Karolinska Institutet
From the Departments of Clinical Science, Huddinge University Hospital
and Woman and Child Health, Stockholm, Sweden

TISSUE PROSTATIC SPECIFIC ANTIGEN (T-PSA):
A WAY TO PREDICT AND UNDERSTAND THE DEVELOPMENT OF PROSTATE CANCER

Mirtha Grande

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To my mother, Anders, Oscar
and
in the memory of Sara and Silvio

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Abstract

Prostate cancer (CaP) is the most prevalent malignancy among men in Sweden. One major problem associated with CaP is the choice of treatment since the disease may develop very differently from patient to patient. Many efforts have focused on the development of markers for prognosis and monitoring treatment affecting the endocrine system of CaP. In this respect prostate specific antigen in serum (S-PSA) is currently the most commonly used marker.

We have evaluated PSA levels measured in fine needle aspiration biopsies from tumor tissue (tissue PSA, T-PSA) for prognosis of CaP outcome and its use as a tool for understanding the development of the disease. T-PSA and S-PSA values in metastasis-free (M0) patients were inversely correlated, indicating that S-PSA levels reflect the degree of leakage of PSA into the blood stream rather than the production of PSA in the tumor. T-PSA measurement at the time of diagnosis was more effective than cytological grade, tumor stage, DNA ploidy and total and free serum PSA as a predictor for the outcome of therapy. Moreover, the change in T-PSA between 12 and 0 months of treatment was an even better predictor in this respect. In patients who did not respond to treatment initial T-PSA values were low but increased dramatically during treatment.

Our patients were treated surgically with bilateral orchidectomy / GnRH analogues (Zoladex™) or with parenteral depot estrogens (Estradurin™). In M0 patients who responded to treatment, T-PSA values were significantly higher in estrogen treated patients than in patients treated by orchidectomy / with GnRH analogues, indicating a stimulatory action of estrogens on PSA synthesis in vivo. Estrogens are used in the treatment of CaP, but may also have a role in the etiology of the disease. We therefore studied the effects of estrogens on vasoactive factors of possible importance in CaP, using hormone sensitive (LNCaP-FGC) and hormone resistant (LNCaP-r) cell line. We found that estrogens stimulated mRNA expression of the vasoactive factor, ecNOS but suppressed mRNA expression of ET-1. Estrogens may therefore influence CaP through alteration of these factors.

Our findings demonstrate that the levels of T-PSA can be directly related to the prognosis of prostate cancer. In the future, protein characterization of the tumors may improve our ability to predict the outcome for patients with this disease.

Key words: tissue PSA, aspiration biopsies, estrogens, ecNOS mRNA expression, ET-1 mRNA expression, LNCaP-FGC, LNCaP-r.

List of Articles

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

I. Stege R., Tribukait B., Carlström K., **Grande M.** and Pousette Å. Tissue PSA from fine-needle biopsies of prostatic carcinoma as related to serum PSA, clinical stage, cytological grade, and DNA ploidy. Prostate 38(3):183-8 (1999).

II. Stege R., **Grande M.,** Carlström K., Tribukait B. and Pousette Å. Prognostic significance of tissue prostate-specific antigen in endocrine-treated prostate carcinomas. Clinical Cancer Research 6(1):160-5 (2000).

III. **Grande M**., Carlström K., Rozell B.L., Stege R. and Pousette Å. Prognostic value of serial tissue prostate-specific antigen measurements during different hormonal treatments in prostate cancer patients. Clinical Cancer Research 6(5):1790-5 (2000).

IV. **Grande M.**, Carlström K., Stege R., Pousette Å. and Faxén M. Estrogens increase the endothelial nitric oxide synthase (ecNOS) mRNA level in LNCaP human prostate carcinoma cells. Prostate 45:232-7(2000).

V. **Grande M.**, Carlström K., Stege R., Pousette Å. and Faxén M. Estrogens affect Endothelin-1 mRNA expression in LNCaP human prostate carcinoma cells. Submitted to European Urology.

Abbreviations

Androgen receptor = AR
4-androstene-3,17-dione= A4
α_1-antichymotrypsin = ACT
Benign prostatic hyperplasia= BPH
Carcinoma of the prostate = CaP
Dehydroepiandrosterone= DHA
Dehydroepiandrosterone sulphate = DHAS
Digital rectal examination = DRE
5-α-dihydrotestosterone = DHT
endothelial nitric oxide synthase = ecNOS
endothelin-1 = ET-1
Estradiol-17β= E2
Estrogen receptor α = ERα
Estrogen receptor β = ERβ
Fine needle aspiration biopsy = FNA
Flow cytometry = FCM
Gonadotropin releasing-hormone = GnRH
Hypoxanthine-guanine phosphoribosyl transferase = HGPRT
inducible NOS = iNOS
Luteinizing hormone = LH
α_2-macroglobulin = AMG
neuronal nitric oxide synthase NOS = nNOS
Nitric oxide = NO
Prostatic acid phosphatase = PAP
Prostate specific antigen = PSA
Tissue PSA = T-PSA
Veterans Administration Co-operative Urological Research Group = VACURG

Background

Prostate cancer

Carcinoma of the prostate (CaP) is the most common malignant tumor in men in Sweden, corresponding to approximately 27% of all male cancer. After lung cancer, prostate cancer is the most common cause of death among men. According to the most recent statistics from the 1999, 5918 men have been diagnosed with the disease and the number of cases has increased by an average of 1.8 percent per year for the past ten years (Socialstyrelsen, Cancer incidence in Sweden 1997, 1999). CaP usually affects elderly men and in 1997 the average age at diagnosis was 74 years.

Due to the dramatic increase in early detection of CaP (Lu-Yao and Greenberg, 1994; Johansson *et al*., 1997) the management of this disease is one of the great challenges of modern urology. One peculiar feature of CaP is the discrepancy between the lifetime risk of clinical cancer and the 26-38% frequency of latent prostate cancer detected at autopsy (Breslow *et al*., 1977; Kabalin *et al*., 1989). This has been taken as an evidence for the existence of two biologically distinct, but histologically indistinguishable forms of CaP. On the one hand we have the clinical CaP, which causes symptoms and eventually kills the patient, and on the other hand a latent disease incapable of aggressive behaviour (Adami and Baron, 1994).

Risk factors

The causes of CaP probably reflect a complex interaction between environmental and genetic factors. It is widely known that there are differences in incidence depending on race and geographical location. The highest incidence is found in north west Europe and in the USA and the lowest in Asian countries. Literacy, age, eating habits and overweight have been suggested as risk factors (Grönberg *et al*., 1996). Furthermore, hereditary CaP appears to be common among relatives to men with early onset disease (Bratt *et al*., 1997). The localisation of a “CaP gene” was reported in 1996 (Smith *et al*., 1996). Coupling analysis showed a significant correlation to a locus on chromosome 1 (1q24-25) and the putative gene was denominated »Hereditary Prostate Cancer 1« (HPC1). During recent three additional candidate genes for hereditary CaP have been identified (Berthon *et al*., 1998; Xu *et al*., 1998; Gibbs *et al*., 1999). Males castrated before puberty do not develop clinical prostate cancer (Wu and Gu, 1991). Underlying causes are incompletely recognised; nevertheless it is known that androgens are required for the origin of the disease and that even estrogen may be involved in this respect (Bosland, 1992; Carlström and Stege, 1997).

Morphology

The human prostate is composed of three major regions – the peripheral, central and transition zones (McNeal, 1988a). The peripheral zone comprises approximately 70% of the glandular component of the prostate. It is characterized by simple glands and loose stroma, and is the site of origin of most prostatic adenocarcinomas (McNeal, 1988b). The prostate functions as a secretory organ and is composed of many different cell types, which can be broadly grouped into two classes: epithelial and stroma cells.

Hormonal regulation

Growth and integrity of human prostatic epithelium is strongly dependent upon the adjacent stroma (Krieg *et al*., 1983; Farnsworth, 1999). Development and function of the prostate is under the control of sex hormones, notably androgens.

Testosterone is the most important circulating androgen and is produced to ≥ 95 % by the testicular Leydig cells. The secretion of testosterone is stimulated by luteinizing hormone (LH) from the anterior pituitary, the secretion of which in turn is mainly controlled by hypothalamic gonadotropin releasing-hormone (GnRH). Most of the testosterone in the circulation is protein bound (98 –99%), either to a medium high affinity sex hormone binding globulin (SHBG) or to albumin. Only free and albumin-bound testosterone are considered as biologically active, that is capable of diffusing into the target cells (Pardridge, 1986). Due to its high affinity to testosterone, SHBG regulates its biological activity. Within the prostate and many other target organs testosterone is converted by 5-α-reductase into 5-α-dihydrotestosterone (DHT) (Maes *et al*., 1979; Monti *et al*., 1998).

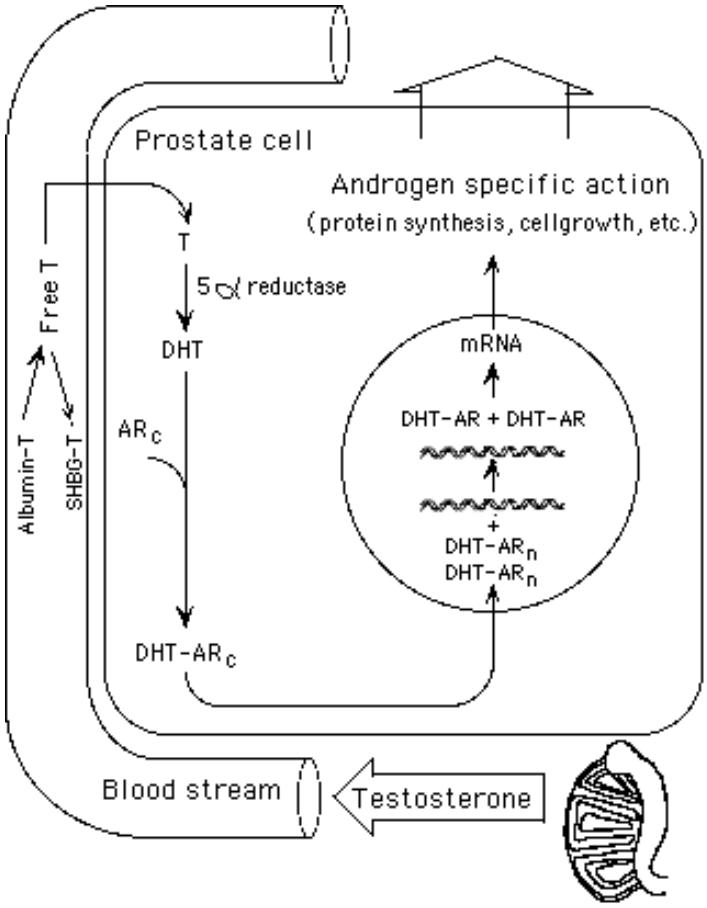

Figure 1. Mechanism of androgen action. Within the cell nucleus, the androgen receptor (AR) complex undergoes dimerization and binding to a hormone responsive element in order to regulate transcription. (T, testosterone; DHT, dihydrotestosterone; ARn, activated nuclear receptor; mRNA, messenger RNA; ARc, inactive receptor).

A very minor part of the androgenic activity in the adult human male originates from the adrenal cortex. Dehydroepiandrosterone (DHA) and its sulphate DHAS and 4-androstene-3,17-dione (A4) are weak androgens secreted mainly from the adrenocortical *zona reticularis*. They exert their activity mainly by conversion to testosterone in peripheral tissue and to testosterone and DHT in androgen target organs (Monti, et al., 1998).

A small amount of circulating estrogen in the adult male is produced by direct testicular secretion of estradiol-17β (E2) while most is formed by the peripheral conversion of androgens, notably A4 and testosterone. Due to their ability to suppress pituitary LH secretion, thereby decreasing the production of testosterone, estrogens at high doses are used in the treatment of CaP. Endogenous estrogen may also play a role in the etiology of prostatic disease (Carlström and Stege, 1997 and references cited therein).

Pathology

Androgens are important for normal prostate development and function and are probably involved in the emergence of CaP. The effect of androgens is mediated via the androgen receptor (AR), which stimulates transcription of androgen sensitive genes. Changes in the AR in prostate cancer have been proposed to take place in three different ways. Amplification of the AR has been seen when so-called hormone-responsive tumors become hormone-independent (Visakorpi *et al*., 1995). Secondly, mutations of the AR are common in recurrent CaP (Taplin *et al*., 1995) and preferably within the hormone-binding domain. Finally, it has been suggested that polymorphisms within certain micro-satellite sequences (short so-called CAG-repetitive sequences) in the AR are associated with the development of the disease (Coetzee and Ross, 1994; Irvine *et al*., 1995).

Over 95% of all CaP are adenocarcinomas. The tumors occur with varying frequencies in the different zones of the prostate and are not directly proportional to the amount of glandular tissue. Seventy percent of CaP occurs in the peripheral zone, 20% in the transition zone, and 5 -10% in the central zone (McNeal, 1988b).

Endocrine treatment and clinical parameters

The principle for endocrine treatment of CaP is to “throttle” the supply of androgens to the cancer cells. Bilateral orchidectomy or medical castration are the two options to eliminate testicular testosterone production. The Nobel Prize in 1966 was awarded to C. Huggins for his discovery together with C.V. Hodge, that castration relieved the symptoms of patients with metastatic CaP (Huggins *et al*., 1941). They also found a similar effect by treatment with estrogen (Huggins and Hodges, 1941).

The effects of estrogens in the treatment of CaP are believed to be primarily indirect via the pituitary-gonadal axis (Alder *et al*., 1968; Jarred *et al*., 2000) (see figure 2). Estrogens also directly block testicular steroidogenesis (Daehlin *et al*., 1985; 1986). Castrated rats, which have been implanted with CaP and treated with testosterone in combination with estrogens, have shown that estrogens inhibit the growth of glandular epithelial cells while stimulating tumor stroma resulting in smaller tumors (Daehlin *et al*., 1987). The effects of estrogens on stromal tissue may be receptor mediated, whereas the inhibitory effect of estrogens on the epithelium may be mediated by some other mechanism (Landström *et al*., 1988).

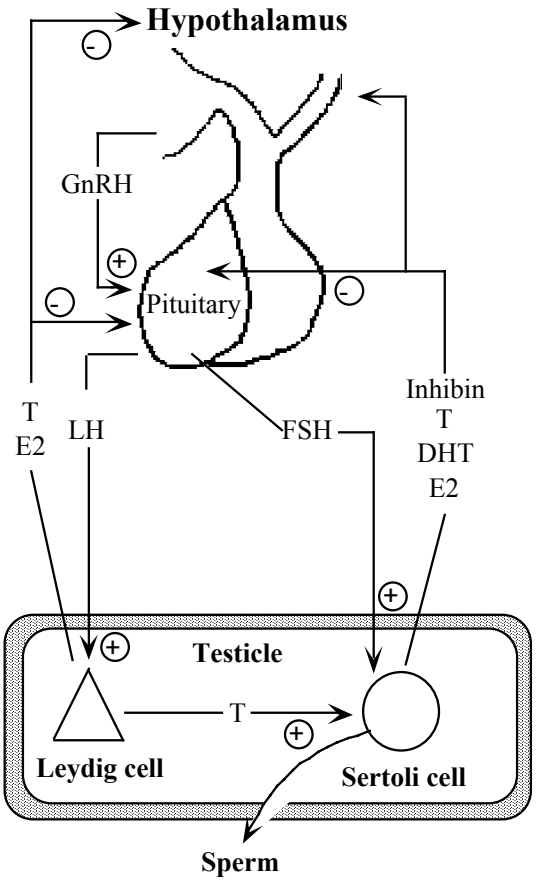

Figure 2. Hypothalamic-pituitary-testicular axis. (GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; T, testosterone; DHT dihydrotestosterone; E2, estradiol; +, positive influence; - negative influence).

In the late 1950s and the 1960s, castration or treatment with oral diethylstilbestrol (DES) or both were tested against placebo in the first Veterans Administration Co-operative Urological Research Group (VACURG) study. No clear differences in survival were found between the two choices of treatment, but it was noted that the number of cardiovascular complications and deaths from cardiovascular disease was high in the DES group. These findings led to a reduction in estrogen dosage to levels that were still effective in the treatment of cancer but had less cardiovascular side effects (Byar, 1973). The cardiovascular side effects are related to the high impact of oral preparations of estrogen on the liver during the first-pass (Goebelsmann *et al*., 1985; Daehlin, et al., 1986; Henriksson *et al*., 1986). Oral estrogens cause profound changes in protein synthesis in the liver, including increased levels of coagulation factor VII, which are likely to be of particular importance (Henriksson *et al*., 1989) since progression of arteriosclerotic disease has been linked to this factor (Meade *et al*., 1980), and as has deterioration of coronary status during oral estrogen treatment (Henriksson, et al., 1989).

Parenteral administration of estrogen avoids the first-pass effect (Goebelsmann et al., 1985). A parenteral estrogen dose of 240 mg intra-muscular polyestradiol phosphate per month has been shown to suppress testosterone production to castration

levels without any major effect on liver proteins, including factor VII (Henriksson *et al*., 1992). Furthermore, no signs of increased cardiovascular morbidity were found in pilot studies (Henriksson *et al*., 1990).

In 1978 Labrie and co-workers reported that continuous treatment with GnRH analogues eliminated testicular testosterone production by suppressing pituitary LH secretion and thus reduced the size of prostate in experimental animals. This gave way to a new form of endocrine treatment of CaP (Belanger *et al*., 1980). They then blocked the remaining adrenal androgen activity by addition of antiandrogens, the so-called total androgen blockade (Labrie *et al*., 1983; 1986). However, several other studies have failed to demonstrate any advantages of this costly form of treatment compared to surgical castration or treatment with GnRH analogues alone or high dose parenteral estrogens (Iversen *et al*., 1997; Eisenberger *et al*., 1998; Hedlund, 1999)

Hormone dependency

Most CaP patients respond to androgen ablation initially, indicating that at least a portion of their cancer cells is androgen sensitive. Unfortunately however, almost all of these patients eventually relapse to a state, which is no longer responsive to androgen withdrawal. The mechanism behind the transition from an initially androgen-sensitive to an insensitive tumor remains regrettably unknown. Overgrowth of a pre-existing androgen-independent tumor cell clone or a cellular adaptation to growth in the absence of testicular androgens are the most common explanations (Isaacs and Coffey, 1981; Labrie, et al., 1983).

Established methods for diagnosis and prognosis of CaP

Standard methods for diagnosis, prognosis and monitoring of treatment of CaP include cytological or histological *grading* of biopsy samples taken from the tumor, *staging* by digital rectal examination and by transrectal ultrasonography, detection of bone metastases by radiological *bone scan* and determination of *serum tumor markers*, commonly prostate specific antigen (PSA).

Grading

Material for microscopic examination of the tumor may be obtained by biopsy with a fine or thick needle, transurethral resection or radical prostatectomy. Among the needle biopsy techniques fine needle aspiration biopsy (FNA) with a 22-G needle (diam 0.7 mm) and core biopsy with an 18-G needle (diam 1.2 mm) have received widespread use. Digitally guided transrectal FNA biopsy according Franzén et al was met with rapid acceptance in Scandinavia and other parts of Europe (Franzén *et al*., 1960). This technique has been shown to be as effective and reproducible as ultrasound guided transrectal core biopsy (Adolfsson *et al*., 1991). FNA biopsy enables minimally invasive collection of samples from prostatic tumors. A necessary prerequisite is the skill of the cytologist or urologist in the sampling procedure.

The material obtained by FNA biopsy is classified according to cytological criteria. The commonly used grading system is based on the criteria scored as defined by “The Uropathological Study Group on Prostatic Carcinoma” (Müller *et al*., 1980; Helpap *et al*., 1985). The three grades of malignancy are identified based on six cellular properties; average nuclear size, variability in nuclear size, average nucleolar size, nucleolar variability (size, shape and number), disturbance of nuclear arrangement and cellular and nuclear dissociation. Tumors are then graded as follows:

Score 1-5 = G0 (dysplastic changes, no malignancy); Score 6-10 = GI (well differentiated carcinoma); Score 11-14 = G2 (moderately differentiated carcinoma); Score 15-18 = G3 (poorly differentiated carcinoma).

One difficult aspect with cytological grade as a prognostic factor is the inter- and intra-observer variability in grading (Müller, et al., 1980). Most of the classifications that could not be reproduced were borderline findings between grades I and II or grades II and III (Böcking *et al*., 1982).

Many investigators require core biopsy in order to be able to use histology for diagnosis. Gleason's grading system, the most widely used classification system, yields a final histopathological score ranging from 2 (very well differentiated) to 10 (poorly differentiated) (Gleason, 1977).

Staging

The precise staging of CaP is absolutely indispensable for correct therapeutic management. When treatment for cure is to be given, most urologists require that the tumor is confined to the organ and that there is no extraprostatic tumor growth (Catalona, 1990). The ultimate goal of staging is determination of tumor severity and exclusion or verification of the presence of metastases in lymph nodes, bone, or soft tissue. Of the various staging systems for prostate cancer, the two most used are presented on page 15 (Table 1).

Bone scan

When bone metastasis is seen on bone scan, the prognosis is poor in most cases with a ten-year survival of less than 10% (Johansson, et al., 1997). The local extension of the primary tumor has no prognostic importance in these cases (Johansson *et al*., 1991). The risk of developing metastasis for patients with organ-confined disease has been estimated to be 10-14% ten years from the time of diagnosis (Adolfsson *et al*., 1997; Johansson, et al., 1997).

Serum tumor markers

As in other malignant diseases, great efforts have been made to find serum markers that indicate the presence of a prostatic tumor. So far, no serum marker specific for tumor cells of prostatic adenocarcinoma has been reported, nor has any available marker been shown to strictly correlate to the degree of histological differentiation. Prostatic acid phosphatase (PAP) and prostate-specific antigen (PSA) are biochemical markers that are normal components of the acinar epithelium of the prostate and are also found in large quantities in its exocrine secretion (Huggins and Hodges, 1941; Wang *et al*., 1979). Both PAP and PSA levels in serum are markedly increased in patients with CaP. Serum PAP was the first to be used as a marker for diagnosis and monitoring of therapy (Simon and Nygaard, 1959). Nowadays serum PAP has been superseded by serum PSA (S-PSA) since S-PSA has been shown to be more sensitive and specific (Oesterling, 1991; Bunting, 1999).

Table 1. Staging systems for prostate cancer

UICC* classification system (Harmer, 1978; Schroder *et al*., 1992)		**AUA**** (Jewett, 1975)	
Category:		Stage:	
T0	No tumor palpable	A	No tumor palpable
T1	Clinically apparent tumor, not palpable nor visible by imaging		A1 Focal A2 Diffuse
T2	Tumor palpable or visible by imaging confined to the prostate	B	B1 Unilobar < 2 cm
T2a	Tumor involves = ½ a lobe		B2 Unilobar >2 cm
T2b	Tumor involves > ½ a lobe but not both lobes.		
T2c	Tumor involves both lobes		
T3	Tumor extends through prostatic capsule	C	C1 + C2 Extending through capsule
T3a	Unilateral extracapsular extension		
T3b	Bilateral extracapsular extension		
T3c	Tumor invades seminal vesicles		C3 Extending to other adjacent organ
T4	Tumor is fixed or invades adjacent structures other than seminal vesicles		
T4a	Tumor invades bladder neck and/ or external sphincter and/ or rectum		
T4b	Tumor invades levator muscle and/ or is fixed to pelvic wall		
N	Lymph node metastases	D	D1 Lymph node metastases
M	Distant metastases		D2 Other metastases

* **UICC**= International Union Against Cancer.
****AUA**= American Urologic Association.

Measurements of S-PSA are now widely used for the diagnosis and monitoring of treatment of patients with CaP. However, increased concentrations of PSA are also found in men with benign prostatic hyperplasia (BPH). Nonetheless, PSA has proved to be the most useful marker of CaP yet found.

PSA was first recognised in human seminal plasma in 1970, but not until 1979 was it isolated and demonstrated to originate specifically from the prostate (Ablin *et al*., 1970a; 1970b; Hara *et al*., 1971; Wang, et al., 1979). PSA is a glycoprotein with a molecular mass of 33 kDa, composed of a single polypeptide chain (Wang, et al., 1979). It is a serine protease and a member of the human glandular kallikrein family. PSA has been shown to participate in the proteolytic degradation of gel-forming proteins secreted by human seminal vesicles (Lilja, 1985; 1987; Lee *et al*., 1989; Christensson *et al*., 1990). Semenogelins I and II are constituents of the gel structure (Lilja *et al*., 1987; 1989; 1992). The PSA mediated fragmentation of these proteins occurs concomitantly with the dissolution of the gel structure of semen and the progressive activation of sperm motility (Lilja and Laurell, 1984; Lilja, 1985).

The prostate epithelium in the glandular ducts secretes PSA and efficiently prevents the escape of the protease into the general circulation. However, small amounts of PSA leak into the circulation and this increases in prostate disease. The ratio between the PSA concentration in seminal plasma (0.5 – 3 g/L) and in serum (<4 μg/L) is approximately 10^6:1 in healthy men (Malm and Lilja, 1995).

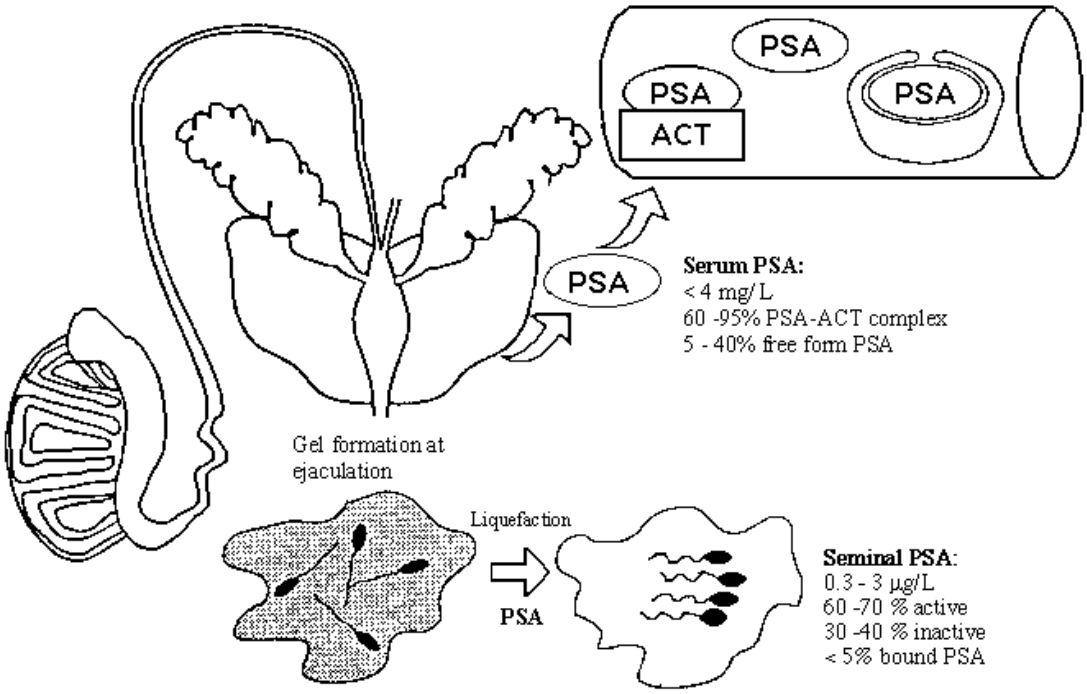

Figure 3. Schematic illustration of the male reproductive organs. The concentrations of PSA in serum and seminal plasma as well as the relative amounts of the different forms of PSA is indicated. Adapted from Malm J & Lilja H (1995).

Christensson and associates reported the ability to distinguish between different molecular forms of PSA (free and protein bound) in both serum and seminal fluids (Christensson, et al., 1990). The enzymatically active single-chain (free) form of serum PSA may be inactivated by complex formation with the protease inhibitors α_1-antichymotrypsin (ACT) and α_2-macroglobulin (AMG), which are present at 10^4- to 10^5- fold molar excess to PSA (Christensson, et al., 1990). In vivo complex formation between PSA and ACT may occur in the prostate since PSA-producing epithelial cells have also been reported to produce ACT (Laurell *et al*., 1992). ACT-bound PSA constitutes approximately 90% of total S-PSA, whereas the remaining 10% is free and unbound (Lilja *et al*., 1991). Whilst the levels of free PSA and PSA-ACT may be measured in commercial PSA assays, there are no accessible PSA epitopes on PSA-AMG, preventing immunodetection of the PSA moiety unless denaturing conditions are used to unfold the PSA-AMG complex (Christensson, et al., 1990; Zhang *et al*., 2000). These distinct molecular forms are present at different concentrations in the serum of men with and without prostate cancer, and their measurement may help to distinguish prostate cancer from BPH (Lilja, et al., 1991; Stenman *et al*., 1991; Morgan *et al*., 1996; Partin *et al*., 1996).

Clinical use of PSA measurement

As previously mentioned, benign conditions may affect the serum concentrations of PSA. Moreover, not all CaP cause a detectable increase in S-PSA concentration. Thus, the usefulness of PSA in early detection of curable CaP is limited due to the overlap in PSA concentrations between patients with BPH and those with CaP. This overlap is most obvious with PSA concentrations of between 4.0 and 10.0 µg/L. It has been estimated that the diagnosis of 30- 40 % of patients with CaP will be missed if diagnosis is based on S-PSA alone, using a cut-off level of 4 µg/L (Brawer and Landge, 1989; Partin *et al*., 1990).

However, measuring the fraction of free PSA may increase the specificity of PSA testing for prostate cancer detection in men with S-PSA concentrations between 4 and 10 µg/L (Van Cangh *et al*., 1996; Wang *et al*., 1996; Partin, et al., 1996; Prestigiacomo and Stamey, 1997; Catalona *et al*., 2000).

In several studies it has been shown that the S-PSA concentration increases in proportion to advancing clinical stage (Hudson *et al*., 1989; Oesterling, 1991; Partin and Oesterling, 1994; Chan and Sokoll, 1997). However, since there is a substantial S-PSA overlap between all clinical stages of the disease, the PSA concentration is not as reliable determinant of clinical stage on an individual basis.

Post-treatment PSA of < 10% of pretreatment values at 2, 3 and 6 months after commencement of treatment has been associated with prolonged survival (Reynard *et al*., 1995). Moreover, a drop in S-PSA as well as the S-PSA value at the lowest level (PSA-nadir) after hormonal treatment of metastatic disease are indicators of progression-free survival (Matzkin *et al*., 1992). The time at which the PSA begins to increase once the nadir is reached may occur before objective evidence of progression (Miller *et al*., 1992).

PSA elevation in hormone refractory prostate tumors has been attributed to: 1) mutations and/or amplifications of AR that broaden its ligand specificity and/or enhances tumor cells responsiveness to androgen, respectively (Culig *et al*., 1993; Elo *et al*., 1995; Taplin, et al., 1995; Visakorpi, et al., 1995); 2) androgen-independent activation of the AR by growth factor signalling pathways like insulin-like growth factor-1 and keratinocyte growth factor, which would elicit AR-mediated transcriptional activation (Culig *et al*., 1994; Reinikainen *et al*., 1996), and/ or 3) the direct stimulatory action by soluble prostate specific autocrine factor(s) secreted by hormone-refractory CaP cells and/ or AR-independent regulatory elements within the PSA (Hsieh *et al*., 1993; Yeung *et al*., 2000).

Other methods for the diagnosis and prognosis of CaP

DNA-ploidy

Since the mid-seventies, cellular DNA ploidy analyses by flow cytometry (FCM) have increasingly been applied to tumors of various sites, including those of the urinary tract (Tribukait, 1993; Wang *et al*., 2000). DNA ploidy has been shown to be of high prognostic value, particularly in low-grade, low-stage tumors (Tribukait, 1993). Furthermore, cell material from FNA biopsies is highly suitable for FCM. However, single samples often contain an insufficient number of cells, particularly in benign lesions, and it is necessary to repeat the puncture of the lesion in order to obtain sufficient material for cytology and FCM. Thus there is a risk of non-representative

sampling, which emphasizes the need for experience in obtaining transrectal aspiration biopsy (Tribukait, 1991).

The ploidy of the tumor, which roughly expresses the total number of chromosomes, is usually very well defined. An important observation is that the ploidy categories (diploid, tetraploid, non-tetraploid aneuploid) change with tumor stage.

The biological significance of the co-existence of diploid tumor cells in aneuploid tumors is unknown. Disturbances of the balance between the diploid and aneuploid tumor cell population, for example in the course of hormonal manipulation, may result in a growth advantage for the aneuploid tumor part (Tribukait, 1993).

The significance of heterogeneity within CaP tumors has been discussed as a problem in obtaining a representative DNA sample from multiple prostate tumors (Greene *et al*., 1994) as well as from one primary tumor (O'Malley *et al*., 1993). It has been shown that underestimation of the aggressiveness of CaP due to tumor heterogeneity can be minimized by simultaneous study of the tumor grade and DNA ploidy in a better way than by increasing the number of biopsies (Wang, et al., 2000).

In most studies a correlation between DNA-ploidy and cytological grade has been found (Adolfsson, 1994). Furthermore, in a multivariate analysis DNA-ploidy was a significant prognostic factor of disease specific survival, when nuclear DNA content was quantified with FCM (Zincke *et al*., 1992).

Tissue PSA

In the late 1980:s Pousette and co-workers studied the transition from hormone sensitive to hormone resistant CaP using a model system consisting of two prostate carcinoma cell lines; the hormone-sensitive LNCaP-FGC and its hormone-resistant variant LNCaP-r. The PSA values in the hormone resistant cell line were found to be lower than in the hormone sensitive cell line. On the basis of these results, a method for quantitation of PSA in FNA biopsies from patients with prostate diseases was developed. This was named tissue PSA (T-PSA) and correlated inversely to both tumor stage and cytological grade, thus highly malignant tumors have low tissue concentrations of PSA (Hasenson *et al*., 1989).

The distribution of PSA in the prostate gland is not uniform (Qiu *et al*., 1990), with 20-25% of all prostate tumors expressing little or no PSA (Stein *et al*., 1982). When the level of PSA mRNA was correlated using Gleason score there was a trend for the poorly differentiated tumors to have lower levels of PSA mRNA than the well-differentiated tumors (Qiu, et al., 1990).

Model systems for CaP

Among the small number of permanent *in vitro* CaP cell lines described the LNCaP (*L*ymph *N*ode *C*arcinoma of the *P*rostate) is a frequently used *in vitro* model of human CaP with properties characteristic of prostatic epithelium (Horoszewicz *et al*., 1980). LNCaP was isolated in 1977 by J.S. Horoszewicz and co-workers (Horoszewicz, et al., 1980), from a needle aspiration biopsy of a lymph node of a 50-year old Caucasian male with a confirmed diagnosis of metastatic CaP. The LNCaP cells have low anchorage potential, grow on plastic surfaces but not on glass and do not tend to produce smooth and uniform monolayers. They exhibit a much slower rate of growth *in vitro* than most “typical” cell cultures and can rapidly acidify culture medium (Horoszewicz, et al., 1980). The unique property of the LNCaP cell line is the

continuous production of PAP and PSA both *in vitro* and *in vivo* in nude mice (Horoszewicz, et al., 1980; 1983).

The LNCaP model system is hormonally responsive and contains ARs. The AR is abnormal with a single point mutation in the ligand-binding domain (Veldscholte *et al*., 1990a; Culig, et al., 1993). It is not androgen specific and androgens, progestagens, estrogens and anti-androgens all bind and activate this mutated AR. Physiological concentration of pregnenolone, the common precursor of all steroid hormones, has also been shown to stimulate LNCaP cells via the mutated receptor (Grigoryev *et al*., 2000). Estrogen receptors (ER) have been found in LNCaP cells by some workers (Castagnetta *et al*., 1995; Lau *et al*., 2000), however but not others (Berns *et al*., 1986; Veldscholte *et al*., 1990b; Brolin *et al*., 1992; Hobisch *et al*., 1997; Hanstein *et al*., 1999). In addition, E2 has been found to provoke a rapid enhancement of calcium influx in LNCaP cells (Audy *et al*., 1996). This influx was insensitive to blockade by flutamide (antiandrogen) as well as tamoxifen (antiestrogen). This suggests that some of the E2 responses might be independent of either androgen- or estrogen receptors (Audy et al., 1996).

The LNCaP-FGC (*f*ast *g*rowing *c*olony) cell line, derived from an early passage of the original culture, has been widely distributed and also commercially available. This subline differs from the parental cells only by its growth rate (König *et al*., 1989). Propagation of LNCaP cells has led to the development of a number of sublines with different hormonal responsiveness (van Steenbrugge *et al*., 1991). The LNCaP-r subline is an androgen independent cell line, which spontaneously derived from the LNCaP-FGC and further characterized by Pousette and co-workers (Hasenson *et al*., 1985).

The effects of E2 on LNCaP cell growth are biphasic. Treatment of LNCaP cells with 10 nM E2 has been reported to stimulate cell growth (Schuurmans *et al*., 1990; Carruba *et al*., 1996). Proliferation was diminished at higher concentrations than 10 nM, and E2 did not affect the LNCaP-r cells (Hasenson *et al*., 1988; Carruba, et al., 1996). It is apparent that 10 nM E2 in the presence of an androgen suppresses rather than enhances LNCaP-FGC cell growth. By contrast, other studies have shown that E2 inhibited growth of LNCaP cells at low concentrations (10 nM) and enhance cell growth at high concentrations (100nM). These bi-directional effects of E2 on LNCaP cell growth have been attributed to the sensitivity of these cells to culture conditions and thus to growth factor interferences (Carruba, et al., 1996; Lupowitz and Zisapel, 1999).

The existence of two rather than one ER, today characterized as ERα and ERβ with 5 isoforms, means that the mechanism of action of E2 is more complex than previously thought (Moore *et al*., 1998). Male reproductive tissue showed expression of ERβ in most tissues, with highest expression in the prostate (Taylor and Al-Azzawi, 2000).

Tumor growth depends largely on blood supply and solid tumors grow rapidly as soon as they become vascularised (Folkman, 1974). Estrogens are widely used in the treatment of CaP. At the same time, however, estrogens are involved in the etiology of the disease. Elevated serum levels of estrogens, together with elevated levels of biologically active testosterone, are present in untreated patients with CaP; there are several reports on an increased frequency of CaP and other prostatic disorders in men exposed to high estrogen levels in the presence of adequate androgen concentrations (Carlström and Stege, 1997). Based on this we chose to study two aspects of tumor growth in LNCaP cells. One peptide involved in vascular

development and tumor growth and the other a peptide with mitogenic and growth-promoting properties, both of which are affected by estrogens.

Biology of nitric oxide

Nitric oxide (NO) plays a key role in physiological as well as pathological processes, including inflammation and cancer. The NO biosynthetic pathway is shown in figure 4.

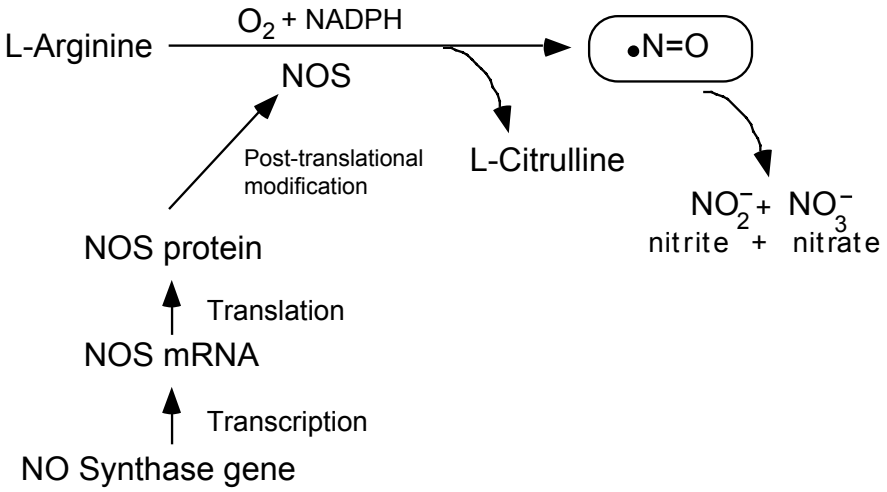

Figure 4. NO biosynthetic pathway. Nitric oxide is produced from L-arginine by the nitric oxide synthase (NOS) enzyme family, forming the free radical NO and citrulline as byproducts. NO has a short-half life and is rapidly oxidized to the stable, inactive end-products, nitrite and nitrate (NO2- and NO3-). NOS expression and activity are regulated by transcriptional and post-transcriptional mechanisms.

NO is produced from L-arginine by the nitric oxide synthase (NOS) a family of enzymes, forming the free radical NO and citrulline as by-products (Nathan, 1992). NO has a short half-life ($t_{1/2}$ = a few seconds) and is rapidly oxidised to stable, inactive nitrite and nitrate.

Nitric oxide synthase enzymes, NOS

Three isoforms of the NOS enzyme have been isolated and represent the products of three different genes. Two of the NOS enzymes are continuously present and are thereby termed constitutive NOS (cNOS). One cNOS enzyme was first identified in endothelial cells (endothelial cNOS, ecNOS) and the second was initially localised to neurons (neuronal cNOS, nNOS), but has also been identified in other tissues (Nakane *et al*., 1993). In contrast to the two cNOS enzymes the third NOS isoform, inducible NOS (iNOS) is not normally present in resting cells.

The classification of NOS isoforms has until now been based on biochemical criteria. They have been known as calcium dependent and constitutive or calcium independent and inducible enzymes (Knowles and Moncada, 1994). However, it is now clear that there are calcium-dependent isoforms, which are inducible (Palmer *et al*., 1992) and calcium-independent isoforms, which are apparently constitutive (Radomski *et al*., 1991).

Different hypotheses have been put forward about NO production in tumors, presenting NO as a factor that inhibits antitumor immune response (Lejeune *et al*., 1994; Jenkins *et al*., 1995), as well as induces tumor cells apoptosis (Cui *et al*., 1994). Of the two constitutive forms of NOS; ecNOS appears to play an important role in vascular development, maintenance of vascular tone and tumor growth (Archer, 1993; Chinje and Stratford, 1997).

NOS in the prostate

NOS immunoreactivity has been localised to the glandular epithelium of the human prostate, with the highest levels in the peripheral zone; the zone from which CaP mainly derives (Burnett *et al*., 1995). Evidence for Ca^{2+}-dependent NOS activity has been showed in the prostate, seminal vesicle and vas deferens (Ehrén *et al*., 1994).

Effects of estrogens on ecNOS activity

Estrogens seem to stimulate ecNOS activity (Gilligan *et al*., 1994; Weiner *et al*., 1994). Physiological concentrations (0.001 nM – 10 nM) of E2 enhance the activity of ecNOS in endothelial cells of cultured blood vessel tissue (Hayashi *et al*., 1995). E2 was suggested to enhance ecNOS via a receptor-mediated system, while high E2 concentrations tended to inhibit ecNOS activity via a receptor-independent pathway (Hayashi, et al., 1995).

Endothelin-1, ET-1

Endothelin-1 (ET-1) is a 21 amino acid vasoconstrictor peptide (Yanagisawa *et al*., 1988) and exerts a very potent and long-lasting contractile response in nonvascular smooth muscle, including prostatic tissue. It also elicits mitogenic and growth-promoting effects on different cell types (Simonson *et al*., 1989; Mazzocchi *et al*., 1990). Human seminal fluid contains the highest concentration of ET-1 of any body fluid studied (Battistini *et al*., 1993) and ET-1 has been detected in human benign prostatic hyperplasia (BPH) and CaP tissue (Nelson *et al*., 1995).

ET-1 in the prostate

Almost every human prostate cancer cell line available expresses ET-1 mRNA and produces ET-1 *in vitro* (Nelson et al., 1995). The peptide has been detected in 100% of primary CaP and in 87% of metastatic sites (Nelson *et al*., 1996). Elevated plasma levels of ET-1 have also been detected in patients with advanced hormone refractory CaP (Nelson et al., 1995), suggesting an involvement of the peptide in the morbidity and/or progression of this ominous disease.

ET-1 has been proposed to be an autocrine/ paracrine growth factor for human cancer cell lines (Shichiri *et al*., 1991; Bagnato and Catt, 1998; Pirtskhalaishvili and Nelson, 2000). Although this has been disputed by others (Grant *et al*., 1997)

The biological effects of ET-1 are mediated by two different receptor subtypes named ETA and ETB (Sakurai *et al*., 1992). Although, ETB receptor expression predominates in BPH epithelium, no binding sites and almost completely undetectable levels of ET receptor mRNA have been detected in human CaP cells (Nelson et al., 1995; Grant et al., 1997).

Effects of estrogens on ET-1 activity

E2 inhibits stimulated ET-1 release in human cultured vascular endothelial cells (Wingrove and Stevenson, 1997; Morey *et al*., 1998) and the effect is inversely related to the concentration of the hormone. This is in agreement with *in vivo* results showing a significant decrease in circulating ET-1 levels in women receiving hormone replacement therapy (Ylikorkala *et al*., 1995). It has been suggested that ET-1 acting together with other angiogenic factors, such as NO, might participate in the neoangiogenesis that occurs during tumor establishment. NO is a feedback inhibitor of ET-1 release (Boulanger and Luscher, 1990) and increased release of NO would be expected to decrease ET-1 accumulation in the media of estrogen-treated cells. It has been confirmed that estrogen is associated with increased expression of NO synthase; however this effect was not concomitant with the observed inhibition of ET-1 release (Wingrove and Stevenson, 1997).

Aims of the Present Investigation

- To evaluate the prognostic value of T-PSA in the development of CaP before and during hormonal treatment.

- To study T-PSA during different regimens of hormonal treatment of CaP in order to gain further insight in its regulation.

- To study the effects of estrogens on vasoactive factors in an *in vitro* CaP model system.

Material and Methods

Patients and treatment

The study material comprised 104 men aged 53- 86, of which 13 had a diagnosis of BPH and 91 had CaP (paper I); 179 patients aged 51- 82 years with CaP (paper II) and 63 patients aged 54- 87 years with CaP (paper III).

In total 179 patients with CaP were included in the studies from January 1986 until September 1991 as well as 13 patients with BPH from a parallel cohort.

The patients in papers II and III were consecutively allocated, in a non-randomized manner, to one of three forms of hormonal treatment depending exclusively on the patient's decision. Ninety-two (paper II) and 30 (paper III) patients were treated surgically with bilateral orchidectomy. Fourteen (paper II) and 11 (paper III) patients were treated with GnRH analogues (3.6 mg of goserelin acetate (Zoladex™) s.c. every fourth week). The remaining 73 (paper II) and 22 (paper III) patients received parenteral depot estrogens (240 mg of polyestradiol phosphate (Estradurin™) i.m. per month). Both regimens of medical treatment suppress circulating testosterone to castration levels (Labrie, et al., 1986; Carlström *et al*., 1997).

At the time of diagnosis, all patients had M_0 disease in papers I and II while 11 of the 63 patients in paper III had M_1 disease. All patients were scanned for bone metastases and / or conventional X-ray every 24 weeks during the entire observation period, as part of the routine monitoring at the Department of Urology.

T-staging

T staging was performed by digital rectal examination (DRE) throughout the entire period of investigation by a senior urologist (R.S.), according to UICC guidelines (Harmer, 1977). Ultrasound equipment for transrectal examination of the prostate was not available at our department when this study was initiated.

Follow-up

The patients were followed and evaluated according to the recommendations of European Organisation for Research on Treatment of Cancer (EORTC). Clinical examinations were performed every 12 weeks, including assessment of prostate size by DRE and location and size of soft tissue metastases. Bone scan and / or conventional x-ray for assessment of bone metastases was performed every 24 weeks during the entire observation period. Objective progression of the disease was defined as an increase of T1 or T2 stage by 2 steps or more compared to the lowest T stage recorded and an increase from T3 to T4. The appearance of skeletal or non skeletal metastases was also recorded as objective progression.

Fine needle aspiration biopsies

Fine needle aspiration biopsies were obtained during routine examination according to the method of Franzén (Franzén, et al., 1960). All biopsies were taken by the same senior cytologist (B.L.R.). Five biopsies from the same tumor area were obtained at each time. When it was necessary to perform aspiration biopsies repeated times in the same tumor area, the pathologist did a meticulous depiction of the primary tumor location for each patient (paper III). A detailed explanation of how the biopsies were then treated is given in paper I and illustrated in figure 5.

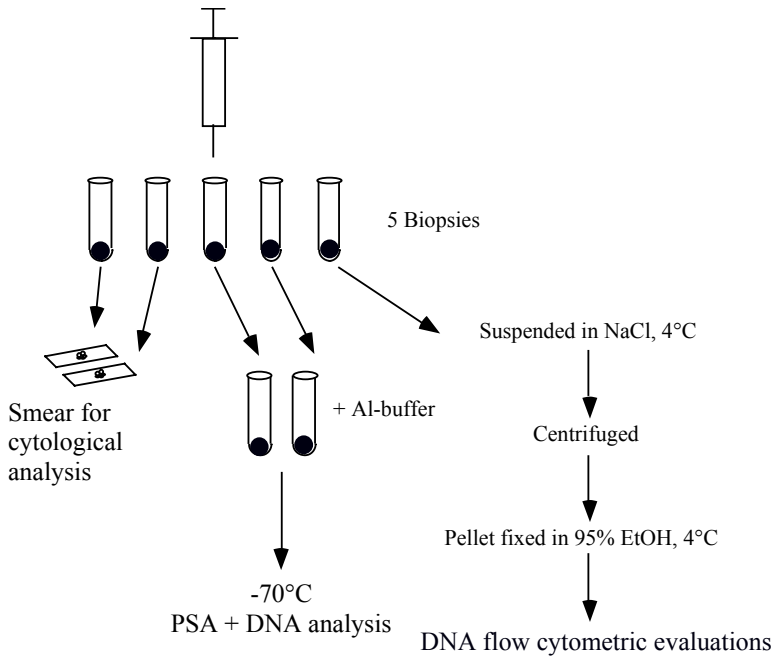

Figure 5. Schematic illustration of how the samples from fine needle aspiration biopsy were handled. Two biopsies were used for cytological review, two for biochemical analysis and one for DNA flow cytometric evaluations.

Cytological sample preparation and evaluation

From the aspirates, smears were prepared, air-dried and stained with May-Grünwald-Giemsa. The grading of the prostatic tumors was based on the criteria expressed in scores as defined by the Uropathological Study Group of Prostatic Carcinoma (Müller, et al., 1980). Cytological grading of the aspirates was performed throughout the entire period of investigation by the same senior pathologist (B.L.R.) without knowledge of the biochemical data.

Flow cytometry

The cells were fixed in ice-cold 96% ethanol. After pepsin and RNase treatment, the cell nuclei were stained and analysed in a flow cytometer as previously described in detail (paper I).

Analysis of tissue and serum PSA

The FNA biopsy samples were thawed and subsequently processed at 4ºC. Homogenisation was carried out using a sonicator (Ultrasonics Ltd. A350G). Sonication was performed six times for each sample, with 10 s sonication and 50 s cooling between each sonication, and the volume was made up to 1000 µL with A1-buffer (0.05 M Tris-HCl, 0.001 M EDTA, 0.001 M dithiothreitol (DTT), 0.01M NaCl, pH 7.4). The samples were then centrifuged at 105,000 g for 18 min. The cytosol was removed and stored at -70ºC until analysis of marker proteins. The pellet was suspended in 300 µL Na-phosphate buffer with EDTA (2M NaCl, 2mM EDTA, and 0.05 M Na_2HPO_4, adjusted to pH 7.4 with 0.05 M NaH_2PO_4) and stored at -70ºC until analysis of DNA.

DNA determination

DNA concentrations in the homogenates were determined by fluorescence spectrophotometry using bis-benzimide, commonly known as Hoechst 33258 (H 33258) dye according to Labarca and Paigen (Labarca and Paigen, 1980). Calf thymus DNA was used as the standard solution. Briefly, 100 µL of the stain (10 µL/mL H 33258) was added to 800 µL of Na-phosphate buffer without EDTA (2 M NaCl, 0.05 M Na_2HPO_4, adjusted to pH 7.35 with 0.05 M Na_2HPO_4) and 100 µL of 0-, 10- or 50-fold diluted pellet suspension. The same buffer without EDTA was used for dilution of the pellets. The vials were protected from light until quantitation. Fluorescence was measured with a Hitachi F-4000 fluorescence spectrophotometer (exc. 356 nm, em. 458 nm, time average 10 s, and response 2 s) and the data obtained related to a standard curve from 0.1 to 3 µg/mL.

H 33258 has been found to be particularly useful in the quantitative determination of DNA in biological materials. It is a simple and sensitive method which utilizes the enhancement of fluorescence seen when the compound binds to DNA. H 33258 binds to the minor groove of DNA. When 365 nm light (long UV) excites this bound dye, its fluorescence at 458 nm can be measured. The procedure can be used directly on crude tissue homogenates in which the deoxyribonuclease protein structure of chromatin has been dissociated, making the DNA fully accessible to the reagent. RNA does not interfere and the method can readily quantitate amounts of DNA as low as 10 ng, which is the amount present in just a few thousand cells.

Quantitations of T-PSA and S-PSA

To determine the level of T-PSA in cytosol, samples were diluted 1:10- 1:10,000 using the zero calibrators provided with the kits. Serial dilutions of cytosols in these systems yielded dilution curves that were perfectly parallel to the standard curves. The amounts of PSA present in the cytosol from the aspirates are given as µg PSA/ µg of DNA.

Commercial radioimmunoassay kits from Diagnostic Products Corp., Los Angeles, CA, were used for quantitation of total PSA when measured both in serum

and in cytosol in papers I and III and for cytosol PSA in paper II. In paper II the levels of total and free S-PSA were measured with a chemiluminescent enzyme immunoassay using commercial kits (Immulite PSA and Immulite Free PSA; Diagnostic Products Corp., Los Angeles, CA).

Cell cultures

All cells were grown (paper IV and V) in similar manner. LNCaP-FGC cells cultured in medium with complete foetal bovine serum for more than 30 passages have been observed to gradually show a better growth response in the steroid-depleted serum (van Steenbrugge et al., 1991). With this in mind, early passages of the androgen sensitive cells were used.

RNA extraction, reverse transcription and DNA amplification (PCR)

All the RNA extractions and reverse transcriptions were done as explained in paper IV. The non-competitive RT-PCR reactions were normalized with Hypoxanthine-guanine phosphoribosyl transferase (HGPRT) as an internal reference control gene. HGPRT is moderately expressed housekeeping gene not found to be up regulated by E2 (Foss *et al*., 1998).

PCR product analysis

The PCR products were analysed on 2% agarose gels and visualized by ethidium bromide. The gels were visualized under ultraviolet light, and further analysed by scanning densitometry using a gel scanner with a computerized video gel documentation system (see paper IV for more details). The intensity of the bands was measured with image analysis software. The semi-quantitative determination of the ecNOS (paper IV) and ET-1 (paper V) mRNA was carried out on the basis of the corresponding HGPRT mRNA level and was presented as the ratio between the intensity of the respective bands. The HGPRT gene was transcribed at a constant level in prostate cells.

Analysis of estrogen metabolites

To study possible formation of E2 from E1 and E1 and E2 from E1S, the culture media were extracted with diethyl ether and ether phase was separated and evaporated to dryness. E1 was analysed using an in-house radioimmunoassay with tritiated tracer, anti-E1 from Diagnostic Systems Laboratory Inc., Webster., Texas (Product No DSL-8710) and dextrane coated charcoal separation. For the analysis of E2, the extracts were dissolved in the zero calibrator supplied by the kit manufacturer and E2 was analysed by radioimmunoassay using a commercial kit (E2 Coat-a-Count®) from Diagnostic Products Corp., Los Angeles, CA.

Statistics

Simple parametric and non-parametric tests were used to compare differences between the groups. Correlations were performed using Spearman's rank correlation test.

In paper II, the interval time to progression and to death were analysed with life table techniques, as well as Cox multivariate analysis with regard to the clinical and biochemical parameters (Cox, 1972; Peto *et al*., 1977).

To be able to easily illustrate the material and discover non-linear relations, we subdivided the patients into three different categories according to the level of T-PSA values. The categories were: 1) patients with low T-PSA values, 2) patients with intermediate T-PSA values and 3) patients with high T-PSA values. The continuous variables were run separately by Cox univariate analysis and the category variables by survival analysis.

Similarly, in paper III survival curves were generated by the life table method and the log-rank test was used to estimate significance (Peto et al., 1977). Because development of prostate cancer regression was "time dependent" after initiation of endocrine treatment, analysis of the prognostic impact of time to progression was performed after an observation period of 6 months. A period of 6 months was chosen for distributional reasons with respect to survival time and time for development of CaP progression. Thus, as many patients as possible could be evaluated with respect to these two factors.

Results

Inverse correlation between S-PSA and T-PSA (I)

The aim of our study was to elucidate the factors affecting the tissue and serum levels of PSA in patients with prostatic disease. For this purpose 104 metastasis-free patients, 13 with BPH and 91 with CaP, were studied. Relationships between cytological grade, T-stage and DNA ploidy are summarized in table 2.

Table 2. Summary of the clinical features of the participating patients in study I.

		T stage			DNA ploidy	
	Total	2	3	4	Diploid	Tetraploid/ aneuploid
Cytological grade						
G0	13	-	-	-	13	-
G1	14	14	0	0	14	-
G2	52	10	42	0	39	13
G3	25	0	14	11	12	13
		24	56	11	65	26

T-PSA was correlated to a battery of other variables including cytological grade, T stage, DNA ploidy, total S-PSA and T-PSA. A highly significant negative correlation ($r_s = -0.55$, $p < 0.001$) was found between S-PSA and T-PSA.

Significantly lower T-PSA concentrations were found in tetra-/ aneuploid tumors compared to diploid CaP tumors. A similar correlation was seen for cytological grade. The highest T-PSA concentrations were found in the BPH material and in diploid G1 tumors and the lowest in tetra-/ aneuploid G3 tumors.

Significant correlations were also found between increasing S-PSA values and increasing tumor stage ($p<0.01$) as well as with cytological grade ($p<0.001$). The lowest S-PSA concentrations were found in BPH and in diploid G1 tumors and the highest in tetra-/ aneuploid G3 tumors.

Tissue PSA as a predictor of clinical outcome (II)

The prognostic value of T-PSA was compared with a battery of other prognostic markers and evaluated with regard to time to progression and time to disease specific death. The study material comprised 24 patients with stage T2 tumors, 130 with T3 and 25 with T4, all with M0 disease at the time of diagnosis. Thirty five tumors were graded as G1 or as well-differentiated carcinomas; 78 as moderately differentiated carcinomas or G2; and 66 as poorly differentiated carcinomas or G3. A total of 113 tumors were diploid and the remaining 66 were tetra-/ aneuploid tumors. Changes in T-PSA with increasing cytological grade, T stage and a more unfavourable DNA ploidy pattern were very much more pronounced than the corresponding changes in total or free S-PSA.

The patients were included in the study over a period of 5 years and 8 months and followed for a further 6 years (76 months). All patients were followed for at least 71 months or until death.

The impact of different factors, including cytological grade, T stage, DNA ploidy, total and free S-PSA, ratio of free: total PSA, T-PSA and age, was calculated by Cox univariate regression analysis, with time to progression and time to disease specific death as end-points. Among these variables T-PSA showed the strongest impact on both time to progression and time to disease specific death (see table 3).

Table 3. Cox univariate analysis with respect to time to progression and time to death in CaP.

Factor*	**n**	χ^2
Time to progression		
T-PSA	179	121.4
Total S-PSA	115	30.4
Free S-PSA	115	29.2
Time to death from CaP		
T-PSA	179	101.3
Total S-PSA	115	24.4
Free S-PSA	115	19.4

*Only significant factors are presented in this table ($p<0.001$).

Background factors were evaluated using Cox stepwise regression analysis and T-PSA was found to be the only factor of significant importance for time to progression ($\chi2 = 121.4$, $p<0.001$) among all the other variables. Furthermore, T-PSA ($\chi2 = 81.8$, $p< 0.001$) and, to a lesser degree, T stage ($\chi^2 = 11.5p<0.003$), were the only significant predictors of time to disease specific death.

A life table analysis was performed using the categories, T-PSA, T-stage, cytological grade and DNA ploidy to group the patients and with time to progression and time to death from CaP as events. The patients were divided into three groups of approximately equal number according to their T-PSA values (Table 4).

Table 4. Grouping of CaP patients according to T-PSA levels.

T-PSA group	**n**	**T-PSA values, µg PSA / µg DNA**
1	60	$\leq$ 0.192
2	60	0.193 – 1.171
3	59	$\geq$ 1.172

At the end of the observation period, 93% of the patients in group 1 had progressed and of these 87% died of CaP. None of the patients in group 3 died of CaP nor had their tumor progressed by the end of the observation period (see figure 6)

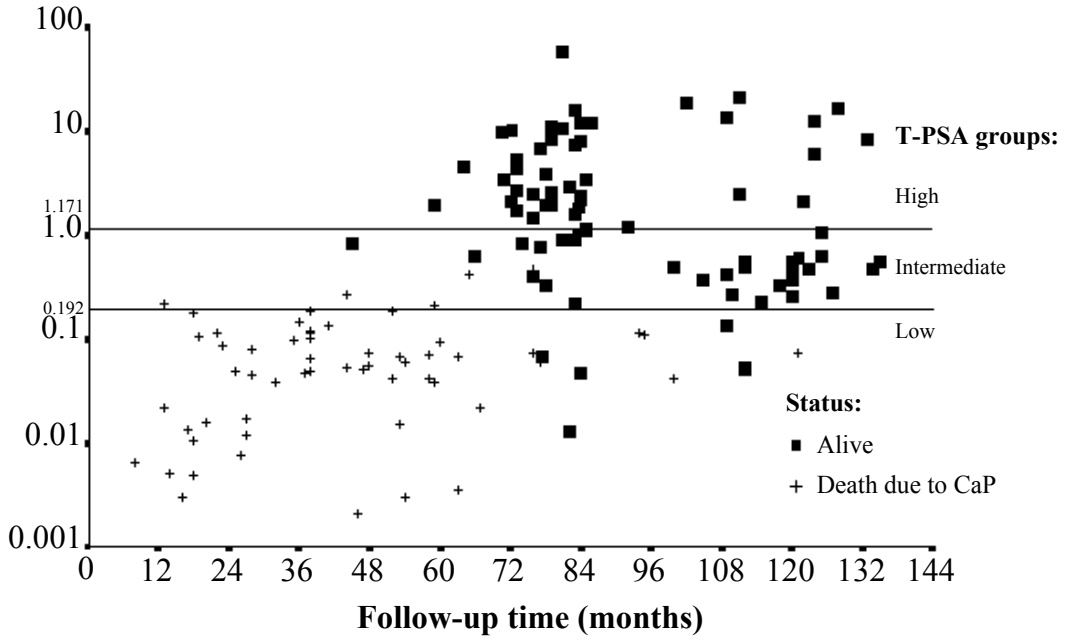

Figure 6. T-PSA values in relation to follow-up time are given in months. The horizontal lines delineate the limits for the three T-PSA groups according to their T-PSA levels. Status of the patients is indicated as (■), alive or (+**),** dead due to CaP.

T-PSA measurements during different treatments (III)

The effects of different treatments affecting the hormone status on T-PSA were followed in a study material comprising 63 patients. T-PSA measurements were performed at the time of diagnosis, and 6, 12 and 24 months after initiation of treatment.

Three of the tumors were staged as T2, 40 as T3 and 20 T4; Thirty-one were graded as G2 and 32 as G3; 52 had M0-disease and 11 had M1-disease. Thirty of the patients were surgically castrated by bilateral orchidectomy, 11 were treated with GnRH agonists (Zoladex®) and 22 with parenteral depot estrogens (Estradurin®).

T-PSA values increase in non-responders (III)

The initial T-PSA levels were significantly lower in non-responders to endocrine treatment therapy compared to responders, but the levels increased dramatically during treatment.

12- to 0-months ratio of T-PSA is a good predictor of clinical outcome (III)

T-PSA measured during treatment may reflect biochemical changes in the tissue. The ratio between T-PSA at 12 months and at diagnosis had the most significant

prognostic value for predicting clinical outcome compared to for example, with tumor stage, cytological grade, T-PSA at diagnosis or after 6 and 24 months.

Estrogens affect T-PSA concentration in vivo (III)

Because bilateral orchidectomy and medical castration with GnRH agonists produce similar sex steroid conditions, these groups of patients were combined in the statistical calculations. Interestingly, however, there was no difference in pretreatment values between the orchidectomized/ GnRH agonist-treated group and the estrogen-treated group, T-PSA values during treatment were significantly higher in the estrogen-treated patients compared to the surgically or GnRH agonist treated group ($0.001 < p > 0.005$).

Estrogens increase ecNOS mRNA expression in LNCaP cells (IV)

We chose to study the effects of estrogens on ecNOS mRNA expression in LNCaP cells, because this endothelial cell-specific form of NO synthase is thought to play an important role in vascular development and tumor growth in human CaP. Furthermore, estrogens have been shown to upregulate ecNOS expression in different human cell culture systems. We used the androgen-sensitive LNCaP-FGC cell line and its androgen-resistant derivative LNCaP-r.

LNCaP-FGC cells incubated for 48 h with 10 and 100 nM E1S showed a significantly ($p< 0.05$) increased ecNOS mRNA expression. Similar results were seen after 2-4 h incubation with E2, irrespective of the concentration used. The only significant increase seen in the androgen-insensitive LNCaP-r was found after 48 h using the highest concentration of E2 (100 nM, $p< 0.05$). Furthermore, ecNOS mRNA expression was significantly lower in untreated LNCAP-r than in LNCaP-FGC cells.

Estrogens affect ET-1 mRNA expression in LNCaP cells (V)

Our purpose was to study the effects of estrogens on ET-1 mRNA expression in the LNCaP cells and furthermore whether the effects of E1S are mediated via conversion to E2. Estrogens have been shown to down regulate ET-1, a mediator of the osteoblastic response of bone to metastatic CaP.

ET-1 mRNA expression in LNCaP-FGC cells was significantly suppressed by E2 and E1S following incubation for 2 - 4 h and after 48 h by E2 only at 1 and 10 nM. In LNCaP-r cells suppression by E2 only was seen at a concentration of 100 nM following 2 – 4 h of incubation. ET-1 mRNA expression was significantly higher in untreated LNCaP-r than in untreated LNCaP-FGC cells. LNCaP-FGC cells efficiently transformed E1 into E2 but very little to E1 and no E2 was formed from E1S.

Discussion

Biopsy procedure

We have used cell material from fine needle aspiration biopsies, which is suitable for cytological evaluation, DNA flow cytometry and now shown to be suitable also for quantitation of PSA. PSA was related to the amount of DNA in the samples as measured by a sensitive DNA specific spectrofluorometric method. By measuring DNA instead of protein we increase the sensitivity since PSA is only related to the nuclear containing components of the aspirate, avoiding the possible influence of other components, mainly erythrocytes. This also means that changes in DNA per cell such as during tumor development should influence the measurement. A major concern has been that the admixture of benign cell material in tumor biopsies may bias the results. In our studies, cytology, flow cytometry and PSA levels are based on analysis from five separate biopsies: Two were obtained for cytology studies, two for biochemistry and one for flow cytometry. The samples from these aspirates are sometimes scanty, particularly in benign lesions and it may be necessary to repeat the puncture of the lesion in order to obtain sufficient material. If this is the case, the sample obtained may not be representative. Thus emphasizing the need for high experience in performing the technique of transrectal aspiration biopsy.

If a significant proportion of normal prostate cells is present in the aspirate, the measured PSA values will be overestimated. However, values are underestimated if a significant proportion of stromal or inflammatory cells are present in the aspirates. It has however been shown that cell material aspirated from the prostate contains a high yield of tumor cells, with decreasing proportion of benign epithelial cells with increasing dedifferentiation of the tumor. In well and poorly differentiated tumors, the average values were 66% and 85%, respectively and fraction of inflammatory cells was 3 – 4% (Tribukait, 1991; Wang *et al*., 1992).

The tissue / serum PSA ratio is in an order of 10^6:1. It can therefore not be excluded that during biopsy procedure PSA from the glandular ducts could be mixed with the sample, and as a consequence contributing to higher T-PSA values.

In spite of these concerns and drawbacks, aspiration biopsy material offers a unique possibility to repeatedly obtain material directly from the tumor and enables us to follow the changes during tumor development and treatment.

Differences between neoplastic and non-neoplastic tissues

In our studies, quantitation of PSA in prostate tissue has shown lower levels of PSA in neoplastic than in non-neoplastic tissue (Pretlow *et al*., 1991). The decreased levels of PSA protein seen in most carcinoma tissues may be the result of a decreased gene transcription, decreased stability of the mRNA product, loss of message translation or increased release and depletion of the cellular stores of PSA. An aberrant PSA mRNA might be synthesised by the tissue in this pathological condition. However, Henttu and colleagues found increased amounts of PSA mRNA in CaP tissues compared to BPH specimens, resulting either from an increased number of cells expressing this mRNA or from high amounts in fewer cells. They suggested that there are differences in the transcriptional regulation of the PSA gene between BPH and CaP tissue. Furthermore, the increase in total PSA mRNA was accompanied by an increase in the percentage of aberrant mRNA, which would produce variant PSA protein if translated (Henttu *et al*.,

1990). This single study indicates that other factors are responsible for the differences between BPH and CaP tissues.

Tissue/ serum relationships

PSA production in the tumor tissue, its translocation into the circulation and clearance of the protein should influence the serum level of PSA. The inverse correlation between T-PSA and S-PSA in M0 patients indicates a minor influence of PSA synthesis and tissue concentration on circulating S-PSA. What the inverse correlation probably reflects is an increased leakage from the tissue and / or an increased transport of the protein to the peripheral circulation. The elevated PSA levels in the blood stream may arise from the tumor cells inability to preferentially excrete the protein into the acinar lumen or the loss of normal ductal structure and obstruction (van Iersel *et al*., 1996).

Prognostic value of T-PSA

Despite the heterogeneity of PSA production in tumor cells, tissue PSA in relation to the DNA content has been shown to be a superior prognostic marker compared to serum levels of PSA.

T-PSA values are probably less dependent on factors such as transport of PSA into the blood and clearance of the protein from the circulation, which are not related to the production of the protein in a given amount of tissue. Consequently, T-PSA is more closely related to cytological grade, T stage, DNA ploidy and histopathology than S-PSA (Hasenson, et al., 1989; Stege *et al*., 1992; Yang *et al*., 1992).

A low T-PSA value was related to an increased malignancy grade and tumor stage and a shift from diploid to tetra-/ aneuploid tumors (Stege, et al., 1992). The relationship of DNA ploidy to T-stage and cytological grade is influenced by the uncertainties in subjective staging and grading, as well as by the time factor in tumor progression and the possibility of changes in the behaviour of tumors as time goes on. It should be noted that the tumors in both of the studies (paper I and II) were characterized by the DNA pattern at diagnosis and that changes in ploidy may occur during tumor development. Repeated FNA in untreated patients observed for more than 20 months has revealed ploidy changes of diploid and tetraploid tumors of about 9% annually (Tribukait, 1991).

Despite these concerns T-PSA showed a high prognostic value. In a Cox stepwise regression analysis, we showed that T-PSA is the dominant variable in predicting the outcome of endocrine treatment compared to tumor stage, cytological grade, DNA ploidy, free and total S-PSA, and free/ total S-PSA ratio. Even the ratio calculated between S-PSA and T-PSA, a theoretical index that should reflect the translocation of PSA, was not superior to T-PSA in the analysis with respect to time to death in our material. This ratio showed significance with respect to time to disease specific death ($p<0.001$) but the χ^2 value was lower compared to T-PSA (55.4 compared to 81.8). An even stronger predictive value was found using the 12- to 0-months T-PSA ratio, which can be considered as a biochemical analysis of the changes that occurred in the tissue.

Our conclusion is that measuring PSA in prostate aspirates improves the predictive value of this well used marker in the prognosis of CaP. It is tempting to speculate that T-PSA reflects the normal protein synthesis of the gland.

Regulation of PSA in the tissue

The synthesis of PSA is considered to be androgen sensitive but other factors also influence its synthesis and secretion. Besides androgens, estrogens stimulate synthesis of PSA at least in LNCaP cells (Henttu *et al*., 1992; Henttu and Vihko, 1992). In the patients who responded to the treatment, T-PSA levels during treatment were significantly higher in those who received estrogens compared to those who were treated with orchidectomy or GnRH analogues (paper III). If this was a result of a direct stimulatory action of estrogens on cellular PSA synthesis in vivo in the presence of castration levels of testosterone, or if it simply reflected a change in the ratio between epithelial and stromal cells, could at first not be determined. However, we have recently analysed these samples for tissue concentrations of prostatic acid phosphatase (T-PAP). While T-PSA values during treatment in these samples were still significantly higher at 6 months and during 6 – 12 months in the estrogen treated patients than in those who were treated by orchidectomy / GnRH agonists, (table 5) no such difference was found for T-PAP. This lack of significant differences in T-PAP speaks against different ratios between epithelium and stroma as the reason for the differences in T-PSA between the estrogen treated patients and ochidectomy and GnRH analogue treated group.

This also indicates a difference in the regulation of the androgen sensitive proteins PSA and PAP. However, it should be underlined that our results are based upon a limited number of observations.

Table 5. Effect of different treatments on T-PAP and T-PSA in M0 patients who responded to treatment after orchidectomy / GnRH agonists or with estrogens.

	ORX/ GnRH	**Estrogens**
T-PAP, µg/ µg DNA		
0 months	0.162 (0.031 – 0.937) [8]	0.275 (0.230-0.657) [5]
6 months	0.025 (0.100 – 0.193) [8]	0.049 (0.005 – 0.479) [5]
12 months	0.032 (0.013 – 0.262) [5]	0.092 (0.033 – 0.464) [5]
6 + 12 months	0.032 (0.005 – 0.263) [13]	0.063 (0.001 – 0.479) [10]
T-PSA, µg/ µg DNA		
0 months	0.362 (0.052 – 1.10) [8]	0.481 (0.262 – 0.804) [5]
6 months	0.031 (0.002 – 0.411) [8]	0.140 (0.041 – 0.241) [5]*
12 months	0.011 (0.002 – 0.411) [8]	0.081 (0.030 – 0.191) [4]
6 + 12 months	0.022 (0.002 – 0.471) [16]	0.101 (0.030 – 0.241) [9]**

Significant differences between treatment regimens are denoted by *= $p<0.05$ and **= $p<0.01$ respectively.

While the initial T-PSA values for the non-responders to endocrine treatment were significantly lower compared to responders to treatment, the T-PSA values of all non-responders increased dramatically during the treatment. This is a paradoxical finding, since we have shown that the T-PSA values decreased with increasing cytological grade, T stage and more unfavourable DNA ploidy patterns. It is clear that the regulation of T-PSA in tumors differs from the normal tissue, probably involving other factors, e.g. growth factors (Hsieh, et al., 1993) and the possibility exists that

PSA synthesis has become androgen insensitive. The regulation of PSA in androgen insensitive CaP cells is still under investigation (Hsieh, et al., 1993; Yeung, et al., 2000).

Effects of estrogens on CaP

For several decades estrogens have been used in the treatment of CaP. However, estrogens may also be involved in the etiology of the disease. Elevated serum levels of estrogens and of biologically active testosterone are present in untreated patients with CaP and an increased frequency of CaP and other prostatic disorders has been reported in subjects who have been exposed to high estrogen levels in the presence of adequate androgen concentrations (Carlström and Stege, 1997 and references cited therein). This suggests a dual action of estrogens on the development of CaP, being stimulatory at lower levels and inhibitory at high concentrations.

The results from our in vitro studies with LNCaP cells (papers IV and V) suggest that estrogens may affect CaP by altering vasoactive compounds. Being responsible for NO synthesis, ecNOS may play an important role in vascular development and tumor growth in human LNCaP (Chinje and Stratford, 1997). Endothelin-1 (ET-1) is not only a potent vasoconstrictor but also serves as an important growth stimulator in various cancers, including breast, cervical pancreatic and CaP (Nelson et al., 1995; 1996; Bagnato and Catt, 1998). It has been hypothesized that in addition to its mitogenic effects ET-1 may also regulate the process of cell apoptosis. The reported finding that ET-1 protects cells from apoptosis has an immediate impact on our understanding of the pathogenic role of ET-1 in cell growth disorders, such as BPH and CaP (Wu-Wong *et al*., 1997).

Both ecNOS mRNA and ET-1 mRNA in LNCaP cells were affected by estrogens. In LNCaP-FGC cells E2 and E1S added at different concentrations, significantly increased ecNOS mRNA and decreased ET-1 mRNA expression while in LNCaP-r cells only E2 at the highest concentration (100 nM) exerted any significant effects. If this latter finding reflects a certain sex steroid sensitivity even on hormone resistant LNCaP-r cells is not known. LNCaP-r cells have been reported to be unresponsive to androgens (100 nM DHT) and the inhibition of cell proliferation by high concentrations of androgens (1 μM DHT) has been explained as a cytotoxic effect (Hasenson, et al., 1985). Others have seen a slight stimulatory effect of androgens on these cells, indicating some degree of androgen sensitivity (Joly-Pharaboz *et al*., 1995). Differences in experimental protocols and in passages of the cells used may account for these discrepancies (Langeler *et al*., 1993).

The terminally biologically active estrogen E2 and the sulfoconjugate E1S exerted similar effects on LNCaP cells. E1S is the most abundant circulating estrogen in non-pregnant subjects and has been shown to serve as a prehormone for E2 in estrogen target tissue (Carlström, 1987). However, while LNCaP cells easily converted E1 into E2, we could only detect a very limited hydrolysis of E1S and no formation of E2 from E1S, even after 48 h of incubation. The mechanism of estrogen effects on ecNOS mRNA and ET-1 mRNA in LNCaP cells remains unclear. One may speculate over non-genomic mechanisms apart from the binding of estrogen to any classical steroid receptor.

There are several reports showing that estrogens can upregulate the expression of ecNOS mRNA in different tissues (Gilligan, et al., 1994; Weiner, et al., 1994; Mikkola *et al*., 1996; Armour and Ralston, 1998; Kleinert *et al*., 1998), but it is the first time that this has been studied in the LNCaP cell model. It is known that

estrogens affect the mRNA expression of ET-1 in a different way than ecNOS expression (Wingrove and Stevenson, 1997). NO, which is regulated by NOS enzymes, has been proposed as a feedback inhibitor of ET-1 protein, and increase in the release of NO would be expected to decrease ET-1 accumulation in the media of treated cells (Boulanger and Luscher, 1990; Wingrove and Stevenson, 1997). Our results confirmed that estrogen is associated with an increase in expression of ecNOS mRNA but in most cases this was not concomitant with the observed inhibition of ET-1 mRNA expression in the LNCaP-FGC cells. However, the relation between ecNOS mRNA and ET-1 mRNA in untreated cells reflect the effect of estrogens on NO and ET-1 levels. One possible mode of action for estrogen treatment may be a reduction of ET-1 mRNA causing decreased tumor cell proliferation and thus growth and maintenance of the tumor. The fact that ET-1 not only acts as a mitogen, but also attenuates apoptosis may also be partly influenced by estrogens (Kyprianou *et al*., 1996; Wu-Wong, et al., 1997).

Conclusions

- A negative association between S-PSA and T-PSA values were found in metastasis-free patients, indicating that S-PSA levels reflect the degree of PSA leakage from the tissue into the blood stream rather than the production of PSA in the tumor.

- T-PSA is shown to be a superior predictor of clinical outcome for hormone treated patients.

- T-PSA measured between month 12 and month 0 is the best predictor of clinical outcome in hormone treated patients.

- The T-PSA values increase in patients with tumors that do not respond to hormone treatment.

- Estrogens stimulate T-PSA values *in vivo.*

- Estrogens can induce ecNOS mRNA expression in LNCaP-FGC cells.

- Estrogens can inhibit ET-1 mRNA expression in LNCaP-FGC cells.

Populärvetenskaplig sammanfattning på svenska

Prostatacancer är mannens vanligaste cancersjukdom i Sverige. Förloppet av prostatacancer är mycket varierande. Man har därför fokuserat ansträngningar på att utveckla markörer för att kunna prognostisera och följa upp hormonbehandlade prostatacancerpatienter. Mätning av prostataspecifikt antigen i blodet (S-PSA) är viktig ur klinisk synpunkt, men har dock stora begränsningar. Vi har utvecklat en metod som genom att mäta nivåer av PSA i finnålsbiopsier från tumörvävnad (T-PSA) bättre förutsäger patientens sjukdomsutveckling.

Studien har gjorts på metastasfria patienter som behandlades med kirurgisk kastration eller hormonellt (med antingen östrogener eller GnRH analoger). T-PSA och S-PSA är inverst korrelerade. Detta tyder på att S-PSA mer indikerar graden av läckage från prostatakörteln ut till blodet än den verkliga produktionen av proteinet i vävnaden.

Genom att bestämma T-PSA vid diagnostillfället kan vi bättre förutsäga sjukdomsförloppet jämfört med andra kliniska parametrar som tumörcytologi, tumörstadium, DNA ploidi, mätningar av total-PSA i blodet samt fritt-PSA. En ytterligare förbättring av förutsägelsen kan göras genom att mäta T-PSA efter 12 månaders behandling och jämföra med värdet vid diagnostillfället.

Patienter som inte svarade på behandling visade ett initialt lågt T-PSA-värde, som ökade dramatiskt under behandlingen. Av de patienter som svarade på behandling hade gruppen som behandlades med östrogener signifikant högre T-PSA-värden jämfört med grupperna som behandlades med kirurgi eller GnRH. Detta tyder på att östrogener kan stimulera produktionen av PSA-proteinet.

Östrogen används för behandling av prostatacancer, men kan också vara involverat i uppkomsten av sjukdomen. Vi har därför också studerat de effekter hormonet har på kärlaktiva faktorer som kan ha inverkan på prostatacancerutveckling. Som modell använde vi en hormonkänslig cellinje (LNCaP-FGC) och en hormon-okänslig cellinje (LNCaP-r). Vi fann att östrogener stimulerar bildandet av kväveoxidsyntas, och att de motverkar produktionen av endotelin-1 på mRNA-nivå. Östrogener kan alltså påverka prostatacancerns utveckling genom att störa balansen av dessa faktorer.

Sammanfattningsvis har vi visat att patienter med låg risk att dö i sjukdomen kan identifieras genom att mäta T-PSA och att man genom att mäta T-PSA kan följa sjukdomsförloppet hos hormonbehandlade patienter. Vi har också visat att effekter på kärlaktiva substanser är en tänkbar verkningsmekanism för östrogener på prostatacancer.

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