Prostasome Modulation of Blood Cascade System and Phosphoprotein Reactions with Focus on Prostate Cancer

ADIL ABDELGADIR BABIKER
Dissertation presented at Uppsala University to be publicly examined in Rudbeck Hall, Rudbeck Laboratory, Uppsala, Monday, May 23, 2005 at 13:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

Abstract

Prostasomes are extracellularly occurring submicron, membrane-surrounded organelles produced by the epithelial cells of the prostate and present in semen. Their precise physiological role is not known, although some of their properties assign them to important physiological and pathophysiological functions. In this thesis, some new properties of seminal and malignant cell line (DU145, PC-3 and LNCaP) prostasomes have been identified.

Differences in the expressions and activities of prostasomal CD59, ATPase, protein kinases and tissue factor (TF) have been characterized. The transfer of prostasomal CD59 to CD59-deficient erythrocytes (rabbit and human PNH erythrocytes) has been established. CD59, protein kinases and TF were overexpressed by malignant cell prostasomes. ATPase activity was highest on seminal prostasomes with minimal expression by malignant cell prostasomes resulting in more residual ATP available for phosphorylation reactions. Several proteins were phosphorylated by prostasomal protein kinases, viz. complement component C3, fibrinogen, vitronectin and E-cadherin. Furthermore, TF was identified as the main endogenous phosphorylation substrate on prostasomes. In addition, prothrombotic effects of prostasomes were established. DU145 and PC-3-derived prostasomes exerted a higher clotting effect on whole blood and plasma compared to LNCaP and seminal prostasomes.

In conclusion, malignant cell prostasomes showed higher ability to interact with the biological system in favor of prostate cancer cell promotion and survival. The roles played by prostasomes in this context may improve the understanding of the mechanisms that help the prostate cancer cells to avoid the complement attack (CD59 transfer and phosphorylation of C3), to promote angiogenesis (TF) and to metastasize. It may also provide a better understanding of some of the complications usually seen in some terminal prostate cancer patients like thrombotic events and tendency to develop disseminated intravascular coagulation.

Keywords: ATPase, CD59, Complement, DU145, Extracellular phosphorylation, LNCaP, PC-3, Prostasomes, Prostate cancer, Protein kinases, Tissue factor

Adil Abdelgadir Babiker, Department of Oncology, Radiology and Clinical Immunology, Akademiska sjukhuset, Uppsala University, SE-75185 Uppsala, Sweden

© Adil Abdelgadir Babiker 2005

ISSN 1651-6206
ISBN 91-554-6238-3
urn:nbn:se:uu:diva-5779 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-5779)
“Of knowledge ye have been vouchsafed but little”

To the memory of my parents
List of Papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.


Reprints were made with the permission of the publishers.
Contents

Introduction ...................................................................................................11
The Prostate Gland ...................................................................................11
  Anatomy ..............................................................................................11
  Histology .............................................................................................12
The Nature of Prostatic Secretion ........................................................13
  The Glandular Epithelia.......................................................................13
Prostasomes ..............................................................................................13
  Prostasomal Membrane Architecture ...................................................14
  The Secretion of Prostasomes ..............................................................14
  Prostasomal Membrane Enzymes ........................................................15
  Functional Role of Prostasomes ..........................................................16
  Seminal Protein Kinases and Protein Phosphorylation .....................19
Prostate Cancer .........................................................................................21
  Epidemiology .......................................................................................21
  Pathology .............................................................................................21
  Staging of prostate cancer .................................................................22
  Diagnostic Techniques ........................................................................22
  Diagnosis .............................................................................................23
Complement System ..................................................................................23
  Introduction .........................................................................................23
  Activation of Complement ..................................................................23
  Regulation of Complement ................................................................25
  Role of Complement in Tumor Surveillance .......................................26
Paroxysmal Nocturnal Hemoglobinuria (PNH) .......................................28
  Definition, Etiology and Pathogenesis ...............................................28
  Clinical Manifestations .......................................................................28
Aims of the Present Investigation .................................................................29
Materials and Methods ...........................................................................31
  I. Preparation of Prostasomes ...............................................................31
  II. Experiments on Prostasomal CD59 ..................................................32
  III. Hemolytic Assay of Complement Alternative Pathway ...............33
  IV. Prostasomal ATPase and Protein Kinases .....................................33
  V. Prostasomal Tissue Factor (TF) [Paper IV] .....................................34
  VI. Prothrombotic Experiments [Paper IV] .........................................35
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Results and Conclusions</td>
<td>37</td>
</tr>
<tr>
<td>Paper I</td>
<td>37</td>
</tr>
<tr>
<td>Paper II</td>
<td>38</td>
</tr>
<tr>
<td>Paper III</td>
<td>39</td>
</tr>
<tr>
<td>Paper IV</td>
<td>40</td>
</tr>
<tr>
<td>Additional Data:</td>
<td>40</td>
</tr>
<tr>
<td>Discussion</td>
<td>42</td>
</tr>
<tr>
<td>Summary</td>
<td>49</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>51</td>
</tr>
<tr>
<td>References</td>
<td>53</td>
</tr>
</tbody>
</table>
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APB</td>
<td>Alternative pathway buffer</td>
</tr>
<tr>
<td>APW</td>
<td>Alternative pathway of complement</td>
</tr>
<tr>
<td>ASIS</td>
<td>Active site inhibited recombinant FVIIa</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>cAMP</td>
<td>3',5'-Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>C1-INH</td>
<td>Complement component 1 (C1) inhibitor</td>
</tr>
<tr>
<td>C3</td>
<td>Complement component 3</td>
</tr>
<tr>
<td>C3b,Bb</td>
<td>APW C3 convertase</td>
</tr>
<tr>
<td>C3b,Bb,C3b</td>
<td>APW C5 convertase</td>
</tr>
<tr>
<td>C4b,C2a</td>
<td>CPW C3 convertase</td>
</tr>
<tr>
<td>C4b,C2a,C3b</td>
<td>CPW C5 convertase</td>
</tr>
<tr>
<td>C5b-9</td>
<td>Membrane attack complex</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CK (I,II)</td>
<td>Casein kinase (I,II)</td>
</tr>
<tr>
<td>CKI-7</td>
<td>N-(2-amoioethyl)-5-isoquinoline-8-sulfonamide</td>
</tr>
<tr>
<td>CPW</td>
<td>Classical pathway</td>
</tr>
<tr>
<td>CR1</td>
<td>Complement receptor 1</td>
</tr>
<tr>
<td>CT1</td>
<td>Corn trypsin inhibitor</td>
</tr>
<tr>
<td>DAF</td>
<td>Decay accelerating factor (CD55)</td>
</tr>
<tr>
<td>DIC</td>
<td>Disseminated intravascular coagulation</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DRB</td>
<td>5,6-dichlorobenzimidazole riboside</td>
</tr>
<tr>
<td>DRE</td>
<td>Digital rectal examination</td>
</tr>
<tr>
<td>DU145</td>
<td>Prostatic cell line from brain metastasis</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetate</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetate</td>
</tr>
<tr>
<td>EIA</td>
<td>Enzyme immunoassay</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosyl phosphatidyl inositol</td>
</tr>
<tr>
<td>GVB</td>
<td>Gelatin veronal buffered saline</td>
</tr>
<tr>
<td>H89-2HCl</td>
<td>N-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulf人们 amide dihydrochloride</td>
</tr>
<tr>
<td>HANE</td>
<td>Hereditary angioneurotic edema</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LNCaP</td>
<td>Prostatic cell line from lymph node metastasis</td>
</tr>
<tr>
<td>LP</td>
<td>Lectin pathway</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
</tr>
<tr>
<td>MAPKs</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>MASP</td>
<td>Mannan-binding-protein-associated serine protease</td>
</tr>
<tr>
<td>MBL</td>
<td>Mannan binding lectin</td>
</tr>
<tr>
<td>MCP</td>
<td>Membrane cofactor protein</td>
</tr>
<tr>
<td>MIRL</td>
<td>Membrane inhibitor of reactive lysis (CD59)</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MRS</td>
<td>Magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>PAP</td>
<td>Prostatic acid phosphatase</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen activator-1</td>
</tr>
<tr>
<td>PC-3</td>
<td>Prostatic cell line from bone metastasis</td>
</tr>
<tr>
<td>PIPLC</td>
<td>Phosphoinositol-specific phospholipase C</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PKs</td>
<td>Protein kinases</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate acetate</td>
</tr>
<tr>
<td>PNH</td>
<td>Paroxysmal nocturnal hemoglobinuria</td>
</tr>
<tr>
<td>PPP</td>
<td>Platelet-poor plasma</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate-specific antigen</td>
</tr>
<tr>
<td>RCA</td>
<td>Regulators of complement activation</td>
</tr>
<tr>
<td>RE</td>
<td>Rabbit erythrocytes</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecylsulfate – polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TAT</td>
<td>Thrombin-antithrombin complex</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue factor</td>
</tr>
<tr>
<td>TRUS</td>
<td>Tansrectal ultrasound</td>
</tr>
</tbody>
</table>
Introduction

The Prostate Gland
Anatomy
The human prostate gland is a small accessory reproductive organ weighing about 25g in adult male. It is located inferior to the urinary bladder neck and above the urogenital diaphragm. It is intimately associated with the urethra, going straight through the gland. The prostate is not easily accessible for examination. The posterior part of the peripheral zone (see figure below) can be accessed by digital palpation via the rectum. This site is also the most common entrance for biopsy taking. The gland cannot be subjected to self-examination by the subjects.

The main arterial supply to the prostate gland is from the prostatic branches of the inferior vesical artery, and is also supplied by small branches from the middle rectal and pudendal vessels. The veins are situated between the ‘true’ and ‘false’ capsules. The lymphatic vessels from the prostate drain into internal iliac lymph nodes.

The human prostate gland receives dual autonomic innervation from both parasympathetic (cholinergic) and sympathetic (noradrenergic) nerves in the prostatic nerve plexus, a part of the pelvic autonomic plexus that lies adjacent to the prostate gland. The pelvic plexus receives its parasympathetic input from the sacral segments of the spinal cord (S2-4) and sympathetic fibers from the hypogastric presacral nerves (T10-L2). The autonomic nerves arising from the pelvic plexus escort the vascular supply. Both cholinergic and noradrenergic fibers innervate the prostate stroma, and cholinergic nerves innervate the smooth muscle of the capsule and the space around the blood vessels and are responsible for the secretory function of the epithelial part. The sympathetic nerves control the prostatic musculature, and their excitation closes the bladder neck during the ejaculation of the seminal fluid into urethra.
Histology

There are four separate zones of the prostate gland: the peripheral, central, transition and peri-urethral gland zones. These zones represent about 65-70%, 25%, 5-10% and 1% of the normal prostate volume, respectively (1-3). The prostate is a collection of 30-50 tubulo-alveolar glands whose ducts empty into the prostatic urethra. A fibro-elastic capsule rich in smooth muscle surrounds the prostate. The gland is subdivided histologically into mucosal, submucosal and main gland. The mucosal and submucosal glands are lined by pseudostratified epithelium, while the main gland is lined by a simple columnar epithelium. The glandular compartment of the prostate (acini and ducts) is built up by basal and secretory, luminal epithelial cells, which are occasionally interspersed with neuroendocrine cells. Hence, these latter cells occur in the epithelial lining of prostatic tissue (4) and constitute a third population of highly specialized cells (5).

The functional differentiation starts during puberty and is characterized by the general development of pseudostratified epithelium with basal and secretory cells in all parts of the prostate gland.
The prostatic secretory cells contain an abundance of rough endoplasmic reticulum, a large Golgi complex, a large number of secretory granules (so-called storage vesicles) and numerous lysosomes.

The Nature of Prostatic Secretion

The prostate gland produces a protease-rich fluid that constitutes about 30% of the ejaculate. This secretion is transported to the urethra via prostatic ducts. It is a slightly acidic (pH 6.5), serous fluid in which several major secretory products can be identified. It contains high concentrations of prostatic acid phosphatase (PAP) and prostate specific antigen (PSA) (2 important markers). It contains also amylase, proteolytic enzymes, fibrinolysin, citric acid, zinc, calcium, magnesium, sodium, potassium, carbohydrates, polyamines, hormones, lipids and growth factors (6,7).

Up to 57 major protein groups, of which 27 are non-serum proteins (i.e. presumably exuded by the epithelial cells) have been identified (8). The major prostate specific proteins are PAP and PSA, as mentioned above, which are expressed at pubertal and adult age. Proteolysis is the major function of prostate secretion, being rich in the exopeptidase and endopeptidase. The most extensively studied is PSA. PSA is a member of the human kallikrein family, which in turn is a subgroup of the serine protease family, and PSA is also known as semenin, seminal protease or chymotrypsin-like protease (9,10). Albumin is present in semen at a higher concentration (0.5-1.4 g/L) than any other plasma protein. The prostate gland is considered to be the major site of albumin transudation (11). Human seminal plasma contains also coagulation and fibrinolytic proteins mainly of prostatic origin.

The Glandular Epithelia

The prostatic epithelia and stromal compartments act together as one functional unit, and evidence suggests that stromal-epithelial interactions play an important role in regulating prostatic development and growth (12,13). The glandular epithelial tissues are formed by cells specialized in producing fluid secretion that is different from blood plasma and intercellular fluid.

Prostasomes

Prostasomes were discovered in late 1970’s by Ronquist et al. (14-16). They are submicron, membrane-surrounded organelles produced by the glandular epithelial cells of the prostate gland and present in human semen (17). A lipid bilayered membrane usually encases those organelles. They have a corpuscular appearance with a mean diameter of 150 nm, range 40-500 nm (18). Intracellularly, prostasomes are mostly encased in bigger storage vesi-
cles together with electron dense material in the prostatic secretory cells (17). The secretory function of human prostate includes both apocrine and merocrine extrusion processes (19). Prostasomes may thereby be released as small intact organelles in the prostatic fluid (and semen) by a merocrine-like mechanism (ordinary exocytosis) involving the membrane surrounding the storage vesicle and the plasma membrane of the prostatic secretory cells (17).

Prostasomal Membrane Architecture

The prostasome membrane architecture is unique in its composition as regards the lipid content. Quantitative analysis of membrane lipids revealed domination of cholesterol over phospholipids. The molar ratio of cholesterol/sphingomyelin/glycerophospholipids was found to be 4:1:1. Thus, the cholesterol/phospholipid ratio, being 2.0 (20), was very high in comparison to most other plasma membranes including that of spermatozoon being 0.83 (21).

The Secretion of Prostasomes

The prostasomes are present as inclusions in storage vesicles of the prostate epithelial cells (17,22). The storage vesicles with the prostasomes precursors, reasonably, originate from the Golgi membranes by a budding process since there were no ultrastructural indications that the prostasomes were produced in the cytoplasm and then incorporated in the storage vesicle (22). The prostasomes, intracellularly encased in the storage vesicle, are then released by an exocytotic mechanism into the acinar lumen of the gland after fusion between the membrane surrounding the storage vesicle and the plasma membrane of the prostate epithelial cell.
Prostasomal Membrane Enzymes

Prostasomal membrane contains several enzymes, the physiological role of most of them being not yet known. Prostasomes show a rather high ATP-splitting activity, which is linked to their membrane (17). The Mg$^{2+}$ and Ca$^{2+}$ stimulated ATPase enzyme of the prostasomal membrane is calmodulin-dependent (23). They contain also $\gamma$-Glutamyltransferase whose activity in seminal plasma is more than 1000-fold higher than in normal serum (24). The prostate is the major source of this enzyme (25) and the most of it is bound to the membrane of the prostasomes (26). Prostasomes also possess arachidonate 15-lipoxygenase activity (27) which catalyses oxygenation at the n-6 carbon of many polyunsaturated fatty acids, leading to the formation of a cis-trans conjugated hydroperoxy fatty acid, which is reduced, enzy-
matically or non-enzymatically, to a conjugated hydroxy fatty acid. Arachi-
donate 15-lipoxygenase seems to be important in the acrosome reaction of
bull spermatozoa (28). Neutral endopeptidase (NEP) is a 100 kDa
prostasome-associated highly glycosylated membrane-bound enzyme (29).
Prostasomes contain the membrane-bound 5′-nucleotidase enzyme (5′-
ribonucleotide phosphohydrolase) (30). It is a phosphohydrolase that spe-
cifically catalyses the hydrolysis of 5′-nucleotides and is widely distributed
in animal and human tissues including bull and human semen. Its activity is
increased in the presence of Mg\(^{2+}\) or Mn\(^{2+}\) and strongly inhibited by Ni\(^{2+}\). No
definite physiological role has been identified.

Dipeptidyl peptidase IV (CD26) activity was found to be extremely high in
prostasomes (31). CD26 is a surface antigen that has been found in several
cell types. It is a type II integral membrane protein (32) and possesses a pep-
tidase activity. It is a highly specific serine-type protease that cleaves N-
terminal dipeptidase from peptides with a proline or alanine at the penulti-
mate position (33). Dipeptidyl peptidase IV was transferred to spermatozoa
upon incubation with prostasomes. Many biologically active peptides are
substrates for dipeptidyl peptidase IV (34) but its exact physiological role in
humans is not yet fully known.

Aminopeptidase N (CD13) is 150 kDa zinc-dependent proteolytic enzyme
usually used as a marker for prostasomes (24,35).

**Prostasomal protein kinase activity:**

Stegmayr et al. first demonstrated the presence of protein kinase activities in
the secretory granule and vesicle fraction of seminal plasma (36). Spermato-
za contain protein kinase A (PKA) that may modulate sperm function (37-
40). It was suggested that, in vivo about half the protein kinase in seminal
plasma was bound to prostasomes (41).

**Functional Role of Prostasomes**

Despite the many biophysiological and biochemical expressions of
prostasomes shown *in vitro*, yet their physiological role is not fully known,
but the results of some works could assign them to some important func-
tions.

**Sperm-prostasomes interaction**

Prostasomes can adhere to and, to some extent, fuse with human spermato-
zoa as shown by free zone electrophoresis and electron microscopy (42),
octadecyl-rhodamine fluorescence self-quenching (43) and immunofluores-
cence staining and confocal microscopy (44). The fusion was shown to be
cation-independent, strictly dependent on pH and quite a sperm- specific
phenomenon. Membrane fusion may represent an important physiological
mechanism to help spermatozoa resist the vaginal acidic milieu. Another
result of this fusion could be the transfer of certain prostasomal enzymes to the spermatozoa, i.e. aminopeptidase N (CD13) (45) and dipeptidylpeptidase IV (CD26) (46). Prostasomes were found to promote the sperm forward motility (47-49).

Prostasomes also increased the number of hyperactivated spermatozoa, which is thought to be an important parameter for the penetration of the zona pellucida and subsequently for the fertilization (50).

**Anti-bacterial and anti-viral effect of prostasomes**
Prostasomes have a bactericidal effect (51). Their antibacterial activity is associated with bacterial membrane deformation. Thus the bactericidal mechanism of prostasomes may be due to the effect of prostasomal proteolytic enzymes, which is different mechanistically from that of neutrophil granulocytes, being dependent on the generation of reactive oxygen species (ROS) for their killing of invading microorganisms.

It has been demonstrated that prostasomes could inhibit viral activity, probably via their membrane cofactor protein, CD46 (52). Kitamura et al attributed this activity to the effect that prostasomes functioned like mock cells, and by taking up the virus, rendered it unable to infect the cells.

**Anti-oxidant activity of prostasomes**
Prostasomes can inhibit superoxide anion production by neutrophils (53). It seems that an exchange reaction of lipids (especially of sphingomyelin and cholesterol) between prostasomes and neutrophils results in the inhibition of the NADPH oxidase activity of the granulocytes and therewith abolition of the free radical formation (54).

**Innate-immunosuppressive properties of prostasomes**
Prostasomes have also been shown to contain the complement regulatory proteins CD46 (membrane cofactor protein, MCP), CD55 (decay accelerating factor, DAF), both being expressed in very small amounts, and CD59 (55,56). The complement-regulatory effect of prostasomes (55), is mainly mediated by their content of CD59, which is present in seminal plasma at a concentration of 20-30 µg/mL (56). Beside their content of complement regulatory proteins, prostasomes are considered roughly immunosuppressive due to their effect on neutrophils (53,57,58).

CD59, also known as protectin or membrane inhibitor of reactive lysis (MIRL), is a regulator of complement activation that is expressed on erythrocytes, leukocytes, epithelial and endothelial cells. It is an 18- to 26-kDa glycosylphosphatidylinositol (GPI)-anchored protein (59). CD59 inhibits complement-mediated lysis by preventing full assembly of the membrane attack complex (MAC) on host cells. It binds to C8 in the C5b-8 complex, preventing the polymerization of C9 during the final step of MAC formation.
It is possible to transfer GPI-anchored proteins of seminal plasma such as CD59 to spermatozoa and guinea pig erythrocytes (56).

**Prostasomal Tissue Factor (TF) and Seminal Clotting**

The potent procoagulant activity of seminal plasma added to human blood plasma was first described in 1942 (61). This potent clotting activity is due to a high concentration of functional tissue factor (62-64). Human tissue factor (TF, coagulation factor III, CD142) is a 43-45 kDa, single chain, transmembrane glycoprotein which serves as an essential cofactor for factor VII in initiating the physiological coagulation cascade of the blood. TF is present in seminal plasma in association with prostasomal membrane at a very high concentration (21 ng/mL) (62), compared to the concentration of the free soluble domain of TF in blood which mounts to 85 pg/mL. Most, if not all, of TF activity in seminal plasma is assignable to the prostasomes. TF, beside its role in activation of coagulation of blood, may play a role in controlling the balance of angiogenic and anti-angiogenic factors (65,66). Angiogenesis, angiogenetic proteins and neo-vascularization are important in cancer as well as non-neoplastic conditions as wound healing where TF is usually overexpressed (67). TF, has also been suggested to play a major role as a morphogenic factor during early embryonic development (68,69). Some researchers speculated about the assignment of seminal TF for other functions rather than blood coagulation due to its very high expression in semen in comparison to other coagulation proteins (64,70-72). Prostasomal TF may protect against anti-sperm antibody development and against transmission of infectious agents (62). It is the major factor in inducing the formation of the seminal coagulum which may be important for the sperm biology. TF may also participate in preparation of endometrium for implantation of the fertilized ovum and placental development (73).

Increased coagulation is frequently seen in patients with advanced prostate cancer (74). Multiple studies demonstrated high expression of TF in both primary prostate tumors and in metastases. It is postulated that TF has a role in regulating prostate cancer progress and angiogenesis (75,76). TF was found to up-regulate the expression of IL-8 on the surface of breast carcinoma cells by binding to factor VIIa, which led to increased cell migration and invasion (77). Normally, hemostasis and angiogenesis are tightly regulated processes both being less regulated in cancer. TF is being discussed both as a useful prognostic marker for patients with metastatic prostate cancer (78) as well as a potential target molecule for specific immunotherapy in prostate cancer (79).

Biochemical studies have shown TF to be palmitoylated and phosphorylated via the cytoplasmic domain. These posttranslational modifications are expected to affect its structural and functional properties (80-82).
Seminal Protein Kinases and Protein Phosphorylation

Phosphorylation is a very fast way of regulating proteins and modifying their properties e.g. enzymatic or co-factor activity, affinity for ligands or susceptibility for degradation. Phosphorylation is an enzymatic reaction carried out by protein kinases. Protein kinases are enzymes that catalyze the transfer of the $\gamma$-phosphoryl group of ATP (or GTP) to an amino acid side chain of a protein substrate in the presence of divalent cations. These enzymes could be subdivided into two main groups based on their ability to transfer the $\gamma$-phosphate of ATP or GTP to (i) alcohol groups on serine/threonine, or (ii) phenolic groups on tyrosine residues of their protein substrates.

The serine/threonine protein kinases can be divided further on the functional basis of being either (83):

I. Second messenger dependent [e.g. cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase, diacylglycerol-activated/phopholipid dependent protein kinase C (PKC), $\text{Ca}^{2+}$/calmodulin-regulated protein kinase].

II. Second messenger independent (e.g. casein kinases CKI and CKII).

Protein kinases which have the tyrosine as the target amino acid include the hormone receptor associated kinases (e.g. insulin receptor and epidermal growth factor) and oncogene products (e.g. Src, Ras and Ab1). Protein kinases with dual specificity can phosphorylate tyrosine and serine/threonine residues [e.g. mitogen-activated protein kinases (MAPKs)]. Protein phosphorylation is implicated in controlling several biochemical events such as metabolism, membrane transport, neurotransmission, genomic activation, transcription and cell proliferation (83-85). Protein phosphorylation is reversibly controlled by phosphatases (dephosphorylation).

Protein kinases and phosphatases are secreted among other proteins by the prostate gland. The presence of protein kinases in testis and accessory gland of male reproductive tract and differential sensitivity of theses enzymes has been described before (37,86). The presence in seminal fluid of a cAMP-dependent protein kinase with a high specificity for histone has also been demonstrated (87). Wilson et al. suggested the presence of more than one protein kinase in the seminal fluid (88). They also concluded that the presence of phosphoprotein phosphatases in seminal fluid, under the experimental conditions they used, did not influence protein kinase reaction towards anionic and cationic protein substrates. Speculations about the source of protein kinases suggested that they derived from the prostate gland, spermatozoa and to a lesser extent form the seminal vesicles (87,89,90).

The presence of protein kinase activities in the membranes of secretory granules and vesicles of prostatic origin in human seminal plasma was first demonstrated by Stegmayr et al. (36). They demonstrated that the protein
kinases in these organelles phosphorylated both serine and threonine residues in histones, phosvitin and endogenous prostasomal proteins. Protein kinase activity, mainly belonging to the PKA group of enzymes, is associated with prostasomes (41,91). Prostasomes also show high ATP-cleavage activity which is linked to their membrane (17). This cleavage activity is due to the Mg$^{2+}$ and Ca$^{2+}$ stimulated ATPase enzyme of the prostasomal membrane which is calmodulin-dependent (23).

![Figure 3: Overview on the Tissue Factor Pathway](image-url)
Prostate Cancer

Epidemiology

Carcinoma of the prostate is the fourth most commonly diagnosed cancer in men worldwide, and the most commonly diagnosed in Swedish men (see below). Generally, the incidence of prostate cancer is higher in Western countries than in the Middle East and Asia. Age seems to be a major risk factor that is higher than in most other malignancies. The incidence of the disease between 65-70 years is nearly 350/100,000 and it increases to 1000/100,000 between 80-85 years of age. Only 15% of cases are diagnosed in men before 65 years, while 95% are diagnosed between 45 and 89 years of age with a median age of diagnosis of 72 years.

The serum testosterone levels decrease after the age of sixty (92) and this change is concomitant with an increase in sex hormone binding globulin and plasma estradiol-17ß concentrations (93) suggesting a complex relationship. The disease does not occur in pubertal eunuchs or true eunuchoids (94). It is justified to state that a continuous supply of androgens is necessary both for the development and growth of prostate cancer (95).

Clinically apparent disease is rare under the age of 50 and increases dramatically with age. The age-adjusted incidence and death rates from prostate cancer vary dramatically from country to country as well as between racial-ethnic groups. In 1989 the incidence rates in the USA were highest in blacks (149/100,000 person per year), intermediate in whites (107/100,000) and lowest in Orientals (Japanese 39 and Chinese 28/100,000).

In Sweden the incidence of prostate cancer was 142/100,000 persons in 1998 and 222/100,000 in 2003 which was estimated to be 29.9% and 35.3% of all cancers in male population, respectively. The percentage distribution of the new cases was 15.1% in 1998 and 18.6 % in 2003. The prostate cancer is the most common cancer in Swedish men with an average annual increase of 2.5%. The trend of annual increase was 4% for the years 1993-2003 (96,97).

Pathology

The vast majority of prostate cancers arise within the peripheral zone (see histology) of the prostate gland. They can be divided into four categories on the basis of their natural history and presentation:

- **Clinical carcinomas:** any case in which a diagnosis of prostate cancer is made clinically and confirmed by microscopic examination.
- **Latent carcinomas:** These are carcinomas found at autopsy in males who had no clinical evidence of prostate cancer.
- **Incidental carcinomas:** These are tumors found in 6-20% of samples of prostatic tissue removed surgically from non-malignant disease.
d. **Occult carcinomas:** The patients present with symptoms and signs of metastatic disease due to cancer of the prostate, before the primary site is detected.

On microscopic examination the tumor is principally an adenocarcinoma, which could be divided into four major patterns:

a. **Cribriform pattern:** Which is predominantly intraductal.

b. **Diffuse infiltrating pattern:** In which the cells are poorly differentiated and stream out individually into the stroma.

c. A carcinoma consisting of medium-sized glands in which cytological changes suggestive of malignancy are often not present.

d. A carcinoma consisting of small glands in which cellular atypia is a prominent feature.

### Staging of prostate cancer

The goals in staging of prostate cancer are: (1) to evaluate prognosis and (2) to direct therapy rationally based on the extent of the disease. The tumor, node and metastases (TNM) system for staging of tumor is widely used since 1975. Clinical staging depends on serum tumor markers, tumor grade and imaging modalities.

### Diagnostic Techniques

1. **Immunohistochemical features of carcinoma of prostate:**

Two monoclonal antibodies that react specifically with and mark the tumor cells are being used for diagnostic purposes (immunohistochemistry and ELISA):

   a. **Prostate-specific antigen (PSA):** a glycoprotein localized to the endoplasmic reticulum, but also found within the gland lumina.

   b. **Prostatic acid phosphatase (PAP):** which is normally localized to the lysosomes.

2. **Serum tumor markers:**

   a. **Prostatic acid phosphatase:** which is raised in 60% of localized prostatic cancers and 80% of those with bone metastases.

   b. **Prostate-specific antigen (PSA):** serum levels of PSA are normally less than 4µg/L but there is a slight “physiological” increase with age. Increases above this level were found in 40-60% of patients with localized cancer of the prostate. Similar increase may be found in 30-50% of patients with prostatitis or with nodular hyperplasia of the prostate. Thus the serum PSA concentration is neither very sensitive nor specific for prostate cancer, but it is used as a screening test, which is however, controversial.
Diagnosis
The most important diagnostic measures to investigate prostate cancer are:

1. **Serum PSA level**: Although PSA test has the highest positive predictive value for cancer of prostate, the use of PSA without DRE is not recommended.
2. **DRE (Digital Rectal Examination)**: Prostate biopsy is recommended for all men who have DRE abnormalities, regardless of the PSA level, because 25% of men with prostate cancer have PSA levels less than 4µg/L.
3. **TRUS (Transrectal Ultrasound)**: Any patient with a DRE suspicious for cancer or a PSA level elevation should undergo prostatic biopsy (TRUS-Directed Prostate Biopsy) regardless of TRUS findings.

Some other diagnostic measures could be taken throughout the course of the disease, e.g. MRI (Magnetic Resonance Imaging), MRS (Magnetic Resonance Spectroscopy), prostatic scintiscan and skeletal scintiscan.

Complement System
Introduction
The complement system is an important part of the body’s innate immune system. It comprises about 30 distinct plasma and cell bound proteins that react with one another to opsonize pathogens and induce a series of inflammatory responses that help fight infection. The complement activates in the absence or presence of antibodies through triggered-enzyme cascades. The complement system achieves its function through main effector mechanisms:

1. Opsonization of pathogens by C3b and C4b;
2. Recruitment of inflammatory cells, mediating chemotaxis and release of anaphylatoxins (C3a and C5a);
3. Lysis of certain pathogens and cells by C5b-9 complex;
4. Recognition and clearance of apoptotic cells;
5. Handling of immune complexes.

Activation of Complement
**The classical pathway (CPW)**
The classical pathway (CPW) was the first complement pathway to be discovered. It consists of three different complement components: the C1 complex (C1q, C1r, C1s), C2 and C4 (98-101). In its non-activated form C1r interacts with C1s to form a Ca$^{2+}$- dependent tetramer (C1s-C1r-C1r-C1s), which binds to C1q to form C1. C1 binds to Fc parts of IgG or IgM mole-
ules already bound to an antigen. Activated C1 cleaves C4 into C4a and C4b. C2 binds to C4b which is cleaved by C1s to form C4b,2a complex, also known as C3-convertase of the CPW, which can cleave and activate C3 into C3a and C3b (102,103).

**The lectin pathway (LPW)**
The lectin pathway (LPW) is the most recently described pathway that is activated by certain carbohydrates on microbial surfaces. The mannan-binding lectin (MBL) is a plasma glycoprotein of thecollectin family (104) which initiates complement activation upon binding to carbohydrates on microorganisms causing their lysis via the activation of C4 and C2 (as described above) thus leading to the recruitment and deposition of C3 and C5b-9 with the generation of C5a (a potent chemotactic factor) (105-110). MBL also regulates the activity and substrate recognition by mannan-protein-associated serine proteases-1,2&3 (MASP-1,2,3) which are homologous to C1r and C1s of the classical pathway of complement activation (111-114).

**The alternative pathway (APW)**
The alternative pathway (APW) was discovered after the classical pathway and is activated in the absence of specific antibodies (115). It represents an important part of innate immunity, due to its ability to differentiate between self and non-self (116). This pathway includes four complement components: C3, factor B, factor D and properdin (factor P) (117-121). C3 is specifically activated by the classical and the alternative pathway convertases into the anaphylatoxin C3a and C3b (122-124). The non-cleaved C3 molecule also called iC3 or C3(H2O) is an activated form of C3 which possesses a C3b-like function (125-131). Factor B is the zymogen of the APW convertase that is cleaved to Ba and Bb fragments. The Bb fragment binds to C3b to form the APW convertase C3b,Bb (132,133). While factor D is the limiting step in the alternative pathway activation, factor P (properdin) stabilizes the APW convertase (134-137). By binding to C3b,Bb factor P extends its half-life (138,139).

**The terminal pathway**
The binding of C3b fragment to classical or alternative pathway convertases alters their specificity from C3 to C5 generations and cleaves C5 into C5a and C5b. C5a is potent chemotactic and anaphylactic component and C5b binds to C6 and then C7 to form a complex able of membrane insertion that binds subsequently to C8. The C5b-8 complex acts as an initiator and accelerator of C9 polymerization. C9 polymerizes and assembles to C5b-8 on the cell membrane by up to 18-25 molecules in the form of a pore. C5b-9 is known as the membrane attack complex (MAC) that forms pores in the cell membrane leading to cell lysis and death (110,140-144). In some cases sublytic concentrations of membrane attack complex have been shown not to
cause cell lysis but rather initiate signals that lead to cell apoptosis (145-147) or stimulate some cell types like the platelets (148).

Regulation of Complement
The activation of complement is under strict control in order to avoid unwanted activation of the complement on host cells. Complement regulators are either soluble or membrane bound regulatory proteins. Important soluble regulatory proteins are C1-INH, factor H and C4bp. C1-INH is a plasma protease inhibitor involved in the regulation of the CPW and reduced function of C1-INH causes the clinical syndrome hereditary angioneurotic edema (HANE). The membrane bound complement regulatory proteins are complement receptor 1 (CR1, CD35), membrane co-factor protein (MCP, CD46), decay accelerating factor (DAF, CD55) and membrane attack complex inhibitory factor (MACIF, CD59), also known as membrane inhibitor of reactive lysis (MIRL) or protectin. Regulation is exerted mainly at the level of C1 complex, C3 convertase and membrane attack complex (MAC) formation.

Membrane co-factor protein (CD46)
Binds C3b and C4b, allowing their degradation by Factor I. The degraded proteins are no longer able to participate in convertase complexes and further complement activation is thus terminated.

Decay-accelerating factor (CD55)
CD55 mediates inhibition of complement by facilitating dissociation of CPW C3 convertase (C4b,2a) into C4b and C2a. It also promotes the dissociation of alternative complement pathway C3 convertases (C3b,Bb) into C3b and Bb as well as inhibition of the C5 convertases (C4b,2a,3b and C3b,Bb,C3b) on the cell surface.

Membrane Inhibitor of Reactive Lysis (CD59)
CD59 is a complement regulatory protein that is found on erythrocytes, lymphocytes, endothelial and epithelial cells. It is detectable in most body fluids. CD59 inhibits MAC formation by binding to sites on C8 and C9. This blocks the polymerization and assembly of multiple C9 molecules into the complex (141). CD59 molecular weighs between 18-26 kDa and it anchors to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor. In a clinical condition known as paroxysmal nocturnal hemoglobinuria (PNH) some clones of erythrocytes may lack CD59 thus become vulnerable to complement-mediated lysis resulting in intravascular hemolysis.

It has been postulated that the protein kinases (PKs) may play a role in complement regulation. Depending on their physiological substrate PKs may be able to inhibit the formation of convertases or MAC thus inhibiting comple-
ment activation. Through this mechanism PKs may play a possible role in the development and/or aggravation of some pathological conditions like cancer. Paas and Fishelson have described tyrosine and serine/threonine ecto-protein kinases on intact cells, e.g. monoblastoid cell line U937, and on microparticles derived from these cells, which among several other proteins phosphorylate C9 and to some extent C3 (149). In another work by Ekdahl and Nilsson, it has been demonstrated that C3 synthesized by monoblastoid cell line U937 was phosphorylated by CKII (150).

Role of Complement in Tumor Surveillance

It has been suggested that the complement system is involved in the immunosurveillance against tumors (151). Complement activation on tumors may occur in response to the formation of immune complexes containing anti-tumor antibodies, through spontaneous activation of the APW in response to altered cell surface expression (152) or through tissue destruction resulting from tissue ischemia and necrosis (153). Various tumors have been shown to use different cooperating and synergistic mechanisms to evade the attack by the complement system, indicating that evasion of complement attack is one of the basic mechanisms in tumor development (154,155). An important mechanism is the over-expression of complement regulatory proteins such as MCP (CD46), DAF (CD55) and CD59, on the tumor cell surface or in the extracellular environment in e.g. thyroid, prostate, gastric, breast, colon and ovarian cancer (151,154-162). Donin and coworkers (154) have shown through antibody blocking that CD59 produces a more complete inhibition of the complement cascade than does CD46 or CD55. They also demonstrated that blocking of CD59 with monoclonal antibody (mAb) sensitizes breast, ovarian and prostate carcinoma cells to complement attack. An interesting observation has been made by Gazouli et al. in that the CD59 gene contains regulatory elements controlled by p53, suggesting that p53 up-regulates CD59 on the tumor cell surface (163).
Figure 4: Overview of Complement Activation Pathways and Their Main Consequences

MBL = mannan binding lectin
CPW = classical pathway
APW = alternative pathway
Paroxysmal Nocturnal Hemoglobinuria (PNH)
Definition, Etiology and Pathogenesis
Paroxysmal nocturnal hemoglobinuria (PNH) is an uncommon acquired clonal hematologic stem cell disorder classified as an intravascular hemolytic anemia. The main etiology of disease is the deficiency of CD59 on PNH cells (164,165). Deficiencies of CD59 and other GPI-anchored proteins on PNH cells may be the result of deficient biosynthesis of the GPI anchor (162,166-170).

Clinical Manifestations
PNH shows usually an insidious onset, while the course tends to be prolonged and constant in all individuals. The classical symptom of discolored urine (nocturnal hemoglobinuria) results from increased hemolysis during sleep probably due to retention of CO₂ with slight fall in blood pH.
Aims of the Present Investigation

The aims of this thesis were to compare prostasomes of seminal and malignant cell origin regarding:

- the expression, function and transfer of the complement regulatory protein CD59 to CD59-deficient autologous, allogeneic and xenogenic cells.
- other mechanisms to regulate the complement system in prostate cancer and the significance of those mechanisms for cancer development.
- the protein kinases profile and their specificity for exogenous and endogenous prostasomal substrates and the patho-physiological impact on the development of prostate cancer.
- the prothrombotic properties in whole blood and plasma, the expression and role of prostasomal tissue factor.
Figure 5. Schematic Representation of Results and Conclusions Included in the Thesis

**PKs**: Protein Kinases (prostasomal), **DIC**: Disseminated Intravascular Coagulation, **TF**: Tissue Factor, **PKA**: Protein Kinase A
Materials and Methods

I. Preparation of Prostasomes

1. Seminal Prostasomes [Papers I-IV]. Prostasomes were isolated from human semen which was left to liquefy and then centrifuged to separate the spermatozoa from the seminal plasma. The seminal plasma was then centrifuged to obtain the prostasomes-containing pellet, which was re-suspended in isotonic and further purified by gel filtration (17). Thereafter the prostasomes marker enzyme aminopeptidase was analyzed to identify the prostasomes containing fractions (17,24) which were pooled and ultracentrifuged and then stored at -70°C.

2. Prostasomes from Prostatic Cancer Cell Lines [Papers II-IV]. Three human prostatic cancer cell lines were used: DU145 from brain metastasis (171), PC-3 from bone metastasis (172) and LNCaP from lymph node metastasis (173).

Cells growing in monolayers were harvested, ultrasonicated on ice, then centrifuged and the supernatant collected. The supernatant, which contained prostasomes, was ultracentrifuged and subjected to further purification as described above. Cell lines prostasome productivity was in agreement with previously obtained data (174). In addition, cell culture medium was harvested, cell debris removed by centrifugation and prostasomes collected by ultracentrifugation as described above.

3. Prostate Gland Derived Prostasomes (Native Prostasomes) [Paper II]. Tissues from prostate glands were obtained from three patients who had undergone vesico-prostatectomy for adenocarcinoma of the urinary bladder with no primary involvement of the prostate gland. The tissue samples were homogenized, cell debris was removed by centrifugation and the obtained supernatants were ultracentrifuged and further purified as described above.

4. Heat Treatment of Seminal Prostasomes [Paper I]. For stability studies seminal prostasomes were heat treated at 60, 70, 80, 90 and 100°C for 30 min.

5. Treatment of Prostasomes with PIPLC [Paper I]. In order to remove CD59 and other GPI-anchored proteins, seminal prostasomes were treated with PIPLC [Phosphoinositol-specific phospholipase C (PIPLC)] as described before (175).

6. Detergent Treatment of Prostasomes [Papers III, IV]. In order to reduce their lipid contents, seminal, DU145, PC-3 and LNCaP prostasomes were
incubated with saponin or n-Octylglucoside solution followed by ultracentrifugation.

7. Protein Composition of Prostasome Preparations [Paper II]. The protein content and composition of each prostosomal preparation was visualized by SDS-PAGE under reducing conditions.

II. Experiments on Prostasomal CD59

1. Expression of CD59 on Prostasomes Detected by Flow Cytometry (FACS) [Paper II]. Expression of CD59 by seminal, DU145, PC-3, LNCaP and native prostasomes was detected using anti-human anti-CD59 antibody and visualized by FACS.

2. Expression of CD59 on Prostasomes Detected by Dot Blot [Paper II]. The precipitates collected from cell culture supernatants were subjected to dot blot analysis using antibody against prostasomes (176) and the expression of CD59 was visualized by anti-CD59.

3. Transfer of Prostasomal CD59 to RE, PNH Erythrocytes and PIPLC or Unmanipulated Prostatic Cancer Cells [Paper II]
   a. Preparation of Erythrocytes for the Transfer and Hemolytic Assay Experiments:
   RE were washed and suspended in GVB-Mg EGTA buffer (below) in accordance with a previously described method (177) [Paper I, II]. Samples, collected from three patients with PNH were washed and suspended in GVB-Mg EGTA buffer [Paper I]. The normal red blood cell control obtained from an apparently healthy individual and subjected to the same washing procedure [Paper I].
   b. Transfer of Prostasomal CD59 to RE and PNH Erythrocytes: [Paper I, II]
   Rabbit erythrocyte (RE) suspension was incubated with serial dilutions of seminal, DU145, PC-3, LNCaP, or native prostasomes then washed with GVB-Mg EGTA buffer. The transfer of prostasomal CD59 to RE was detected with the appropriate antibodies and visualized with FACS. PNH erythrocytes suspension was pre-incubated with seminal prostasomes. The transfer of prostasomal CD59 was detected as described above.
   c. Treatment of Cancer Cells with PIPLC and subsequent Transfer of Prostasomal CD59: [Paper II]
   DU145, PC-3 and LNCaP cells were treated with PIPLC to remove their CD59. After washing incubation with of DU145, PC-3 or LNCaP prostasomes followed. The transfer of prostasomal CD59 was detected as described above.
   d. Transfer of Prostasomal CD59 to Unmanipulated Prostatic Cancer Cells: [Paper II]
Cancer cells (DU145, PC-3 or LNCaP) were incubated with DU145, PC-3 or LNCaP prostasomes and each type of cells with seminal prostasomes. The transfer of prostasomal CD59 was detected as described above.

III. Hemolytic Assay of Complement Alternative Pathway
1. Normal Sera and Buffers [Paper I, II]
The APW buffer (GVB) [ethylene glycol tetraacetate (EGTA)], which contained gelatin. To inactivate the CPW MgCl₂ was also added. Fresh human serum collected from apparently healthy donors. After pooling and recentrifugation serum aliquots were stored at -70°C. During subsequent experiments, each aliquot was thawed only once.
2. Complement-Mediated Hemolysis [Paper I, II]
To test the APW of the complement system RE were incubated with serum. The serum concentration was adjusted by dilution in APB to give approximately 50-70% lysis of the total amount of RE. The incubation was stopped by addition of cold EDTA buffer, followed by centrifugation. The optical density of the supernatant was monitored in a spectrophotometer at 405 nm as a measure of hemolysis.
3. Pre-incubation of Rabbit and PNH Erythrocytes with Prostasomes [Paper I, II]
RE or PNH were pre-incubated with prostasomes in serial dilutions. The erythrocytes were then washed remove the prostasomes before incubation with serum and the reaction was stopped and read as described above. Alternatively heated or PIPLC-treated prostasomes were incubated with RE [Paper I].
RE suspension was pre-incubated with PC-3 prostasomes. After washing to remove the prostasomes RE were incubated with anti-CD59 antibody. The RE were washed to remove excess antibody before incubation with serum in the appropriate concentration. The reaction was stopped and read as described above.

IV. Prostasomal ATPase and Protein Kinases
The determination of ATP stability was important because of the presence of ATPase on the prostasomes. Since ATP is a substrate in the phosphorylation reaction, the breakdown of ATP is anticipated to limit the rate of the phos-
phorylation reaction. ATP was incubated with seminal, DU145, PC-3 and LNCaP prostasomes in conditions similar to those used for PKA, PKC and CK phosphorylation experiments. The remaining ATP was measured by luciferin-luciferase technique. Detergent-treated prostasomes were analyzed in parallel.

2. Detection of Prostasomal ATPase and Protein Kinases by Flow Cytometry

[Paper III]

Seminal, DU145, PC-3 or LNCaP prostasomes were incubated with anti-CKIIα, anti-PKAγ, anti-CKIΔ and anti-ATPase. After the addition of FITC-conjugated secondary antibody samples were analyzed with FACS.

3. Enzymatic Characterization of Prostasomal Protein Kinases [Paper III]

a. Assay for Protein Kinases A, C and Casein Kinase (PKA, PKC, CK):

Seminal, DU145, PC-3 and LNCaP prostasomes were incubated with histone or Tris buffered saline in the presence of Mg\(^{2+}\) and cAMP (PKA), Ca\(^{2+}\) (PKC) or Mn\(^{2+}\) (CK) and \(^{32}\)P ATP. The reaction was interrupted by the addition of electrophoresis sample buffer followed by boiling. All samples were subjected to SDS-PAGE followed by Coomassie staining of the gels. The level of radioactivity determined by Phosphor Imager and images obtained were analyzed using image analysis software.

b. The Effect of PMA and Protein Kinase Inhibitors:

Detergent-treated prostasomes were used to test the effect of PMA and protein kinase inhibitors H89 (PKA), staurosporine (PKC), CKI-7 (CKI) and DRB (CKII) on prostasomal kinases. Detergent-treated prostasomes were used in this test to avoid the hydrophobic behavior of PMA and protein kinase inhibitors and to reduce the ATPase activity of prostasomes. PMA and protein kinase inhibitors in the appropriate concentrations were pre-incubated with detergent-treated seminal, DU145, PC-3 and LNCaP prostasomes. Thereafter, phosphorylation substrate, ions and \(^{32}\)P ATP were added and the analysis was completed as described above.

4. Phosphorylation of Fibrinogen, Vitronectin, E-cadherin and Complement Component (C3) by Prostasomal Protein Kinases

Fibrinogen or C3 [Paper III], vitronectin [Paper IV] or E-cadherin (summary) were phosphorylated using detergent-treated seminal, DU145, PC-3 and LNCaP prostasomes in the presence of Mg\(^{2+}\) and cAMP, Ca\(^{2+}\) or Mn\(^{2+}\) and \(^{32}\)P ATP. The reaction was completed as described above.

V. Prostasomal Tissue Factor (TF) [Paper IV]

1. Expression of TF Prostasomes Detected by Flow Cytometry

Seminal, DU145, PC-3 or LNCaP prostasomes were incubated with FITC-labelled monoclonal anti-human TF antibodies. Samples were analyzed with FACS.

2. Immunoprecipitation of Phosphorylated TF from Solubilised Prostasomes
Phosphorylation was performed under conditions optimized for PKA activity. After phosphorylation polyclonal anti-TF antibody was precipitated with Protein G-Sepharose prior to SDS-PAGE as described above.

3. Enzyme immunoassay (EIA) for TF
Native or solubilised prostasomes were coated onto ELISA plates. Thereafter TF was detected by HRP-conjugated anti-TF. The TF content in lysed cellular fractions (see below) was also analyzed.

VI. Prothrombotic Experiments [Paper IV]

1. Clot Formation Induced by Prostasomes in Re-Calcified Plasma and Whole Blood
Clot formation in the presence and absence of seminal, DU145, PC-3 or LNCaP prostasomes in citrate, re-calcified plasma or whole blood was measured by clot assays. In both cases the clotting time (end point) is given in seconds.

2. Turbidimetric Assessment of Fibrin Formation in Re-calcified Plasma
Intact (nonsolubilised) prostasome-induced fibrin gel formation in re-calcified plasma was monitored in polystyrene cuvettes at 600 nm, in spectrophotometer as previously described (178). In some cases prostasomes were pre-incubated with ATP, Mg$^{2+}$ and cAMP under conditions optimized for PKA activation (see above). In other experiments the prostasomes were pre-incubated with the TF inhibitor ASIS (active site inhibited recombinant FVIIa), an inhibitory anti-TF antibody, or the contact activation pathway inhibitor CTI (Corn Trypsin Inhibitor) prior to the addition of plasma.

3. Enzyme Immunoassays (EIA) for Coagulation and Fibrinolysis Markers
   a. Factor XIIa-antithrombin (FXIIa-AT):
      Complexes formed between Factor XIIa and AT were measured in plasma according to a previously described method (179).
   b. VIIa-antithrombin (FVIIa–AT):
      Plasma factor VIIa-antithrombin (FVIIa–AT) complexes were quantified using capture anti-FVIIa and a biotinylated detecting anti-AT antibody followed by HRP-conjugated streptavidin.
   c. Thrombin-Antithrombin (TAT):
      Plasma levels of thrombin-antithrombin complexes (TAT) were measured using antibodies. Anti-human thrombin was used as capture antibody detected with HRP-coupled anti-human antithrombin antibody. Values were expressed as mg/L.
   d. D-dimer:
      D-dimer was analyzed using the Imuclone D-dimer ELISA kit.

4. Incubation of Prostasomes in Whole Blood
PC-3 or DU145 prostasomes were incubated with citrate blood. The blood was then subjected to a series of centrifugation and filtration procedures to
obtain three cellular fractions, erythrocytes, leukocytes and platelets. Ali-
quots of the three cellular fractions were incubated with FITC-conjugated chicken anti-prostasome antibodies and analyzed by FACS as described above. In addition, other aliquots of cells from prostasome-incubated blood were solubilised and ultrasonicated. The cell debris was removed by cen-
trifugation and the TF content of the supernatant and the platelet-poor plasma (PPP) were analyzed by EIA.
Results and Conclusions

Four different studies have been carried out. In the first paper we studied the transfer of CD59 from seminal prostasomes to CD59-deficient erythrocytes (rabbit erythrocytes and human PNH erythrocytes). In the second paper we extended the study of CD59 transfer to include prostasomes from malignant cell lines, namely DU145, PC-3 and LNCaP. We studied the transfer of CD59 to RE, malignant cells stripped of CD59 by PIPLC and unmanipulated cells. We also studied the expression of CD59 by different types of prostasomes. In the third work we characterized the prostasomal protein kinases, ATPase activity and we identified two physiologically relevant substrates for prostasomal kinases. In the fourth paper we studied the expression of TF by different prostasome preparations. We characterized the effect of prostasomes on clotting time of plasma and whole blood. We also identified TF as the most abundant endogenous substrate for prostasomal PKA.

Paper I

In the first paper we investigated the possibility of transfer of prostasomal CD59 to CD59-deficient erythrocytes. We used RE and human erythrocytes obtained from PNH patients. The RE pre-incubated in different concentrations of seminal prostasomes acquired a dose-dependent resistance to complement lysis during the subsequent incubation with serum. The control erythrocytes pre-incubated in buffer lacking seminal prostasomes remained susceptible to lysis during the subsequent incubation with serum. The saturation point of prostasomal protection was obtained at a low prostasome concentration of about only 40µg/mL with a very little additional effect of prostasomes up to a concentration of 200µg/mL. When heated at 100°C for 30 min, prostasomes lost their ability to protect the RE from complement-mediated lysis. The loss of efficacy of prostasomes was directly proportional to temperature. The RE incubated with unheated prostasomes gained maximum protection.

The RE pre-incubated with prostasomes already pre-treated with PIPLC gained protection against subsequent complement lysis to a lesser extent compared with erythrocytes pre-incubated with prostasomes without this pre-treatment. The RE pre-incubated with prostasomes and then treated with PIPLC lost most of the protection already gained.
The PNH erythrocytes pre-incubated with prostasomes acquired resistance to complement lysis nearly to the level of normal erythrocytes while those pre-incubated with buffer lacking prostasomes remained susceptible to lysis. The PNH cells pre-incubated with prostasomes and then treated with PIPLC remained protected by prostasomes. The PNH cells pre-incubated with prostasomes already pre-treated with anti-CD59 antibodies showed a lesser resistance to subsequent complement lysis than those pre-incubated with unmanipulated prostasomes.

The flow cytometry results showed that CD59 was transferred from prostasomes to RE during pre-incubation. The amount of CD59 acquired by the RE was less when pre-incubated prostasomes were heated at 80°C. The PNH erythrocytes acquired the CD59 from the prostasomes in the same fashion as the RE.

These results support the idea that the transfer of CD59 from prostasomes to autologous or allogeneic cells can protect them from lysis elicited by C5b-9. It is therefore likely that prostasomes are the source of CD59 that can provide spermatozoa with further protection against the complement attack. This effect may be also extendable to the ovum once entering the uterus due to strong interaction between prostasomes and spermatozoa.

Paper II

In the second paper clear differences were demonstrated in protein composition of prostasomes of different origins, although all prostasome preparations contained similar amounts of protein.

The presence of variable amount CD59 was established by flow cytometry in all the types of prostasomes. Low CD59 expression was seen in seminal prostasomes and native prostasomes with only few particles of detectable levels of CD59. In contrast, prostasomes derived from the malignant PC-3 and DU145 cell lines contained a higher amount of CD59 than did the prostasomes from malignant LNCaP cells or prostasomes of nonmalignant origin.

FACS cytometric analysis showed that CD59 from all types of prostasomes tested (seminal, DU145, PC-3, LNCaP, and native) was transferable to RE and PIPLC-treated DU145, PC-3 and LNCaP cells (to the pre-treatment level), in addition to the unmanipulated LNCaP cells.

RE pre-incubated in different concentrations of PC-3, DU145, or seminal prostasomes acquired a dose-dependent resistance to complement lysis during the subsequent incubation with human serum (unlike RE pre-incubated in different concentrations of LNCaP and native prostasomes). The protective effect of prostasomes on RE could be counteracted by a subsequent incubation of prostasome-treated RE with an anti-CD59 mAb.
These results suggest that CD59 is over-expressed by prostatic malignant cell lines, especially DU145 and PC-3, and may be released into the extracellular space in association with prostasomes. In conclusion, we suggest that malignant cells use prostasomes as a versatile defense system against the complement attack.

**Paper III**

In the third paper the expression and activity of ATPase and all protein kinases (PKA, PKC and CK) were studied using non-solubilised and solubilised prostasomes. Seminal prostasomes degraded the added ATP almost completely during incubation. LNCaP prostasomes expressed negligible amounts of ATPase activity while higher and similar activity was found associated with the other prostasomes of different origins. This would indicate that LNCaP protein kinases will be active at much lower concentrations of free ATP compared to the others. These results were confirmed using flow cytometry. PKA, PKC and CK activities were detected in all prostasome preparations when measured using non-solubilised and solubilised prostasomes. Prostasomes of malignant origin showed significantly higher PKA activity and moderately increased CK expression compared to those from seminal fluid. In contrast, similar low PKC-activity was seen for all preparations. The profile of protein kinase expression differed between the preparations. The amount of endogenous substrate phosphorylation present in the prostasome preparations varied considerably with significantly higher amounts in all the preparations of malignant origin compared to seminal prostasomes. The differences in the composition of prostosomal proteins between the four prostasome preparations used in this study may play a role in the profile of these results as reported previously (180).

By using flow cytometry it was demonstrated that all types of prostasomes of malignant origin expressed higher levels of PKA and CKII compared to seminal prostasomes. PKC could not be demonstrated at all on seminal prostasomes and was found in negligible amounts on malignant cell prostasomes. Fibrinogen which has a role in tumor angiogenesis and metastasis and complement component C3 which is the key protein in the complement cascade were identified as two biologically and highly interesting substrates for prostasomal kinases.
Paper IV

In the fourth paper we studied the prothrombotic properties of prostasomes. Using flow cytometry we demonstrated that all types of prostasomes of malignant origin expressed considerably higher levels of TF compared to seminal prostasomes. These data were confirmed by EIA on intact or solubilised prostasomes where the highest levels of TF were detected on DU145-derived prostasomes closely followed by PC-3 prostasomes and LNCaP prostasomes. The TF content in seminal prostasomes was appreciably lower. All prostasome preparations shortened the clotting time of re-calcified plasma and whole blood significantly, especially by the addition of DU145 and PC-3-derived prostasomes followed by LNCaP and seminal prostasomes.

Under conditions favoring PKA activity and using immunoprecipitation and SDS-PAGE and Phosphoimage analysis, TF was established as a predominant endogenous substrate by phosphorylation of all prostasome preparations of malignant origin. Phosphorylated TF was also precipitated from the samples containing seminal prostasomes with very much lower activity in seminal prostasomes compared to the others.

Exogenously added vitronectin was identified as a substrate for prostasomal protein kinases as it was phosphorylated to a high extent by prostasomal PKA and CKII and to a lower degree by PKC, by all prostasomal preparations with more intense phosphorylation elicited by all prostasomes of malignant cell origin compared to seminal ones.

The FACS detection of prostasomal antigens on blood fractions preincubated with PC-3 or DU145 prostasomes showed a low expression of antigens from PC-3 prostasomes and a higher expression of antigens from DU145 prostasomes on RBCs. A subpopulation of WBC, most likely corresponding to granulocytes expressed moderate to high amounts of antigens from both prostasomal preparations with no prostasomal antigens detected in PPP in any of the samples.

The parallel samples analyzed by EIA for TF showed that TF was found in WBC from blood incubated with DU145 and PC-3 prostasomes, high level of TF was detected in PPP and the amount in lysed RBC was close to the detection limit for the assay. No TF was detected in control samples (RBC, WBC or PPP from blood which had not been in contact with prostasomes).

Additional Data:

We tried to identify more substrates for prostasomal protein kinases with physiological relevance. E-cadherin, a 38 kDa transmembrane glycoprotein, is one of the most important matrix proteins and plays a crucial role in cell-
to-cell adhesion. The phosphorylation of E-cadherin affects the cells aggregation and adhesion. The preliminary results of experiments on E-cadherin identified it as a possible exogenous substrate for prostasomal PKA. The degree of phosphorylation was much higher with malignant cell prostasomes (Fig.6).

**Figure 6: Phosphorylation of exogenous E-cadherin by Prostasomal PKA (Lanes 1,3,5,7). Lanes 9 and 10 show non-phosphorylated E-cadherin and phosphorylated casein, respectively. Lanes 11 and 12 show Coomassie stained gel corresponding to lanes 9 and 10.**
Discussion

Prostasomes possess a pluripotency in reproduction by which they ensure the spermatozoa abilities to pass and survive in the lower and upper female genital tract, to penetrate the zona pellucida and successfully fertilize the ovum. These potencies of prostasomes are, however, a double-edged sword. It is a long-known principle that tumor cells tend to exploit the host’s physiological systems in order to get support in terms of, for example, nutrition, growth or metastasis. Hence, we wanted to investigate whether the prostosomal abilities that were favourable for the reproduction process also could be favourable for the prostasome-producing, poorly differentiated neoplastic prostate cells to survive as metastases. Our hypothesis was based on the abundant occurrence of CD59 in the prostasomal membrane and on our initial finding that this membrane-bound CD59 could be released and transferred to CD59-deficient red blood cells, which were herewith rendered resistant to a subsequent complement attack and hemolysis. These unambiguous effects on the otherwise unprotected red blood cells against a complement attack tempted us to further study these phenomena in prostasome-producing prostate cancer cells and their ability to counteract the immune defense. This was also justified by the fact that prostate cancer cells were indeed able not only to synthesize but also to release prostasomes to their close extracellular environment.

In this study (Paper II) we found that prostasomes produced by cells of malignant and non-malignant origin (including seminal prostasomes) showed different expressions of CD59 and different abilities of complement regulation. Although all types of prostasomes share the property of being able of inhibiting the complement system (Paper I, II), this property was augmented in prostasomes of malignant cells. Flow cytometric analysis of the CD59 content on the different types of prostasomes suggested that the expression of prostasomal CD59 is increased when the glandular epithelial cells of the prostate turn malignant. Both PC-3 and DU145 cells produced abundant amounts of CD59-positive prostasomes; DU145 derived prostasomes showed a higher mean intensity while PC-3 produced a subpopulation with the highest expression of CD59. In contrast, LNCaP produced prostasomes with a much lower mean CD59 intensity. Interestingly, those results closely resemble those obtained by Jarvis et al. (159), who using FACS on intact cells detected high levels of CD59 on PC-3 followed by DU145, and with much lower expression on LNCaP.
On the whole, the malignant cell lines produced proportionally more CD59-positive prostasomes than did non-malignant cells, and the prostasomes produced by one of the malignant cell lines also expressed a much higher concentration of CD59 than did the others.

A previous work of Rooney et al. (55) showed that spermatozoa and guinea pig erythrocytes could acquire CD59 of seminal plasma and prostasomes. In papers I and II we present data showing that erythrocytes lacking CD59, and therefore susceptible to complement-mediated lysis, acquired resistance to lysis after pre-incubation with purified seminal prostasomes. The RE are activators of the APW of the human complement and they do not have a functional CD59 against the human complement system. Our data from paper I suggest that RE became resistant to human complement-mediated lysis by acquisition of human CD59 from seminal prostasomes. This property of prostasomes was dose dependent. At a prostasome concentration between 40-100µg/mL, RE reached their maximum level of resistance to complement lysis most probably because of the saturation of the CD59 acceptor sites on the erythrocyte membrane.

Transfer of CD59 from malignant cell prostasomes to RE also rendered the erythrocytes less susceptible to complement lysis indicating a preserved functionality after transfer (Paper II). In agreement with the increased amount of CD59-positive prostasomes produced by PC-3 and DU145 cells, the prostasomes from these cells exhibited the most pronounced inhibitory effect. The limited antihemolytic effect of the native and LNCaP prostasomes could be related to the low proportion of CD59-positive prostasomes they produced (Paper II).

The pre-incubation of PNH erythrocytes with seminal prostasomes led to what could be described as a normalization of the erythrocytes as regards their resistance to the complement attack in nearly the same way as shown by normal human erythrocytes (Paper I). As was indicated with flow cytometry, the normalization was the result of the transfer of prostosomal CD59 to the PNH erythrocytes. This was underlined by the blocking experiments with anti-CD59 antibodies and we concluded that the complement regulatory effect of prostasomes was indeed due to the transfer of CD59. The blocking effect of anti-CD59 antibodies increased the susceptibility of the normal erythrocytes to complement lysis. The inhibition of CD59 by the specific antibody resulted in cells resembling the PNH cells, which concurred with data from a previous study (181).

We also studied the effect of heat and PIPLC as physical and biochemical perturbance on the protective capability of prostasomes (Paper I). Heating did not completely inhibit the protective effect of prostasomes except at boiling point, probably due to denaturation of proteins. This resistance to heat may be due to the unique membrane architecture of prostasomes with a very high molecular ordering (20). PIPLC from Bacillus cereus was known to facilitate the release of proteins from their GPI-anchor (175). The effect of
PIPLC on the complement-regulatory property of prostasomes was unambiguous. This was interpreted as the release of CD59 by the action of PIPLC on the GPI-anchor and its recovery in the supernatant after ultracentrifugation. The released CD59 seemed to still retain its ability to bind to RE and to exert its regulatory function. This could also be verified (by inclusion of supernatant in incubation medium) by the slight, but still inhibitory effect on the APW. Whether the CD59 retained in the supernatant had the same physico-chemical properties as that structurally linked to the prostasomes remains to be elucidated.

The acquired resistance of rabbit erythrocytes pre-incubated with prostasomes to complement-mediated lysis was also affected by PIPLC-treatment of the pre-incubated erythrocytes, most likely due to its action on the GPI anchor of CD59 (Paper I). This effect was not applicable to similarly treated human PNH erythrocytes, which may be because of a different site of insertion of the GPI anchor into the cell membrane and inaccessibility of PIPLC to this anchor. These results concur with previous results (164) demonstrating that PIPLC could cleave only 10% of CD59 from the normal human erythrocytes and hence, this was most probably because of the inositol phosphatide association being inaccessible to PIPLC. This suggests that the inability of PIPLC to release CD59 acquired by the human PNH erythrocytes from prostasomes (Paper I) was a result of the insertion of CD59 in erythrocyte membrane rather than adherence, which in turn resulted in apparent normalization of the erythrocytes.

The transfer of prostasomal CD59 to PIPLC-treated malignant cells (Paper II) indicated that prostasomes could serve as a source of CD59 for malignant cells in vivo. However, the unmanipulated DU145 and PC-3 cancer cells did not acquire additional CD59 when incubated with prostasomes. This finding suggests that all the acceptor sites on these cells were already saturated with CD59 (possibly due to the transfer from endogenous prostasomes), a conclusion that is in agreement with previous studies showing that CD59 is over-expressed on prostatic cancer cells (159). In contrast, the untreated LNCaP cells still had unoccupied CD59-binding sites and were in this respect comparable to the PIPLC-treated DU145 and PC-3 malignant cells.

The transfer mechanism, i.e. the release of CD59 from prostasomes and its insertion in the membrane of acceptor cells, is not known. Väkevä et al noticed that isolated CD59 was transferred to RE in vitro by a mechanism involving high density lipoproteins (HDL) as carriers (182). This mechanistic approach was further elaborated by Sloand et al who showed that CD59 could be transferred to PNH erythrocytes in vitro using HDL preparations rich in CD59 (183). Kooyman et al demonstrated that proteins containing (GPI)-anchor could undergo a transfer between membranes of cells in vivo (184). Since CD59 is a GPI-anchored protein in prostasomes (55,56), a hydrolysis is anticipated to occur before acquisition of CD59 in acceptor cell membranes. This idea is in line with our present demonstration of release of
CD59 after incubation with PIPLC and their subsequent (re-)saturation with CD59 upon incubation with prostasomes. Prostasomes contain a very active phospholipase (185) but PIPLC has so far not been reported in prostasomes. A transfer also requires a close interaction between prostasomes and acceptor cells. Such an interaction has been demonstrated by different techniques to be valid for prostasomes and sperm cells (42,186), and it is possible that similar mechanisms are working between the prostasomal and acceptor cell membranes in this study. Hence, the transfer of CD59 from prostasomal membranes to acceptor cell membranes may be the result of cooperative forces between these two membrane systems. This would be somewhat in analogy with a previous study of phosphorylation reactions concerning prostasomes and spermatozoa. Incubations of spermatozoa and prostasomes together resulted in a 10 -fold increase in total protein phosphorylation compared to the level of phosphorylation achieved when either component was incubated alone (36).

In paper III, we demonstrate that all types of prostasomes express PK (A, C) and CK. All prostasomes of malignant origin had significantly higher PK activity compared to seminal ones. It is particularly intriguing that LNCaP prostasomes which were associated with the highest PKA activity were devoid of ATPase activity, suggesting that prostasomes with these properties would be capable of substantial phosphorylation of available biomolecules, if trapped in a secluded milieu. The low ecto-ATPase activity on prostasomes of malignant cell origin concurs with a previous observation on ecto-ATPase activity on malignant human glia cells which was low in comparison with normal glia cells (187). Some previous works have speculated about the source of recruitment of ATP for such phosphorylation, which may be due to direct association of nucleotides to prostasomes, such as GTP proteins (8). The occurrence of both ADP and GDP in human prostasomes have been reported (188). Some previous works also showed that malignant cells have higher ecto-ATP synthesizing activity (187,189,190).

Unlike prostasomes in seminal plasma, prostasomes produced by metastatic cells of prostate cancer will be exported to the extracellular space surrounding the metastatic cells and will remain trapped within or in close vicinity to the individual cells of the tumor (191). This will create a micro-environment where prostasomes in their extracellular location protect the prostatic malignant cells against immune attack and thereby promote their metastasizing ability.

This indicates that the protein kinases, depending on their physiological substrate, may play a possible role in tumor pathogenesis. Paas and Fishelson (149) have described tyrosine and serine/threonine ecto-protein kinases on intact cells, e.g. monoblastoid cell line U937, and on microparticles derived from these cells, which among several other proteins phosphorylate C9 and to some extent C3. In a previous work by Ekdahl and Nilsson (150), it was
demonstrated that C3 synthesized by monoblastoid cell line U937 was phosphorylated by CKII. Phosphorylation is one of the mechanisms by which the modification of C3 function occurs. Phosphorylation of complement component C3 by PKA has previously been demonstrated to make it inaccessible to physiological activation (192). The observation that C3 is phosphorylated by malignant prostasomal PKA is strong evidence that these prostasomes may indeed have the capability to disarm complement activation by regulatory phosphorylation therewith achieving an advantage of survival to the malignant cell that secreted the prostasomes. The observation that fibrinogen is phosphorylated by all three kinases (PKA, PKC and CKs) is equally intriguing since fibrinogen has been implicated to play a role in tumour angiogenesis and migration of tumour cells, e.g. breast and bladder cancer (193,194), and since it has been reported earlier that several of its functions are affected by phosphorylation with different protein kinases (195).

TF the main activator of the extrinsic pathway of coagulation is present in huge amounts in prostasomes (62). We identified in paper IV TF as the dominating endogenous substrate of prostasomal PKA on both malignant cell-derived and seminal prostasomes. TF, which we showed to be overexpressed by malignant cell prostasomes, is known of playing additional biological functions, including the promotion of tumour angiogenesis (65,196), cell adhesion (197), cell migration (198) and tumour cell invasion (199). Furthermore, it binds to plasminogen with high affinity (200) as effector which may play a role in the enhancement of tumour growth and metastasis (201,202). Suppression of fibrinolysis is important for metastatic prostate cancer. In addition to the overexpression of TF, this work points to another mechanism by which this suppression of fibrinolysis may be achieved, namely by prostasome-mediated extracellular phosphorylation of key proteins within the coagulation and fibrinolytic systems. Phosphorylation of fibrinogen has previously been shown to greatly enhance the resistance of the formed fibrin network to plasmin degradation (203). Phosphorylation of vitronectin decreases binding of PAI-1 (plasminogen activator inhibitor-1) and plasminogen leading to suppression of fibrinolysis (204). Previous studies have speculated about the TF phosphorylation site (the 21 amino acid cytoplasmatic region), and its involvement in the signal transduction of TF (80). However, PKA-mediated phosphorylation in the extracellular domain of TF has not been reported previously and its impact(s) of TF function remains to be clarified. Our incubation experiments with [γ-32P] ATP and investigation with flow cytometry establish that TF has a domain on the surface of the prostasome membrane. The clotting ability of prostasomes, seemingly in a dose-dependent fashion, may be related to the extent of their expression of TF. This finding is contradictory to another study revealing no difference in the plasma level of soluble TF detected in either prostate cancer patients or controls (205). It is anticipated that prostasomes are released into
the blood circulation at some point during the development of prostate cancer. Therefore, the TF activity registered in whole blood of prostate cancer patients might be prostasomal-derived. On the other hand, it is not a matter of course that these prostasomes appear as free organelles in blood plasma, since they occur associated to leukocytes as indicated previously (206), which concurs with our findings in addition to their association to erythrocytes. This study suggests a major role for prostasomes in thrombotic events that may occur in advanced prostate cancer favoring prostate cancer growth and development.

The understanding of the protective mechanisms utilized by the metastatic prostate cancer cells in order to avoid attack by complement and other parts of the innate immune system will help to identify suitable targets for pharmaceutical intervention. Possible targets may include GPI-anchored proteins and specific prostasomal protein kinases present at high concentrations within close vicinity of metastatic prostate cancer cells. If the over-expression of RCAs and these prostasomal protein kinases could be controlled or counteracted it could also be used to potentiate other types of immunotherapy.

Also, better understanding of mechanisms involving the coagulation events and the occurrence of DIC (disseminated intravascular coagulation) seen in some terminal prostate cancer patients may provide better chance for prevention and/or control of these serious clinical conditions.

Vitronectin stabilizes the inhibitory form of PAI-1 that modulates fibrinolysis. It also promotes cell adhesion (207) and inhibits the lytic activity of complement (208). It may have a possible role in malignancy, as it is expressed at high levels in tumors (209). Previous work showed that PKA can phosphorylate vitronectin at serine 378, herewith affecting its conformation (210,211). By this phosphorylation, vitronectin was converted from an antifibrinolytic agent that prevents the occurrence of undesired fibrinolysis into a profibrinolytic form that initiates the solubilisation of blood clots (204). Vitronectin can also be phosphorylated by PKC at serine 362 which attenuates its cleavage by plasmin, thus regulating plasminogen activation (204,211,212). A recent work has shown that vitronectin can be phosphorylated by CKII at threonine 50 and 57 leading to promotion of cell adhesion and spreading (211-213). We demonstrate here that vitronectin is an ample substrate especially for prostasomes derived from malignant cells, for prostasomal protein kinase-catalyzed phosphorylation reactions that may influence the antifibrinolytic-fibrinolytic equilibrium in prostate cancer.

The cadherin family of transmembrane glycoproteins plays an important role in cell-to-cell adhesion and cadherin dysregulation is strongly associated with prostate cancer progression (214-216). Experimental disruption of E-cadherin function stimulated migration and invasion of DU145-E and other E-cadherin-positive prostate cancer cell lines (217). A previous study has shown that phosphorylation of E-cadherin by PKD1 (PKCmu) was associ-
ated with increased cellular aggregation and decreased cellular motility in prostate cancer (218). In this study we concluded that the phosphorylation of E-cadherin may be a mechanism used by metastatic cell to aggregate and achieve cell to cell adhesion.
Summary

The results in this work have shown that:

- CD59 from all types of prostasomes is transferable to other cells but the mechanism of transfer of prostasomal CD59 to acceptor cell membrane is not yet known.
- The transfer of prostasomal CD59 to autologous or allogeneic cells can protect them from complement-mediated lysis elicited by C5b-9.
- Since a complete complement system is present in the uterus and cervix, it is most likely that seminal prostasomes are the source of CD59 that gives the spermatozoa further protection against this potential threat.
- Under physiologic conditions not only the spermatozoa might be acceptors of the CD59 but also the ovum might need additional protection against complement lysis once entering the uterus.
- CD59 is over-expressed by prostatic malignant cell lines, especially DU145 and PC-3, and may be released into the extracellular space structurally linked to prostasomes.
- Given that the metastasized cells continue to produce prostasomes in situ, we hypothesize that prostasomes in their extracellular location surrounding the malignant cells protect them from complement attack and thereby promote their metastasizing ability.
- The identification and interruption of the reaction sequence that controls the transfer of prostasomal CD59 may obstruct the ability of the prostatic malignant cells to metastasize.
- The prostasomes contain protein kinases (PKA, PKC and CK) able to phosphorylate several endogenous and some exogenous substrates and the prostasomal protein kinase activity increases in malignant cell prostasomes.
- The ample phosphorylation of C3 by malignant prostasomal PKA is strong evidence that prostasomes may indeed have the capability to disarm complement activation by regulatory phosphorylation.
- The significance of fibrinogen being phosphorylated by all three prostasomal kinases should be further investigated since fibrinogen has been implicated to play a role in tumor angiogenesis and metas-
tasis and since it has been reported earlier that several of its functions are modified by phosphorylation with different protein kinases.

- The significance of vitronectin phosphorylation by all three prostasomal kinases should be further investigated since vitronectin plays an important role in cancer biology.
- Overexpression of TF by prostatic cancer cells plays an important role in cancer angiogenesis, cancer cell aggregation and metastasis.
- The significance of the phosphorylation of the external domain of TF by prostasomal PKA should be further investigated.
- The phosphorylation of E-cadherin by prostate-derived metastatic cancer cell prostasomes of the prostate may be an important mechanism needed by those cells to aggregate and adhere to each other.
Acknowledgements

This study was carried out in the section of Clinical Immunology, Department of Oncology, Radiology and Clinical Immunology as well as in the section of Clinical Chemistry, Department of Medical Sciences, Faculty of Medicine, Uppsala University. This work was supported by Lion’s Cancer Fund, Uppsala, Sweden; The Swedish Heart and Lung Association; The Swedish Medical Research Council (grant number 5647) and by faculty grants from the University of Kalmar.

This work wouldn’t have been possible without the help of many persons to whom I wish to express my sincere thanks and gratitude.

First I would like to thank my supervisors, Prof. Kristina Nilsson Ekdahl for her keen follow up, for sharing her vast knowledge in the immune system, biochemistry, protein chemistry and for sacrificing her valuable time in setting up the experimental work, helping in laboratory work and analysis of results. Prof. Gunnar Ronquist for setting up the research project and giving me the chance to take part in it, sharing his unchallenged knowledge in prostasomes and reproductive physiology and for his careful scrutiny of work. Assoc. Prof. Bo Nilsson for sharing his precious knowledge in complement system, hemostasis and biomedical research and for his state-of-the-art ideas and perfect planning.

Prof. emeritus Ulf Nilsson for helping start successfully this project, participating in the first paper, sharing his boundless knowledge on the complement system and complement laboratory research and for a discussion without which I wouldn’t have been able to proceed further.

I also wish to thank Dr. Lena Carlsson for teaching me the ABC of prostasomes chemistry and for keeping an uninterrupted help and technical support. Dr. Javier Sanchez and Osama Hamad for the valuable participation in the analysis of results and editing of the fourth paper, and in this context, I like to extend my thanks to Dr. Garcia Elguez and Susanne Lindblom for their technical support. Assoc. Prof. Gehad Elghazali for all the assistance, the valuable discussion on paper II and for the hospitality.

The practical laboratory part of work wouldn’t have been possible without a secure flow of materials and an excellent technical support which were prompt and perfect under the control of Lillemor Funke, I appreciate much your readiness to help all the time.

I also wish to thank the followings for the technical assistance: Mrs. Marriette Sjunneskog, Dr. Jonas Andersson, Dr. Anna Björkland, Dr. Jaan Hong,
Dr. Peter Schmidt, Anna-Karin Lidehäll and Björn Carlsson for the providing necessary information.
I also thank Prof. Thomas Tötterman, Prof. Per Venge, Dr. Magnus Ess and Dr. Anders Larsson and Prof. Sten Nilsson.
I’m also very grateful for the friendly atmosphere created by all colleague researchers and workers in Rudbeck laboratory: Prof. Rolf Larsson, Dr. Johan Rönnelid, Dr. Olof Sjöberg, Dr. Lisa Moberg, Linda, Jenny and Helena and all workers in the routine laboratory, especially Elizabeth Wijkström for her continuous concern. I also thank my colleagues who shared with me the writing room, Chengyuan, Linshu, Kawa, Elin and Teet. I express my sincere thanks to the secretary in both departments, Mona Persson and Barbro Bjurhäll.
I also would like to express my sincere gratefulness to a group of people who exerted a great effort so that I could start my training and post-graduate studies in laboratory sciences. First I thank Dr. Khalid Habbani the former director of Khartoum Teaching Hospital (KTH)–Sudan; for his great-hearted support, enthusiasm and for believing in me. Prof. Eltom Sirageldin the founder and former director of the Diagnostic and Research Unit in KTH, for his support and for arranging my training in Germany. Prof. Michael Krieg, the director general of the Institute of Clinical Chemistry, Transfusion Medicine and Laboratory Medicine, Bergmannsheil, Bochum University Hospital- Germany, for his generous assistance, patience and for his appreciable support. Dr. Abdallah Sid Ahmed, the former minister of health - Khartoum state and his coworkers for the assistance.
I thank Mrs. Maj Britt Holmberg and her family for the hospitality and for considering me a family member. I appreciate your showing concern about “din femte son”.
I would like to express my sincere appreciation, gratitude and love for my family in Sudan, my sisters and especially my brother Shihab who offered the maximum support and encouraged me to proceed with my studies, who always endorsed me, took care of the family and filled the positions of a loving father and a caring brother.
I also would like to take this opportunity to pay tribute to three memorable persons in my life, for whom all words of appreciation and gratitude wouldn’t be enough to honor, who did not have the chance to see the result of their long lasting efforts, who showed the maximum dedication, altruism, wholeheartedness and support for me to complete my graduate and post-graduate studies, my late parents and my late cousin Eltayeb Abdelrazig. Finally I thank the Sudanese communities in Uppsala and in Maastricht for making me part of their families.
References

36. Stegmayr B, Brody I, Ronquist G. A biochemical and ultrastructural study on the endogenous protein kinase activity of secretory granule membranes of

54


56
100. Thielens NM, Illy C, Bally IM, Arlaud GJ. Activation of human complement serine-proteinase C1r is down-regulated by a Ca(2+)-dependent intramolecular control that is released in the C1 complex through a signal transmitted by C1q. Biochem J 1994;301(Pt 2):509-516.


122. Schreiber RD, Pangburn MK, Lesavre PH, Muller-Eberhard HJ. Initiation of the alternative pathway of complement: recognition of activators by bound


Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 34

Editor: The Dean of the Faculty of Medicine

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".)