealed a negative correlation between ADAMTS1 and MVD, indicating that ADAMTS1 may function as an anti-angiogenic factor in the tumors. Since ADAMTS1 has been shown to directly bind and sequester $VEGF_{165}$,[197] downregulation of ADAMTS1 in LNCaP-19 may compensate for the low VEGF levels and result in more bioavailable VEGF. However, ADAMTS1 can also inhibit angiogenesis by regulating the availability of other factors such as TSP1 and 2.[185]

Versican is a widely expressed ECM proteoglycan that is an anti-adhesive factor that promotes motility of prostate cancer cells [223,224] and elevated levels of versican in prostate tumors is associated with disease progression.[225] Since versican is a substrate for ADAMTS1 proteolysis, loss of ADAMTS1 has been suggested to be associated with the accumulation of versican.[176] We therefore investigated whether there was any association between ADAMTS1 expression and versican proteolysis in LNCaP and LNCaP-19 tumors. An antibody specific for the neoepitope of versican that is generated by ADAMTS1 or ADAMTS4 cleavage was used, and the amount of cleaved versican was analyzed by Western blotting. In spite of large

differences in ADAMTS1 expression between LNCaP and LNCaP-19, there was no difference in the amount of cleaved versican. The levels of TIMP2 and TIMP3 were also analyzed, in order to study if the lack of difference could be due to an increased inhibition of the proteolytic activity of ADAMTS1 in LNCaP tumors. However, the total levels of these two ADAMTS1 inhibitors were not obviously higher in LNCaP tumors. Altogether, this indicates that altered levels of ADAMTS1 do not seem to affect versican proteolysis in this model system.

In conclusion, the expression of a number of genes associated with angiogenesis was altered in AI prostate cancer cells compared to AD cells. One of the most deregulated genes was the anti-angiogenic factor ADAMTS1, which was significantly downregulated in LNCaP-19. The expression of ADAMTS1 correlated negatively with MVD in the tumor xenografts, but was not associated with versican proteolysis.

PAPER III

In paper II, ADAMTS1 was identified as a down-regulated gene in AI LNCaP-19 cells compared to AD LNCaP cells. Since the role of ADAMTS1 in prostate cancer is largely unknown this study was undertaken to investigate the expression and significance of ADAMTS1 in prostate cancer specimens from patients. The expression of ADAMTS1 was investigated by immunohistochemistry and a semi-quantitative scoring system was used to evaluate the staining. The material analyzed consisted of TURP samples from patients with early untreated tumors and from patients with recurrent disease after ADT.

ADAMTS1 was highly expressed in epithelial cells of benign glands. The expression was localized to the luminal epithelial cells, while basal epithelial cells were negative. In contrast, ADAMTS1 was significantly down-regulated in tumor cells, indicating that loss of ADAMTS1 may be involved in the development and progression of prostate cancer. Decreased expression of ADAMTS1 has previously been reported in human breast, pancreatic, hepatocellular and lung cancer compared to benign tissue,[203-205] while the expression of ADAMTS1 in human prostate cancer is mainly uninvestigated. However, in a recently published review by Ricciardelli et al, they report preliminary studies indicating an increased expression of ADAMTS1 in prostate tumors compared to non-cancerous tissue.[226]

We next investigated whether there was any difference in ADAMTS1 expression between hormone naïve and castration resistant tumors. However, no obvious difference in the ADAMTS1 levels could be observed between these two tumor types. Furthermore, ADAMTS1 did not correlate with Gleason score of the tumors. Altogether the results indicate that downregulation of ADAMTS1 occurs early during human prostate cancer progression. Although downregulation of ADAMTS1 was observed as an early event in prostate cancer, ADAMTS1 seems to be of importance in very advanced prostate cancer. ADAMTS1 has the capacity to inhibit metastasis in experimental cancer models[191,192] and in this study lower levels of ADAMTS1 were observed in tumors from patients with metastatic disease compared to patients without metastases in the castration resistant group.

Several experimental studies have shown that AI tumors are more angiogenic than AD tumors, but there are only few studies describing the MVD in human castration resistant tumors.[221,222] In accordance with previous studies, MVD was found to be significantly higher in castration resistant tumors compared to hormone naïve tumors and correlated to Gleason score. Interestingly, the increased MVD observed in castration resistant tumors was only observed in tumors expressing low levels of ADAMTS1. In castration resistant tumors still expressing relative high levels, the MVD did not differ from hormone naïve tumors. This indicates that ADAMTS1 actually may function as an angiogenesis inhibitor in prostate cancer and that

downregulation of ADAMTS1 is important for increased angiogenesis during progression. However, in hormone naïve tumors there was no relation between ADAMTS1 expression and MVD, suggesting that without other pro-angiogenic alterations associated with androgen-independency, decreased levels of ADAMTS1 alone is not enough for increased angiogenesis.

In summary, ADAMTS1 is downregulated in prostate cancer compared to benign prostate tissue. This seems to be an early event in prostate cancer progression, since decreased expression was observed already in early untreated tumors. Still, ADAMTS1 may function as an anti-angiogenic and anti-metastatic factor in castration resistant tumors, where lower levels of ADAMTS1 were associated with increased angiogenesis and metastasis.

PAPER IV

According to our previous data ADAMTS1 is downregulated during prostate cancer progression, and it was suggested that ADAMTS1 may function as an inhibitor of angiogenesis and tumor growth. However, the role of ADAMTS1 in cancer is complex and ADAMTS1 has also been described to promote tumor growth.[191,201]

To further elucidate the functional role of ADAMTS1 in AD and AI prostate cancer the expression of ADAMTS1 was modified in LNCaP and LNCaP-19 cells. LNCaP cells were stably transfected with a shRNA vector in order to downregulate ADAMTS1, while ADAMTS1 expression was reintroduced in LNCaP-19 cells by transfection with an expression vector containing the full-length ADAMTS1.

Modified expression of ADAMTS1 did not affect the proliferation rate *in vitro* in either of the two cell lines. However, the *in vivo* growth as subcutaneous tumors was indeed affected by altered ADAMTS1 expression, which indicates that ADAMTS1 mainly regulates tumor growth indirectly. Upregulation of ADAMTS1 in LNCaP-19 resulted in a markedly slower tumor establishment, suggesting that ADAMTS1 initially may function as a tumor inhibitor of these AI prostate cancer cells. However, after a while the cancer cells seem to escape from this inhibitory effect, and once tumors were established increased ADAMTS1 expression had no inhibitory function on further tumor growth rate. In contrast, downregulation of ADAMTS1 in LNCaP had no impact on tumor establishment, but strongly reduced the tumor growth rate, indicating that ADAMTS1 rather act as a tumor promoting factor in these xenografts. It has been demonstrated by others that the function of ADAMTS1 is dependent on whether ADAMTS1 is present in its full-length form or in processed forms.[191] However, in this study the discrepancy in growth regulation of LNCaP and LNCaP-19 tumors cannot be ascribed this explanation, since ADAMTS1 only was detected in its latent form and in its full-length active form in both cell lines.

The effects of ADAMTS1 on tumor blood vessels were also investigated. Surprisingly, modified ADAMTS1 expression did not affect the MVD in the xenografts, but an obvious difference in blood vessel morphology was observed. In paper I, we described that the transition of LNCaP into LNCaP-19 was associated with altered blood vessel morphology from large and round vessels with a visible lumen into smaller and thinner vessels mainly without a lumen. Interestingly, knockdown of ADAMTS1 in LNCaP resulted in a blood vessel phenotype characteristic of LNCaP-19, and by reintroducing ADAMTS1 in LNCaP-19 the vessel morphology could be reversed into an LNCaP like phenotype. Since LNCaP tumors have a higher frequency of pericyte stabilized blood vessels than LNCaP-19,[215] the pericyte coverage in the tumors was analyzed to investigate whether

altered vessel stabilization could account for the changed vessel morphology observed. However, modified ADAMTS1 expression did not affect pericyte coverage of blood vessels in the tumor xenografts.

Interestingly, ADAMTS1 was found to influence the levels of TSP1 in the tumors. Downregulation of ADAMTS1 in LNCaP was associated with elevated levels of TSP1 and upregulation of ADAMTS1 in LNCaP-19 resulted in decreased levels of TSP1. Since TSP1 also is a potent inhibitor of angiogenesis this compensatory effect could be one possible explanation to why the MVD was unaffected by modified ADAMTS1 expression. In addition, TSP1 is known from other studies to affect blood vessel morphology, where the absence of TSP1 is associated with larger tumor vessels,[154,227] which is in accordance with our results. ADAMTS1 has previously been reported to cleave TSP1, which results in the release of anti-angiogenic fragments from ECM.[185] However, we could not observe any difference in the amount of cleaved TSP1 when ADAMTS1 expression was modified.

In this paper, we found that ADAMTS1influenced the growth of AD and AI tumor xenografts differently. Furthermore, modified expression of ADAMTS1 affected the TSP1 levels and the blood vessel morphology in this experimental model of prostate cancer.

DISCUSSION

Castration therapy, as demonstrated by Huggins several decades ago, has greatly improved the treatment of advanced prostate cancer,[51,52] but eventually the tumor cells escape this treatment and AI tumor growth develops. In spite of extensive research, castration resistant prostate cancer is still an incurable disease and there is nothing to offer these patients but palliative treatment. A major challenge in the prostate cancer research field is therefore to develop new and better treatment strategies for highly aggressive, castration resistant prostate cancer. This requires an increased knowledge about cellular and molecular mechanisms that are important for the growth and survival of AI cancer cells. However, most studies regarding prostate cancer are conducted on tumors from hormonally untreated patients, which results in limited knowledge about AI prostate cancer.

LNCaP AND LNCaP-19 AS MODEL SYSTEM FOR PROSTATE CANCER

Since AI prostate cancer most often relapses as distant metastases and not locally in the prostate, a major problem is to get access to human material for studies on this terminal stage of the disease. This research field is therefore highly dependent on model systems. However, one major limitation has been the lack of experimental models that represent the whole progression from androgen-dependency into androgen-independency. Except from in humans, prostate cancer only arises spontaneously in dogs and rarely in rats.[88] The access to model systems of naturally occurring prostate cancer is therefore restricted. One of the most used models of spontaneous prostate cancer is the Dunning rat model that originates from a prostate tumor in an inbred Copenhagen rat.[228] Through further transplantation and propagation in rats, this tumor has subsequently developed into several sublines with increasing aggressiveness.[229] There are also transgenic mouse models, for example the transgenic adenocarcinoma of mouse prostate (TRAMP) model that represents the whole disease progression from premalignant lesions to metastatic disease.[230] However, a great limitation with these models is that the murine prostate anatomy differs a lot from humans and it is questionable how representative these tumors are for human prostate cancer. Most research groups therefore use xenograft models to study human prostate cancer *in vivo* by implantation of human prostate cancer cells or tumor tissue in mice. Due to great difficulties in establishing permanently growing cell lines from prostate tumors that still are AD, most of the

available cell lines are AI. The problems with establishing AD cell lines are probably due to that these cancer cells still are quite highly differentiated and still require a complex interplay with the surrounding stroma in order to survive and grow. Two of the most commonly used AI cell lines are DU 145 and PC-3.[231,232] LNCaP was for a long time the only AD cell line that could be propagated *in vitro* and is therefore the absolutely most used cell line for studies of AD prostate cancer.[210] Through further propagation *in vitro* and *in vivo* several AI sublines with different characteristics have arisen from this cell line.[233]

To study the characteristics associated with transition into androgen-independency we established a new AI subline to LNCaP, called LNCaP-19. Together with its parental cell line, this new cell line provides the opportunity to study and compare AD and AI prostate cancer. There are several advantages with this model system. First, it originates from a human prostate cancer and since LNCaP-19 is a subline to LNCaP they both originate from the same patient. Differences identified between the two cell lines are therefore not due to biological heterogeneity, but to progression of the cancer cells. Furthermore, this experimental model allows studies both *in vitro* and *in vivo* and tumors can be grown either subcutaneously or orthotopically in the prostate.[234] LNCaP-19 grows independently of androgens both *in vitro* and *in vivo*. In contrast to DU 145 and PC-3,[88,233] LNCaP-19 retains AR and PSA expression and is still stimulated by androgens. This androgen responsiveness resembles the situation in most patients where castration resistant tumors increase their growth rate when ADT is withdrawn.[211,212] Further characterization of LNCaP-19 demonstrated several characteristics that are associated with more aggressive and castration resistant tumors. The rapid development of tumors after implantation in both intact and castrated mice indicates that LNCaP-19 is more malignant than its parental line. LNCaP-19 tumors also displayed a more than 2-fold increase in MVD compared to LNCaP tumors (paper I). More recent studies have also shown that LNCaP-19 has acquired several proinvasive properties,[235] and has the ability to form metastases (unpublished data). Interestingly, when implanted in tibia LNCaP-19 forms osteoblastic lesions, which is typical for bone metastases of prostate cancer. Altogether, our results indicate that this is a useful model system for studies on prostate cancer, but there are of course also limitations to take into consideration. Even if LNCaP is dependent on androgens for growth this cell line is isolated from a lymph node metastasis in a patient with castration resistant prostate cancer and thereby probably represents a rather late stage of AD prostate cancer.[210] The LNCaP cells also contain a mutated AR that can be activated by estrogens, progesterone and anti-androgens.[74] A general drawback with xenograft models is the use of immunodeficient mice that excludes important interactions between the immune system and the tumors. Furthermore, tumors are often grown subcutaneously for practical reasons and not orthotopically in their correct anatomical site which might be more relevant. Even though LNCaP-19 shares several characteristics with castration resistant prostate

tumors, it is important to remember that this is still a model system, representing only one individual.

TRANSITION INTO ANDROGEN-INDEPENDENCY

By removing androgens, a factor promoting proliferation but also differentiation is removed. Interestingly, proliferation and production of secretory proteins is not similarly regulated by androgens in LNCaP and LNCaP-19 cells. As shown in paper I, the stimulation of growth reaches its maximum at 0.1 nM R1881, while PSA and VEGF secretion is further stimulated by higher doses of androgen. This dissociation of androgen responses is common among prostate cancer cell lines, and demonstrates that androgen regulation of tumor growth and expression of specific secretory proteins are independently regulated.[236,237] This could indicate that high levels of androgens keep the cancer cells in a differentiated state, while lower levels stimulate proliferation, which could be one explanation to why clinically relevant prostate cancer becomes more prevalent in elderly men when testosterone levels start to decline.[238]

The mechanisms behind the relapse of castration resistant prostate cancer have been a subject for discussion for a long time. It is not known whether the outgrowth of AI cells is due to cellular adaptation or to the selection of preexisting AI cells, even if most studies support the latter theory.[239-242] Whether these preexisting AI cells are cancer stem cells present already at cancer initiation or if the cancer cells gradually acquire an AI phenotype through genetic alterations during the disease progression has also been discussed.[89,242] The transition of LNCaP into LNCaP-19 does not seem to rely on either cellular adaptation or an originally heterogeneous cell population, since LNCaP cells from an earlier passage was unable to proliferate in the absence of androgens even after a year of continuous culturing. Our experiments rather indicate that transition into androgen-independency is a gradual conversion of initially AD cells that can occur spontaneously even in the presence of androgens. When androgens subsequently are removed these cells will selectively continue to grow. That transition into androgen-independency can occur independently of androgen status has been reported in several experimental studies.[243,244] However, in human prostate tumors all of these mechanisms can be relevant since human prostate tumors are composed of a more heterogeneous collection of cancer cells.

The ability to grow independent of androgens can rely on several mechanisms, both AR-dependent and AR-independent. However, the fact that most recurrent tumors still express the AR supports the idea that AR activity is important also in this stage of the disease.[67,87] Thus, castration resistant prostate cancer is a more correct

description of recurrent prostate cancer than AI or hormone refractory cancer. However, experimental studies have shown that transition into androgen-independency can result in at least three different phenotypes; androgen-responsive, androgen-insensitive and androgen-repressed cell lines.[245] These different phenotypes probably also exist in castration resistant human prostate tumors and the ratio between them may alter during disease progression and treatment. LNCaP-19 is an AI cell line that still is androgen-responsive. However, even if the AR is expressed and obviously is activated in the presence of androgens we do not know if AR activity is important for the growth and survival of these cells in the absence of androgens. The low levels of androgen regulated genes, like PSA and VEGF, in the absence of androgens (paper I) would not suggest that LNCaP-19 has a higher AR activity than LNCaP under these conditions.

ANGIOGENESIS IN AI PROSTATE CANCER

Several studies have demonstrated that the prostatic vasculature is dependent on androgens. Castration results in a rapid destruction of blood vessels followed by a reduction in blood flow and thereafter involution of the prostate gland.[28-33] This indicates that vascular regression is an important mediator of the castration effects on the normal prostate as well as in prostate tumors.[26,27] Tumor growth and metastatic dissemination is critically dependent on angiogenesis and as described in paper II, III and by others, prostate cancer progression into androgen-independency is associated with increased angiogenesis.[221,222,246,247] This means that induction of angiogenesis no longer requires circulating androgens in this stage of the disease. However, the knowledge about regulation of angiogenesis in castration resistant prostate cancer is very limited, since most studies are performed on hormonally untreated tumors. In paper II, we found a number of angiogenesis related genes that were differentially expressed between LNCaP and LNCaP-19, including ADAMTS1, ephrin-A5, fibronectin 1, midkine, neuropilin 1 and VEGF. The importance of these genes for angiogenesis in castration resistant prostate tumors is not known, but highlights the importance of studying regulation of angiogenesis in AI tumors, since it may differ from AD tumors.

VEGF is one of the most important factors that induce angiogenesis in both normal tissue and in tumors.[130,131] VEGF levels are significantly higher in prostate tumors than in benign tissue and correlate with MVD and Gleason score.[139-141] In addition, serum levels of VEGF are higher in patients with metastatic disease compared to patients with localized prostate cancer.[143] However, there are only few studies describing the VEGF expression in castration resistant tumors.[216-219,222] In line with other studies we show that VEGF is positively regulated by androgens in prostate cancer cells that are androgen-responsive,[32,147] but surprisingly we could not detect

elevated levels in the more angiogenic cell line LNCaP-19 compared to LNCaP (paper I).[220] In accordance with our study, decreased levels of VEGF have been reported in several other AI cell lines, while other studies describe elevated expression of VEGF.[159,220,222,248] Thus, it seems that angiogenesis also can be driven by other factors in AI prostate cancer.[159] Also in human prostate tumors data are conflicting, which may indicate a great heterogeneity regarding VEGF expression in castration resistant tumors.[216-219,222]

The increased angiogenesis observed in prostate cancer is not only associated with an upregulation of pro-angiogenic factors, but also to the loss of anti-angiogenic factors. Decreased levels of TSP1 have been described in prostate tumors compared to benign tissue.[159-162] In addition, TSP1 is regulated by androgens and androgen deprivation results in increased levels of TSP1.[33,160] Initially, this upregulation is probably one reason to the vascular regression observed after castration. However, as demonstrated by Colombel et al, the tumors eventually escape from the inhibitory role of TSP1 and start to grow again in spite of continuous high levels of TSP1.[33] In line with this we found an elevated expression of TSP1 in LNCaP-19 tumors compared to LNCaP tumors (paper IV), indicating that elevated levels of TSP1 are associated with AI tumors. This has also been demonstrated in prostate cancer patients.[249]

ADAMTS1 IN PROSTATE CANCER

Most experimental studies have described ADAMTS1 as an anti-angiogenic and anti-tumorigenic factor.[171,191,192,200] However, more recent studies have also demonstrated that ADAMTS1 can function in the opposite direction.[191,201] Also in human malignancies data show inconsistency regarding ADAMTS1.[203-209] These conflicting results are not surprising, since ADAMTS1 is a multi-domain protein with several functions.

ADAMTS1 was identified in paper II as a factor that was downregulated during transition into androgen-independency in the LNCaP/LNCaP-19 model. Further analysis of ADAMTS1 in clinical samples verified that ADAMTS1 is significantly downregulated in human prostate tumors compared to benign prostate tissue. The mechanism behind the downregulation of ADAMTS1 is not known, but there are studies showing that hypermethylation of the gene promoter can be responsible for loss of ADAMTS1 expression in other malignancies.[206,250] However, methylation of the ADAMTS1 gene promoter could not be observed in prostate tumors.[250] In the progression of human prostate cancer down-regulation of ADAMTS1 seems to be an early event, since the major decrease was observed already in early hormone naïve tumors. We could not observe any significant difference between the

hormone naïve and castration resistant tumors and there was no correlation with Gleason score. In a microarray study by Tamura et al, a minor increase (1.5 times) in ADAMTS1 mRNA expression was found in castration resistant tumors compared to hormone sensitive tumors.[251] Other microarray analyses of human prostate tumors have not identified ADAMTS1 as a differentially expressed gene between AD and AI tumors.[249,252]

In paper III we demonstrate that low levels of ADAMTS1 is associated with increased angiogenesis and metastasis in castration resistant tumors, which could indicate that ADAMTS1 may function as an anti-angiogenic and anti-metastatic factor in these tumors. This is also supported by the results from paper II, where transition into the more malignant cell line LNCaP-19 was accompanied by downregulation of ADAMTS1 and higher MVD. Increased expression of ADAMTS1 has also been demonstrated in LNCaP-19 tumors that are growth suppressed in the prostate.[234] Furthermore, in paper IV reintroduction of ADAMTS1 in LNCaP-19 greatly delayed tumor establishment subcutaneously in nude mice. Altogether our studies indicate that ADAMTS1 might function as a suppressor of AI prostate tumors. That ADAMTS1 expression is associated with a less malignant phenotype of AI prostate cancer has also been described by Ifon et al. He reported that transfection of the highly aggressive AI cell line PC-3 with the tumor suppressor gene U94 resulted in a markedly slower tumor growth together with an increased expression of ADAMTS1.[253]

However, even if our results indicate that ADAMTS1 might be an inhibitor of AI prostate cancer, this does not seem to be the case in AD tumors. Knockdown of ADAMTS1 in LNCaP cells severely impaired the growth rate of these tumors. ADAMTS1 has been shown to promote tumor growth in other cancer models by proteolytic release and activation of different growth factors.[191] If this is the mechanism behind the tumor promoting effect in LNCaP tumors is only speculative, but release of bFGF from ECM by activation of heparan degrading enzymes has been demonstrated to be essential for androgen stimulated growth of LNCaP *in vitro*.[254]

ADAMTS1 is a potent inhibitor of angiogenesis in experimental models, and several mechanisms have been proposed to be responsible for this inhibitory function.[185,197] That ADAMTS1 is a regulator of tumor blood vessels was also evident in LNCaP and LNCaP-19 tumors. However, ADAMTS1 did not primarily affect the number of blood vessels in these tumors, but rather the morphology and appearance of the vessels. The mechanism behind this ADAMTS1 mediated alteration of blood vessel morphology is unknown, but it could possibly be due to the altered TSP1 levels that were observed in the tumors. In paper IV, we found that decreased expression of ADAMTS1 in LNCaP tumors was associated with increased TSP1 and a switch from large, round vessels into small and thin vessels,

while overexpression of ADAMTS1 in LNCaP-19 resulted in loss of TSP1 and a switch back from small and thin vessels into larger, rounder vessels. This is in accordance with previous studies demonstrating that mammary tumors in TSP1-null mice display significantly larger blood vessels than tumors in wild-type mice.[154,227] However, lumen formation and diameter of blood vessels can be regulated by several factors, including MMP activity and the presence of VEGF, which is of special interest in the context of ADAMTS1.[255] The significance of this altered morphology for angiogenesis remains to be clarified, but to speculate the presence of ADAMTS1 may result in blood vessels that are more mature and less apt to respond on angiogenic stimuli. Thus, loss of ADAMTS1 might be a prerequisite for increased angiogenesis but is probably not sufficient. In accordance with this, our data from prostate cancer patients shows that increased MVD was only observed in the group of castration resistant tumors expressing low levels of ADAMTS1, while decreased levels of ADAMTS1 was not sufficient to induce an increased angiogenesis in the hormone naïve group.

Taken together the role of ADAMTS1 in cancer development and progression is complex and currently a subject of controversy. The C-terminal region of ADAMTS1 inhibits tumor angiogenesis, tumor growth and metastasis, while the metalloproteinase domain can have both tumor promoting and tumor inhibiting activity.[191,192,200] The total effect of ADAMTS1 probably depends on the balance between these conflicting actions. Furthermore, since ADAMTS1 is influencing tumor growth indirectly mainly by affecting the availability and activity of growth regulatory factors, the effect of ADAMTS1 is probably also largely dependent on which substrates that are present in the environment. ADAMTS1 has been identified in several expression analyses as a gene that is differentially expressed under different conditions (paper II),[195,234,251] but there are still very few studies regarding the actual function of ADAMTS1. In order to further elucidate the role of ADAMTS1 in prostate cancer development and progression more functional and mechanistical experiments are needed.

ANTI-ANGIOGENIC TREATMENT

Cancer can be treated either by targeting the cancer cells directly or indirectly, and one possibility would be to interfere with the blood supply of the tumor. The critical role of angiogenesis in cancer has led to a great interest in developing anti-angiogenic therapies. In prostate cancer increased MVD is related to clinical stage, progression, metastasis and survival, which highlights the importance of angiogenesis for disease progression.[105-108] Targeting cancer indirectly by anti-angiogenic treatment has several advantages. Vascular endothelial cells are directly accessible via the circulation, which makes drug delivery easier. Drug delivery out

of the circulation into the tumor tissue is often inefficient due to high interstitial pressure.[256] A single capillary supplies a large number of tumor cells which should result in an amplification of the anti-tumorigenic effect. Since angiogenesis is a rare occasion in adult men, targeting of angiogenesis theoretically should lead to minimal side effects. In addition, endothelial cells are more genetically stable than the cancer cells and are thus less susceptible to develop drug resistance.[257,258] However, even if anti-angiogenic treatment has been proven to efficiently inhibit tumor growth in several experimental studies, there are also several limitations and consequences to consider. Targeting the tumor vasculature renders the tumors more hypoxic, which is known to promote tumor progression into a more aggressive phenotype.[259] Moreover, there are several studies describing that tumor cells eventually escape angiogenesis inhibition by compensatory upregulation of other pro-angiogenic factors and switching to alternative pathways to promote angiogenesis.[260-262] It is therefore critical to investigate the interaction between different angiogenesis regulating factors in order to develop efficient anti-angiogenic treatments.

In clinical trials single agent efficacy of anti-angiogenic therapy has been disappointing, but has shown promising result in combination with chemotherapy.[263] This additive effect is probably due to several mechanisms. First, anti-angiogenic treatment results in normalization of blood vessels and thereby to a more efficient delivery of the cytotoxic drugs into the tumor.[256,264] Inhibition of angiogenesis is also believed to slow down tumor growth during drug free periods. The anti-angiogenic treatment may also be amplified by chemotherapy that does not only target tumor cells but also dividing endothelial cells. Furthermore, low dose metronomic chemotherapy has been shown to decrease VEGF expression and to induce endogenous angiogenesis inhibitors such as TSP1 in experimental tumor models.[265-267]

Several anti-angiogenic agents are currently evaluated in phase I, II and III clinical trials for their therapeutic efficacy. Most focus has been on the VEGF system as a target of anti-angiogenic agents.[268] Bevacizumab, a monoclonal antibody against VEGF, has so far shown promising results[57] and treatment with bevacizumab in combination with docetaxel and prednisone is now evaluated in a large randomized phase III study in men with metastatic castration resistant prostate cancer.

Early experiments showed that ADAMTS1 was a potent anti-angiogenic protein that suppressed angiogenesis more efficiently than both TSP1 and endostatin.[171] However, the possible role of ADAMTS1 as a future anti-angiogenic drug is difficult to establish. The complexity of the protein together with the lack of effect on MVD shown in the experimental studies in this thesis, indicates that ADAMTS1 may not be optimal as a single agent. However, the effect on blood vessel morphology together with the correlation to decreased MVD and metastasis in AI

prostate cancer indicate that ADAMTS1 still might have an anti-angiogenic potential. Thus, if this specific function of ADAMTS1 could be identified and isolated it might be utilized in future anti-angiogenic strategies. This, of course, is only speculative and needs further investigations.

FUTURE PERSPECTIVES

Castration resistant prostate cancer is today an incurable disease, and the best thing to offer patients in this stage of the disease is docetaxel treatment that can prolong survival with a few months.[54,55] Even if castration resistant prostate cancer may be an incurable disease it should not be untreatable. However, it is important to realize that prostate cancer in general and castration resistant prostate cancer in particular, is a very heterogeneous disease. The heterogeneity is not only between patients, but also within the same patient. Large differences in expression profiles between metastases in the same patients have been demonstrated.[269,270] It is therefore unreasonable to think that such a heterogeneous disease can be treated homogeneously. In order to develop effective treatment strategies this is an important thing to consider in trial design. A drug that is effective for a subgroup of patients may go unnoticed due to dilute effects in an unselected population. By trial designs that enrich for a molecular target or predictive biomarker this can be avoided. In addition, there has been much attention on studying individual angiogenic growth factors, but there are several more complex questions that need to be addressed. How should different anti-angiogenic agents be combined in order to avoid development of resistance or at least prolong time to relapse? What combinations of anti-angiogenic treatment would be optimal for each individual patient? When to apply anti-angiogenic treatment, early or late? If we can answer these questions anti-angiogenic treatment has great potential to transform AI prostate cancer into a chronic disease.

CONCLUSIONS

- LNCaP-19 is a suitable model for studies regarding AI prostate cancer. This newly established cell line displays several properties that are characteristic of AI prostate cancer, including the ability to grow without androgens, enhanced malignancy, decreased PSA levels and increased MVD. (Paper I)

- Transition into androgen-independency in experimental prostate cancer is accompanied by altered expression of a number of genes involved in angiogenesis regulation. Among these, a significant decrease was found for ADAMTS1, whose expression correlates negatively with MVD. (Paper II)

- ADAMTS1 is down-regulated in human prostate cancer compared to benign prostate tissue. Furthermore, low expression of ADAMTS1 is associated with high MVD and metastasis in castration resistant tumors. (Paper III)

- ADAMTS1 affects the growth of AD and AI tumor xenografts differently. In addition, modified ADAMTS1 expression results in altered blood vessel morphology and TSP1 levels. (Paper IV)

POPULÄRVETENSKAPLIG SAMMANFATTNING

Prostatacancer är den vanligaste formen av cancer bland män i Sverige. Sjukdomen drabbar framförallt äldre män, och varje år upptäcks ca 9 000 nya fall. I många fall är tumören beskedlig och växer långsamt, men ungefär en fjärdedel av de patienter som får prostatacancer kommer att dö av sin sjukdom.

I de fall då tumören växer långsamt och inte har spridit sig utanför prostatan kan man operera bort tumören eller ge patienten strålbehandling. Ibland väljer man att avvakta med behandling och istället noga följa och kontrollera tumörens tillväxt. Vid mer aggressiva former av prostatacancer växer och sprider sig tumören utanför prostatan och kommer så småningom bilda dottertumörer, s.k. metastaser, i framförallt lymfkörtlar och skelettet. Det går då inte operera eller stråla bort tumören utan patienter med spridd prostatacancer behandlas istället med kemisk eller kirurgisk kastration. Eftersom cancercellerna till en början är beroende av det manliga könshormonet testosteron för att växa, utnyttjar man detta i kampen mot cancern. Vid kastration avstannar testosteronproduktionen i kroppen och tumörtillväxten bromsas därmed i de flesta fall upp. Tyvärr är effekten av kastrationsbehandlingen övergående och efter något år börjar tumörerna att växa och sprida sig igen på ett mer aggressivt sätt. Cancercellerna är då inte längre beroende av testosteron för sin tillväxt och prostatacancern sägs därmed vara androgen-oberoende. Idag finns inga bra behandlingsmetoder för detta stadium av sjukdomen och prognosen för patienten är dålig. Det är därför av yttersta vikt att utreda hur tumörernas egenskaper förändras vid övergången till androgen-oberoende tillväxt för att kunna utveckla nya och mer effektiva behandlingsmetoder.

Nybildning av blodkärl, s.k. angiogenes krävs för att en tumör skall kunna växa och spridas. Utan nybildning av blodkärl får de inre delarna av en tumör inte tillräcklig tillgång till syre och näringsämnen, och tumörcellerna får svårare att sprida sig till andra organ. Angiogenes regleras av flera ämnen som utsöndras från cancercellerna. Dessa ämnen kan vara stimulatoriska eller hämmande. En obalans av dessa ämnen leder till en ökad mängd blodkärl som kännetecknar aggressiva tumörer. Genom att blockera angiogenes skulle man kunna förhindra fortsatt tillväxt och spridning av tumörer. Detta kräver dock en ökad förståelse för hur tumörangiogenes regleras i olika skeden av cancerutvecklingen varför syftet med detta avhandlingsarbete var att studera hur cancercellernas påverkan på blodkärl förändras då de blir androgen-oberoende.

För dessa studier krävs experimentella modellsystem som speglar den kliniska situationen så bra som möjligt. Inledningsvis utvecklades därför ett modellsystem som möjliggör jämförande studier av androgen-beroende och androgen-oberoende prostatacancer både i cellodlingssystem och i försöksdjur. Genom att odla cancerceller som fortfarande är androgen-beroende i frånvaro av androgener etablerades en ny cellinje som inte längre behöver androgener för att växa. Dessa cancerceller härstammar från en patient med prostatacancer. Studier av den nya cellinjen visade på flera egenskaper som är typiska för androgen-oberoende tumörer, såsom snabbare tillväxt då de växer som tumörer i möss samt fler blodkärl i tumörerna. Sammantaget visar resultaten att det etablerade modellsystemet är väl lämpat för studier av androgen-oberoende prostatacancer då det i mångt och mycket liknar den kliniska verkligheten.

För att undersöka hur cancercellerna stimulerar den ökade nybildningen av blodkärl som kännetecknar de androgen-oberoende tumörerna undersöktes i nästa studie nivåerna av 96 kända ämnen som är involverade i regleringen av angiogenes. Ett antal ämnen som uppvisade förändrade nivåer i de androgen-oberoende cancercellerna identifierades, där ibland ett protein som heter ADAMTS1. ADAMTS1 är ett protein som kraftigt hämmar angiogenes och tumörtillväxt i andra experimentella system. De androgen-oberoende cancercellerna producerade påtagligt lägre nivåer av detta hämmande protein, vilket skulle kunna vara en förklaring till att dessa tumörer bildar fler blodkärl.

Därefter studerades nivåerna av ADAMTS1 i tumörer från patienter med prostatacancer för att se om detta protein även är av betydelse i humana tumörer. Nivåerna av ADAMTS1 visade sig vara markant lägre i tumörvävnad jämfört med i normal prostatavävnad. Det var dock ingen skillnad mellan androgen-beroende och androgen-oberoende tumörer, vilket tyder på att nivåerna av detta protein minskar i ett tidigt skede under cancerutvecklingen i humana tumörer. Vidare visade sig de androgen-oberoende tumörerna, liksom i modellsystemet, innehålla fler blodkärl än de androgen-beroende tumörerna. Höga nivåer av ADAMTS1 var associerat med färre blodkärl och färre metastaser i de androgen-oberoende tumörerna, vilket kan innebära att ADAMTS1 fungerar som en tumörhämmande faktor i human prostatacancer.

För att få en ökad förståelse för vad ADAMTS1 egentligen gör i tumörerna modifierades produktionen av detta protein i de båda cellinjerna. Nivåerna sänktes i de androgen-beroende cellerna som normalt producerar höga nivåer av ADAMTS1, och höjdes i de androgen-oberoende cellerna som normalt endast producerar låga nivåer av detta protein. Det visade sig att ADAMTS1 tycks ha olika funktion i de två olika tumörtyperna. I de androgen-beroende tumörerna främjade ADAMTS1 tillväxten, medan proteinet hade en hämmande effekt på androgen-oberoende tumörer. Antalet blodkärl påverkades inte men utseendet på blodkärlen förändrades

tydligt. Dessutom påverkade ADAMTS1 förekomsten av ett annat angiogenes-hämmande protein som heter TSP1. Sammantaget tyder detta på att ADAMTS1 påverkar blodkärlen i tumörerna, men betydelsen av detta är fortfarande inte utredd.

Sammantaget visar detta avhandlingsarbete att det etablerade modellsystemet är lämpligt för att studera androgen-oberoende prostatacancer, samt att detta skede av sjukdomen är associerat med fler blodkärl i tumörerna vilket troligtvis är en bidragande orsak till en mer aggressiv tillväxt och spridning av cancern. Vidare tyder resultaten på att ADAMTS1 är ett protein som är involverat i utvecklingen av prostatacancer och som tycks ha en hämmande effekt på androgen-oberoende tumörer.

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