Formation, Storage and Secretion of Prostasomes in Benign and Malignant Cells and Their Immunogenicity in Prostate Cancer Patients

GÖRAN SAHLÉN
Dissertation presented at Uppsala University to be publicly examined in Rudbeck Hall, Rudbeck Laboratory, Dag Hammarskjölds väg 20, Uppsala, Friday, March 16, 2007 at 13:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in Swedish.

Abstract

Prostasomes are submicron-sized, membrane-bound organelles produced by the epithelial cells of the prostate and normally found in the secretion in the gland ducts. Their physiological role is in the promotion of sperm-function in human reproduction. This thesis contains four papers dealing with the production of prostasomes and some possible applications in clinical urology of the prostasome.

Paper I and II provided an ultrastructural description of the synthesis, storage and secretion of prostasomes in benign as well as in malignant tissue. Most notable were the extracellular appearances of prostasomes in metastatic lesions whereby the prostasomes become exposed to the immune system of the patient. This supported findings in earlier studies in which patients with advanced prostate cancer had elevated levels of anti-prostasome antibodies. The results of paper III reinforced the view of the prostate-unique origin of the prostasome. In particular, there were no indications in SDS-PAGE patterns or flow-cytometric studies of material from seminal vesicle secretion that it contained components that could be associated with a production of prostasomes.

Some possible clinical functions of the prostasomes were investigated in paper IV. Exposure of prostasomes to the immune system through mechanical and thermal trauma to the prostate did not induce an evident formation of anti-prostasome autoantibodies. Furthermore, the serum levels of anti-prostasome antibodies registered by assays with preparations of prostasomes from seminal plasma as antigen did not correlate with existing prostate cancer. Seminal prostasomes seemed not to function as substitute markers for prostate cancer in the test kit used. A possible explanation could be underestimated differences in antigen properties between seminal or prostate gland-derived prostasomes and prostasomes from tumor tissue.

Keywords: Prostasomes, prostate cancer, antibodies, origin, seminal vesicles

Göran Sahlén, Department of Surgical Sciences, Akademiska sjukhuset, Uppsala University, SE-75185 Uppsala, Sweden

© Göran Sahlén 2007

ISSN 1651-6206
ISBN 978-91-554-6802-0
urn:nbn:se:uu:diva-7511 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-7511)
To

Klara and Lovisa
Original papers

Paper I
Ultrastructure of the Secretion of Prostasomes From Benign and Malignant Epithelial Cells in the Prostate.
Reprinted with permission of John Wiley & Sons, Inc.

Paper II
Prostasomes are Secreted From Poorly Differentiated Cells of Prostate Cancer Metastases.
Reprinted with permission of John Wiley & Sons, Inc.

Paper III
Ultrastructural and Biochemical Differences in Tissue and Secretion from Seminal Vesicles and Prostate Gland Indicating the Exclusive Prostatic Origin of Prostasomes.
Göran Sahlén, Lena Carlsson, Anders Larsson, Bo Johan Norlén, B. Ove Nilsson and Gunnar Ronquist.
In manuscript.

Paper IV
Evaluating Seminal Prostasomes as a Possible Prostate Cancer Marker by Monitoring Anti-Prostasome Antibodies in Urological Inpatients and Patients Subjected to Surgical Trauma to the Prostate.
In manuscript.
Contents

Original papers ................................................................. 5
Contents ............................................................................. 7
Abbreviations ................................................................. 9
Introduction ...................................................................... 11

The prostate and the seminal vesicles and their secretory products ..11
  Anatomy and morphology of the prostate ......................... 11
  Anatomy and morphology of the seminal vesicles ............ 13
  Seminal plasma ............................................................. 13

The prostasome .............................................................. 14
  Background .................................................................... 14
  Biochemical Characteristics of the Prostasome ................. 17
  Function of seminal plasma and prostasomes .................. 19

Prostate cancer .............................................................. 21
  Background .................................................................... 21
  Diagnosis and Gleason grading .................................... 22
  Prostate specific antigen ............................................. 24
  Other markers of prostate cancer ................................. 25

Aims of the papers ........................................................ 26

Paper I ........................................................................... 26
Paper II ........................................................................... 26
Paper III .......................................................................... 26
Paper IV .......................................................................... 26

Material and methods ..................................................... 27
  Microscopy .................................................................... 27

Patients and Methods .................................................... 29
  Paper I .......................................................................... 29
  Paper II .......................................................................... 29
  Paper III ......................................................................... 30
  Paper IV .......................................................................... 31

Results and conclusions ................................................ 32
  Paper I .......................................................................... 32
  Paper II .......................................................................... 37
  Paper III ......................................................................... 40
  Paper IV ......................................................................... 44
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAU</td>
<td>Arbitrary antibody units</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosid tri phosphatase</td>
</tr>
<tr>
<td>AZ</td>
<td>Anterior zone</td>
</tr>
<tr>
<td>Ca</td>
<td>Calcium</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CZ</td>
<td>Central zone</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton, unit of molecular mass (1.66 x 10^{-24} g)</td>
</tr>
<tr>
<td>DU</td>
<td>Distal urethra</td>
</tr>
<tr>
<td>EPCA</td>
<td>Early prostate cancer</td>
</tr>
<tr>
<td>hK2</td>
<td>human kallikrein 2</td>
</tr>
<tr>
<td>HSP70</td>
<td>Heat shock protein (70kDa)</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>Mab</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
</tr>
<tr>
<td>Mag</td>
<td>Magnification</td>
</tr>
<tr>
<td>Mg</td>
<td>Magnesium</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>N-terminal</td>
<td>The amino group (NH₂-) - carrying end of a protein.</td>
</tr>
<tr>
<td>PAP</td>
<td>Prostate acid phosphatase</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate specific antigen</td>
</tr>
<tr>
<td>PSMA</td>
<td>Prostate-specific membrane antigen</td>
</tr>
<tr>
<td>PU</td>
<td>Proximal urethra</td>
</tr>
<tr>
<td>PZ</td>
<td>Peripheral zone</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide-gel electrophoresis</td>
</tr>
<tr>
<td>SV</td>
<td>Seminal vesicle</td>
</tr>
<tr>
<td>T-cell</td>
<td>Lymphocyte differentiated in the thymus gland.</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue factor</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumour Node Metastasis Tumour staging system.</td>
</tr>
<tr>
<td>TUMT</td>
<td>Transurethral microwave treatment</td>
</tr>
<tr>
<td>TZ</td>
<td>Transitional zone</td>
</tr>
<tr>
<td>Zn</td>
<td>Zinc</td>
</tr>
</tbody>
</table>
Introduction

The prostate and the seminal vesicles and their secretory products

Anatomy and morphology of the prostate

The prostate belongs to the male sex accessory glands together with the seminal vesicles, the ampullae of the vasa deferentes and the bulbourethral glands. They each produce specific components of the ejaculate and require androgen stimulation to do so [1]. The normal adult human prostate gland is about the size of a plum (3 x 4 x 2cm) and it weighs about 18g. It is located directly under the urinary bladder and it surrounds the urethra as the urethra runs down to the pelvic floor, see figure 1. The prostate is enclosed in a fibromuscular capsule and is proximally integrated with strands of smooth muscle in the bladder floor and distally, the prostatic apex is continuous with the distal urethral sphincter [2]. Approximately 70% of the prostate consists of glandular elements that produce the prostate secretion, which is emptied into the gland ducts. The remaining 30% of the prostate is a fibromuscular stroma enveloping the glandular elements to

Fig. 1.
express secretions from the prostatic ducts into the urethra during ejaculation. The prostate has a zonal anatomy described by McNeal [3] and seen in figure 2. In the normal prostate the peripheral zone makes up 70% of the secretion-producing tissue and forms the dorsal aspect of the prostate: 70% of prostatic carcinomas arise in the peripheral zone. The transition zone surrounds the urethra and constitutes 5-10% of the normal gland, and gives rise to 20% of the prostatic malignancies. It is in the transition zone that benign prostatic hyperplasia may develop. The central zone runs from the base of the bladder to the colliculus and it encloses the ejaculatory ducts. These ducts are formed by the seminal vesicles and the ampulla of the vas from each side: they open into the urethra on either side of the colliculus, see figure 1. The central zone represents 25% of the glandular tissue, but only 1-5% of carcinomas develop there. Finally, in the anterior zone of the prostate, a sheet of fibromuscular stroma, without glandular components, extends from the ventral aspect of the bladder neck to the urethral sphincter in the pelvic floor: a cancer very rarely develops in the anterior zone.

![Fig. 2.](image)

The zonal anatomy of the prostate. Schematic illustration in which PZ represents the peripheral zone, TZ=transition zone, CZ=central zone, AZ=anterior zone, DU=distal urethra, PU=proximal urethra/bladder neck, SV=seminal vesicle and vas deferens entering the prostate. © Printed with permission of RSNA, from Lee F, Torp-Pedersen ST, Siders DB, Littrup PJ and McLeary RD. Transrectal ultrasound in the diagnosis and staging of prostatic carcinoma. Radiology 1989; 170:609-615.

The glandular epithelium producing the prostatic secretions is composed of three major cell types: secretory cells, basal cells and neuroendocrine cells [4]. The secretory cells stand on the basal membrane, side by side, lining the glandular ducts. A normal secretory cell has a polarized intracellular organization with the nucleus at the base of each cell, the middle section of the cell dominated by Golgi apparatus and with the apical part of the cell filled with secretory products moving into the ducts. In cancerous secretory cells, the internal architecture deteriorates with loss of polarity, deformation of the nucleus and the appearance of prominent nucleoli in the nucleus and, as a sign of dedifferentiation, the cell might loose function with seizure of secretion. The basal cells, resting on the basal membrane, are wedged in between the secretory cells and are considered to be stem cells to the secretory cells. Basal cells have very little
secretory activity and are almost always missing in prostatic cancer tissue; this is used as a diagnostic tool [5]. The neuroendocrine cells of the prostate are sparsely spread out among the secretory cells of the epithelium, but are also found in the urothelium of the prostatic part of the urethra. They are assumed to take part in the regulation of secretory activity and cell growth in the prostatic tissue surrounding them. They are a cell-lineage of their own, with a neurogenic origin, and they lack androgen receptors [6].

The prostate gland residing deep in the human pelvis is not easy to examine, at least not to the individual himself. However, by digital rectal exploration, the gloved finger of the physician can reach a large part of the surface of the peripheral zone, as it faces the rectal wall. Most cancers develop in the peripheral zone and digital palpation of the prostate is still a very important maneuver when evaluating a patient with a possible prostate cancer. Transrectal imaging by ultrasound is a valuable tool when assessing the shape and size of the gland and it is also used for guiding transrectal biopsies of the prostate, but it is not reliable for detecting a cancer.

Anatomy and morphology of the seminal vesicles
The seminal vesicles are 5cm long, coiled tubes lying behind the prostate and under the bladder. Together with the vas deferens on each side, they form the bilateral ejaculatory ducts, which open into the urethra at the colliculus, see figure 1. They have a columnar secretory epithelium that depends on androgen hormonal stimulation for its function [1], and they secrete and store the largest part of the ejaculate volume. In spite of their name, the seminal vesicles do not store spermatozoa.

Seminal plasma
The seminal plasma (semen without the spermatozoa) of the human ejaculate is a mixture of fluids from the accessory sex glands (see above) added to by secretions from the epididymides and the vasa deferentes. It plays an important, but in its details poorly understood, role in the human fertilization process. The average volume of seminal plasma in a human ejaculate is 3ml, of which the largest contributions come from the seminal vesicles (60%) and the prostate (30%). During ejaculation the fluids are released in a sequential manner. In a split-ejaculate test the secretions from the prostate are identified in an early portion together with the spermatozoa, whereas the contribution from the seminal vesicles is expelled in a later fraction [7, 8]. The chemical composition of the different fractions are characteristic of the organ from which they emanate. For instance, the prostate has a secretion very rich in citric acid, calcium, magnesium, zinc, and proteins such as prostate specific antigen (PSA), prostatic acid phosphatase (PAP) and albumin [7, 9]. The seminal vesicles produce a fluid characterized by a high content of fructose, prostaglandins and semenogelins [7, 10, 11].

An additional secretory product of the prostate, and the crucial one in this presentation, is the prostasome.
The prostasome
Background
In 1978, Ronquist et al. [12-14] first described a small (40-500nm), membrane-surrounded organelle, secreted from the prostate epithelium, and later referred to as the prostasome. Prostasomes were isolated from semen and seminal plasma by differential centrifugation and preparative ultracentrifugation in several steps, until no further fractioning could be achieved, see figure 3. The resulting substance of purified prostasomes was subjected to chromatography with subsequent silica density gradient centrifugations revealing a main band with the density of 1.03 which coincided with high activity of enzymes unique to prostate fluid indicating the prostate-specific origin of the prostasomes [15]. The pellet with material isolated from seminal plasma was also prepared for electron microscopy which showed membrane-bound granules, i.e. prostasomes, and vesicles [13]. Further electron microscopy studies of non-malignant prostate tissue depicted multiple prostasomes in larger storage vesicles in the cytoplasm of the secretory cells and prostasomes secreted into the gland ducts, mostly free but also found still retained in intact storage vesicles [14], see figure 4. Based on the electron microscopic findings, two mechanisms of secretion of the content of the storage vesicles into the ducts were postulated. Either the storage vesicle

![Fig. 3. Separation of storage vesicles and prostasomes from human semen and prostatic fluid by differential separation and Sephadex G-200 chromatography. From: Ronquist G and Brody I. The prostasome: its secretion and function in man. Biochimia et Biophysica Acta 1985; 822: 203-218, fig. 1, page 205. © Elsevier.](image-url)
Fig. 4.
membranes fuse with the cell membrane of the epithelial cell after which the storage vesicle content of prostasomes is emptied into the ductal lumen by way of exocytosis or, the storage vesicle itself is secreted in toto into the ducts, a mechanism named diacytosis by the authors [14]. Possibly both mechanisms are functional at the same time.

The mode to purify prostasomes described by Ronquist is still the gold standard [16]. In this fashion, prostasomes have been harvested not only from seminal plasma, but also from homogenized prostate tissue and bone metastases of prostate cancer [17], and their characteristics reinforce the notion of the prostasomes originating only in prostate tissue. All three types of prostasomes showed a similar size-distribution in flow cytometry and displayed a similar but not identical banding pattern in gel electrophoresis (SDS-PAGE). Nine different monoclonal antibodies against prostasomes from seminal plasma recognized common surface antigens in all three types of prostasomes. A characteristic ratio was outlined in the prostasome membrane between cholesterol and phospholipids being about 2:1 in all three types.

Cluster of differentiation (CD) molecules are proteins that are recognized as antigens by a series, or cluster, of different antibodies. Four such CD-molecules (CD10, CD13, CD26 and CD46) serve as markers for prostasomes. In Carlson’s [17] study, they were represented in all three types of prostasomes, but to a varying degree.

In a study by Nilsson et al. [18], a monoclonal mouse-derived anti-prostasome antibody, mab78, recognized prostasomes in the apical parts of secretory cells in light microscopy of paraffin sections of prostate epithelium. It also labeled secretions in the glandular duct lumina, but did not stain the cell nucleus. Prostate specific antigen (PSA) and prostatic acid phosphatase (PAP) are also secretory products of the prostate epithelium but the anti-prostasome antibody mab78 did not label blots with PSA or PAP [18, 19], see figure 5.
Biochemical Characteristics of the Prostasome

Membrane lipid composition
The prostasome is surrounded by a bi-layered (sometimes multi-layered) membrane as revealed by transmission electron microscopy [14]. The membrane has a characteristic lipid composition with cholesterol dominating over phospholipids in a ratio of about 2:1, whereas, the ratio of the same lipids in other plasma membranes is slightly below 1:1. This gives the membrane great resilience to thermal and mechanical stress [20].

Membrane proteins
The membrane protein composition of prostasomes is complex. Utleg et al. [21] identified 139 proteins associated with the prostasome membrane: 128 had not previously been described in prostasomes. The proteins of the prostasomes have physiological functions that are only partly known, even so, membrane-bound proteins serve as markers for prostasomes. Some examples follow below.
ATPase. An ATPase depending on Mg$^{2+}$ and Ca$^{2+}$ for stimulation was described by Ronquist in 1978 as the first type of enzyme linked to prostasomes [12]. It may be involved in the concentrative accumulation of divalent cations in prostasomes. This ATPase has a much higher activity in prostatic secretion than in seminal plasma and at least 85% of the activity is linked to prostasomes [22]. Another ATPase, this one depending on Zn$^{2+}$, is also associated with prostasomes [23]. The three divalent cations zinc, calcium and magnesium are linked to the prostate secretion and prostasomes in seminal plasma [9, 24, 25].

Neutral endopeptidase (CD10). A polyclonal antibody against prostasomes also binds to this enzyme, which is restricted to the secretory cells of the prostate epithelium and their secretion [26].

Aminopeptidase N (CD13). A zinc ion-dependent enzyme linked to the prostasome membrane [27]. It is one of the membrane-bound peptidases of prostasomes that may serve as negative loop in controlling concentrations of bioactive peptide–signaling pathways. CD 13 catalyses the removal of N-terminal amino acids from peptides, preferentially those with neutral residues, but with broad specificity in the cleavage of acidic and basic residues.

Dipeptidyl Peptidase IV (CD26). The activity of this prostasome membrane-bound enzyme in seminal plasma originates strictly from the prostatic secretion. It is absent in fluid from the seminal vesicles [28, 29] It is also the antigen of a monoclonal anti-prostasome antibody [30] and is an integral membrane protein with an asymmetrical orientation exposing the catalytic site at the external surface. A 110kDa protein identical to the T-cell activator molecule CD26, it is a serine-type peptidase that cleaves aminoterminus dipeptides with either L-proline or L-alanine at the penultimate position. CD 26 has been implicated in a variety of reactions including protease/sperm binding via adenosine deaminase [31].

Transmembrane protein (CD46). The protein CD46 is an inhibitor of complement system activity. All of the seminal plasma-CD46 has been associated with prostasomes [32].

Tissue factor. This is a cell-membrane bound protein found subendothelially in the vessels throughout the human body. It is the initiating molecule of coagulation in blood. In case of damage to a vessel, it is responsible for starting a cascade of reactions, called the secondary hemostasis, which via formation of thrombin finally results in fibrin strands reinforcing the blood platelet plug of the primary hemostasis. Very high levels of tissue factor are associated with prostasomes in seminal plasma [33].
Function of seminal plasma and prostasomes
Once the Rubicon has been crossed and the ejaculate has changed hosts, the spermatozoa are in foreign territory. The new environment is demanding and they face an "antigen-nightmare" [34] in a new host with defenses at best indifferent, at worst formidable. What are the options?

The semen-coagulum
Almost immediately after emission, the human ejaculate coagulates and immobilizes the spermatozoa. It has been postulated that the role of this phenomenon is to prevent semen from being lost out of the female vagina. The actual process in the coagulum is unclear, but after 10-20 minutes, the coagulum liquefies and the sperm become motile again. The coagulum is formed not mainly with fibrin, but with seminal vesicle derived semenogelin I as the dominating substance. The liquefying enzyme that dissolves the coagulum is a protein specific to the prostatic secretion: the prostate specific antigen (PSA) [35].

The blood-clotting tissue factor (TF) is amply represented in the ejaculate, where it is bound to the prostasome membrane. The process behind the gelation of the ejaculate-clot might be different from that of blood-clotting [35, 36] but components of the haemostatic system active in semen, presumably with tissue factor (TF) as the starting molecule, have been identified [37]. The conspicuously high concentration of tissue factor in seminal plasma, mostly associated with prostasomes, could have other functions. It is speculated whether, in the event of bleeding during intercourse, rapid blood-clotting seals access to the female blood stream making it less likely that an anti-sperm antibody formation occurs which would endanger future fertilizations. Rapid sealing of abrasions might also prevent infectious agents from gaining entrance to the woman's blood circulation [33].

Sperm-protection I
Released from the liquefying coagulum and free to move again, the spermatozoa now need support. The fructose provided by the seminal vesicles is the main source of energy to the spermatozoa (most other cells of the body prefer glucose as energy fuel) and the seminal vesicle derived prostaglandins are described as an important, perhaps the most important, factor shielding the spermatozoa from the immunological defenses of the female genital tract [38, 39].

Sperm-protection II
The prostasome plays more than one role in the protection of the sperm. In vitro studies indicate various prostasomal factors working in favor of sperm-function [40]. Interaction is a basic requirement for the favorable influences of prostasomes on spermatozoa. In free-zone electrophoresis, spermatozoa and prostasomes interacted with each other during the run and did not dissoc-
ate. This finding was confirmed by electron microscopy [41]. The interaction between sperm and prostasomes explains how the activity of prostasome-associated enzymes can be acquired by spermatozoa [42, 43], it is interesting in this context as prostasomes contain several proteins participating in the regulation of the complement system.

The complement system is a cascade of enzymes that can be activated by, for instance, invading microbes. The complement cascade will then attract and activate phagocytic cells and mast cells involved in the acute inflammatory response; however, prostasomes can limit the activity of stimulated phagocytes [44]. The activated complement system can also result in the formation of the membrane attack complex (MAC). The MAC is an executive factor, as it assembles on cellular membranes forming a trans-membrane channel permeable to water and electrolytes; thus causing lysis of cells. The MAC-inhibitory protein CD59 can protect from MAC-mediated lysis of cells [45], and is one of the complement-regulators bound to the membrane of prostasomes [46]. Other complement regulators associated with prostasomes are CD55 and CD46 which inhibit the actual activation of the system [32]. Normally, spermatozoa express CD59, but it can be cleared away and the inhibition of MAC declines. A transfer of complement-regulating capacities would increase the chances of the spermatozoa avoiding the defenses of the new host. Through fusing prostasomes carrying CD59 with CD59-depleted spermatozoa, the MAC-inhibitory CD59-activity of the sperm was restored [46].

Prostasomes from malignant cell lines have higher CD59 contents than seminal or native (from homogenized prostate tissue) prostasomes. CD 59 from prostasomes from cancer cell lines can be transferred to CD59-free tumour prostasomes and rabbit erythrocytes; thus indicating a potent complement inhibiting capability retained in prostate cancer tissue [47].

**Sperm-protection III**

Prostasomes have an anti-bacterial effect and cause growth inhibition in bacterial cultures of Bacillus Megaterium. The effect, with disruption and perforation of the bacterial membrane, is registered by electron microscopy and seen in cultures with other bacteria but to a lesser extent [16]. Neutralization of measles virus by prostasomes has been described [48].

**Sperm-promotion**

A different example of sperm receiving support from prostasomes through fusion is the in vitro finding that spermatozoa immobilized by buffer washings could regain and prolong their forward motility if prostasomes were added to their swim-up medium [49, 50].
Prostate cancer

Background

Prostate cancer is the most commonly diagnosed malignancy in Swedish men and the incidence is steadily increasing. Approximately 9500 men were diagnosed with prostate cancer in 2004 (data from the Swedish National Prostate Cancer Register), an increase from 7100 diagnosed in 1999 [51]. In most men with prostate cancer, the clinical course of the disease is uneventful with no or only minor problems to the patient, but because of the high prevalence of the disease, progressive disease still results in a substantial number of deaths. In 2003, 2600 Swedish men died of prostate cancer [52]. Sweden is among the top-five countries in the world ranking for prostate cancer incidence. In North American autopsy data of men dying in accidents, prostate cancer was already identified in men in their 20’s and the prevalence gradually increased until 80% of men in their 70’s were found to have prostate cancer [53]. In Sweden the age at diagnosis is dropping and the number of diagnosed non-palpable and localized tumors is increasing. This is reflected in an increasing rate of attempted curative treatments; the number of radical prostatectomies doubled between 2001 and 2004 (approximately 2400 procedures) [51].

Although the prostate is the most common site for cancer in the male, there are almost no cancerous tumors at all in the other sex accessory glands, most notably the seminal vesicles. There is no prostate cancer development without androgens, but the androgens fueling prostate cancer are also accessible to the other sex accessory organs. The prostate has a complex embryology and there is a distinction to be made in the embryological origin of the different organs. The outer zone of the prostate, i.e. the peripheral zone of the prostate which gives rise to 70% of diagnosed prostate cancer, generates from the endoderm-derived urogenital sinus which also forms the urethra and the bladder. The inner zone of the prostate develops out of the so called mesonephric ducts which are mesodermal in origin, and the seminal vesicles are derived from the Wolffian duct [1].

One hypothetical explanation for the discrepancy in cancer occurrence is that the specific secretory products characterizing the glands could make a difference. The physiological potency of the prostasome, for instance the immunosuppression and complement inhibition exemplified in vitro and described earlier, could serve a younger man’s reproduction but might be detrimental to an older man’s ability to stave off developing tumours with an adequate immunological response [54].
Diagnosis and Gleason grading

The current diagnosis of prostate cancer is achieved with transrectal biopsies of the prostate. An ultrasound probe is placed in the patient’s rectum, held against the prostate to provide an image of the gland so that core biopsies can be taken via a biopsy channel in the probe. The procedure is performed under local anaesthesia. The ultrasound examination of the prostate is not diagnostic but serves to localize the prostate, reproduce the shape and size of the gland and to guide the biopsies. Generally 8-10 biopsies are taken and delivered for histo-pathological evaluation, see figure 6.

The biopsies are prepared and examined under a light-microscope and are routinely graded according to the Gleason system. The grading is done in a magnification that normally would not allow detailed evaluation of intracellular structures. Rather, the principles behind the Gleason system primarily consider the shape and the borders of the tumor, possible stromal invasion and arrangement of the cells in glandular fashion, i.e. attributes possible to visualize with
light-microscopy. The grading, (Gleason grade 1-5), describes the similarity of the tumor to normal prostate glandular tissue. A low Gleason grade refers to tumor tissue that is well differentiated with a close similarity to normal structures. A high Gleason grade refers to poor differentiation of the tumor with only little or no resemblance to benign tissue, see figure 7. Prostate cancer is often multifocal, with different grades in different lesions in the gland. The Gleason system handles this by adding the two most common Gleason grades represented in the tumor to a Gleason score which has become an important parameter when appraising the clinical significance of a tumor. Together with a patient’s PSA-level and his TNM stage (in which “T” describes palpable tumor extension, “N” records possible lymph-node metastases and “M” states the existence of bone metastases), the Gleason score is the most important factor prognosticating disease progression [55, 56].

Fig. 7.
Gleason-drawing of growth patterns of prostate cancer. Grades 1-5 with grade 1 very similar to normal prostate glandular patterns and grade 5 almost without glandular formations. In: Amin MB et al. (eds.): Gleason grading of prostate cancer: a contemporary approach. Philadelphia, Lippincott Williams & Wilkins, 2004; page 6, fig. 1-2. © 2004 Lippincott Williams & Wilkins.


**Prostate specific antigen**

The incidence of prostate cancer has increased during the last decade, much due to the widespread use of prostate specific antigen (PSA) as a tumor marker in serum. Prostate specific antigen is an enzyme secreted by the prostate epithelium into the gland ducts. However, it is unclear how the enzyme ends up in serum, but the assumption is that disrupted basal membranes and malformed collecting systems in cancerous lesions leak PSA into the surrounding connective tissue and from there into capillaries of the blood and lymph circulation. Physiologically, PSA has a role in semen as a liquefier of the coagulum formed after ejaculation. Although not specific for prostate cancer, PSA has by virtue of its organ-specificity come to be used as a prostate tumor marker. However, there are no definitive cut-off levels of serum PSA to either rule out prostate cancer or to correctly identify the stage of existing disease. Serum PSA together with digital rectal examination and transrectal ultrasound are all part of the standard procedure when prostate cancer is suspected, but the positive predictive value, even with the three methods combined, might be as low as 40-60% when compared with subsequent prostate biopsies [57]. At present, the high rate of PSA-testing results in a great number of prostate biopsies being taken, many of them unnecessary, as any PSA level, in the range below 10µg/L, is more specific of prostate hyperplasia than of cancer [58]. However, a high occurrence of cancer, even tumors of an aggressive kind, has been detected in patients with a PSA below 4µg/L, a level often regarded as normal [59]. It seems “…any excuse to biopsy the prostate has an excellent, age-dependent chance of being positive” [58]. Wide-spread screening-like behaviour, based on PSA measurements, results in many cancer diagnoses and anxious patients wanting counseling and treatment. This puts pressure on the clinician and his patient since it is not obvious that every tumor should be treated.

The disease-specific 10-year survival in moderately- to well-differentiated, localized, prostate cancer comes close to 90% even without treatment [60]. This would speak in favor of a conservative approach as curative treatments, such as radical prostatectomy or the most common alternative, radiation, carry with them side-effects of impotence and urinary incontinence. On the other hand, a recent study on localized prostate cancer has for the first time shown that in patients younger than 65 years of age, radical prostatectomy reduces disease-specific mortality and the risk of local progression and metastases. There was also an indicated improvement in overall mortality [61].
**Other markers of prostate cancer**

Despite the shortcomings of PSA as a prostate cancer marker, especially its poor specificity [58], there are no other markers that can rival PSA as the foremost clinical instrument in the detection and monitoring of prostate cancer. For the time being, the position of PSA has been strengthened through the use of percentage free PSA, i.e. the ratio of free PSA in serum to total PSA (primarily PSA bound to $\beta_1$-antichymotrypsin), with increased specificity and fewer negative biopsies among patients with a PSA lower than 10µg/L [62, 63].

Human kallikrein 2 (hK2), a prostate specific enzyme of the same family as PSA (also designated hK3) and detectable in serum in patients with prostate cancer, is a potential cancer marker that can predict the presence of prostate cancer before a prostate biopsy but only in men who have an elevated PSA [64]. Early prostate cancer antigen (EPCA), a matrix protein from the nucleus of prostate cells, assumed to be associated with neoplastic alterations in the nucleus, is found in significantly higher plasma levels in patients with prostate cancer compared with controls [65]. Prostate-specific membrane antigen (PSMA), a protein overexpressed in prostate epithelium, is detectable in serum in patients with prostate cancer, and appears to correlate to disease stage [66]. Although the antigen is used in the development of a radiolabeled agent intended for imaging of metastases [67] it has not surpassed PSA in the detection of localized disease. A recent review of molecular markers has been published by Bradford [68].

Urine-based tests, aimed at detecting prostate cancer specific proteins or nucleic acids have been proposed. However, a review by Müller and Brenner of 21 various markers in 34 different studies, concluded that the early promise of many of the markers had to be confirmed in larger, prospective studies before any test could be considered to be reliable [69].

The possible diagnostic value of determining a serum autoantibody profile of prostate cancer was emphasized in a study by Wang [70], claiming a better performance than PSA in distinguishing prostate cancer patients from controls. Prostasomes have already been proven to be competent immunogens in animal studies. Polycolonal antibodies have been derived from rabbits [71] and chicken [72]. Monoclonal antibodies have been obtained from models in which mice were immunized with prostasomes harvested from seminal plasma [18]. In clinical urology the validity of a serum anti-prostasome antibody assay has been previously demonstrated for generalized prostate cancer associated with elevated titres of anti-prostasome antibodies [73]. The study of antigens associated with prostasomes, could reveal further applications of the prostasome in clinical urology. This thesis presents results adding new information to this field of research.
Aims of the papers

**Paper I**
To describe the ultrastructure of the secretory components, specifically the prostasomes, of the prostatic secretory epithelium in biopsy specimens from two groups of patients, one group with benign prostate hyperplasia and the other with prostate adenocarcinoma. The Prostate 53:192-199(2002).

**Paper II**
To demonstrate that dedifferentiated cells in vertebral metastases of prostate cancer, have grossly retained their ability to produce and export prostasomes to the extracellular space, although having been disconnected from their glandular situation. The Prostate 61:291-297(2004).

**Paper III**
To characterize differences between the seminal vesicles and the prostate in terms of the secretory cells and their secretory products. In manuscript. ("Ultrastructural and Biochemical Differences in Tissue and Secretion from Seminal Vesicles and Prostate Gland Indicating the Exclusive Prostatic Origin of Prostasomes").

**Paper IV**
To survey the occurrence of anti-prostasome antibodies in serum of patients with urological diseases including prostate cancer of different stages. To investigate in a prospective study, a possible relationship between the generation of anti-prostasome antibodies and two different forms of prostate tissue destruction and furthermore, to evaluate the relationship between the level of anti-prostasome antibodies and prostate biopsy results. In manuscript. ("Evaluating Seminal Prostasomes as a Possible Prostate Cancer Marker by Monitoring Anti-Prostasome Antibodies in Urological Inpatients and Patients Subjected to Surgical Trauma to the Prostate").

**Ethical considerations**
All four papers in this thesis were approved by the Ethics Committee at the University Hospital, Uppsala.
Material and methods

Microscopy

In Papers I, II and III of this thesis, microscopy in general and electron microscopy in particular were important methods.

The limits to what can be seen are set by magnification and resolution. Resolution is the shortest distance between two objects at which they can still be seen as separate objects with a maximum of resolution in a microscope at about half the wavelength of the illuminating beam. Increased magnification without improved resolution is of limited value [74, 75].

Light microscopy. In light-microscopy, the light observed passes through thinly sliced and colour-stained preparations of, for instance, prostatic tissue, and then through magnifying glass lenses before it reaches the eye. The light-microscope can magnify an object a few thousand times. With a wavelength of visible light between 0.4 and 0.7µm, there is a maximum resolution of 0.2µm which makes it possible for epithelial cells of the prostate to be seen, but intracellular components, such as mitochondria are barely discernible.

Electron microscopy. In electron microscopy, the wavelengths of visual light are exchanged for a beam of electrons directed at the object. The beam of electrons has a wavelength inversely proportional to the speed of the electrons. The speed depends on the voltage accelerating the electron. The electron-beam is generated in an electron gun and electromagnetic lenses focus the beam onto the specimen. When the electrons hit the object they are deflected, depending on their energy and the nature of the object. Electron microscopy can magnify the object many ten thousand times. There are different ways of visualizing the results.

In transmission electron microscopy, the electron gun is at the top of the microscope and it sends the electron beam through a series of electromagnetic lenses that focus the beam on the object. The electrons are deflected depending on the elementary weight of the substance that is hit by the beam. As the common substances in the human body are light-weight, causing little deflection, the contrast needs to be increased. To achieve this, heavy metals such as lead can be fixed to the specimen. The heavier the element added is, the greater is the deflection of the electrons: this results in a darker area on the visualizing screen which is coated with phosphorous that will fluoresce when hit by electrons. A transmission electron microscope achieves a resolution of 0.2nm by which it becomes possible to examine cellular components such as the Golgi apparatus and cellular membranes.
Fig. 8.
Similarities and differences between a light microscope and a transmission electron microscope. Courtesy of Prof G. Roomans.
Patients and Methods

Paper I

The patients were referred to the Department of Urology at the University Hospital in Uppsala, on the suspicion of prostate cancer. They were examined as outpatients with transrectal ultrasound and six standard core biopsies were taken and sent for routine light microscopy. The prostate was biopsied bilaterally at the apex, mid-laterally and at the base of the gland. In addition, two biopsies corresponding to the mid-lateral positions were taken for morphological studies by electron microscopy. These biopsies were rapidly fixed in 2.5% glutaraldehyde, cut into small pieces and embedded in Epon plastic. From a larger number of patients, seven patients were selected according to their light microscopy pathology report (three patients with benign prostate hyperplasia and four with prostate cancer of moderate to poor differentiation (Gleason score 7-9)). The selection of the patients was based on the quality of the biopsy results with unambiguous diagnosis. In order to ascertain that the gland structures examined represented benign or malignant tissues, the plastic blocks were first cut on a microtome into 2µm sections, which were stained in toluidine blue and reviewed by an experienced pathologist who indicated suitable areas for further investigation. Then, the corresponding areas on the plastic blocks were trimmed and cut into finer sections of 50nm, placed on slot grids, contrasted with lead citrate and uranyl acetate and then examined with an electron microscope.

Paper II

Biopsies of vertebral prostate cancer metastases were harvested from the operating field in eleven patients undergoing spinal surgery with laminectomy due to threatening neurological symptoms of tumor pressure on the spinal cord. The best biopsies from six patients were chosen and they were achieved when the biopsies came from a soft tumor “cuff” growing in the spinal canal and not containing any bony material. The biopsies were taken with a biopsy forceps normally used in endoscopic procedures. This provided small biopsies which were instantly put into a 2.5% glutaraldehyde fixative, embedded in Epon plastic and trimmed and cut for light microscopic identification of areas with neoplastic prostate tissue. The same areas on the plastic blocks were cut again into 50nm sections, put on slot grids for contrasting with lead citrate and uranyl acetate in preparation for examination by electron microscopy.
Eleven patients were included in this study. They were included consecutively as patients scheduled for major open surgery, which would expose the seminal vesicles. The median age of the eleven patients at operation was 67 (range 57-76) years. Ten of the patients underwent radical retropubic prostatectomy because of prostate cancer: pre-operatively, they were regarded as having localized disease. Seven patients had no palpable tumor at all (T1c) and three patients had palpable but to the prostate restricted tumor (T2). In the post-operative histology reports, two cases of extra capsular growth were identified but none of the ten cases displayed tumor-growth of prostate cancer into the seminal vesicles. The median pre-operative PSA-level among the ten patients with prostate cancer was 7.0 (4.7-9.2) µg/L. None of the patients with prostate cancer were receiving hormonal treatment at the time of surgery. The 11th patient underwent a cysto-prostatectomy with curative intent due to a urothelial bladder tumour. In such an operation the prostate and the seminal vesicles are removed en-bloc with the urinary bladder. In the present case, the prostate and the seminal vesicles were without any type of cancer in the post operative report.

The sampling of the seminal vesicle secretion was undertaken as the seminal vesicles were exposed during surgery in the operating field, but before the vascular supplies to the vesicles were severed. To obtain “pure” seminal vesicle secretion, fluid was aspirated with a large-bore syringe directly from the vesicles. The material from the different patients was pooled and processed after the method established by Ronquist in 1978 for purifying prostasomes from seminal plasma. The same procedures were applied to the seminal vesicle material with differential centrifugation and Sephadex G-200 chromatography resulting in a pellet of substance of seminal vesicle-specific origin [16]. Pellets from seminal vesicle secretion were analyzed by SDS-polyacrylamide gel electrophoresis. In addition, seminal vesicle secretion was examined by flow cytometry using monoclonal antibodies against prostasome-marking cluster of differentiation (CD) proteins.

Biopsies from the seminal vesicle epithelium were fixed in 2.5% glutaraldehyde, embedded in Epon-plastic, cut in 50nm sections, contrasted with lead citrate and uranyl acetate for electron microscopy. The biopsies were examined to illustrate the ultrastructure of seminal vesicle epithelium with focus on comparisons with earlier findings regarding the secretory epithelial cells in prostate tissue.
Paper IV

There were two groups of patients included in the study. In the first group, 150 male inpatients were surveyed for elevated serum titres of anti-prostasome antibodies. Sixty of the patients had prostate cancer of varying stages; the remaining 90 patients carried diagnoses ranging from urethral calculi to advanced bladder cancer. Controls were 56 male blood donors.

In the second group, two sub-groups of urological outpatients were investigated. In one sub-group, 36 patients with symptomatic benign prostate hyperplasia were evaluated and were subjected to transurethral microwave treatment (TUMT procedure) in order to alleviate troublesome voiding. In this procedure, microwave induced heat was applied to the prostate via a transurethral catheter resulting in considerable tissue destruction. Before treatment, serum samples of PSA and anti-prostasome antibodies were taken from each patient. Within a few hours after the treatment, renewed registrations of PSA levels were made. During a 3 months postoperative time of observation, new measurements of serum levels of PSA and anti-prostasome antibodies were made after 6 weeks and again after 3 months. In the second sub-group, 89 patients were evaluated: the patients were referred for investigation with transrectal ultrasound guided core biopsies of the prostate on the suspicion of prostate cancer. Eight to ten biopsies were taken in this routine investigation. Before the biopsy, serum samples of PSA, IgG and anti-prostasome antibodies were taken. The same tests were measured again at 3 and 6 weeks and finally at 3 months after the prostate biopsy.
Results and conclusions

Paper I

The secretory mechanism of prostasomes in benign and malignant prostatic was determined as similar in both types of tissue, see figures 9 and 10. The prostasomes and their intracellular storage vesicles appeared to emanate from the Golgi apparatus of the secretory cells. Small vesicles seemed to bud from the Golgi apparatus and then be filled with granular material. Ribosome-like particles appeared to adhere to the vesicular membranes. Closer to the apex of the cell, the vesicles were larger and in general stored multiple prostasomes as described in earlier research on benign tissue [14]. Prostasomes could be visualized in the gland ducts in both types of tissue, see figures 11 and 12.

The conclusion was that prostasomes were synthesized and secreted not only by cells in benign prostatic tissue, but also in a similar fashion in neoplastic tissue in the prostate gland, see figure 13. Figures 9-13 from paper I reprinted with permission of John Wiley & Sons, Inc.

Fig. 9.
Apical parts in cells from a prostate with benign hyperplasia. Several storage vesicles with a content varying in amount and structure present in the cytoplasm. Paper I.
Fig. 10.
Golgi apparatus in a cell from a patient with benign prostate hyperplasia. Small, empty vesicles with granulated membranes are budding from the Golgi membranes and similar larger vesicles are present in the vicinity of the Golgi apparatus. Vesicles with dense contents or various textures are scattered in the Golgi areas. Several vesicles have cytoplasmatic invaginations containing ribosomes and small parts of endoplasmic reticulum. Mag. 33,800X. Paper I.
Fig. 11.
Luminal parts of well-differentiated neoplastic cells from a patient with prostate cancer. Some cells bordering a duct lumen are seen. The cells demonstrate storage vesicles containing an electron-lucent substance and small vesicles and dense bodies, that is, prostasomes. Similar circular structures are noticed in the duct lumen, although some of the slightly dense vesicles represent cross-sectioned microvilli. Mag. 13,600X. Paper I.
Fig. 12.
Luminal parts of well-differentiated neoplastic cells from a patient with prostate cancer. The lumen contains a flocy substance with many dense bodies (prostasomes) and circular structures (mostly cross-sectioned microvilli). Mag. 17,400X. Paper I.
Fig. 13.
Poorly differentiated neoplastic cells from a patient with prostate cancer. The lower supranuclear parts of the cells contain several large, irregularly outlined, nearly empty vesicles, suggesting that these neoplastic cells have ceased to produce normal storage vesicles. Only a few matured storage vesicles are present in the subluminal region of the cells. In the lumen, many prostasome-like bodies are still present. Mag. 3,500X. Paper I.
Paper II

Electron microscopy revealed prostate cancer cells in groups surrounded by connective tissue with thin-walled blood vessels. Intracellularly, these poorly differentiated cells had conventional organelles albeit not always as well developed as in normal tissue, see figures 14 and 15. Nevertheless, Golgi-areas with prostasome-like particles in nearby storage vesicles could be seen and prostasomes could also be visualized in the connective tissue surrounding the agglomerates of secretory cells, see figure 16.

The conclusion of this investigation was that prostasomes were synthesized in metastatic prostate cancer tissue and were secreted extracellularly where the prostasomes may become accessible to the immune system of the patient. Figures 14-16 from paper II reprinted with permission of John Wiley & Sons, Inc.

Fig. 14.
Groups of neoplastic cells in vertebral metastases from a prostate cancer. The cell groups are surrounded by loose connective tissue containing thin-walled vessels and various blood cells. Epon section prepared for light microscopy. Mag. 600X. Bar 50µm. paper II.
Fig. 15.
Part of a neoplastic cell of a vertebral metastasis from a prostate cancer. The cytoplasm contains the conventional organelles although some of them differ in structure from those of the normal prostate epithelial cell. For instance, the large Golgi apparatus is replaced by several smaller Golgi areas (G). Some vesicles (storage vesicles) contain differently shaped dark granules (prostasomes) (arrow). Electron micrograph. Mag. 15,000X. Bar 1µm. Paper II.
Fig. 16.
Peripheral part of a neoplastic cell. The cell surface possesses microvilli and protrudes into a connective tissue space filled with an amorphous substance containing prostasomes. Some irregularly shaped aggregates of prostasomes are observed in the upper part of the figure. Electron micrograph. Mag. 33,000X. Bar 0.5µm. Paper II.
Paper III
SDS-Polyacrylamide-gel electrophoresis of seminal vesicle material resulted in banding patterns in which three prominent prostasome-marking CD proteins were missing but with two other proteins distinctly expressed. These bands were later identified by flow cytometry and monoclonal antibodies to be heat shock protein (HSP70) at 70kDa and clusterin at 55kDa, see figures 17 and 18.

The ultrastructure of the seminal vesicle gland revealed secretory vesicles in the epithelial cells. The secretory vesicles appeared smaller and contained only one secretory granule compared with the storage vesicles in prostate tissue, which generally contained multiple prostasomes. The secretory granules found in the seminal vesicle gland ducts were sparsely distributed in a dense amorphous substance, see figures 19 and 20.

There was no indication in the present study that prostasomes originated in the seminal vesicles.

Fig. 17.
SDS-Polyacrylamide-gel electrophoresis patterns of seminal prostasomes (P) and vesicular seminalis secretion (VS) in two different concentrations. Molecular markers in lane 1 (MW). Prostasome marking CD proteins (at 150, 120 and 90kDa) encircled in the SDS-PAGE patterns lanes marked P. HSP70 and clusterin (at 70 and 55kDa) encircled in SDS-PAGE pattern lanes marked VS.
Fig. 18.
Flow cytometric detection of membrane-bound proteins on particles from vesicula seminalis secretion. Two common prostasome–bound CD antigens (CD13 and CD26) and clusterin and HSP70. A. Forward scatter (FS) versus fluorescence (FL1). B. Fluorescence (FL) versus number of events (counts).
Fig. 19.
Apical part of a cell synthesizing secretion granules. The supranuclear cytoplasm is filled with homogeneous secretion granules, each one included in a slightly larger vesicle. Changes during the preparation process have caused dilated Golgi sacks and swollen mitochondria. Mag. 39,000X. (20mm – 0.5µm).
Fig. 20.
Part of the glandular lumen and apical parts of epithelial cells. In the lower part of the picture, a lipofucsin granule is observed. One cell, cut at its bulging surface, have long microvilli and possesses some secretory granules. In the lumen, released secretory granules and lipofucsin granules are seen lying within a flocky, slightly dense secretion. Mag. 18,000X. (9mm – 0.5µm).
Paper IV
In the survey group, the urological inpatients had higher median serum titres of antibodies against seminal prostasomes than both blood donor controls and patients of the Biopsy subgroup, but the diagnosis of prostate cancer was not a prerequisite for an elevated level, see figure 21. Among the patients subjected to a diagnostic prostate biopsy, the median anti-prostasome antibody titres were equal compared with controls. The pre-biopsy serum level of antibodies could not be used to predict whether the patient would prove to have a prostate cancer or not, see figure 22.

Trauma to the prostate gland, regardless of whether it was mechanical (a prostate biopsy with a limited amount of tissue destruction) or thermal (microwave induced heat destruction of several grams of prostate tissue), did not instigate the development of antibodies against seminal prostasomes, see figure 23.

The conclusion was that seminal prostasomes could not serve as a proxy to localized prostate cancer and that exposure of prostasomes to the immune system through trauma did not evoke an autoimmune response.

Fig. 21.
Median serum anti-prostasome antibody titre in the patients subjected to prostate biopsy compared with control groups of male blood donors and urological inpatients without and with prostate cancer (w pca).
Fig. 22.
Median pre-biopsy antibody titre levels, expressed in arbitrary antibody units (AAU), in patients with benign (left) and malignant (right) prostate disease at the time of biopsy.

Fig. 23.
Anti-prostasome antibody serum levels, expressed as arbitrary antibody units (AAU), over time in patients with benign disease in the biopsy report (left), and with malignant disease (right), through the 4 visits (V1-V4) of the time of observation.
Discussion

Against a background of biochemical and morphological descriptions of the prostasome and a wide expanse of in vitro research which has unraveled a multitude of prostasome-associated effects within reproductive physiology, the investigations presented in this thesis explored possible applications of the prostasome in clinical urology.

In Paper I, prostasomes synthesized and secreted in a similar fashion in both benign and malignant prostate gland tissue were demonstrated. The well known tendency of prostate cancer to multifocal growth in the gland made it essential that the ultrastructural studies in the electron microscope dealt with glandular structures of an unambiguous diagnosis of either a benign or a malignant nature. The diagnosis of prostate cancer in routine histology relies on the Gleason principles, classifying the tumor according to the growth pattern of the cells under light microscopy. Uncertainties can be circumvented by applying aids such as immunohistochemical staining of basal cells in the epithelium but this stratagem does not always work well when the tissue is fixed in glutaraldehyde and embedded in Epon-plastic for electron microscopy. In this study, the final designation of the tissue samples as benign or cancerous depended on the pathologist’s experience in recognizing malignant cells in a stepwise sectioning and evaluation of toluidine blue stained specimens. There are changes in cancer cells other than the pattern of growth that guide the pathologist. Intracellular factors such as the size, shape and location of the nucleus in the secretory cell, the increased occurrence of eccentric nucleoli in the nucleus, increased rate of mitosis, diminished secretory activity second to loss of differentiation and identification of perineural growth of epithelial cells are alterations indicative of malignant transformation [76].

The ultrastructural findings in electron microscopy were consistent with earlier descriptions of prostasomes in benign tissue [14] but in our material, prostasomes were also found in storage vesicles in the cytoplasm of malignant cells and in the acini of neoplastic glandular formations. In a previous study of samples from benign as well as cancerous prostate tissue fixed in formaldehyde and embedded in paraffin for light microscopy, anti-prostasome antibodies labeled the apical parts of the epithelial cells and secretion in the gland ducts [18]. (We attempted to pin-point the prostasomes in electron microscopy with electron immunohistochemistry in the same fashion using antibodies against prostasomes in a gold labeling anti-body, anti-body reaction on our biopsies embedded in Lowicryl plastic, but the results were inconclusive, perhaps due to the fixative, which was glutaraldehyde.)

A plausible mode of prostasome synthesis, similar in both benign and malignant cells was described. The storage vesicles formed in the Golgi areas and their content of prostasomes and the secretion in the gland ducts appeared similar in both types of cells. Thus, prostasomes were secreted also by malignant cells, a finding interesting in itself and also supporting the hypothesis that properties
of the prostasomes could play a role in patho-physiological considerations concerning prostate cancer [47, 54].

In Paper II, there was no doubt as to the nature of the tissue examined. Very little benign prostatic tissue can be found around the spinal cord but to rule out other malignancies, the biopsies were double-checked with routine histological evaluation.

In one preceding study, patients with advanced prostate cancer disease with high PSA levels had elevated levels of anti-prostasome antibodies compared with controls [73]. In another study, prostasomes were retrieved from homogenized metastatic prostate cancer tissue and studied biochemically [17]. The results from these studies indicated that if prostasomes (that normally should be found only in the seclusion of the prostate, the prostate gland ducts and the urethra in the male body) in some way were exposed to the patient’s immune system they could give rise to anti-prostasome antibodies. Such antibodies might in their turn be used as markers not only for grossly metastatic disease, as indicated by the study mentioned above [73], but also for extraprostatic disease in other clinical situations such as locally advanced tumor growth, that has not yet spread to local or regional lymph nodes or local recurrence after radical prostatectomy. The prostasome (40-500nm) is much smaller in size compared with a malignant prostate cell. Hypothetically, leakage of prostasomes to the lymphatic or blood circulation (similar to the way PSA presumably leaks from malignant lesions in the prostate) might stimulate subsequent immunological reactions to prostasomes by the host, indicating the existence of a localized cancer.

The results of this study supported at least the first part of this line of reasoning. In the metastatic tissue, the neoplastic cells formed small groups of cells with secretory activity resulting in secretion of prostasome granules into the extracellular spaces in the connective tissue.

Prostasome-associated effects such as immunosuppression and complement inhibition probably aid the spermatozoa in their task to reach and fertilize the egg, as discussed earlier. The same effects in cancer-derived prostasomes secreted extracellularly might also promote tumor progression in prostate cancer metastases [77].

Paper III considered the proposal in an earlier article by Renneberg et al. [71] stating that the prostasomes might not be an exclusive prostatic gland product but rather a compilation of components from different sources in the male urogenital tract. The reason for this proposal would be that the polyclonal rabbit-derived anti-prostasome antibody used in the study reacted both with the secretory cells of the prostate epithelium and the secreted substance in the gland ducts, and also to a varying degree with epididymides (but not testes) and seminal vesicles and, in addition, with some cells outside the urogenital organs, for instance bile canaluculi and the parotid gland. The immunogen in Rennebergs paper was prostasomes purified from seminal plasma that had been exposed to secretions from testes, epididymides, vasa deferentes, seminal vesicles, prostate gland, the bulbo-urethral glands as well as urothelium (but hardly from bile
canaliculi). Cross-reactions occurring when using a polyclonal antibody seem not unlikely given the complex array of proteins associated with purified prostasomes described by Utleg [21]. Investigations with a monoclonal mouse-derived antibody showed no cross-reactions in the male uro-genital organs except for a weak labeling in seminal vesicle epithelium [78]. The suggested contribution of components to the prostasomes from the epididymides had already been proven unlikely as the activity of prostasome-associated enzymes in seminal plasma did not diminish after vasectomy [79].

Studies of the pelleted seminal vesicle material with SDS-PAGE gel electrophoresis resulted in banding patterns in which some of the prostasome-marking CD proteins were missing. But two other proteins, heat shock protein (HSP70) at 70kDa and clusterin at 55kDa, were strongly expressed and labeled with monoclonal antibodies as demonstrated in flow cytometry, whereas these proteins were hardly discernible in prostasomes.

Heat shock protein (HSP70) and clusterin are so called chaperone proteins which normal cells express as a response to stress of different kinds, for instance heat (even fever or physical exercise) or metabolic derangement (diabetes mellitus). One of their functions is to prevent aggregation of intracellular proteins that might cause cell death, apoptosis. The over-expression of chaperone proteins that exists in many tumors has been interpreted as a retained capacity which is advantageous to the cancer as it limits apoptosis of tumor tissue. The occurrence of HSP70 and clusterin in the seminal vesicle pellet concurred with previous research [80] finding large amounts of positive clusterin-staining in normal seminal vesicle epithelial cells and secretion whereas normal prostate tissue and secretion showed no or very little staining. However, HSP70 is expressed by prostate cancer tissue [81] and most prostate cancer tissue specimens express clusterin. Furthermore, the expression rate of clusterin is correlated to the Gleason score [82]. In vitro, drug induced suppression of HSP70 in prostate cancer cell lines leads to an increased rate of apoptotic cell death [83]. Recently, HSP70 and clusterin have both been identified as parts of the antigenic profile of seminal prostasomes recognized by the serum of patients with high anti-prostasome antigen titres [84]. It is unknown whether this expression of HSP70 and clusterin in seminal prostasomes is different in prostasomes originating solely in tumor tissue.

The differences in ultrastructure between seminal vesicle and prostate tissues were illustrated by electron microscopy of the seminal vesicle epithelium which demonstrated secretory vesicles in the epithelial cells. They contained only one secretory entity compared with the storage vesicles in prostate tissue which generally contain several prostasomes. When secreted into the gland ducts, prostasomes retain their shape but the secretory granule in the seminal vesicle specimens seemed to fall apart and disperse in the amorphous substance dominating the duct lumen.

In Paper IV, the auto-immunogenicity towards seminal prostasomes was tried with a test kit in different clinical settings. In the survey of 150 male urological
inpatients, the median level of anti-prostasome antibodies in serum was higher than among blood donor controls. However, counter to the assumption prior to the study, there was no difference in median antibody titer level between the 60 inpatients with a prostate cancer and the 90 inpatients without a manifest prostate cancer. Among the 89 patients in the study-subgroup who came for diagnostic prostate biopsies, the pre-biopsy serum level of anti-prostasome antibodies did not differ from those of controls, and the results showed that the pre-biopsy titer could not prognosticate who would have a benign biopsy result and who would be diagnosed with a prostate malignancy, regardless of grade or stage. There was also no correlation between the level of anti-prostasome antibodies and either PSA-level or prostate size.

As urological problems are common in the health-history of middle aged and older men (manifest in this study by a high rate, around 40%, of previous urological morbidity among all patients coming for diagnostic prostate biopsy), the possibility that unspecific prostate tissue destruction, other than prostate cancer, might give rise to “false-positive” results, was investigated. However, neither the rather timid trauma of a prostate biopsy nor the drastic heat destruction of many grams of prostate tissue during a TUMT-procedure evoked a lasting increase in anti-prostasome antibodies (or in general IgG levels in the case of the biopsy patients). Hence, it appears doubtful, again counter to the hypothesis, that seminal prostasomes are auto-immunogenic in the assumed fashion.

The conclusions were that seminal prostasomes, which served as antigen in the assay monitoring the serum antibody levels, must be regarded as an insufficient proxy to localized prostate cancer and, furthermore, that seminal prostasomes did not have auto-immunogenic properties in a situation of thermal or mechanic destruction of prostate tissue.

The clinical conditions under which to expect a measurable, or elevated, titer of auto-antibodies against prostasomes are enigmatic. In two studies published recently [85, 86], the correlation between prostate cancer expressions and the level of anti-prostasome antibodies was evaluated. In both studies, low grade cancer, i.e. well differentiated tumors with no, or limited spread, tended to have higher titer levels than high grade tumors, i.e. poorly differentiated cancers, some with advanced stage. The suggested explanation was that well-differentiated tumour cells produce more prostasomes resulting in a greater antigen challenge to the immune system. In neither study was there a correlation between antibody titre and PSA levels. In one of the studies [85], the antibody titer levels were higher among the patients with well-differentiated cancers (but not poorly differentiated cancers) than in the patients with benign disease.

PSA, one of the best tumor marker in medicine, is nevertheless hampered in its utility by poor specificity in the situation where it matters most: in a screening situation ultimately aiming to find the still localized but aggressive cancer and at the same time excluding the harmless tumour. Among the 89 patients who came for a diagnostic prostate biopsy in paper IV we did not find differences in pre-biopsy titer levels that could prognosticate the outcome of the procedure in
reference to a cancer diagnosis, regardless of tumor stage. There was no correlation between the patients’ PSA levels and anti-prostasome antibody titers in this study. There were no tangible differences among the patients in the other clinical parameters (prostate size and age) registered either. The patients were referred on the whole due to a moderately elevated PSA concentration and thus mimicking a screening situation.

PSA is organ- but not cancer-specific. The same appears to apply to seminal prostasomes being an antigen for anti-prostasome auto-antibodies. The majority of prostasomes purified from seminal plasma and utilized as antigen reasonably originates from non-neoplastic secretory cells. Our results from the trauma group in the study indicated that prostasomes from prostate secretion and tissue are not immunogenic in the assumed fashion. It is therefore uncertain to what extent the circulating antibodies detected by an assay with purified seminal prostasomes as antigen matches the antigen profile of prostasomes secreted by prostate cancer secretory cells. The protein profile of seminal prostasomes described by Utleg [21] would perhaps look different in prostasomes derived solely from metastatic tissue.

Future
The role of the prostasomes in human reproduction is under continuous research whereas in the context of clinical urology, the role of the prostasome-organelle has not been evaluated to the same extent. The clinical role of the prostasome with its array of unique characteristics, such as membrane composition and associated proteins, needs to be investigated further.

Prostate cancer in all of its aspects, constitutes a formidable challenge for the future in the field of urology. Given the ever increasing incidence of the disease, the cardinal wish of the urological clinician would be a screening marker discriminating the aggressive but still localized (therefore curable) cancer from the harmless tumor. Short of that, he or she would value a reliable tool to target extraprostatic tumor growth. Approximately 25% of all prostate cancer patients who have been through surgery or radiation with curative intent will have a disease-relapse indicated by a recurrent and/or rising level of PSA [87]. The cause being either local recurrence due to inadequate surgery or radiation, or an understaging of the disease before treatment. Theoretically, the tumor might still be curable with adjuvant radiation in these situations, but there is no reliable method of imaging to guide the radiation-oncologist and PSA is unhelpful in the actual localization of the cancerous tissue. This is definitely a situation in which a locally expressed tumor specific prostasome-associated test would be ideal for playing the role of a diagnostic tool.

In the series of four articles presented in this thesis, noteworthy results are the findings in electron microscopy that prostasomes are secreted not only by neoplastic cells in the prostate gland but also by prostate cancer cells in metastatic tissue. Furthermore, prostasomes are secreted into the extracellular spaces
around cells in spinal metastases. Less encouraging but nevertheless important is the realization that prostasomes from seminal appear insufficient as proxy for prostate cancer, at least when it is still localized. Whether this would change if prostate cancer specific prostasomes, e.g. metastasis derived (which have biochemistry and SDS-PAGE patterns different from those of seminal prostasomes), were used as antigens in the development of antibody-clones and assays remains to be tried. Possibly, such prostasomes might prove to be appropriate as cancer proxies in future studies.

Fig. 24.
Peripheral area of a group of three neoplastic cells with their neighboring connective tissue in a vertebral metastasis of prostate cancer. The cell surfaces are bulging into a narrow connective tissue space, which is filled with an amorphous substance containing prostasomes and aggregates of prostasomes. Mag. 6,000X. Bar 2µm. Paper II.
Summary

Prostasomes are small (40-500nm), membrane enclosed, secretory products of the prostate epithelial cells. Prostasomes, first purified from prostate fluid (expressmate) and seminal plasma in the late 1970’s have also been identified in homogenized prostate gland tissue and metastatic tissue of prostate cancer. Their physiological role appears to be the promotion of sperm function in different ways in human reproduction. In *in vitro* studies, prostasomes have improved sperm motility and have shown immune suppressive, antibacterial and complement inhibitory effects that enhance sperm function in the female reproductive tract. In the context of clinical urology, there are studies associating prostate cancer with elevated titers of anti-prostasome antibodies in serum. In this thesis, four papers are presented which have explored some of the urological aspects of prostasomes.

In the first and second papers, utilizing electron microscopy, the secretory machinery of prostasomes was outlined with the synthesis, storage and secretion of prostasomes depicted in core biopsies from benign as well as malignant prostate gland tissue (Paper I). Even poorly differentiated cells of prostate cancer tissue in spinal metastases retained the capacity for producing prostasomes and also to secrete them extracellularly into the surrounding connective tissue (Paper II). This makes the prostasomes accessible to the immune system of the patient, making an anti-prostasome antibody generation more likely. This reinforces findings in earlier studies of elevated titers of anti-prostasome antibodies in patients with advanced disease.

The third paper in this thesis evaluated earlier postulations that prostasomes are not a prostate gland unique product, but rather a compilation of material originating from different parts of the male urogenital tract, especially the seminal vesicles. To that purpose, pelleted material from seminal vesicle secretion was isolated. SDS-PAGE gel electrophoresis of the pellet-material resulted in pattern profiles in which three prostasome marker proteins were lacking in the banding. On the other hand, two proteins, later identified in flow cytometry as heat shock protein 70 and clusterin, were clearly expressed in seminal vesicle substance but hardly in prostasomes. In addition, differences in secretory ultrastructure between seminal vesicle epithelium and prostate secretory epithelial cells were illustrated with electron microscopy. The conclusion of the study was that prostasomes had no obvious origin in the seminal vesicles.
Finally, in the fourth paper, a survey of a group of urological inpatients with different diagnoses including prostate cancer revealed higher median serum levels of anti-prostasome antibodies compared with blood donor controls. However, inpatients with prostate cancer did not have higher titers than patients without prostate tumor. Among patients who came for a prostate biopsy, the pre-biopsy serum level of anti-prostasome antibodies did not discriminate between the patients who were later diagnosed with a prostate cancer and those who were not. The possible generation of anti-prostasome antibodies second to prostate tissue destruction was investigated in patients who either underwent a prostate biopsy on the suspicion of prostate cancer or were submitted to microwave treatment of benign prostate hyperplasia. Over a three-months’ time of observation no lasting increase in anti-prostasome antibody titre was registered after either procedure. The results in the fourth paper indicated that prostasomes derived from seminal plasma could not serve as proxy to localized prostate cancer and that thermal or mechanical destruction of prostate tissue did not induce production of anti-prostasome antibodies.

**Concluding remarks**

The results presented in this thesis reinforce the view of the prostate-unique origin of the prostasome and provide an ultrastructural description of the synthesis, storage and secretion of prostasomes in benign as well as in malignant tissue, most notably the extracellular appearance of prostasomes in metastatic lesions. However, in a clinical setting, exposure of the prostate gland through trauma did not induce formation of anti-prostasome antibodies using seminal prostasomes as antigen. Furthermore, the serum levels of anti-prostasome antibodies did not correlate with different expressions of existing prostate cancer. Seminal prostasomes appear not to be proper antigens for possible auto-antibodies in connection with prostate cancer. A possible explanation could be underestimated differences in antigen properties between seminal or prostate gland-derived prostasomes and prostasomes from tumor tissue.
Sammanfattning på svenska

Prostasomer är små (ca 150µm i diameter) membranomslutna granulae vilka produceras i prostatakörtelsns epitel och utsöndras till körtelgångarna. De isolerades ur sädessvärk och prostatasekret och beskrevs för första gången i slutet av 1970-talet. Prostasomernas fysiologiska roll är att främja spermiernas funktion i reproduktionsprocessen. Forskningsstudier har visat att prostasomerna ökar spermiernas rörlighet, de har antibakteriella egenskaper och de skyddar spermierna mot immunologiska och cellulära försvarsmekanismer. Studierna i den nu föreliggande avhandlingen undersöker egenskaper hos prostasomerna som skulle kunna ha klinisk betydelse i urologin.

I delarbete I och II presenterades resultaten från elektronmikroskopiska undersökningar av benign och malign prostatavävnad samt skelettmetastaser av prostatacancer. Dessa undersökningar visade att prostasomer produceras och utsöndras i malign prostatavävnad på liknande sätt som i benign vävnad och att även celler i metastasvävnad kan syntetisera prostasomer och, vilket är särskilt värt att notera, att prostasomer utsöndras extracellulärt till omgivande bindväv runt metastaserna. Detta ger en stor exponering av prostasomer för patientens immunförsvar vilket skulle kunna resultera i ett svar från patienten med autoantikroppar. De finns också studier sedan tidigare i vilka man har funnit auto-antikroppar mot prostasomer i blodet hos patienter med avancerad prostatacancer (se ref. 74).

I delarbete III bemöttes påståenden om att prostasomerna inte skulle vara en prostataspecifik produkt utan fastmer en sammanbakning av ämnen från olika delar i det manliga urogenitala systemet, i första hand sädessläsorna. Elektrofores och flödescytometri av isolerat sekretionsmaterial från sädessläsorna visade en tydligt annorlunda proteinuppsättning jämfört med den från prostasomer. Tre proteiner typiska för prostasomer saknades medan två andra typer av proteiner var kraftigt uttryckta i sädessläsorna. En elektronmikroskopisk undersökning visade också att sekretionsprodukterna skiljer sig tydligt mellan de sekretoriska cellerna i prostataeptel jämfört med de i sädessläsorna. Slutsatsen blev att man inte kunde finna hållpunkter för att prostasomer, helt eller delvis, produceras i sädessläsorna.
I delarbete IV undersöktes förekomsten av antikroppar mot prostasomer hos olika grupper av urologiska patienter. Dessutom undersöktes om diagnostisk prostatabiopsi eller transuretral värmebehandling av prostataförstoring resulterar i att patienterna utvecklar ett anti-kroppssvar mot prostasomer från den exponerade prostatavävnaden. Resultaten visade att det visserligen var högre nivåer av anti-kroppar mot prostasomer hos urologiska patienter i slutenvård jämfört med en kontrollgrupp av blodgivare, men att patienter med prostatacancer inte hade högre nivåer än patienter utan prostatacancer. Bland patienter vilka remitterats för prostatabiopsi på misstanken om prostatacancer var nivåerna av antikroppar mot prostasomer inte högre hos de patienter som visade sig ha cancer i biopsivänen jämfört med de patienter som inte fick en cancerdiagnos. Varken prostatabiopsierna eller värmebehandlingen av prostata resulterade i någon påvisbar ökning av nivåerna av anti-prostasom antikroppar hos patienterna under en 3-månaders uppföljningsperiod. Resultaten i delarbete IV skulle kunna förklaras av skillnader i struktur och biokemisk sammansättning mellan prostasomer från olika typer av prostatavävnad och som man redan känner till från prekliniska undersökningar (se ref. 17). De instrument som använts i studien för att mäta nivåerna av prostasomantikroppar hade prostasomer utvunna ur sädessvätiska som antigen. Resultaten tydde på att dessa seminala prostasomer inte kunde fungera som ställförerträdare för prostatacancer. För att komma närmare en förklaring bör skillnader mellan prostasomtyperna kartläggas mera noggrant för att sedan prövas i kliniskt relevanta situationer, ett arbete som påbörjats (se ref. 84).
Acknowledgements

I wish to express my sincere gratitude to the individuals and institutions that have made it possible for me to complete this thesis:

Prof. **Gunnar Ronquist**, principal supervisor, discoverer of the prostasome, profoundly knowledgeable in clinical chemistry and what makes creation tick in general. Unceasingly optimistic and patient. Prefers a thesis to be – in latin!


Prof. **Bo Johan Norlén**, supervisor, mentor and unique role-model in urology for whom nothing is impossible. Always supportive and encouraging in his easygoing way. Equally comfortable indoors doing research, as outdoors, hunting in a clearing, awaiting the approach of beast or fowl.

Co authors: Prof. **Anders Larsson**, generous with crucial advice and practical manoeuvres. **Lars Egevad** MD. PhD, world-class pathologist. **Lena Carlsson**, PhD, experienced scientist with total command of the laboratory. **Anders Ahlander**, B.Sc, mentor in the ways and means of electron microscopy.

**Eva Beckman** R.N, **Monica Andersson** R.N. and **Marita Lind**, coordinating the flow of urological patients in the paper IV study. **Hans Garmo** PhD. and **Johan Lindbäck** B.Sc., influential statisticians. **Enayat Mavadati**, B.Sc. protocol-producer. **Håkan Pettersson**, desktop publisher.

My **Colleagues**, looking oddly happy despite the extra workload. I should do this again!!

**Muriel**, a life-time award, **Karin** and **friends**. The resource.

My mother **Margareta** and my late father **Gösta**, sisters **Karin** and **Anneli**. The source.

**Uppsala University**, for providing the environment and resources essential for the project.

The **Foundation Johanna Hagstrand and Sigfrid Linnér’s Minne** and the **Swedish Medical Research Council** for generous financial support.

*No man is wise enough by himself.*

Titus Maccius Plautus (254 BC - 184 BC)
References


14. Brody I, Ronquist G, Gottfries A: Ultrastructural localization of the prosta-
some - an organelle in human seminal plasma. Upsala J Med Sci 1983; 88:
63-80.
Characteristics of human prostatesomes isolated from three different sources.
18. Nilsson BO, Meishan Jin, Ronquist G: Immunolocalization of prostatesomes
prostasome mAb 78 binds to an antigen distinct from PSA and PAP. J Urol
branes exhibit very high cholesterol/phospholipid ratios yielding high molec-
22. Ronquist G. Effect of modulators on prostasome membrane-bound ATPase
23. Ronquist G. Zinc ion stimulation of ATP cleavage by prostatesomes from
of calcium, zinc, and magnesium in benign nodular hyperplasia of the human
47: 323-328.
25. Stegmayr B, Berggren PO, Ronquist G, Hellman B. Calcium, magnesium
and zinc contents in organelles of prostatic origin in human seminal plasma.
26. Renneberg H, Albrecht M, Kurek R, Krause E, Lottspeich F, Aumüller G,
Wilhelm B : Identification and characterization of neutral endopeptidase
(EC 3.4.24.11) from human prostatesomes - localization in prostatic tissue and
in prostatic organelles present in seminal plasma. Clin Chim Acta. 1982; 126:
161-170.


45. Rooney IA, Davies A, Morgan BP: Membrane attack-complex (MAC) -mediated damage to spermatozoa: protection of the cells by the presence on their membranes of MAC-inhibitory proteins . Immunology 1992; 75: 499.


51. NCPR. www.roc.se 2006

52. www.socialstyrelsen.se 2006


A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)