TINT

Tumor Indicating Normal Tissue
New field of diagnostic biomarkers for prostate cancer

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ABSTRACT

Background  Prostate cancer is the most common cancer in Sweden. Due to its highly variable behavior, multifocal nature, and insufficient diagnostic methods, prostate cancer is difficult to diagnose and prognosticate. Some patients have an aggressive lethal disease, but the majority of prostate cancer patients have slow-growing, non-lethal disease with long expected survival without treatment. Current diagnostic methods—serum levels of prostate-specific antigen (PSA) and histological grading of biopsied prostate tissue—often do not give the information required to be able to safely differentiate indolent tumors from potentially lethal ones. Many prostate cancers are difficult to detect by imaging, so tissue biopsy cannot be safely guided towards the tumor, and particularly not towards the most aggressive forms.

To overcome this problem, multiple needle biopsies are taken from the organ, but biopsies are small and they sample less than 1% of the whole prostate. In this thesis, we explore the non-malignant prostate tissue adjacent to tumors, which is always sampled in biopsies, and we study adaptive changes in this tissue, which may provide new diagnostic and prognostic markers for prostate cancer. We have therefore proposed that this type of tissue should be termed TINT (Tumor Instructed/indicating Normal Tissue).

Methods  In our studies, we used orthotopic rat prostate cancer models with tumors of different aggressiveness. We also used clinical materials from patients diagnosed with prostate cancer at transurethral resection (1975-1990); the majority of these men were followed with watchful waiting. Analyses were performed with whole-genome expression array, quantitative real-time PCR, immunohistochemistry, and western blotting.

Results  Using the animal model, we found that the presence of a tumor induces changes in gene expression in the surrounding tumor-bearing organ (TINT). The gene signature of TINT was linked to processes such as extracellular matrix organization, immune responses, and inflammation. We also showed that some of these adaptive TINT changes appear to be related to the aggressiveness and metastatic potential of the growing tumor, such as increases in macrophages, in mast cells, in vascular densities, and in vascular cell-proliferation. Some of these findings were confirmed by our observations in patient samples. We found that high staining of the extracellular matrix component hyaluronan in the stroma of the non-malignant prostate tissue was prognostic for short cancer-specific survival. We also found that an elevated proportion of C/EBP-beta positive epithelial cells in non-malignant (TINT) prostate tissue was associated with a good prognosis.
Conclusions Using animal experiments and patient samples, we showed that the presence of prostate cancer induces changes in the tumor-bearing organ, alterations associated with tumor aggressiveness, and that grading of these changes in TINT can be used to predict outcome in prostate cancer patients.
POPULÄRVETENSKAPLIG SAMMANFATTNING


Prostatacancer diagnostiseras för närvarande genom nålprovtagning från prostata. Eftersom tumörerna inte syns med någon ”röntgenmetod” vet man inte var i prostata man skall ta vävnadsprov. För att minska osäkerheten tar man många nålprov, men eftersom varje enskilt prov är mycket litet och endast motsvarar 1/1000 av prostatans volym, är underökningen fortfarande osäker. Det vanligaste resultatet är att man trots ökat PSA (Prostata Specifik Antigen) i blodprov, som tas vid misstanke om prostatacancer, inte hittar någon tumör. Detta kan bero på att provet tagits på fel ställe eller att det faktiskt inte finns någon tumör. Behandlande läkare vet då inte om patienten skall utsättas för ytterligare provtagning eller om c Ancermisstanken kan avskrivas. Även om en tumör hittas så kan det finnas fler och kanske mer aggressiva tumörer någon annanstans i prostata. I de fall där en tumör hittas, väljer man ofta att avvakta med terapi men följer patienten med nya biopsier för att se om tumörerna verka bli farligare.

För att kunna växa och spridas behöver tumörer påverka närliggande och mer avlägsna vävnader i kroppen. Sannolikt behöver aggressiva tumörer påverka omgivningen mer än ofarliga. Om det fanns kunskap om vilka förändringar i normalvävnad som indikerar närhet till en tumör, så skulle man kunna diagnostisera prostatacancer även om biopsierna inte träffar tumören. Ännu bättre vore ifall särskilda biomarker för närvaro av aggressiva tumörvarianter kunde identifieras. I så fall skulle denna nya kunskap kunna leda till bättre diagnos- och prognosmetoder för prostatacancer.

I denna avhandling studeras den till synes normala prostatavävnad som omger eller ligger nära intill en tumör. Vävnadsmaterial från patienter samt material från djurmodeller används för att undersöka adaptiva förändringar i prostatavävnaden vilka induceras av närvaron av en tumör och då särskilt aggressiva tumörformer. Kunskapen om sådan ”normal vävnad” kommer att öka förståelsen av tumörväxt och spridning av prostatacancer, samt ge oss möjligheten att hitta nya diagnos- och prognosfaktorer för prostatacancer. Därför kallar vi denna...
"normalvävnad" för TINT (Tumour Instructed/Indicating Non-malignant Tissue).

Delarbete I i denna avhandling, visar med hjälp av en djurmodell att närvaron av en tumör framkallar förändringar i genetik i TINT. Dessa förändringar visar sig vara kopplade till biologiska processer såsom omorganisation av vävnad (i så kallad extracellulär matrix), immunrespons och inflammation.

I delarbete II, undersöktes en av de extracellulära matrix komponenterna (hyaluronan) i TINT-vävnad hos cirka 300 patienter med prostatacancer. Vi fann att ökad hyaluronan i TINT var associerad med dålig prognos dvs. kort cancerspecifik överlevnad. Även av djurexperimenten framgick att tumörcellerna växte snabbare när hyaluronan sprutades in i tumören.

I delarbete III, visas att vissa av de adaptiva morfologiska TINT-förändringarna huvudsakligen är relaterade till aggressivitet och metastatisk potential hos den växande tumören. Exempel på sådana förändringar i TINT är ökad antal makrofager, mastceller, och blodkärl och vaskulär tillväxt, som alla visade sig vara kopplade till aggressiva tumörer.

I delarbete IV, undersöktes uttrycket av en transkription faktor C/EBP-beta i TINT-vävnad hos cirka 300 patienter med prostatacancer. Ökad C/EBP-beta positiva epitelceller i TINT visade sig vara associerad med en god prognos. Ytterligare ett fynd var att ökad C/EBP-beta positiva epitelceller i TINT var korrelerade med ökning av en viss typ av makrofager i TINT, som har en anti-tumör funktion.

Sammanfattningsvis visar studierna att en prostatatumör förändrar den omgivande normala prostatavävnaden, både på gen- och morfologisk nivå. Resultaten innebär att det finns möjlighet att med våra TINT-markörer kunna utveckla metoder som kan identifiera de patienter som har en så aggressiv prostatacancer att den kräver behandling. På så sätt besparas också patienter med en "snäll" tumör behandlingsrelaterande biverkningar.
ORIGINAL PAPPERS

1) Characterization of a Gene Expression Signature in Normal Rat Prostate Tissue Induced by the Presence of a Tumor Elsewhere in the Organ. PLOS ONE. 15 June, 2015.


4) Prostate tumors induce C/EBP-beta expression in epithelial cells in the surrounding tumor-bearing organ and the magnitude of this is related to tumor aggressiveness and patient outcome. Manuscript.

Papers that I have participated in during my doctoral education
(Not included in this thesis)


Nilsson M, Adamo H, Bergh A, Halin Bergstrom S.

Extratumoral heme oxygenase-1 (HO-1) expressing macrophages probably promote primary and metastatic prostate tumor growth. Submitted.
LIST OF ABBREVIATION

Gleason score (GS)
Prostate cancer (PC)
Androgen receptor (AR)
Tissue micro array (TMA)
Phosphorylated epidermal growth factor receptor (pEGFR)
platelet derived growth factor receptor beta (PDGFRβ)
Post-inflammatory atrophy (PIA)
prostate specific antigen (PSA)
Prostatic intraepithelial neoplasia (PIN)
Transurethral resected (TUR)
tumor microenvironment (TME)
Tumour indicating normal tissue (TINT)
Vascular endothelial growth factor receptor 1 (VEGFR1)
CCAAT element binding protein beta (C/EBPβ)
lysyl oxidase (LOX)
Extracellular matrix (ECM)
Cancer Associated Fibroblasts (CAFs)
Tumor Associated Macrophages (TAMs)
Heme oxygenase-1 (HO-1)
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INTRODUCTION

The prostate

Anatomy
The prostate gland is part of a man’s urinary and reproductive system. The prostate surrounds the urethra at the base of the bladder. The actual size of the prostate varies, mostly based on age, from the size of a walnut in young men to the size of small apple in older men. The human prostate gland is divided into 3 zones: peripheral, transitional, and central. Approximately 75% of all prostate tumors are found in the peripheral zone. In rodents, the prostate is divided into 4 lobes: the anterior, the dorsal, the peripheral, and the ventral.

Function
The main function of the prostate is to produce the fluid part of semen. The glandular epithelial cells within the prostate produce a thin fluid rich in proteins and minerals. These proteins are important for sperm motility and survival. Although the prostate is involved in fertility, it is not required for reproduction. The most well-known secretory protein that is produced by the prostate glands is prostate-specific antigen (PSA), also known as kallikrein III, it is a serine protease.

Prostate cancer

Incidence and mortality
Prostate cancer is one of the most common cancers in the world. In 2012, an estimated 1.1 million prostate cancer cases were diagnosed worldwide. The incidence of prostate-cancer varies more than 25-fold around the world. On the Caribbean island of Martinique, 1 man in 4 has the chance of being diagnosed with prostate cancer by the age of 74—the highest incidence rate in the world. But in Bhutan, only 1 man in 714 is at risk. The incidence of prostate cancer (PC) between and within the countries is influenced by the age and ethnic mix of a population, and also by the trends in diagnostic testing.

Today in Sweden, PC is the most common cancer; around 11,000 new cases were diagnosed in 2014. The incidence of PC has been increasing since the 1990s. This increase in incidence rate started with the introduction of prostate-specific antigen (PSA) testing. For a long time, PC-related mortality in Sweden was around 70 deaths per 100,000 men per year. Since the beginning of the 2000s, this number fell to about 60 deaths per 100,000 men. In 2014, 2,398 deaths of prostate cancer were recorded in Sweden, 75% of them in men aged 75 years or more.
**INTRODUCTION**

**Diagnosis and prognosis**

Diagnostic tests for PC are usually done when there are relevant symptoms, such as any changes in bladder habits. The diagnostic procedure starts with physical examination—a digital rectal examination (DRE), to feel the prostate gland through the wall of the rectum. The physician checks for any lumps or changes in size, shape, or consistency. The next step is PSA testing. Serum-PSA helps to assess the risk of having prostate cancer. PSA, which is produced in prostatic glandular epithelial cells, normally leaks into the blood in small amounts. A s-PSA level of < 3 ng/ml is considered normal. A s-PSA level of > 10 ng/ml indicates a substantial risk of having PC. Serum PSA levels are also elevated in cases of urinary tract infection, inflammation, and benign hyperplasia. In other words, elevated PSA is not cancer-specific.

In case of elevated PSA, ultrasound guided biopsies are taken from the prostate. A pathologist examines biopsies microscopically and scores tumor-containing biopsies according to the Gleason score system. The modified Gleason score, which is currently used, describes the grade of the most common area and the highest grade present, using a differentiation scale ranging from 1 to 5. A score of 5 represents the poorest differentiated tumor area (grades 1 and 2 are rarely found in the prostate biopsies, and therefore in practice patients are scored as having tumors ranging from Gleason score 6 to 10). The Gleason score (GS) is currently acknowledged to be as the most informative predictor of outcome of PC that is available today. The GS is a good predictor of outcome for low-grade tumors (GS < 6) and high-grade tumors (GS 8–10). In intermediate-grade tumors (GS 6–7), the prognostic value of GS does, however, appear to be lower and the outcome is very variable—especially when about 80% of the tumors detected are scored as Gleason 6 or 7.

In order to determine the spread of the PC, TNM staging is used in the next step of prostate cancer diagnosis:

- **T1** — clinically unapparent tumor, not palpable or visible by imaging.
- **T2** — the tumor is confined within the prostate. Divided into T2a (where the tumor involves one-half of one lob or less). T2b (where tumor involves more than half of one lobe but not both lobes), and T2c (where tumor involves both lobes).
- **T3** — the tumor extends through the prostatic capsule.
- **T4** — the tumor is fixed or invades adjacent structures other than the seminal vesicles.

Computed tomography (CT), magnetic resonance imaging (MRI), and bone scan (BS) are used to evaluate nodal and bone metastases. This will determine whether the PC is local (T1–T2), locally advanced (T3, No), or advanced (metastasized).
Localized prostate cancer is divided into risk classification groups: low-risk PC (PSA < 10ng/mL and GS < 7, and T1-2a), intermediate-risk PC (PSA 10–20 ng/mL and GS 7, and T2b), and high-risk PC (PSA > 20 ng/mL, GS > 7, and T2c). Ten years after being diagnosed as having localized prostate cancer, patients have a PC-specific mortality rate of 4.5% for low-risk disease, 13% for intermediate-risk disease, and 29% for high-risk disease with non-curative treatment. In men with locally advanced PC that was managed with non-curative intent, the PC-specific mortality ranged from 28% to 64% (based on Gleason score) 8 years after diagnosis of PC. Median survival for PC patients with metastases at diagnosis is about 2.5 years.

**Treatment of prostate cancer**

*Curative treatment*

Localized PC can be treated with radical prostatectomy or radiotherapy, both have curative intention. Radical prostatectomy can be done with open retropubic, laproscopic, or robotic assisted technique. Radiotherapy can be given either externally or in combination with brachytherapy. The third option is active monitoring, which imply no treatment, but instead close surveillance with DRE, PSA tests, and recurrent core biopsies. Active monitoring is recommended for patients with low-risk localized PC, and life expectancy is less than 10 years. The aim of this is to reduce over-treatment of indolent tumors.

In this year, researchers are expected to publish results from the British trial (PROTECT), with 10-year follow-up of men who were diagnosed with localized PC using PSA tests and randomly assigned to one of three treatment options.

The best way to treat locally advanced PC is still unclear. An ongoing randomized clinical trail is taken place in Scandinavia, to determine whether primary radical prostatectomy with postoperative radiotherapy improves prostate cancer-specific survival in comparison with primary radiation treatment and hormonal treatment in patients with locally advanced PC.

*Palliative treatment*

Advanced and metastatic PC cannot be cured. Palliative treatment in the form of hormone castration can be offered. This can be achieved with either surgical orchiectomy or injections of GnRH-agonist. The side effects of hormone therapy are hot flushes, loss of libido, increased risk for cardiovascular diseases, and diabetes mellitus type II.

Watchful waiting—also termed “deferred treatment”—is an alternative in palliative treatment. Watchful waiting developed in the pre-PSA testing era; it refers to conservative management of the disease without treatment until the
development of disease-related symptoms. Later treatment in this situation has mostly been some kind of hormonal therapy. Watchful waiting is still considered to be an option, mostly in elderly patients with a high incidence of comorbidity and other causes of mortality.

**The prostate cancer dilemma: to treat or not treat?**

Prostate cancer is an extremely common, variable, and largely unpredictable disease. The incidence rate of PC is increasing all around the world in all age groups. Microscopic latent prostate tumors are surprisingly common in young, middle-aged, and elderly men. More than 50% of elderly men have foci of cancer in their prostates. Most PCs are clinically insignificant, i.e. they are non-aggressive, slow-growing tumors and are unlikely to spread. Men harboring such tumors live for many years without symptoms from them, and eventually die from some other cause.

The main question is not how to diagnose PC, but how to predict which patients need to be treated and which patients are better off left alone. This is why screening for PC is one of the most controversial subjects in the urological literature. PSA testing has a substantial impact on PC diagnosis, and screening followed by treatment results in a reduced number of deaths from PC, but to achieve this many men are over-treated. PSA is organ but not cancer specific marker. PSA is an organ-specific marker rather than a cancer-specific marker. Elevated PSA can be caused by conditions other than PC, such as prostate hypertrophy and prostatitis.

Today, diagnosis of PC is based on histological evaluation of prostate needle biopsies, which sample only a minute fraction of the prostate. PC is generally multifocal; 60–90% of prostates were found to contain 2 or more widely separate tumors by the time of clinical diagnosis, which makes accurate clinical grading and staging difficult. Even when cancer is detected in biopsies, the most aggressive tumor foci present may still have remained undetected—particularly since current imaging methods cannot guide biopsy towards suspected tumors. To overcome this problem, multiple biopsies are taken from different parts of the organ. However, as biopsies sample less than 1% of the total volume of the prostate, it is impossible to know whether the most aggressive tumor present has been sampled. When no tumor is found, we do not know whether this is because it has been missed or whether no tumors are present.

Novel diagnostic and prognostic markers that identify men with PC and grade the aggressiveness of their cancers at an early stage are urgently needed.
**The microenvironment of the tumor**

Not so long ago, cancer was seen as a just mass of malignant cells—and was treated accordingly. Now we understand that tumors have more complex structure, consist of leukocytes, fibroblasts, endothelial cells, and other stromal components, referred to as the tumor microenvironment (TME). Non-malignant cells of the TME can comprise >50% of the mass of primary tumors and their metastases

The hallmarks of cancer accentuate the importance of the tumor microenvironment (TME). Tumor cells cannot survive alone, let alone manifest the disease. Cancer cells recruit and demoralize normal stromal cells to establish a tumor microenvironment, also called tumor stroma, that serves the tumor during establishment, local invasion, and metastasis. Studies have shown that tumor epithelial cells influence the microenvironment directly, by secretion of growth factors and exosomes, and indirectly, by attracting fibroblasts and inflammatory cells such as mast cells and macrophages.

All these alterations in tumor stroma are surprisingly similar to the wound-healing process, so tumor stroma has been described as being reactive stroma. In PC, alteration in the reactive stroma has been demonstrated from increased remodeling of the extracellular matrix (ECM), increased recruitment of inflammatory cells, and increased angiogenesis.

Importantly, these changes in reactive stroma have been found to be related to prostate cancer prognosis. Accumulation of myofibroblasts, accumulation tumor associated macrophages (TAMs), and reduced androgen receptor levels in the tumor microenvironment have all been found to be associated with increasing prostate tumor grade and poor outcome.

**TINT**

The impact of the TME on tumor growth and progression is now a main focus of cancer research. While tumor stroma has now been fully appreciated and is a broad field of investigation, the potential occurrence of adaptive changes in the tumor-bearing organ, i.e. the normal tissue adjacent to and further away from to tumors is far less studied—and often forgotten. Adaptive changes have almost been considered to be exclusive to the tumor stroma (microenvironment), whereas adjacent “normal benign tissue” have not received much attention. Developments in tumor biology during the last decade have clearly shown that in order to grow and spread, tumors need to influence not only the TME (tumor stroma) but also more remote organs and tissues, i.e. the tumor “macroenvironment”. For example, tumor-derived factors instruct the bone marrow to deliver cells necessary for tumor growth and to prepare distant sites...
for subsequent metastasis\textsuperscript{37,38}. As many distant organs are adapted to the needs of aggressive and metastatic tumors, it is quite likely that the tumor-bearing organ is also adapted—and that the magnitude of such changes is more pronounced in the “macroenvironment” of potentially lethal tumors than it is around more indolent tumors\textsuperscript{39}. If so, this knowledge could be used to translate the new developments in tumor biology into novel ways of indirectly diagnosing cancer—by examining its effect on other tissues.

As described above, in order to grow and spread, tumors need to induce supportive alterations in the TME, such as increasing tumor angiogenesis and extracellular remodeling\textsuperscript{26,40,41}, which have been well studied in tumor stroma. Using the same logic, such adaptive changes and interactions might not only affect tumor stroma, but also reach far out into normal surrounding tissues including normal stroma and normal epithelium\textsuperscript{39}. Particularly as tumor cells not only interact with the closely adjacent tumor stroma, but also with distant organs such as the bone marrow and those with pre-metastatic niches\textsuperscript{21,36,42}.

Studies in our research group showed that prostate tumor cells influence both tumor stroma and the stroma in the surrounding tumor-bearing organ, in a way that induces changes in those tissues, changes that might be related to patient outcome. For example, increased levels of PDGFRβ in both tumor stroma and non-malignant normal stroma have been found to be associated with shorter cancer-specific survival in PC patients\textsuperscript{43}. Also, reduced androgen receptor (AR) levels in tumor stroma as well as in normal stroma were found to be related to a poor prognosis in PC\textsuperscript{35}. Changes in normal stroma and tumor stroma do not always follow a similar pattern; cells have more complicated functions, with both pro- and anti-tumor effects. Studies have shown that an increasing number of mast cells lying in the normal stroma tissue is related to vascular and tumor growth in animal models and in patients, and is associated with a poor prognosis. In contrast, accumulation of mast cells in tumor stroma is associated with a good prognosis in PC patients\textsuperscript{25}. In addition to these changes in the normal stroma, studies have shown that the glandular epithelium in normal non-malignant prostate tissue is also altered. For example, the level of phosphorylated epidermal growth factor receptor (pEGFR) is elevated in the tumor-bearing organ and is associated with a poor outcome\textsuperscript{44}.

Based on the growing evidence that the epithelial and stromal compartments of the surrounding normal tissue in the tumor-bearing organ undergo adaptive alterations, the magnitude of these changes could be related to tumor aggressiveness. Our group has therefore proposed that tumor-adjacent non-malignant prostate tissue composed of normal prostate stroma and glands should be termed tumor indicating/instructed normal tissue (TINT). The term TINT describes morphologically normal-appearing epithelium and stroma that is not in direct
contact with the cancer epithelium, and it should not be confused with tumor stroma or the microenvironment of the tumor (TME).

![Figure 1. Illustration of the prostate gland, and the concept of “TINT”](image)

**Changes in TINT as markers of aggressiveness of PC**
Prostate cancer is a common and multifocal disease, but the diagnostic methods available are insufficient for accurate diagnosis, due to the low sensitivity in DRE and TRUS (transrectal ultrasonography) and the low specificity of PSA testing. In order to roll out or verify the presence of cancer, histological assessment of biopsies from the prostate is necessary. One major problem is that the volume of prostate sampled by biopsy is relatively small, and it can be easy to miss prostate tumors. About 20–40% of men with subsequently confirmed PC initially have false-negative biopsies. To handle this problem, multiple needle biopsies are taken. However, the ideal number of biopsies that would be necessary to accurately detect cancer foci is a controversial issue.

In this thesis, I will make a case for the potential clinical value of TINT in prostate cancer diagnosis. By studying adaptive changes in morphologically normal-appearing prostate tissue (TINT) induced by prostate cancer, and related to tumor aggressiveness, we could identify potential biomarkers of PC that might predict the presence of prostate cancer despite negative biopsies, and might also serve as prognostic markers. The diagnostic value of “normal” prostate tissue in negative biopsies is not new. It has been proposed that markers of field cancerization (see general discussions) could be possible indicators of prostate cancer. Also, the present of pre-cancerous lesions such as proliferative post-inflammatory atrophy (PIA) and high-grade prostatic intraepithelial neoplasia (HGPIN) may indicate an increased risk of developing cancer, or that a cancer is already present elsewhere in the prostate, but such changes cannot be used to predict the aggressiveness of the cancer elsewhere in the organ.
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Figure 2. By using biomarkers of TINT, diagnosis of prostate cancer might be improved and the rate of negative biopsies would be reduced.

**Hallmarks of cancer in TINT**

In 2000, Douglas Hanahan and Robert Weinberg published “The hallmarks of cancer” to explain the complexities of the disease, by introducing underlying principles that are acquired or enabled for cancer development and progression. These principles include:

- Insensitivity to anti-growth signals
- Evasion of apoptosis
- Limitless replicative potential
- Induction of angiogenesis
- Tissue invasion and metastasis
- Sustainment of proliferative signaling

In 2011, based on new observations in cancer research, “Hallmarks of cancer: the next generation” was published, with 4 additional principles:

- Avoidance of tumor destruction
- Deregulation of cellular energetics
- Tumor-promoting inflammation
- Genome instability and mutation

With these principles, the authors revealed that the biology of cancer can no longer be understood simply by studying the tumor cells. This emphasizes the importance of the tumor microenvironment for tumorigenesis. In our studies, we have extended the “hallmarks of cancer” to include not just TME but also its macroenvironment—and in particular changes in the tumor-bearing organ, i.e. the tissue tinted (colored) by the presence of a tumor. In this thesis, I examine markers involved in angiogenesis, inflammation, proliferative signaling, and extracellular matrix remodeling in TINT.
Tumor angiogenesis and arteriogenesis

Angiogenesis, the formation of new capillaries from pre-existing ones in the vascular network, is an essential part of physiological processes such as wound healing and the female reproductive cycle. In 1971 and 1972, Judah Folkman published 2 articles, based on his own observations and those of other researchers, introducing the importance of tumor angiogenesis in the development and metastatic spread of tumors, and of how therapeutic inhibition of such angiogenesis might be a new and novel treatment for cancer. Folkman’s hypothesis was that primary solid tumors can probably grow to the maximum size ~1–2 mm in diameter. Up to this size, tumor cells can obtain the necessary oxygen and nutrient supplies that they require for growth and survival, by simple passive diffusion. He also proposed that tumor masses could switch on angiogenesis by secreting a growth factor, to form new blood vessels growing towards the tumor. Years later, new evidence indicated that the angiogenesis switch is regulated by both activator and inhibitor molecules and shows interactions between the tumor and vascular compartments. Vascular endothelial growth factor (VEGF) is a powerful angiogenic agent in both neoplastic tissues and normal tissues. Hypoxia induces the expression of VEGF and its receptor via hypoxia-inducible factor-1α (HIF-1α). Under the influence of certain cytokines and other growth factors, VEGF is expressed not only in cancerous tissue but also in the adjacent stroma. Stromal cells in the TME have a critical role in “switching on” and sustaining a chronic form of angiogenesis. In order to ensure a sufficient blood supply due to increased intratumoral angiogenesis during tumor growth, expansion of upstream arterioles and downstream venules in the tumor-bearing organ is required; this process is referred to as arteriogenesis. Arteriogenesis is better known in cardiovascular diseases and—in contrast to angiogenesis—involves the remodeling of an existing artery to increase the dimensions of the lumen in response to increased blood flow. While angiogenesis is stimulated by tissue hypoxia, arteriogenesis is controlled by fluid shear stress under normoxic conditions.

One essential component of angiogenesis and arteriogenesis is mural cells; they associate and coat the endothelial tube. Mural cells are commonly divided into vascular smooth cells and pericytes. In contrast to normal angiogenesis, angiogenesis in tumors in generally leads to the formation of a poorly organized vasculature—characterized by tortuous and leaky vessels. One explanation of abnormal characteristics of tumor vessels is the failure of tumor vessels to recruit a normal coat of mural cells. In breast cancer, pericyte ablation leads to increased vessel permeability and poor vessel integrity, which inhibits tumor growth, while at the same time favoring invasion of blood vessels by tumor cells and ensuing metastatic spread.
Arteriogenesis depends on the availability of mural cells, which differentiate to smooth muscle cells also on recruitment of monocytes/macrophages; The later induce remodeling of vascular wall and proliferation of vascular wall cells. Evidence in breast cancer research suggests that macrophages and their chemo-attractants promote enlargement of feeding vessels (tumor arteriogenesis) supplying the expanding tumor capillary bed.

In prostate cancer, there are indications that increased tumor vascularity may be associated with risk of metastasis. Prostate tumor angiogenesis is regulated by inducers of angiogenesis, e.g. VEGF, TGF, and MMPs, and inhibitors of angiogenesis e.g. TSP-1, PEDF, PSA. Over-expression of pigment epithelium-derived factor (PEDF) led to reduction in vascular growth both in the tumor and in the surrounding normal tissue; it also slowed tumor growth and reduced lymph node metastasis. Remarkably, studies on the role of angiogenesis in cancer have generally explored the development and function of micro-vessels within the tumor, but increased delivery of oxygen and nutrients to en expanding tumor mass cannot be accomplished unless the arterial and venous parts of the vasculature are also expanded—and they are situated outside tumors. Studies in our group have, however, shown that growth of the vasculature, including larger blood vessels, extends outside the tumor microenvironment. This has also been noted in tumor-bearing non-malignant prostate tissue (TINT). Growth of the vasculature in TINT is probably necessary to ensure the increasing demand for the supply of blood to and drainage from the growing tumor. In line with this, reduction of blood flow through the tumor-bearing organ retarded tumor growth. The vascular growth in TINT is in part mediated by macrophages and mast cells accumulating in the tumor-bearing organ, particularly in the peri-tumoral region. Depletion of these macrophages and inhibition of the mast cells retards tumor growth.

The extracellular matrix and hyaluronan
The extracellular matrix (ECM) is more than simply a stable structure with only a supportive role in maintaining tissue morphology. It contains key growth factors such as angiogenic factors and chemokines. The characteristic properties of the ECM contribute to its importance in the invasion and spread of cancer. The ECM is highly dynamic and is constantly being remodeled by enzymes such matrix metalloproteases (MMPs) secreted by malignant cells, CAFs (Cancer Associated Fibroblasts) and TAMs (Tumor-Associated Macrophages). Tumors are usually stiffer than normal tissues, owing to an increased interstitial pressure and stiffening of the ECM. Collagen and elastin fibers are reoriented and cross-linked by Lysl oxidase (LOX) in the tumor microenvironment. Increased extracellular LOX activity therefore results in a stiffer microenvironment that promotes tumor progression, metastasis, and invasion.
In prostate cancer, tumor stroma shows fundamental alterations in the ECM, such as elevated level of collagen I, increase expression of HYAL-1, and accumulation of hyaluronic acid in the stroma. Alterations in the extracellular matrix components have been associated with outcome in PC.

One component of the ECM is hyaluronan (HA), a glycosaminoglycan composed of repeating disaccharide units, D-glucuronic acid and N-acetyl-D-glucosamine. HA synthesis takes place at the plasma membrane by a transmembrane HA synthase (HAS) with 3 isoforms (HAS1, HAS2, and HAS3), which synthesize different-sized polymers of HA at different rates. Degradation of HA is by hyaluronidases, HYAL-1, -2, -3. HA is important for cell division, cell migration, and angiogenesis during embryogenesis, inflammation, and wound healing. HA is a major part of the extracellular matrix and increases in many tumor types. Degradation of HA leads to a matrix that favors tumor cell invasion, epithelial to mesenchymal transition, angiogenesis, cell proliferation, and recruitment of bone marrow-derived inflammatory and progenitor cells to tumors.

In PC patients, accumulation of HA in tumor stroma is associated with poor outcome. Also, altered expression of hyaluronic acid synthase (HAS) and hyaluronidase (HYAL-1) in tumor epithelial cells are associated with increased cell proliferation, invasion, and metastasis.

**Tumor-promoting inflammation**

Inflammation is considered to be an essential component of tumor development—the seventh hallmark of cancer. Tumors have been described as wounds that do not heal, and inflammatory cells of both the innate immune system and adaptive immune system are attracted to tumors. Immune cells supply direct mitogenic growth mediators that stimulate proliferation of neoplastic cells, by stimulating angiogenesis or by inhibiting tumor immune surveillance. At the same time, the immune system can also play a central anti-tumor role. Evidence in patients with breast cancer has shown that activation of innate immunity after conventional radiation or chemotherapy can trigger anti-tumor immunity. This was explained by involving TLR4 signaling, which is required for crosspresentation of antigens from apoptotic tumor cells on MHC class-I to generate anti-tumor cytotoxic T cell (CTL) responses. Thus, the role of inflammation in cancer has been described as a double-edged sword, and is not fully understood.

On component of the immune system that reflects the concept of “double-edged sword” is tumor-associated macrophages (TAMs). Macrophages are classified into 2 major phenotypes, M1 and M2. M1 macrophages suppress cancer progression and have tumoricidal activity, while M2 macrophages promote it by expressing an immunosuppressive phenotype and display several pro-tumoral functions, including promotion of angiogenesis and matrix remodeling.
The classically activated TAMs, the M1 phenotype, can be activated by interferon γ (IFNγ), and they express high levels of pro-inflammatory cytokines (TNF-α, IL-1, IL-6, IL-12, or IL-23) and inducible nitric oxide synthase, which kills cancer cells. The alternatively activated TAMs, the M2 phenotype, can be activated by transforming growth factor β (TGFβ), and can release growth factors such as epidermal growth factor (EGF) and fibroblast growth factor (FGF), promote growth of tumors, and promote tumor angiogenesis by releasing vascular endothelial growth factor (VEGF). Although most TAMs are considered to have an M2 phenotype and most studies have shown a correlation between increased TAM infiltration and poor prognosis, macrophages show high plasticity, which could explain the conflicting evidence supporting both pro- and anti-tumoral functions of macrophages.

Different studies in prostate cancer have also shown conflicting results; macrophage infiltration in human PC has shown both positive and negative association with cancer progression and clinical outcome. Studies in our group have shown that accumulation of macrophages in the surrounding non-malignant tissue in rat prostate was positively associated with tumor size and extra-tumoral vascular proliferation. Depletion of these macrophages represses tumor growth and angiogenesis, both in the tumor and in the surrounding non-malignant tissue. Also, over-expression of PEDF increased the fraction of M1 macrophages in TAMs in orthotopic rat prostate tumors and suppressed tumor growth, angiogenesis, and metastasis.

C/EBPβ

The CCAAT element binding protein beta (C/EBPβ) is a member of the family of transcriptional factors (C/EBPs) that consists of at least 6 members characterized by the basic leucine zipper domain that can bind as a homodimer or as heterodimers to certain DNA-regulatory regions. C/EBP-beta is generally involved in control of cellular proliferation, differentiation, inflammation, and metabolism. It is expressed in various cell types, particularly in macrophages, where it is an important regulator of macrophage differentiation and cytokine gene expression. C/EBPβ exists in 3 different isoforms: liver activator proteins LAP1 (LAP*) and LAP2 (LAP), and liver inhibitory protein LIP, with different biological roles. Both LAPs are transcriptional activators, whereas the LIP isoform lacks the transactivation domain and part of the regulatory domain. The LAP:LIP ratio is important in determining effects. Studies on human cancers found that the expression levels of C/EBPβ in the epithelial cells correlated with ovarian epithelial cancer progression and the invasiveness of colorectal cancer. In the human prostate, chronic inflammation upregulates expression of C/EBPβ and downregulates expression of the androgen receptor in glandular epithelial cells, and C/EBPβ is highly over-expressed in PIA lesions in the prostate. In prostate cancers, C/EBPβ is upregulated in castration-resistant...
cases and it stimulates metastasis-associated genes. C/EBPβ downregulates the androgen receptor (AR), and AR signaling in turn represses C/EBPβ; also, C/EBPβ-deficient prostate cells were found to be significantly more susceptible to killing by cytotoxic chemotherapy following androgen deprivation.
AIMS

The overall aim of the work of this thesis was to study adaptive changes in the tumor-adjacent non-malignant prostate tissue, which we have termed TINT (tumor instructed/indicating normal tissue). By investigating TINT, it is hoped that new diagnostic and prognostic markers for PC will emerge. Changes in TINT could also provide possible new targets for therapy.

Specific aims

**Paper I**
To study tumor-induced changes in the tumor-bearing organ, by comparing prostate morphology and the gene expression profile of tumor-bearing normal tissue (TINT) with that normal prostate tissue without tumor, in an orthotopic rat model for PC.

**Paper II**
To study hyaluronan distribution in malignant and non-malignant tissue adjacent to tumor (TINT) in PC patients who were followed with watchful waiting, and to determine any correlation with cancer-specific survival.

To assess the effects of hyaluronan on tumor growth in an orthotopic rat model for PC.

**Paper III**
By implanting 3 different Dunning rat prostate tumor cell lines into the prostates of immune-competent rats, we wanted to determine whether the nature and magnitude of morphological alterations in (TINT) might be related to tumor size and aggressiveness, and metastatic capacity.

**Paper IV**
To study C/EBPβ expression levels in malignant and non-malignant glands adjacent to tumor tissue (TINT) in PC patients who were followed with watchful waiting, and to evaluate its potential association with cancer-specific survival.
To determine whether the expression of C/EBPβ (as a TINT marker) in tumor-adjacent prostate tissue was related to the distance to tumor in an orthotopic rat model for PC.
MATERIALS AND METHODS

Cells, animals and patients

**Dunning cells**

The dunning sublines were derived from a spontaneous tumor in the dorsolateral prostate in a 22-months old inbred Copenhagen male rat. The original tumor called R3327, was discovered by W. F. Dunning in 1961. Following serial passages of the original R3327 tumor gave rise to sublines with different characteristics. In this thesis we used some of these sublines, G, AT-1 and MatLyLu. They all give rise to poor differentiated tumors, but the tumors differ in metastatic ability, growth rates and androgen-responsiveness (table 1).

| Table 1. Tumor size and proliferation of different orthotopic Dunning rat prostate tumors. |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                | G               | AT-1            | MatLyLu         |
| Metastatic capacity            | Non            | Low            | High           |
| Paper                          | III&IV         | III            | III&IV         |
| Cells injected (n)             | 2x10³          | 2x10³          | 2x10³          |
| Days after tumor cell injection| 49             | 42             | 7              |
| Tumor weight (mg)              | 49 +/- 21      | 250 +/- 164*   | 15 +/- 4.5     |
| Tumor cell proliferation BrdU labeling (%) | 23 +/- 3.3 | 25 +/- 3.4 | 30 +/- 3.7 |
|                                | 23 +/- 3.9†‡   | 41 +/- 8.3     | 41 +/- 6.6     |

Values are means +/- SD, * significantly different than G tumors at day 49 (p<0.05), †significantly different than corresponding tumor at day 7 (p<0.05), and ‡ significantly different than the corresponding tumor at day 10 (p<0.05).

Dunning rat prostate AT-1, MatLyLu and G tumor cells were grown in culture consist of RPMI with 10% fetal calf serum, and 250 nM dextamethasone in 37°C and 5% CO₂. Before inoculation the cells were grown to about 75% confluence, trypsinized, counted in a Burker chamber and diluted in RPMI to the appropriate concentration.

**IN VIVO**

In our studies we used adult Copenhagen rats (Charles River, Sulzfeld, Germany). The operation was carried out in anesthesia. First, an incision in the lower abdomen was made. Then AT-1, MatLyLu, or G cells (see table 1 for details) were carefully injected into one lobe of the ventral prostate using a Hamilton syringe.

We used different controls in our studies, rat ventral prostates, injected with RPMI medium, heat-killed tumor cells (100°C, 30 minutes in RPMI), or left intact (non-operated, non-injected) ventral prostate.
At sacrifice, the animals were injected with bromodeoxyuridine (BrdU, 50 mg/kg body weight; Sigma-Aldrich, Oslo, Norway) to label proliferating cells, and pimonidazole (Hypoxyprobe, 60mg/kg body weight; Millipore, MA, USA) to label hypoxic tissue. For RNA and protein analysis, the prostate tissue was removed, weighed, and stored at -80°C.

To examine whether injected HA affects prostate cancer growth, 2000 AT-1 cells were injected into the ventral prostate and at day 8 the ventral prostates were injected with either 400 μg HA in 40 μL saline (Hyalgan; Nycomed, Stockholm, Sweden) or 40 μL saline alone. The experiment was ended 4 days later, the prostate was examined and tumor size was determined by histological evaluation.

All of the animal work was approved by the Umeå ethical committee for animal research (permit A110-12) and strong efforts were made to minimize animal discomfort and suffering.

**Figure 3.** Section of the rat prostate 10 days after AT-1 tumor cells injection. An established AT-1 tumor on the right, and next to it the tumor-adjacent normal prostate tissue (TINT)

**Patients**

Between 1975 and 1995, samples were collected at the hospital of Västerås (Sweden), from 404 patients with voiding symptoms diagnosed with prostate cancer after transurethral resection (TUR) of the prostate. The mean age at diagnosis was 74 years (range, 53 to 95 years). Staging was performed at the time of surgery; local clinical stage was determined by digital rectal examination
and radionuclide bonescan was performed for detection of metastases, but no lymph node staging was performed. Because this series was collected before the PSA era, information on serum PSA was not available.

A substantial number of our patients (n 295) had not received any cancer therapy before the TUR of the prostate and were managed with watchful waiting. The median overall follow-up period was 5.9 years (range, 0 to 25.5 years). The cause of death was determined by examination of patient records.

Sample from the transurethral resection of the prostate were formalin-fixed, paraffin-embedded, and tissue microarrays (TMAs) were the constructed. The TMAs contained 5 to 8 tumor cores and 4 nonmalignant tissue cores per patient (figure 4).

**RNA analysis**

*Tissue preparation and RNA extraction*

Five-μm thick cryostat sections of the VP lobe were taken for pathological evaluation, in order determine the size and location of the tumor and the surrounding non-malignant prostate tissue in the samples, and verify that the VP lobe from control animals was free of tumors and other pathologies. Surrounding non-malignant prostate tissue and prostate tumor tissue were dissected with a margin of 0.5 to 1 mm to avoid contamination from each other. When sufficient tissue had been collected an additional cryo-section was cut to verify that the tissue dissected contained only the intended tissue type.

Total RNA from tumors, TINT, normal prostate controls, and cells was extracted using TRIzol according to the manufacturer’s instructions (Invitrogen, Stockholm, Sweden). The concentration of total RNA from each sample was measured with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). The integrity of the RNA was determined using an Agilent 2100 BioAnalyzer (Agilent, Willmington, DE).

*Preparation of cRNA and hybridization*

Biotin-labeled cRNA was synthesized from 200 ng total RNA using the IlluminaTotalPrep RNA Amplification kit (Applied Biosystems, Austin, TX) according to the manufacturer’s protocol. The quality of labeled cRNA was verified using a Nanodrop ND-1000 spectrophotometer. A total of 750 ng biotin-labeled cRNA from each sample was loaded onto the 12-sample RatRef Illumina BeadChip gene expression array (Illumina, San Diego, CA) according to the manufacturer’s protocols. The arrays were scanned and fluorescence signals measured using the Illumina BeadArray Reader (Illumina, San Diego, CA, USA).
Figure 4. Example of patient tissue-micro array (A) that include 8 patients with 5 tumor samples and 4 TINT samples. (B) High magnification of one tumor sample. (C) High magnification of one TINT sample.
**Real-time RT-PCR**
The RNA was DNase-treated (DNase 1; Ambion) to remove contaminating DNA. reverse transcription was performed using superscript III (Invitrogen, Carlsbad, CA). Real-time qRT-PCR was performed using the Applied Biosystems 7900HT Real-Time PCR System (Applied Biosystems, Foster City, CA) and Taqman assays with gene-specific primers and probes set for (Hmox1, Lox, Cdad68, Lpl, Cebp-beta, Cyr61, Mmp3, S100a4, Tgf-bi, Mme, and Gtsm1). The relative values for each gene were normalized using beta-actin (paper IV) or Psmc4 (paper I) as reference gene. The result was analyzed in Taqman Analysis Software SDS2.4 (Applied Biosystems, Foster City, CA). The Mann-Whitney U test was used for comparisons between groups and any p-value < 0.05 was considered significant.

**Protein analysis**

**Tissue preparation and protein extraction**
Frozen human prostate tissues (taken from radical 6 prostasectomy specimens) and frozen rat prostate tissues, were sectioned and stained with haematoxylin and eosin to identify tumor and non-malignant tissue. Frozen sections of non-malignant tissue and tumor tissue were dissected out, (although dissected tumor tissue were not totally free of normal tissue). The dissected tissues were cryo-sectioned into five-μm thick sections and homogenized with a syringe. Lysis buffer containing 0.5% NP-40, 0.5% NaDOC, 0.1% SDS, 50 mM Tris (pH 7.7), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1mM NaF and protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) were added to the homogenized tissues. The samples were mixed and incubated on ice for 30 minutes followed by centrifugation at 20 000 g in 4°C for 10 minutes. The supernatants were is isolated and the protein concentration was determined by using the BCA protein assay reagent kit (Pierce Chemical Co. Dallas, USA).

**Western blot:**
Protein samples were mixed with electrophoresis sample buffer containing 2% SDS and 5% beta-mercaptoethanol and boiled 5 min. Samples protein (10 μg) were separated by electrophoresis on 10% SDS polyacrylamide gels and transferred to western blot membrane using Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, California). The membranes were blocked in Odyssey blocking buffer (Li-Cor, Nebraska USA) for 1h. Then incubated with the primary antibody, rabbit polyclonal IgG against C/EBP-beta (C-19, Santa Cruz), diluted 1:200, overnight at 4°C. After washing in PBST, membranes were incubated with dye-conjugated secondary antibodies for 1h. Proteins were detected using Odyssey CLx imaging system (Li-Cor, Nebraska USA). Actin (Sigma, Stockholm, Sweden) was used as control to confirm equal loading.
**Immunohistochemistry**

**Antibodies**
Sections were stained using primary antibodies against CD68 (AbD Serotec), CD163 (AbD Serotec), factor VIII (Dako), BrdU (Dako), hypoxyprobe (Millipore), and C/EBPβ (Santa-Cruz). For localization of hyaluronan (HA) in the tissue sections, we used a HA binding protein probe, HABP. For isolation and biotin labeling of the HA binding protein procedure (see paper II for details), this method detects different molecular sizes of HA.

**Stereology**
5-μm thick sections were immunostained using primary antibodies against CD68, CD163, factor VIII, BrdU, hypoxyprobe, C/EBPβ and with toluidine blue.

The volume densities of hypoxyprobe stained prostate epithelium, factor VIII-stained blood vessels, CD68 and CD163 positive macrophages, toluidine blue stained mast cells were evaluated using a point counting method. Using a lattice pattern in the eye-piece of a light microscope, the numbers of intersections falling on each tissue compartment were counted in randomly chosen fields.

The number of BrdU-labeled endothelial cells per 100 blood vessel profiles (endothelial BrdU labeling), and the number of BrdU labeled vascular mural cells per 100 vascular profiles of non-capillary blood vessels, i.e. small arteries and veins (mural cell BrdU labeling) were measured by counting hits falling on vascular lamina in the non-malignant parts of the ventral prostate lobe.

The volume density of tumor tissue was determined on hematoxylin eosin-stained sections using also point-counting method. Total tumor weight was then estimated by multiplying the volume density with prostate weight.

**Hyaluronan scoring**
In patient samples, staining of the stroma was evaluated for distribution and intensity. Distribution was evaluated as none (0), 10% (1), 10% to 50% (2), 50% to 90% (3), or more than 90% (4). Intensity was evaluated as none (0), faint (1), moderate (2), strong (3), or very strong (4). The product of staining intensity and staining distribution was calculated (with score values between 0 and 16). Each patient was represented by the mean value from the tumor and nonmalignant tissue cores respectively. HA staining in the epithelium was only scored by intensity.

In rat samples stroma HA staining was scored for distribution (the fraction of stroma volume stained, from 0 to 1) and the intensity of staining (none 0, moderate 1, or strong 2). The intensity and distribution values were multiplied to obtain a HA staining score.
**C/EBP-beta scoring**
In the patient TMA the fraction of normal glands with C/EBP-beta positive epithelial cell nuclei was scored using a 6-tier scale (0=none, 1=up to 2%, 2=2.1-25%, 3=25-50%, 4=50-75%, 5=>75%).

In the rat samples the percentage of C/EBP-beta stained epithelial cell nuclei was measured at random sites in the tumor bearing organ, but also in the peri-tumoral zones 0-0.5mm, 0-5-1mm and 1-1.5 mm outside the tumor.

**Data analysis**
The array data were analyzed with GenomeStudio software (version 2009.2; Illumina). Rank invariant normalization was used to remove or minimize non-biological systematic variation. Differences in gene expression between TINT, tumor, or cell line samples and normal prostate control reference samples were compared using the Mann-Whitney U test. by using the Benjamini and Hochberg procedure, P-values for each gene were adjusted to minimize false-positive results. We performed average linkage clustering with Pearson correlation on the whole dataset of 35 samples (11 TINT, 8 tumors, 15 normal prostate controls, and 1 AT-1 cell line), to examine similarities in gene expression in the different samples. Fold changes in gene expression were calculated by dividing the mean signal for each probe in the TINT group by the mean signal for each probe in the control group.

To identify strong candidate genes that characterize TINT, and are differentially expressed compared with control prostate, we selected genes that had (a) a p-value of < 0.05, (b) ≥2-fold variation in expression, and (c) a probe signal of at least twice the background signal in at least one of the two groups.

Those genes that were significantly expressed in TINT were further analyzed with GeneGo MetaCore software, for enriched biological processes and pathways. GeneGo software includes a manually annotated database of biological pathways and processes obtained from the scientific literature. The software uses algorithms to create lists of networks and pathways, ranked according to calculated statistical significance.

**Statistics**
Please see the respective papers’ statistical paragraphs. Bivariate correlations were calculated using the Spearman’s rank correlation test. The level of statistical significance was defined as P <0.05 (two-sided). Statistical analysis was performed using the SPSS 23.0.0 (SPSS Inc., Chicago, IL, USA) or statistical software Statistica 12.0 (StatSoft, Tulsa, OK, USA).
RESULTS AND DISCUSSION

**Paper I**

In order to study the effect of a tumor on the surrounding normal prostate tissue (TINT), we implanted rat AT-1 prostate tumor cells into the prostates of immune-competent rats and sacrificed the animals at day 10—when the tumors were still surrounded by normal prostate tissue. In this animal model, TINT is the tumor-adjacent non-malignant rat prostate tissue, containing both morphologically normal-appearing epithelium and stroma. We used a genome-wide expression microarray to compare gene expression in TINT to that in normal control prostate tissue (RPMI injected) from tumor-free animals.

We identified 5,888 genes with significantly different expression in TINT compared to control samples (p < 0.05). To identify strong candidate genes that characterize TINT, we selected genes with >2-fold change, p < 0.05, and a probe signal at least twice the background signal in at least 1 of the 2 groups. Altogether, 461 genes were identified; of these, expression of 423 genes was upregulated and expression of 38 was downregulated in TINT relative to normal prostate tissue.

To characterize TINT and determine what biological processes the 461 candidate genes selected were associated with, we preformed a gene ontology analysis using GeneGo MetaCore software. As could be predicted from our previous findings in this rat model, many of the genes altered in TINT were related to processes such as inflammatory responses and organization of the extracellular matrix (ECM).

Furthermore, we visualized the differential expression in TINT relative to that in normal prostate tissue, with 461 significantly altered genes, in a clustering-based heatmap (Fig. 3 in paper I). The heatmap also included the expression levels of selected candidate genes in AT-1 tumor tissue relative to controls. Hierarchical clustering of all samples resulted in 3 major groups of gene expression profiles. Three major gene clusters were identified: (A) genes downregulated in TINT and tumor relative to normal tissue, (B) genes mainly upregulated in both TINT and tumor tissue relative to normal tissue, and (C) genes exclusively upregulated in TINT relative to normal control tissue.

In this study, we found that implanting rat prostate tumor cells into the prostates of immune-competent rats induced changes in gene expression in the tumor-adjacent non-malignant rat prostate tissue (TINT). Some of the changes in gene expression in TINT were probably due accumulation of inflammatory cells, having been attracted by factors secreted from prostate tumor epithelial...
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cells. For example, expression of genes encoding markers of macrophages (Cd68) particularly of the tumor-stimulating “M2 type” (Cd163, Mrc1, Mgl1, Folr2, and Hmox1), lymphocytes (Cd8a), and mast cells (the mast cell chymase gene Cma1 and the gene for mast cell antigen 32, Mca32) was all upregulated in TINT. Implanting prostate tumor appears to affect the fibromuscular stroma and ECM within the surrounding TINT. We found in this study that expression of genes encoding stroma-related factors such as S100A4, periostin, Sparc, CXCL12, various collagens (Col1a1, Col1a2, Col3a1, Col4a1, Col5a1, Col5a2, Col6a1, Col6a3, Col8a1, Col14a1, Col15a), vimentin (Vim), elastin (Eln), fibronectin (Fn1), and lysyl oxidase (Lox) was higher in TINT than in control tissue.

Also transforming growth factor β1 (Tgfb1) mRNA expression was elevated in TINT and tumor tissue. Tgfb1 is key factor that can induce changes in stroma cells in wounds and tumors. An additional factor that would explain changes in gene expression in TINT is hypoxia. Rapid growth of a tumor inside the prostate may result in some degree of hypoxia in the surrounding normal tissue. We evaluated prostate tissue hypoxia using Hypoxyprobe, which showed that the percentage of hypoxic prostate epithelial cells in TINT was greater than in controls injected with medium. Although most of the hypoxia regulation of HIF-1α does not occur at the mRNA level, Hif-1α expression was upregulated 1.7-fold in TINT relative to normal prostate tissue.
**Paper II**

In line with our findings in the first paper with our animal model, recent studies have shown that the stroma in the non-malignant parts of a prostate with cancer is also altered, indicating that the reactive stroma extends far beyond the borders of the tumor. One change noted in TINT in our animal models was alteration in the ECM.

In this paper we studied hyaluronan (HA), which is a glucosaminoglycan and is a part of the extracellular matrix. HA is important for cell division, cell migration, angiogenesis during embryogenesis, inflammation, and wound healing.

With this background, our objective was to investigate whether the presence of a tumor increases hyaluronan (HA) levels in surrounding prostate tissues and whether this extra-tumoral HA influences tumor growth and outcome. From a series of 287 men diagnosed with PC after transurethral resection (TUR) between 1975 and 1995, and followed up with watchful waiting, tissue microarrays were constructed, stained, and scored for HA.

This study showed that a high HA staining score in the non-malignant stroma prostate tissue (TINT) was associated with increased risk of death from PC. It also showed that HA staining score in the stroma of the non-malignant prostate tissue (TINT) was positively correlated with Gleason score, estimated tumor volume, and tumor cell proliferation.

In our orthotopic rat prostate cancer model, HAS-1 (hyaluronic acid synthase-1) mRNA levels were higher in the non-malignant prostate tissue surrounding AT-1 tumors than in controls. Also, immunostaining not only showed strong HA staining in the rat prostate AT-1 tumor stroma, but also a moderate to strong staining in the stroma of the surrounding non-malignant prostate tissue. We also found that intraprostatic injection of HA in orthotopic AT-1 tumor stimulated the growth of the tumor.

The mechanisms responsible for the development of elevated HA levels in tumor and non-malignant stroma prostate tissue (TINT) are largely unknown, but tumor cell secretion of tumor growth factor is one likely mechanism. The expression of tumor growth factor is elevated in human PCs, and it was also elevated in our animal model (paper I). An additional explanation of increased HA levels would be hypoxia. PCs are hypoxic; hypoxia makes tumors more aggressive and it stimulates HA synthesis.

Based on findings in this paper, the presence of a tumor increased the hyaluronan levels in the surrounding morphologically normal prostate tissue (TINT) — and the magnitude of this was associated with tumor aggressiveness and the risk of prostate cancer death. We suggest that a higher HA staining score could be an additional TINT marker associated with prostate cancer aggressiveness.
Paper III

Our findings in papers II and I indicated that the presence of a prostate tumor induces responses in the normal tissue adjacent to the tumor (TINT). In this paper, we determined whether the nature and magnitude of these responses in TINT were related to tumor size and aggressiveness.

We used our Dunning prostate tumor model, consisting of G, AT-1, and MatLyLu tumors, which are all poorly differentiated tumors but differ in metastatic ability and growth rates. We established orthotopic tumors with different tumor sizes from each tumor type, either by following them over time (AT-1 and MatLyLu) or by injecting a different number of cells (G) into one ventral prostate lobe. In this way, we could compare changes in TINT with the different tumor types by adjusting for size, and in addition examine how tumor size would affect the adjacent normal tissue for each tumor type. We used different controls: animals injected with vehicle or heat-killed tumor cells into the prostate. This gave us the prospect of excluding immune response that could be unspecific to the presence of a growing tumor.

This study showed that all tumor types induced increases in macrophage, mast cell, and vascular densities and in vascular cell proliferation in the tumor-bearing prostate lobe compared to controls. These increases occurred in parallel with tumor growth. The most pronounced and rapid responses were seen in the prostate tissue surrounding MatLyLu tumors. Even when small, they were particularly effective in attracting macrophages and stimulating the growth of not only micro-vessels but also small arteries and veins compared to the less aggressive AT-1 and G tumors.

The nature and magnitude of tumor-induced changes in the tumor-bearing organ are related to tumor size but also to tumor aggressiveness. These findings, supported by previous observations in patient samples, suggest that one additional way to evaluate prostate tumor aggressiveness could be to monitor the effect of the tumor on adjacent tissues.
Paper IV
Our previous findings showed that implantation of rat prostate cancer cells into the normal rat prostate results in tumor-stimulating adaptations in the tumor-bearing organ. Similar changes can be seen in PC patients, and related to outcome. In paper I, one factor that was found to be upregulated in the non-malignant part of a tumor-bearing prostate lobe in rats was the transcription factor CCAAT/enhancer-binding protein beta (C/EBPβ). Ontology analysis showed that transcription factor C/EBPβ affects on many of the genes altered in TINT (Figure 5). C/EBPβ is generally involved in regulating key biological processes, including cellular growth and differentiation, and its increased expression correlates with tumor invasiveness $^{139-141}$. In PCs, C/EBPβ expression is upregulated in castration resistant cases and it stimulates metastasis-associated genes $^{129-131}$. The functional role of C/EBPβ in non-malignant tumor-bearing prostate tissue (TINT) is unknown.

To investigate this, we used tissue microarray (TMA) constructed from a series of 390 men who were diagnosed with PC between 1975 and 1995 after transurethral resection (TUR) and followed up with watchful waiting. The tissue microarrays were stained and scored for C/EBPβ. We also used animal models with different tumors: slow growing non-metastatic Dunning G, rapidly gro-
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wing poorly metastatic Dunning AT-1, and rapidly growing highly metastatic Dunning MatLyLu rat prostate tumors. C/EBPβ expression was examined by quantitative RT-PCR and by immunohistochemistry in normal rat prostate tissue surrounding the different tumors (TINT).

In patients with PC, we found that high expression of C/EBP-beta in glandular epithelial cells in the normal prostate tissue (TINT) was associated with accumulation of M1 macrophages (iNOS+) and a favorable outcome. On the other hand, high C/EBP-beta in tumor epithelial cells was associated with a high Gleason score, high tumor cell proliferation, the presence of metastases at diagnosis, and a poor outcome. We also found a synergistic relationship between phosphorylated-EGF receptor and C/EBPβ expression. Patients with high levels of pEGFR and C/EBP-beta in tumor epithelial cells had a 4-fold higher risk of death from PC (compared with the added separate relative risks). On the other hand, patients with high immunoreactivity of pEGFR and low fraction of C/EBP-beta staining in non-malignant epithelial cells (TINT) had a 3-fold higher risk of prostate cancer death (compared with the added separate relative risks).

In animals, C/EBP-beta mRNA expression was upregulated in prostate tumors and in the surrounding tumor-bearing prostate lobe. In tumors and in the surrounding non-malignant prostate tissue, C/EBP-beta was detected by immunohistochemistry in some epithelial cells and in infiltrating macrophages. The magnitude of glandular epithelial C/EBP-beta expression in the tumor-bearing prostates was associated with tumor size, distance to tumor, and tumor cell metastatic capacity.

In this paper, our observations in prostate rats suggested that the presence of an aggressive and potentially metastatic tumor could perhaps be indirectly detected by measuring elevated C C/EBPβ in the epithelium in the adjacent non-malignant parts of the tumor-bearing organ. But when we examined C/EBPβ expression in benign epithelial cells in the tumor-bearing organ in patient samples, we found that high C/EBPβ expression in TINT was associated with lower tumor Gleason score and tumor size and with a favorable outcome. So what could be the explanation for these apparently contradictory findings in patients and in the animal model?

Western blot analysis of patient and rat samples showed that the different roles of C/EBPβ in the non-malignant parts of the prostate between the rat model and patients could not be explained by a major shift in LAP/LIP ratio. Instead, we found that C/EBPβ staining in the TINT epithelium was correlated to macrophage density, both in rats and humans, but in somewhat different ways. Tumor-associated inflammation—when dominated by accumulation of M1-type macrophages—leads to inhibition of tumor growth, whereas inflammation characterized by M2-macrophages stimulates prostate tumor growth.
RESULTS AND DISCUSSIONS

(see Introduction). The tumor-induced inflammation in TINT in our rat model with aggressive metastatic tumors was dominated by M2 (CD163+) macrophages and increased C/EBPβ expression, but in patients increased C/EBPβ was related to accumulation of M1 (iNOS+) macrophages. This may explain why increased C/EBPβ in the tumor-bearing human prostate was associated with a favorable patient outcome, and might also explain the different roles of C/EBPβ in TINT between the rat model and patients. However, the reasons to these different inflammation responses are unknown.
CONCLUSIONS

Paper I
Using an animal model, we found that histologically normal prostate tissue adjacent to a tumor has a unique gene expression signature relative to normal tumor-free prostate tissue. This shows that the presence of a tumor induces changes in gene expression in the surrounding tumor-bearing organ. Studies are now under way to determine the kinetics of this response, and determine how it differs in prostate tissue surrounding tumors with different aggressiveness. The ultimate aim is to identify candidate genes that could possibly serve as novel diagnostic or prognostic markers and/or therapeutic targets for prostate cancer.

Paper II
In this study, we found that the presence of a tumor increased the HA staining score in the surrounding morphologically normal prostate tissue and that the magnitude of this was associated with tumor aggressiveness and the risk of prostate cancer death. Also, exogenous administration of HA stimulated tumor growth in an animal model.

Paper III
Earlier studies showed that the tumor-bearing organ is influenced by the growth of a prostate tumor. In this paper, using our animal model with differentiate aggressive prostate tumors, we found that implantation of tumor cells into the rat prostate not only results in adaptive—and presumably tumor-promoting—changes in the tumor-bearing organ, but also some of these adaptive TINT changes appear to be mainly related to tumor size whereas others are also related to the growth and metastatic potential of the growing tumor.

Paper IV
In this paper, we studied the role of C/EBPβ in non-malignant tissue surrounding the tumor (TINT) using TMA from PC patients and animal models. We found in PC patients that high expression of C/EBPβ in glandular epithelial cells in the normal prostate tissue (TINT) was associated with accumulation of M1 macrophages (iNOS+) and a favorable outcome. In rats, the magnitude of glandular epithelial C/EBPβ expression in TINT was associated with tumor size, distance to tumor, and tumor cell metastatic capacity.
TINT vs. field cancerization; what is what?

The term “field cancerization” was introduced in 1953 by Slaughter when he studied oral squamous cell carcinoma. The term was used to explain that the occurrence of multiple tumors was due to the existence of generalized carcinogens that induce early genetic changes in the epithelium, rather than the spread of pre-existing cancer cells. Much later, field cancerization was proposed for other epithelial tumors and the molecular signatures of field cancerization have been documented even in prostate cancer. Notably, and in line with the definition, field cancerization has been associated with cancer, but not with its aggressiveness.

Prostate cancer tends to present as multifocal disease, but the mechanism behind it is still unknown. Multifocality of PC is compatible with the “field cancerization” concept; multifocal tumors arise from prostate gland that are genetically altered by a particular carcinogen. Although relatively little work has been done on field cancerization in the prostate compared to other organ systems, including breast, some evidence of field cancerization in PC has been reported. Methylation in the GSTP1 and RARbeta2 genes was found in tumor stroma and adjacent normal stroma and glands close to tumor tissue in PC patients. Methylation of these genes was absent in normal epithelium in patients with benign prostatic hyperplasia.

Pre-malignant epithelial changes induced outside a developing tumor by the carcinogenic agent can be explained as being the result of a cancer field effect. On the other hand, adaptive changes in the normal stroma and the glandular epithelium in the surrounding normal-appearing prostate tissue caused by paracrine influences from the tumor epithelium cannot be explained as being the result of an epithelial field effect. This justified the introduction of the term “TINT”, which describes the adaptive changes in tumor-surrounding normal tissue, changes that are related to tumor aggressiveness. Tissues can thus be tinted (colored) by tumors.

Adaptive (TINT) changes and cancer field effects (pre-cancerous epithelial lesions) are probably partly overlapping conditions that explain the changes in the epithelial compartment of tumor-bearing organs. Changes in surrounding tissue are not restricted to the epithelium, but also occur in the stroma, and are due to signals from the growing tumor, which needs to influence and interact with adjacent and more remote cells, tissues, and organs. It is therefore proposed that this type of tissue with adaptive changes should be termed TINT—tumor indicating normal tissue.
The potential of our rat model
In order to study the effect of a tumor on the surrounding normal prostate tissue (TINT), apart from material from PC patients, we used a short-term rat tumor-implantation model. In this model, we implanted rat prostate tumor cells into the prostates of immune-competent rats and sacrificed the animals after 1–2 weeks, in order to examine TINT—the tumor-adjacent non-malignant rat prostate tissue—which in this model contains both morphologically normal-appearing epithelium and stroma. The differences between this model and PC patients are not insignificant. In patients, tumors grow for more than 10 years before they become clinically significant, and in our models the tumors grow for approximately 10 days. Also, PC in patients is often heterogeneous; i.e., different tumors of different size and differentiation grade are often found in the same prostate, and in our animal model we only have one poor differentiated tumor in one prostate.

Regardless of the obvious differences between the rat model and patients, our study in paper I showed altered gene expression in histologically normal tumor-adjacent tissue relative to that in normal prostate tissue that was free from tumor in our rat model. In line with our findings in the rat model, gene expression in tumor-adjacent prostate tissue in patients is also characterized by processes such as inflammation and wounding. Also, similar findings in breast cancer patients have been reported, where gene expression in tumor-adjacent tissue was characterized as a wounding response.

Candidate genes
In our first paper, we identified 461 candidate genes that were differentially expressed in TINT compared to normal prostate tissue that was free from tumor. Many of those genes but not all, showed a similar expression pattern in both TINT and tumor tissue. Of the 461 significantly altered genes, 38 genes were downregulated and 307 genes were upregulated in both TINT and tumor samples relative to the controls. This finding suggests that tumors and TINT may actually share common factors—which indicate that the current strategy for screening for prostate cancer markers has probably been insufficient in many studies, as TINT samples are currently used as controls. Consequently, only factors that are altered in tumors relative to the surrounding (normal) tissue are detected and considered to be potentially useful. This strategy may actually overlook some really useful markers, those that are highly altered in both the tumor and the tumor-bearing organ.

Several particular genes in the list of candidate genes were interesting and deserved further study. One highly upregulated factor was heme oxygenase 1 (HO-1 from the Hmox1 gene), which has been shown to be regulated by cellular
stress caused by, for example, hypoxia and inflammatory cytokines \(^{151}\). Based on this and our finding in paper I that Hmox1 expression was upregulated 14 fold in TINT compared to normal tissue, our group has conducted a study that showed HO-1\(^+\) macrophages accumulating in the tumor-bearing organ, and at the tumor-invasive front in the animal model. In prostate patients, accumulation of HO-1\(^+\) macrophages was seen at the tumor-invasive front almost exclusively in high-grade tumors, and was correlated to the presence of bone metastases \(^{152}\).

Another interesting factor that we identified in paper I was lysyl oxidase (LOX), which is an enzyme that is stimulated by TGF-β1 and hypoxia, and causes cross-linkage between collagen and elastin (which were also found to be upregulated in TINT) \(^{94, 153-155}\). In this way, LOX may increase matrix stiffness and facilitate tumor growth \(^{93}\). Further studies on LOX have been published recently by our group. The first one showed that, men with low levels of LOX in the non-malignant prostate epithelium (TINT) had significantly longer cancer-specific survival than men with high levels of LOX. Also, in radical prostatectomy specimens, LOX immune-staining and LOX mRNA levels were found to be similar between tumor and adjacent non-malignant areas, but significantly elevated in bone metastases samples \(^{156}\). The second study showed that inhibition of LOX enzymes that was initiated before implantation of tumor cells in an animal model reduced tumor growth. On the other hand, if the treatment was started after the tumors were established, it did not affect tumor growth. This was explained by a decrease in collagen fiber, which is a target for LOX, in tumors and in the tumor-adjacent prostate tissue (TINT) \(^{157}\).

**The Västerås patient material**

The TMA samples were constructed from a consecutive series of patients with voiding symptoms who were diagnosed with PC between 1975 and 1995 after transurethral resection (TUR) of the prostate. Because this series was collected before the PSA era, information on serum PSA was not available. This implicate that our TUR-diagnosed PCs could differ from current PSA-detected tumors. Also, this TMA material was mostly composed of samples from the central parts of the prostate and did not represent the whole prostate. Another disadvantage of this patient material was the lack of spatial information, which means that we could not determine how far the response we studied reached into the adjacent tissue. Thus, further studies of our markers of prostate cancer are required; these should be examined in radical prostatectomy specimens to clarify this.

On the other hand, the strength of this patient material was the long follow up time (up to 25 years) of patients after diagnosis of PC. This is especially important in prostate cancer research, due to the slow-growing nature of the disease. Also, this material included PC patients who did not receive treatment independently
of the tumor’s Gleason score. These reasons made the material suitable for the study of PC biomarkers and their association with prostate cancer outcome. Importantly, this material makes it possible to find not only markers for aggressive PC, but also markers for “hurtless” tumors that do not require any treatment.

**Future aspects of TINT**

There is a large clinical need for diagnostics that allow us to predict the presence of cancer by analyzing changes in the surrounding non-malignant “normal” tissue obtained by the negative biopsies.

In this thesis and in other projects of our group, we have provided growing evidence that the tumor-adjacent non-malignant prostate tissue (TINT), composed of normal prostate stroma and glands, is altered. And these changes in morphologically normal-appearing prostate tissue can be related to the aggressiveness of the cancer, probably because they facilitate tumor growth and metastasis. TINT—as defined by us—has diagnostically useful changes in the “normal” tissue adjacent to cancer, related to the presence and aggressiveness of cancer elsewhere in the organ.

Although research on adaptive TINT-related changes in prostate cancer is relatively new, the list of TINT-factors in PC that are associated with tumor aggressiveness and patient outcome (see Table 5 in Paper III, and others such as C/EBPβ, LOX, and HOMX1) is already long. TINT markers could give us crucial information for deciding about further action, especially the choice between active surveillance and more aggressive therapeutic intervention.

In order to really evaluate the usefulness of TINT-related factors in clinical practice, and in particular when using negative biopsies, the findings require verifications in different and larger patient materials. Also, we need to ask questions regarding how the tumor epithelium or other cells in a tumor induce changes in normal prostate tissue. Also, through which mechanisms are these changes related to the aggressiveness of the cancer, and how do they facilitate tumor growth and metastasis?

By answering those questions, we might be able to understand the biology of TINT-related changes, and TINT markers might not only serve as prognostic markers but also as therapeutic targets. We need to investigate the distance from a tumor that each TINT marker can be detected. We must also determine how the magnitude of the TINT effect is related to distance to the tumor and its aggressiveness, such as, for example CEBPβ. Biomarkers with varied expression patterns could be of considerable value for location of aggressive tumors.
One of the factors that were downregulated in rat TINT and prostate tumors was microseminoprotein-beta (Msmb), a factor produced almost exclusively by prostate epithelial cells. Reduced Msmb level in serum is a useful diagnostic and prognostic indicator in prostate cancer. But why are the levels reduced in blood when only a minor proportion of the prostate (the tumor) stops producing it? Or data might suggest that the best biomarkers for cancer could actually be those that are altered in TINT and tumor at the same time. Is Msmb a marker of this type? Have other markers been missed when using tumor-adjacent tissue as control?

In the work for this thesis, we used morphological evaluation and gene-expression analysis to study changes in normal-appearing prostate tissue (TINT) that are related to the aggressiveness of prostate tumor. Could this TINT-effect be measured by other methods? In paper III, we suggested that in order to ensure that there is a sufficient blood supply to an expanding tumor, the growth of arteries and veins in the tumor-bearing organ (TINT) is induced. Consequently, if any increase in size of the arteries and veins could be measured by imaging techniques such as ultrasound or MR, that would also facilitate diagnosis of prostate cancer.

Another aspect that should be considered is whether the TINT-effect extends outside the prostate. Could we measure the TINT-effect in other organs (i.e. measure changes in protein levels or in tissue morphology—that are induced by a prostate tumor—in other organ than the prostate)? Our studies have shown that most of the TINT-related changes are associated with inflammation response, which suggests that regional lymph nodes should be activated by the presence of a tumor in the prostate. Studies are being carried out investigate whether activation of and changes in these regional lymph nodes could be related to prostate cancer and whether they could be different from those in a common infection.

"It's your turn soon, doctor"
Children of Deraa
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