THE APPLICATION OF PROGNOSTIC TISSUE MARKERS TO PROSTATE CANCER: CLINICAL AND EXPERIMENTAL STUDIES

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THE APPLICATION OF PROGNOSTIC TISSUE MARKERS TO PROSTATE CANCER: CLINICAL AND EXPERIMENTAL STUDIES

De toepassing van prognostische weefsel markers bij prostaat carcinoom: klinische en experimentele studies

PROEFSCHRIFT

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opgedragen aan mijn ouders

CONTENTS

LIST OF ABBREVIATIONS

5-HT	serotonin
ABC	avidin-biotin complex
ACTH	adreno-corticotrope hormone
AJCC	American joint committee on cancer
APES	aminopropyl-trietoxy-silane
APUD	amine precursor uptake and decarboxylation
AR	androgen receptor
bp	base pairs
BPH	benign prostatic hyperplasia
BrdU	bromo-deoxy uridine
cDNA	complementary DNA
CgA	chromogranin A
CgB	chromogranin B
CGRP	calcitonin gene-related peptide
CI	95% confidence interval
CT	calcitonin
DAB	3,3'-diaminobenzidine-hydrochloride
DCC	dextran coated charcoal
dNTP	deoxynucleotide-triphosphate
EGF	epidermal growth factor
EGF-R	epidermal growth factor-receptor
FGF	fibroblast growth factor
FISH	fluorescence in-situ hybridization
GRP	gastrin-related peptide
GSS	Gleason sum score
H&E	hematoxylin & eosin
ISH	in-situ hybridization
LH-RH	luteinizing hormone-releasing hormone
M-MLV	moloney-murine leukemia virus
MRI	magnetic resonance imaging
NE	neuroendocrine
NRF	nuclear roundness factor
NSE	neuron specific enolase
PAM	peptidyl glycine-α-amidating mono-oxygenase
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase-chain reaction
PI	prognostic index
PIN	prostatic intraepithelial neoplasia
PSA	prostate specific antigen
ROC	receiver operating characteristic curve
RT-PCR	reverse-transcriptase polymerase-chain reaction
SCPC	small cell prostate cancer
SMS	somatostatin
SPF	S-phase fraction
TGF	transforming growth factor
TNM	tumor, nodes and metastasis
TRUS	transrectal ultrasound
TSA	tyramide signal amplification
TUR	transurethral resection
UICC	Union internationale contre le cancer
VEGF	vascular endothelial growth factor
WHO	World Health Organization

CHAPTER I

PROGNOSTIC MARKERS IN PROSTATE CANCER

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INTRODUCTION

The incidence and prevalence of prostate cancer have dramatically increased during the last two decades[1-3]. It is now the most prevalent malignant non-skin tumor in the western male population and its mortality is second only to that of lung cancer[4]. Potosky *et al.*[1] studied the incidence of prostate cancer in an analysis covering about 6% of the USA male population. A linear increase was found from 1973 to 1986, which became exponential from 1987 to 1991. The exponential increase can be largely attributed to the introduction of prostate specific antigen (PSA), transrectal ultrasound (TRUS) and, more recently, to the introduction of population based screening programs as advocated by the American Cancer Society and American Urological Association[5-7]. However, a very recent study indicates that as from 1991 the age adjusted incidence rate started to decline as fast as it increased before (Figure I.1)[8]. This is explained by the fact that the application of PSA, TRUS and screening programs enabled the diagnosis of cases that would otherwise have been either diagnosed later or even missed completely. These cases are progressively depleting the population of potential new cases. Consequently, the exponentially rising incidence did not reflect the true incidence and will fall to a new, more realistic steady state. This phenomenon also occurred following the introduction of mammography as a screening test for breast cancer[9]. The linear increase in age adjusted prostate cancer incidence rates as described above might be caused by changes in risk factors but also by an increased life expectancy. Whether the observed exponential increase is superimposed on a continuing linear trend can only be concluded if a new steady state is reached.

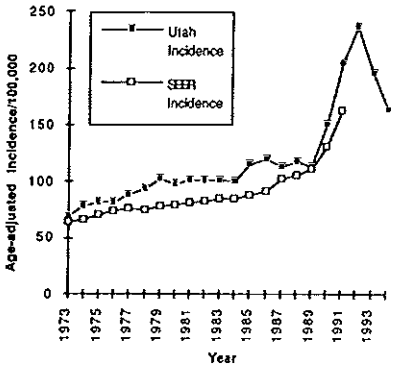

FIGURE I.1
Age adjusted incidence of prostate cancer per 100,000 in two American study populations. Reprinted form: Stephenson RA *et al.*, Cancer 1996;77:1342-8, with permission of the publisher.

Data from the American National Cancer Database[10] show that the increased prostate cancer incidence rates coincided with a shift towards younger patients with more favorable clinical parameters. The percentage of clinically localized tumors

increased from 19% in 1986 to 49% in 1993 and the percentage of moderately differentiated cancer increased from 39 to 58% during the same period. As a result, the fraction of newly diagnosed patients eligible for, and treated by, radical prostatectomy increased from 10 to 29%[10]. The results of this study may have been biased by the fact that the percentage of cases with unknown stage and grade declined during the study period. However, these trends were also described by others[2, 3, 8].

It has been known for years from autopsy studies and studies of radical cystoprostatectomy specimens removed for bladder cancer that the prevalence of latent prostate cancer increases dramatically with age to more than 50% in men over 80 years. In a recent, relatively small study using whole mount prostate sections Sakr *et al.*[11] found prostate cancer foci already in 28 and 34% of men who died in their thirties or forties, respectively. Furthermore, most prostate cancers are growing very slowly[12]. It has been estimated that no more than about 1 out of 4 prostate cancers becomes clinically apparent and that about 1 out of 3 patients with clinical prostate cancer will eventually die of it[13]. For 1996 about 317,000 new prostate cancer cases and 41,400 prostate cancer deaths have been predicted for the USA (incidence/mortality ratio = 7.7:1)[14]. This ratio will probably increase further. One may therefore argue that detection of clinically unapparent, slowly growing prostate cancers may lead to over-diagnosis, over-treatment and may mean an avoidable psychological burden to the 'patient'[15]. On the other hand, prostate cancer can kill patients (and is with that respect only surpassed by lung cancer) and therefore we have to answer the following questions: which tumors are significant, which patients should be treated and how should they be treated? The question whether or not screening for prostate cancer is beneficial goes beyond the scope of this thesis and will not be addressed.

NATURAL COURSE

The natural course of prostate cancer is highly variable, difficult to predict and has not been studied extensively. Furthermore, interpretation of data from screening-studies and prognostic follow-up studies is seriously hampered by the absence of adequate knowledge of the natural course of the disease. Whitmore and Warner[16, 17] selected from more than 4000 patients a group of 75 untreated patients with well or moderately differentiated prostate cancer who showed no tumor progression after one year of follow-up and these patients were managed conservatively. The disease free survival rate of this highly selected patient-group at 10 years was 91%, but decreased to 67 and 37% at 15 and 20 years, respectively. In another long-term follow-up study it was found that 59% of the patients who were considered radical prostatectomy candidates, but were

managed conservatively eventually died of prostate cancer[18]. In studies from Sweden including patients with T_{1-2} tumors, the opposite was found (10% prostate cancer deaths after a mean follow-up period of 12.5 years)[19-21]. These studies have been criticized for a number of favorable selection criteria applied to the study population[22]. Chodak *et al.*[23] performed a meta-analysis on the results of a total of 828 patients published in 6 studies, including the Swedish studies. Tumor specific survival for grade 1 and 2 tumors was identical to the life-expectancy of the general population, but decreased significantly for grade 3 tumors. Metastasis free survival at 10 years was about 70%, 55%, and 20% for grade 1, 2, and 3 tumors, respectively. This meta-analysis was based on data from studies that all suffered from methodologic problems and it is questionable if definite conclusions can be drawn from this study. It, however, appears that the majority of prostate cancers have the potential to progress, but also that it may take considerable time to occur. It is clear that in older patients with small, well differentiated tumors the likelihood of tumor progression is determined mainly by factors not related to the primary prostate tumor (such as the presence of cardiovascular diseases, diabetes mellitus or other malignancies).

TREATMENT

Regarding the choice of treatment it is important to realize that patients with prostate cancer generally are of advanced age, but also that most prostate cancers will progress, be it after a considerable period of time. A number of patients, especially older patients with small tumors, can be managed conservatively[23], which means a careful follow-up program in which (endocrine) treatment will start only at the moment that progression is noted. Most patients with clinically localized prostate cancer (i.e., a tumor that is not growing beyond the prostatic capsule) are offered a form of curative treatment: external beam radiotherapy[24] or radical prostatectomy[25]. For years the choice between those two has been the subject of debate (reviewed by Hartford and Zietman[26]). In a number of studies, surgical therapy was found to be superior to radiotherapy, but patients referred for radiotherapy in general showed unfavorable characteristics (high age, larger tumors and an unknown lymph node status). A very recent study has shown that co-morbidity is a significant and independent prognostic marker for patients with clinically localized prostate cancer treated with endocrine therapy[27]. Since healthier patients are more likely radical prostatectomy candidates, another bias is probably introduced. Some long-term studies did not find differences between the two curative treatments. Randomized clinical trials comparing the two types of treatment have not been completed, however. It is at present not very well known whether definite treatment for clinically localized

prostate cancer is beneficial to the patient. Only one randomized study that included 142 patients compared radical prostatectomy with 'placebo'[28]. At 15 years of follow-up there was no difference in survival between the two groups in 95 evaluable patients. The study used, however, overall survival as an endpoint, the two treatment arms were not balanced according to grade and stage and the statistical power was insufficient to make a comparison between the treatment arms. For these reasons it is not possible to draw a valuable conclusion from this study.

Tumors that are either metastasized or locally extensive cannot be cured anymore and these patients usually receive endocrine treatment. Endocrine treatment by means of a bilateral orchiectomy or with antiandrogens gives a response in about 40-50% of the patients (partial and complete response), but almost all tumors will eventually progress and tumor specific survival rates are in general not improved[29, 30]. Symptoms of urinary obstruction can be handled by a trans-urethral resection (TUR). Androgen independent or hormone refractory prostate cancer (that is a tumor which is able to grow despite castration levels of androgen) is difficult to treat. Prostate cancer is resistant to most cytotoxic drugs, although some cytostatic treatment regimens seem promising[31]. Symptomatic metastatic bone lesions can be treated by radiotherapy.

PROGNOSTIC MARKERS

A large number of authors has recognized the need to distinguish the relatively indolent prostate cancers which will not kill the patient from the potentially lethal tumors (often described by the metaphor 'the pussy cats and the tigers'). This classification only considers tumor characteristics, but it will be clear that the prognosis also depends on patient based factors like age and general health condition.

The term prognosis for patients with established primary or metastatic prostate cancer can be defined as the prediction of future behavior of the tumor, either in the absence of or after application of therapy[32]. With the assessment of prognostic markers attempts are being made to predict the clinical course of the disease in a specific patient. A prognostic marker can be defined as a qualitative or quantitative alteration or deviation from normal of a molecule, substance or process that can be detected by some kind of assay and that is correlated with prognosis[32]. Ideally, knowledge of a prognostic marker should lead to clinical decisions that in turn should result in improved clinical outcome as defined by overall survival, disease-free survival, quality of life or costs of care.

It is important to distinguish prognostic markers from response markers and surrogate end-point markers (although some markers may fit to several categories).

Response markers are markers that usually can be determined repeatedly and that can be used to monitor response to a certain therapy (for example the extent of bone metastasis during endocrine treatment). Surrogate end-point markers are markers that are strongly correlated with and can replace 'hard' end-point markers like clinical progression or tumor related death (for example a rising serum PSA level after an initial decline following radical prostatectomy). In this review the term prognostic marker will refer to the above described definition.

Several variables are strongly correlated with prostate cancer patient outcome and are indeed used to make clinical decisions, notably: tumor-stage and to a lesser extent tumor-grade. Nevertheless, it is as yet for most patients difficult, if not impossible, to predict disease outcome on an individual basis. This is mainly due to a large heterogeneity among prostate tumors, even if from the same stage and grade. It also implies that the clinical course of the disease cannot be predicted on an individual basis by the assessment of only one or two prognostic variables. Moreover, with increasing knowledge about the biology of the disease other (adjuvant or neo-adjuvant) treatments might become available that make current prognostic schemes inadequate or even useless for certain patient categories. For this reason a substantial part of prostate cancer research is dedicated to the search and application of new prognostic tumor markers.

The College of American pathologists divided prognostic markers in several categories (working classification for prognostic markers)[33]. **I.** Markers well supported by clinical literature and generally used in patient management. **II.** Markers extensively studied biologically and/or clinically. **III.** Markers that currently do not meet criteria for category I or II. Category II was sub-divided in **IIa.** tested in clinical trials, and **IIb.** biological and correlative studies done, few clinical outcome studies. It is clear that only the categories I and IIa represent true prognostic markers (that is, markers that can be used for clinical decision making). Most of the prognostic markers investigated at present fall into categories IIb or III.

It is important to investigate new prognostic markers together with established markers using multi-regression analysis techniques, since most markers are found to be strongly correlated with especially tumor grade. Given the slow growth and progression of prostate cancer, follow-up studies should be of long-term to ensure the inclusion of sufficient numbers of events. Archival material of patients with long-term follow-up should be used with special concern. The patients that have been followed longest (for more than 10 years) determine the long-term prognosis and are probably different from contemporary patients due to the stage and grade shift which occurred over the past decade[10]. Thus, especially at the long-term, progression and tumor-specific death rates may be overestimated.

Since this thesis focusses on prognostic tissue markers, emphasis will be given to these markers. Not all prognostic markers will be discussed extensively, neuroendocrine differentiation will be discussed in full detail in chapter II and V.

TUMOR EXTENT, STAGE

The stage of prostate cancer is assessed clinically by a combination of digital rectal examination, TRUS, CT- and MRI-scanning, and X-ray photos. The tumor, nodes and metastasis (TNM) 1992 classification[34] has been advocated by the UICC (international union against cancer) and the AJCC (American joint committee on cancer). It is the most widely used staging system for prostate cancer at the moment (Table I.1).

TABLE I.1

TNM classification of prostate cancer

T	T_x	primary tumor cannot be assessed
	T_0	no evidence of primary tumor
	T_1	clinically unapparent tumor, not palpable, nor visible by imaging
		T_{1a} incidental histologic finding in ≤5% of tissue resected
		T_{1b} incidental histologic finding in >5% of tissue resected
		T_{1c} tumor identified by needle biopsy
	T_2	tumor confined within the prostate
		T_{2a} ≤ half of one lobe
		T_{2b} > half of one lobe
		T_{2c} both lobes
	T_3	locally extensive prostate cancer
		T_{3a} unilateral capsular extension
		T_{3b} bilateral capsular extension
		T_{3c} invasion of seminal vesicle(s)
	T_4	tumor is fixed or invades adjacent structures other than seminal vesicles
		T_{4a} invasion of bladder neck, external sphincter and/or rectum
		T_{4b} invasion of levator muscles or fixation to pelvic wall
N	N_x	regional lymph nodes cannot be assessed
	N_0	no regional lymph node metastasis
	N_1	metastasis in a single regional lymph node, ≤2cm
	N_2	metastasis in a single regional lymph node, 2-5cm or multiple regional lymph nodes ≤5cm
	N_3	metastasis in regional lymph node(s), >5cm
M	M_x	presence of distant metastasis cannot be assessed
	M_0	no distant metastasis
	M_1	distant metastasis
		M_{1a} non-regional lymph nodes
		M_{1b} bone(s)
		M_{1c} other site(s)

Basically, the system is developed as a clinical staging system. If a certain stage has been assessed pathologically (for example, because of the presence of tumor in a bladderneck biopsy (T_4) or pathological examination of a radical prostatectomy specimen) that category is preceded by a p (pT_4). Categories with approximately the same prognosis have been grouped together in stage groups (Table I.2). Tumor grade (G) has been incorporated in the stage grouping system to distinguish between stage 0 and 1 because well differentiated T_{1a} tumors had a far better prognosis than moderately or poorly differentiated T_{1a} or T_{1b} tumors (2% progression for the former group and 32% for the latter at 4 years of follow-up[35]). At long-term follow-up the difference between T_{1a} and T_{1b} tumors seems to disappear, however[36]. In most of the radical prostatectomy specimens removed for stage 0 or I disease significant residual tumor was present[37]. It is probably this group of patients that shows progression at long-term follow-up. As yet, they cannot be identified clinically prior to making decisions on treatment.

TABLE I.2

Stage grouping

Stage 0	T_{1a}	N_0	M_0	G_1
Stage 1	T_{1a}	N_0	M_0	G_{2-4}
	T_{1b-c}	N_0	M_0	G_{1-4}
Stage 2	T_2	N_0	M_0	G_{1-4}
Stage 3	T_3	N_0	M_0	G_{1-4}
Stage 4	T_4	N_0	M_0	G_{1-4}
	T_{1-4}	N_{1-3}	M_0	G_{1-4}
	T_{1-4}	N_{1-3}	M_1	G_{1-4}

Several radical prostatectomy studies showed a reasonable overall correlation between tumor stage and the likelihood of progression. pT_2 tumors showed disease free survival rates of 90-95% at 5 years, 76% at 10 years and 47% at 15 years following radical prostatectomy, whereas these figures were respectively 59-77%, 54% and 11% for pT_3 tumors[38-40]. The same applied to patients treated by external beam radiotherapy[24]. These figures also underscore the heterogeneity of the disease. Despite proven localized disease 25% of the patients with a pT_2 tumor experienced progression within 10 years of follow-up. Numerous studies based on radical prostatectomy specimens found that tumor invasion into one or both seminal vesicles (pT_{3c}) is a poor prognostic sign. In a large study with 812 patients with clinical T_3 tumors treated by radical prostatectomy, however, a 50% disease free survival at 15 years was found[41]. Of the patients in this latter study 17% were down-staged to pT_2 and 33% had lymph node positive disease. In

addition, 60% of these patients received adjuvant treatment (radiation therapy, endocrine therapy or both). Of interest, van den Ouden *et al.*[38] found that the prognosis of patients with clinical T_3 tumors was identical to that of patients with T_{1-2} tumors if the poorly differentiated tumors were excluded. This can also be inferred from the above mentioned study[41].

The concordance between the clinical and pathological T-category has been investigated in several studies. Of the patients with T_{1C} prostate cancer (most often screening patients) treated by radical prostatectomy, 20-49% are finally classified as having a pT_3 tumor[42-44]. For T_{2a-b} and T_{2c} tumors the figures are 25-46% and 48-78%, respectively[25, 45-47]. Thus, a patient with clinically localized prostate cancer has a substantial risk of extra prostatic extension and, consequently, of disease progression following radical treatment. Huland *et al.*[48] tried to improve the results of clinical staging by combining clinical stage with biopsy data and pre-operative PSA level in 257 consecutive patients with T_{1c} or T_2 prostate cancer. Digital rectal examination could not predict pathological stage or PSA progression. Biopsy results and PSA levels correlated well with pT stage, but only in 30% of the clinical T_2 patients the clinical outcome (detectable post-operative PSA) could be predicted.

Like T-stage, the assessment of N-stage by clinical means is also unreliable. For this reason, in most institutions a radical prostatectomy is preceded by a pelvic lymph node dissection. If tumor is found in the lymph nodes on pathological examination of frozen sections, the radical prostatectomy is canceled. It has, however, been suggested that the probability of lymph node metastasis can reasonably well be predicted clinically in subsets of patients by taking into account variables like T-category, tumor grade or PSA[49-51]. The deletion of the pelvic lymph node dissection would be beneficial for the patients with positive lymph nodes who would not have to undergo a major surgical procedure anymore and would also improve cost efficacy of radical prostatectomies.

TUMOR EXTENT, VOLUME

For many tumor types tumor volume is part of the TNM classification system. Due to several factors, this is more complicated for prostate cancer. 1. It is often difficult to recognize prostate cancer macroscopically. 2. Because prostate cancer most often invades and grows in between benign glands clinical assessments are unreliable. 3. Prostate cancer is multifocal in about half of the cases[52]. 4. Estimation of prostate cancer volume requires the investigation of whole mount prostate sections, processed according to a standard protocol making use of sophisticated computerized equipment.

Tumor volume estimated in such a way has been shown to correlate with tumor grade and stage[53-56]. The relationship between tumor grade and tumor volume is influenced by the localization of the tumor. Centrally located tumors can be large at presentation, but are generally more differentiated. For this reason, McNeal *et al.*[55].

suggested that the volume of the poorly differentiated part of a tumor would predict the clinical course best. Many papers established the relationship between tumor volume and other prognostic markers, but only few of them investigated the prognostic value following radical prostatectomy. Epstein *et al.*[57] studied this issue in the radical prostatectomy specimens of 185 patients with $pT_{<3c}pN_0$ prostate cancer and found a prognostic value of tumor volume at univariate analysis, but only of Gleason grade at multivariate analysis. A strong prognostic value of tumor volume was found in two earlier studies[58, 59]. These studies made use of prostates that were not completely embedded and the tumor volume was expressed as a percentage of total prostatic volume.

The possibility to predict total tumor volume using prostate biopsies has been investigated by several authors. The volume of a single biopsy only makes up a very small fraction of total prostatic volume, thus sampling errors may severely inhibit prediction of tumor volume. This might be less a problem in patients with a palpable lesion, compared to the random biopsies from patients with a T_{1c} tumor. Peller *et al.*[53] simply counted the number of biopsies containing cancer in the sextant biopsies of 102 patients treated by radical prostatectomy. Strong correlations were found between the number of positive biopsies and several pathological parameters. On the one hand this finding supports the presumption that larger tumors will be present in more biopsies. On the other hand tumor multifocality and the irregular growth pattern of prostate cancer would suggest a more equivocal relationship. Häggman *et al.*[60] systematically sampled 60 radical prostatectomy specimens with 10 biopsies taken with a special biopti-gun and found a significant correlation between biopsy and prostatectomy tumor volume. Cupp *et al.*[61] also found a significant correlation in the material of 130 patients, but based on their results they stated that on an individual basis biopsy parameters cannot predict total tumor volume reliably (standard error of estimate 6.1ml). In this study the percentage of biopsy length occupied by tumor showed the strongest correlation. Terris *et al.*[62] came to the same conclusion. Thus, although biopsy assessed tumor volume is significantly correlated with total tumor volume, it can probably not be used individually.

Yet another issue that has received considerable attention is the ability to predict the presence of insignificant prostate cancer (the real pussy cats) using biopsy parameters. Dietrick *et al.*[63] concluded from a study of 110 prostatectomy specimens that the presence of less than 3mm of tumor in one of the 6 biopsies reliably identified insignificant cancer (defined as a tumor of <0.5cc). In another study from the same institution in which the same definitions were applied, only 30% of the 'insignificant' cancers at biopsy had a volume of ≤ 0.5ml at radical prostatectomy[64]. Likewise, from the study of Cupp *et al.*[61] it can be calculated that only 2 of 15 patients (13%) that fulfilled this criterion had a tumor volume of <0.5cc. Several authors combined different parameters (biopsy grade, calculated tumor volume, clinical stage, tumor doubling time, age, and serum PSA) to enhance the prediction of insignificant prostate cancer[62, 65, 66]. Although, a combination of parameters better correlated with the presence

of insignificant cancer, it could not be used on an individual basis[62] or was applicable only to a small number of patients[65]. Dugan *et al.*[66] included time related variables like patient-age and tumor doubling time in their model. Insignificant cancer was defined as a tumor that would have grown not larger than $20cm^3$ by the time of expected patient death as predicted from life-tables. It was concluded that most men treated with radical prostatectomy in their institution have significant cancer.

TUMOR HISTOLOGY, GRADE

It is important to realize that different malignant processes can be identified within the prostate histologically. The World Health Organization (WHO) classification[67] is shown in Table I.3. Adenocarcinoma is by far the most common malignant tumor type (>95%). The major part of this thesis will focus on adenocarcinoma and will be referred to as prostatic carcinoma or just prostate cancer. Neuroendocrine tumors (small cell prostate cancer and prostatic carcinoid) will be discussed in more detail in chapter II and V.

TABLE I.3

Histological classification

Epithelial tumors
A. Benign
B. Malignant
1. Adenocarcinoma
2. Transitional cell carcinoma
3. Squamous cell carcinoma
4. Undifferentiated carcinoma
Non-epithelial tumors
A. Benign
B. Malignant
1. Rhabdomyosarcoma
2. Leiomyosarcoma
3. Others
Miscellaneous tumors
1. Neuroendocrine tumors
2. Carcinosarcomas
3. Others
Secondary tumors
Unclassified tumors
Tumor-like lesions and epithelial abnormalities

Numerous prostate cancer grading systems have been developed over the past decades. The grading systems attempt to predict clinical patient outcome based on tumor characteristics like tissue architecture and cellular variables. Boecking *et al.*[68] described 3 criteria that a grading system should fulfill: 1. each diagnostic criterion should be correlated with biological behavior and prognosis. 2. it should display sufficient reproducibility. 3. Grading of random biopsies should be representative for the whole tumor.

Of the many prostate cancer grading systems that have been developed the Gleason grading system is at present the most widely used and most often reported in the (American) prostatic literature[69]. It is also the grading system that was used for the studies described in this thesis and therefore the discussion will focus on this grading system only. The Gleason system is unique in that it takes into account the histological heterogeneity of prostate cancer. The system considers only histological characteristics and recognizes 5 primary growth patterns representing the transition from well differentiated (pattern 1) to undifferentiated (pattern 5) prostate cancer (Figure I.2). The scores of the two most common patterns are added, yielding a Gleason grade or Gleason sum score that may range from 2-10. An advantage of the Gleason system is that it enables the incorporation of tumor heterogeneity in all kinds of studies, for example: genetic studies, studies on the expression level of certain compounds and morphologic studies.

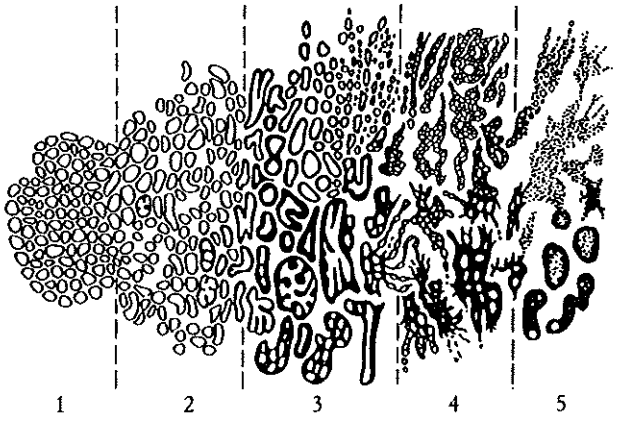

FIGURE I.2
Simplified drawing of the Gleason grading system.

Gleason grade correlates with tumor stage and the independent prognostic value of the Gleason system has been established in numerous studies among which several large series[45, 70]. The prognostic value was found to be largest in tumors with either the

lowest (2-4, excellent prognosis) or highest (8-10, worst prognosis) scores[70]. Tumors with moderate Gleason scores (5-7) are the most common, however, and in these patients the Gleason score cannot distinguish the 'pussy cats' from the 'tigers' on an individual basis. In high-grade tumors, T-stage becomes the most important prognostic marker[71]. Thus, although Gleason grading is a strong prognosticator, its practical use is limited in the largest group of patients.

Because the Gleason grading system is based on histological criteria, it is necessary to investigate several tumor glands and their inter-relationship. This might cause problems if it concerns a small tumor in a biopsy specimen. It has been shown that in about 80% of the biopsy specimens (14- as well as 18-gauge), the Gleason grade differed one point or less from that of the matching radical prostatectomy specimen (reviewed by Bostwick[72]). As expected, it was demonstrated that grading of the primary growth pattern was more reliable than the secondary pattern and that the correlation between biopsy and prostatectomy grade was less for small or low grade tumors. In a study with 60 radical prostatectomies from which 10 systematic biopsies (special constructed biopti-gun) were taken both from the posterior and anterior prostate, the grading concordance was only 65% (± 1 digit) with a 25% risk of undergrading (≥ 2 digits)[60]. Thickman *et al.* found that radical prostatectomy Gleason grade was within 1 digit of biopsy Gleason grade in 75% of the cases if 4 biopsies were investigated[73]. This figure did not improve if all six biopsies were investigated. The authors concluded that biopsy Gleason grade cannot reliably predict the grade of the whole tumor. As expected, in all these studies the risk of undergrading was largest in low-grade tumors on biopsy.

Since tumor grading is subjective, inter- and intra-observer variability may play a role. The reproducibility of different grading systems has been the subject of only few studies. Ten Kate *et al.*[74] concluded from a study in which the inter-observer variability of 5 grading systems (among which the Gleason system) was investigated with 50 radical prostatectomies graded independently by 5 pathologists that none of the grading systems did well. On the other hand, the intra-observer variability of the Gleason system appears to be fairly good[75] with 85% of the scores within 1 digit of the first assessment. In most reported studies tumor grading is performed by one pathologist or is reviewed by a reference pathologist eliminating at least the inter-observer variability.

PROSTATE SPECIFIC ANTIGEN

An enormous body of literature has proven the value of serum prostate specific antigen (PSA) levels as a prostate cancer marker[76] and as a surrogate end-point for clinical studies of locally confined prostate cancer[77]. Methods to enhance the diagnostic value of PSA like PSA-density (PSA level divided by prostatic volume)[78, 79], the ratio of free (not protein bound) to total PSA (free PSA + α_1-chymotrypsin bound PSA)[80] and PSA doubling-times[81] will not be discussed.

PSA at the tissue level as identified by immunohistochemical staining methods has been the subject of fewer studies. PSA is strongly expressed in the glands of the normal and hyperplastic prostate[82]. Most prostate tumors express PSA[83] and it is generally accepted that the immunohistochemically defined expression level is inversely correlated with tumor grade[84], although not all studies confirmed this[85]. The authors of the latter study also did not find a correlation between tissue expression and serum levels, whereas others did[86]. Lymph node metastasis express PSA in about 90%[82]. In a (male) patient with a metastasis of an unknown primary, this can be helpful to establish the diagnosis. A complicating factor is that several recent studies have shown that PSA is not entirely prostate specific, but can also be expressed by, among others, breast tissues[87, 88], salivary gland tumors[89] and non-prostatic neuroendocrine tumors[82].

The prognostic value of tissue PSA expression has been studied on biopsy specimens[90-92] and TUR specimens containing T_{1b} prostate cancer[93]. In 80 patients with advanced prostate cancer PSA was a significant prognostic marker at univariate analysis but not at multivariate analysis ($p=0.48$)[90]. Stege *et al.*[91] found PSA to be a significant prognostic marker at the univariate and multivariate level (together with cytologic tumor grade) in 67 consecutive patients who all received endocrine therapy. In a large study from Norway with patients with locally confined ($n=150$) or metastatic ($n=116$) tumors, who were treated with endocrine or radiation therapy, tissue PSA expression had no prognostic value[92]. This study used overall survival as end-point, which is probably inappropriate. In 13 out of 18 T_{1b} prostate cancer patients who were managed conservatively, PSA immunohistochemistry of the tumor in the TUR specimen could predict the clinical outcome[93].

The studies used different methods to quantify the PSA content and also the patient groups and outcome variables varied considerably. Thus, the prognostic value of tissue PSA levels is not clear. Since most authors found tissue and serum PSA levels to be correlated and since serum is much easier to obtain (repeatedly), tissue PSA is unlikely to become an important prognostic tissue marker.

A new issue currently receiving considerable attention is the demonstration of circulating PSA producing cells by the reverse-transcriptase polymerase-chain reaction (RT-PCR) to detect PSA-mRNA (sometimes erroneously called a micrometastasis detection assay)[94]. Men with BPH or no prostatic disease at all showed negative results whereas about 40% and 80% of the patients with localized and metastatic prostate cancer, respectively, showed positive results[94]. In more recent studies from the same institution the RT-PCR assay better predicted pT-stage than the pre-operative serum PSA level, with an overall sensitivity and specificity of 73 and 90%, respectively[95, 96]. The presence of circulating PSA-producing cells was of independent prognostic value in predicting PSA progression following radical prostatectomy in 94 patients with short follow-up[96]. Others obtained, however, contradictory findings. In a study with 107 prostate cancer patients only 1 out of 7 (14%) and 21 out of 57 (37%) blood samples from metastasized untreated and androgen independent tumors, respectively, showed positive results[97]. In an experimental study employing a nested RT-PCR method several

non-prostatic cell lines were found to express PSA mRNA[98]. PSA RT-PCR can also be applied to bone marrow aspirates or lymph node sections.

The presence of PSA-mRNA in the peripheral blood is very probably a risk factor for the development or presence of metastatic disease. Clearly, the sensitivity and specificity of the assay should be determined and optimized more precisely before the clinical utility can definitely be assessed. Because RT-PCR is a very sensitive technique that is prone to all kinds of technical problems, multi-center studies employing well defined and standardized consensus techniques should be conducted.

NUCLEAR DNA CONTENT

Nuclear DNA content (ploidy) can be studied by flow cytometry and image or static cytometry which both have specific advantages and disadvantages. Falkmer[99] has studied the methodology and potential problems of applying these techniques to prostate cancer. According to this study, many differences among published series were of methodologic origin. Since the advantage (large number of measured cells) and disadvantages of flow cytometry (expensive equipment and excellent technical support required, no simultaneous histopathologic control and risk of measuring non-malignant cells) are opposite to the advantages (relatively easy to perform and only measurement of tumor cells) and disadvantage of static cytometry (small number of measured cells) outweigh each other, she recommended that the two measurements be combined. Most studies found both methods to be fairly well correlated, however[100]. For practical purposes, the results from studies using the different methods will be discussed together.

The first papers on the prognostic value of DNA ploidy have been published already 30 and 23 years ago[101, 102]. In these studies from Portugal two important findings were described: prostate cancer patients with diploid tumors have a significantly better prognosis and respond better to estrogen therapy than patients with triploid or hexaploid tumors. More than 100 papers on this subject, among which large series from Sweden (Karolinska Institute) and America (Mayo Clinic), have since been published. Adolfsson[103] conducted a critical literature review of 115 papers containing prognostic information on DNA ploidy in prostate cancer which were published between 1973 and 1993. From repeated papers on apparently identical series, only the most recent paper was considered. Eventually, 44 papers were reviewed. DNA content was correlated with tumor grade in 23 out of 28 reports and with stage in 14 out of 20. Sixteen out of 18 studies with patients at various disease stages showed univariate prognostic value of DNA ploidy. In 8 of these a multivariate analysis was performed. Five found DNA ploidy to be of independent prognostic value (415 patients totally) and 3 did not (365 patients totally). Two of these latter three found ploidy to be a prognostic marker at univariate analysis. Ten studies were restricted to one specific stage. In 1 and 2 studies on localized ($\leq pT_3$) disease DNA ploidy was a dependent or independent prognosticator, respectively. In metastatic disease, 4 studies showed

prognostic value at univariate analysis and 3 did not. All 3 studies that included a multivariate analysis found independent prognostic value of ploidy.

Ploidy assessments were of independent prognostic value in 3 studies of patients with clinically localized prostate cancer treated with external beam radiation therapy[104-106]. Another study did not confirm this[107]. The findings of the first studies on ploidy in hormonally treated patients have been confirmed[108-111]. In two of these ploidy was of independent prognostic value[109, 110]. In one study neither tumor grade nor ploidy was of prognostic significance[112].

Several authors described the concordance between biopsy and surgical specimen based tumor ploidy[113-115]. As can be expected, the concordance was highest if an aneuploid population was found in the biopsy specimen. Tumor heterogeneity may also pose a problem. But, overall the correlation was high.

Two recent consensus meeting reports describe the clinical applicability and limitations of ploidy assessments in prostate cancer: the WHO conference on early diagnosis and prognostic parameters in localized prostate cancer[100] and the college of American pathologists conference on clinical relevance of prognostic markers in solid tumors[33]. It is important to realize that most of the authors/contributors appearing on these reports belong to the ploidy 'supporters'. The WHO report[100] discusses only T_2 and T_3 tumors and the contributors agreed that if surveillance is a treatment option, knowledge of ploidy is of clinical value. Furthermore, it was strongly advised to study DNA ploidy in clinical trials, particularly in patients with localized disease. The American pathologists conference concluded that DNA ploidy can be used for clinical decision-making only in patients with T_3 or pN+ tumors who are offered subsequent hormonal therapy[116]. The two reports agreed on the prognostic impact of DNA ploidy but clearly disagreed on its current clinical position.

A more sensitive method of measuring DNA ploidy is DNA in-situ hybridization (ISH) of interphase cells[117]. Both fluorescence (FISH) and non-fluorescence methods have been developed. Basically, these techniques visualize individual chromosomes by specific binding of a labeled probe to a particular DNA sequence (mostly localized at the centromere region). It can thus be used to investigate loss or gain of single chromosomes or even parts of chromosomes. FISH has been used on cell suspensions and paraffin-embedded tissues. The quantification of (F)ISH spots in paraffin-embedded tissue sections is somewhat complicated because not all spots in a nucleus need to be present in a tissue section. Thus, the distribution of the real number of spots may interfere with the distribution of countable spots. Persons *et al.*[111] studied the correlation between the three methods described above in the paraffin-embedded specimens of 34 prostate cancer patients. In 28 cases the three methods agreed, whereas FISH (probes for centromeres of chromosome 8 and 12) identified 2 additional aneuploid cases. Other studies confirmed that (F)ISH and flow cytometry are reasonably well correlated, but that (F)ISH is more sensitive[118, 119]. Alers *et al.*[120] have shown that ploidy as defined by probes for chromosome 1 and Y may vary between the Gleason patterns of one tumor, within one Gleason region and even within one

of the p21 gene, the protein product of which (ras-p21) in turn inhibits proliferation and 'decides' whether the cell will die by apoptosis or will repair its DNA damage. Aberrant (i.e., mutated or truncated) p53 has lost its cell-cycle control function and may thus play a role in tumor biology. The Li-Fraumeni syndrome, an inherited autosomal disease, characterized by the development of several malignancies during infancy, was shown to be caused by a germ-line mutation in the p53 gene[134]. DNA analysis of several clinical tumors subsequently identified the presence of mutations in the p53 gene in a fraction of the investigated specimens suggesting that p53 may play a role in tumor biology[135]. Antibodies against p53 were soon developed and enabled the study of p53 expression in more detail[136]. The p53 antibodies recognize wild-type p53 as well as most mutant forms. Since mutated p53 has a much longer half-life than the wild-type molecule, immunohistochemical identification of p53 has usually been associated with the mutant phenotype. But, results of immunohistochemical studies need to be analyzed with care since false-positive and false-negative results have been described, especially with highly-sensitive staining methods[137]. It has been recommended to verify the immunohistochemical data with DNA analysis[137].

The first papers on p53 in prostate cancer identified mutated DNA in 5 out of 29 tumors (17%) whereas no mutations were found in 34 BPH specimens[138, 139]. The presence of mutated p53 correlated with neither stage nor grade. Most immunohistochemical studies found comparable low percentages of untreated primary tumors with p53 mutations (<20%) [140-147]. Others described higher percentages, reaching from 22% to 80%[148-152]. In one of these immunoreactivity was in general cytoplasmic[148], which is at variance with other reports. In the studies in which the immunohistochemical results were confirmed by DNA analysis an excellent correlation was found[147, 153, 154]. There is no agreement on whether tumor-stage or grade is associated with p53 mutations in untreated tumors. Several authors found such correlations[140, 145, 153], while others did not[138, 139, 142, 147, 151]. The frequency of p53 mutations increased from primary to metastatic cancer to androgen independent and irradiation insensitive cancer[144, 150, 155-157]. Especially the association between p53 mutations and androgen resistance[150, 156] and radiation insensitivity[157] is interesting since pre-treatment analysis might stratify patients in those who will and those who will not benefit from these treatment modalities. But, clinical trials have to substantiate this. The presence of p53 mutations in primary tumors had no predictive value for the presence of lymph node metastasis[143].

The prognostic influence of p53 mutations has been investigated by several authors. In a study of Visakorpi *et al.*[140] patients with a p53 mutation had a higher progression rate with a relative risk of 12. This study contained 137 patients who were treated by radical prostatectomy, radiotherapy or endocrine therapy. A multivariate analysis was not performed. Thomas *et al.*[146] found a prognostic value of p53 mutations on the time to progression and tumor related death in 68 patients. The presence of mutations was correlated with stage and grade. Since a multivariate analysis was not performed, the prognostic value may also be attributed to these relationships. In 45

patients with a T_{1a} tumor with Gleason grade 4 or less, a trend towards a worse prognosis (crude survival) of patients with a p53 mutation was found ($p < 0.08$ at univariate analysis)[141]. Vesalainen found no relationship between p53 mutation and prognosis in the biopsy specimens of 139 patients with $T_{1-2}M_0$ tumors[142]. Half of the patients were managed conservatively, the others received radical therapy (n=6) or endocrine therapy (n=32). Thus, the patient sample was rather heterogenous. Another study made use of an even more heterogenous patient population with clinical stages varying from T_{1a} to N+[158]. At univariate analysis the presence of p53 mutations was a prognosticator for time to progression only in patients with Gleason grade 2-7 tumors. Bubendorf *et al.*[145] studied the issue in 137 patients treated by radical prostatectomy. The presence of p53 mutations was related to grade and proliferation rate (Ki-67% immunostaining) but showed no prognostic significance. In a relatively small study with 40 patients treated with a radical prostatectomy, p53 mutations had prognostic value on the time to PSA progression[151]. In this study 80% of the tumors showed p53 immunostaining which is an extremely high figure compared to other studies. The authors explained this by the application of a strict quality control scheme and newer antigen retrieval techniques. It can, however, not be excluded that ultra-sensitive p53 assays identify wild-type p53 as well. Another study with 175 patients treated by radical prostatectomy found p53 expression in 65% of the cases[152]. Expression of p53 was an independent prognostic marker for PSA progression.

Mirchandani *et al.*[144] studied the heterogeneity of p53 mutations at the DNA level. In the case of multifocality lesions with and without p53 mutations were often found and even within one lesion, areas with wild-type and mutant p53 could be identified in a number of cases. The authors conclude that this heterogeneity may explain the large differences in published series on p53 in prostate cancer. Additional explanations are possible. A number of (polyclonal) antibodies were not very well characterized. The immunohistochemical procedures, methods of quantification and definitions of cut-off levels also differed among studies. Finally, most studies performed so far used patient groups from several stages and receiving different treatments. The correlation between biopsies and matching prostatectomies has not been studied.

Ras-p21 expression was increased in prostate tumors as compared to benign prostatic tissues[159-162] and a correlation between its expression level and tumor grade or stage was found in three of these studies[159, 160, 162]. Sumiya *et al.*[159] found no prognostic influence of ras-p21 in 62 patients with metastasized prostate cancer receiving endocrine therapy. In contrast, Agnantis *et al.*[162] found a prognostic value in surgically treated patients by comparing the 5-year crude survival rates of patients with and without ras-p21 expression which is statistically an inappropriate method. Moreover, the relationship between ras-p21 expression and tumor differentiation could possibly explain this finding.

The number of prognostic studies on ras-p21 in prostate cancer is too limited to draw conclusions at this moment. It can also not definitely be concluded whether p53 can be used as a prognostic marker in prostate cancer. The studies differ too much,

both in terms of patients and methodology, and in only a small number of studies multivariate analysis on the prognostic impact has been performed. It might be a useful marker for the selection of patients to be treated with either hormonal or irradiation therapy. Since ras-p21 and p53 interact at a functional level, it could be useful to combine ras-p21 and p53 in prognostic studies as well.

EPIDERMAL GROWTH FACTOR RECEPTOR AND C-ERBB-2

The epidermal growth factor receptor (EGF-R) is a key modulator of normal prostatic homeostasis (reviewed by Steiner[163]). Stimulation of the receptor by the epidermal growth factor (EGF) or transforming growth factor-α (TGF-α) promotes proliferation which in the normal prostate is balanced by the inhibitory activity of TGF-ß[163]. In prostate cancer EGF related peptides appear to play a functional role in tumor growth with TGF-α as the most important peptide (reviewed by Sherwood and Lee[164]). C-erbB-2 is a receptor that shows considerable homology with EGF-R[165], but does not need EGF to be activated. Although the ligands of these receptors might be quite different, most prostatic studies combine the two and for this reason they will be discussed together.

The first study on EGF-R in the prostate quantified receptor content of BPH and cancerous tissues[166]. The expression was lower in prostate cancer as compared to benign tissues and decreased with increasing grade. Immunohistochemically a similar pattern was found[138, 167]. The studies on C-erbB-2 differ considerably, some authors found no immunoreactivity at all[156, 167] or only in a very small minority of cases (4/266)[92] whereas others described 36 to 100% of the investigated tumors to be positive[168-170]. Differences among the antibodies and visualization techniques may explain the disagreements, although 2 studies with completely different outcome used the same polyclonal antibody (NCL CB11) and staining method[92, 169]. Only one study correlated C-erbB-2 immunostaining with tumor grade[168], a significant relationship was not found. The expression level of the two most important ligands of the EGF-R has been studied[171, 172]. EGF was present in 6% of 52 BPH specimens, in 40% of 45 localized tumors and in all 20 metastatic tumors[171]. TGF-α was present in 15% of benign tissues and in 53% of the tumors[172].

In 45 patients with T_{1a} tumors, EGF-R showed no prognostic value, whereas C-erbB-2 did (at the univariate level)[169]. Visakorpi *et al.*[167] studied 147 patients and found a prognostic value of EGF-R at univariate analysis (progression and tumor specific survival) but not at multivariate analysis. In a study of 124 radical prostatectomy specimens with $pT_{<3c}$ prostate cancer, C-erbB-2 was of independent prognostic value together with Gleason grade, DNA ploidy and nuclear morphometry[125]. Moul *et al.*[173] studied 105 radical prostatectomy specimens and found no correlation between EGF-R expression and tumor stage, grade or prognosis.

Although biologically an important role of these molecules could be expected in prostate cancer, this is not reflected by a clear cut relationship with clinical factors such as tumor grade, stage and prognosis. As a consequence EGF and EGF-R will probably not be used as prognostic markers clinically. Illustrative is the fact that only two papers were published in 1996, all the other mentioned papers were published before 1995.

E-CADHERIN AND A-CATENIN

E-cadherin is a member of a family of calcium dependent intercellular adhesion molecules. E-cadherin is located at the cellular surface and is coupled to catenin and thereby connected to the cytoskeleton. Immunohistochemically, normal prostatic glands showed a very intense membranous staining of E-cadherin while the expression in tumors was in general decreased[174-176]. Most studies described 3 patterns of immunostaining in prostate cancer: a pattern comparable to that found in benign glands, a totally negative and a heterogenous staining pattern. A decreased expression in tumors implies a deranged intercellular attachment and an increased metastatic potential of the tumor. All authors described an inverse relationship between E-cadherin expression and tumor grade and stage[174-178]. In one of these, biopsy specimens of 56 patients who subsequently underwent a radical prostatectomy were investigated[178]. Although a correlation with stage and grade was found, the pattern of E-cadherin staining of the biopsy could not predict the presence of lymph node metastasis.

Immunohistochemistry of E-cadherin using formalin-fixed, paraffin embedded material is possible, although after more than 12 hours of formalin fixation the results become unreliable (E. Ruiter and J.A. Schalken, personal communication). This impairs the applicability of E-cadherin to archival prostatectomy specimens since in most institutions the duration of formalin fixation is unknown and variable, but regularly extends 12 hours. In a recent study, a microwave based antigen retrieval procedure was used on routinely fixed material and the authors claimed good results with this method, however[176].

Umbas *et al.*[177] used frozen sections from 42 radical prostatectomy specimens, 29 primary and 18 palliative TUR specimens. In the former two patient groups E-cadherin expression was a statistically significant prognostic marker for time to progression at univariate analysis. A multivariate analysis was not reported by the authors, probably due to the heterogenous patient sample and the relatively small numbers. Since a strong association with both tumor-grade and stage was found, the prognostic value of E-cadherin may be the consequence of these strong associations.

Because E-cadherin is functionally coupled to catenin, its presence at the intercellular membranes does not necessarily imply a functional complex. Decreased or altered catenin expression may also be responsible for an abnormal function. Two studies that have only be published as abstract combined E-cadherin and α-catenin immunohistochemistry in prostate cancer[179, 180]. Umbas *et al.*[179] investigated 20 radical

prostatectomy specimens and 32 advanced tumors. Overall, in 16 of these 52 tumors E-cadherin and α-catenin expression was normal, whereas in 23 tumors the expression of both molecules was abnormal. Likewise, the prognosis of these patients was best and worst, respectively. In 13 locally advanced tumors with normal E-cadherin staining, an abnormal α-catenin staining was related to an intermediate prognosis, but the numbers were small. In the other study the biopsy specimens of 44 surgically treated patients were used[180]. Abnormal E-cadherin staining was found in 18 cases and was an independent predictor for time to PSA progression together with pre-operative PSA and biopsy Gleason grade. E-cadherin and α-catenin expression were concordant in more than 90% of the cases, but the prognostic value of α-catenin was not mentioned.

Although only a limited number of studies have been published as yet the overall agreement between the studies, the straight forward correlation with tumor biology and the convenient way of assessments of the results (normal or abnormal in most studies) make these markers potentially useful. But, more studies need to be performed to determine first an independent prognostic value and second the clinical applicability.

PROLIFERATION

Because one of the hallmarks of malignancy is uncontrolled growth, intuitively one would assume that assessments of the proliferative activity of a tumor would bear prognostic significance. Several methods to measure the proliferative activity are available: counting of mitotic figures[181], determination of the S-phase fraction (SPF) with flow cytometry, labeling of replicating DNA with tritiated thymidine or bromo-deoxyuridine (BrdU), and finally, antibodies to proliferation related antigens (Ki-67[182], MIB-1[183], and proliferating cell nuclear antigen (PCNA)[184]). Ki-67 and MIB-1 are both directed against the same antigen. The Ki-67 antigen as well as PCNA are expressed only during the G_1, S, M, and G_2 phases of the cell cycle, but not during G_0. PCNA has a long half-life and is expressed during DNA repair as well. Assessment of SPF is cumbersome, and although a prognostic value has been reported in prostate cancer[185, 185], it will not be discussed. Likewise, the study of BrdU incorporation requires either the infusion of BrdU to a patient[186] or incubation of the surgical specimen with BrdU. BrdU may have mutagenic effects[187] and the ex vivo incubation method is not reliable. At present most studies use immunohistochemical methods to identify proliferating cells, and the practical use of BrdU labeling and SPF determination in a clinical setting is not recommended.

Several studies compared the proliferation rate in BPH with that of prostate cancer and found, independent of the technique used, an increased rate in cancerous tissues[188-192]. The mean prostate cancer Ki-67, MIB-1 and PCNA labeling indexes among all studies were about 4%, 7.5% and 19%, respectively. The correlation between Ki-67 and PCNA indexes was only weak (0.32 - 0.8) and depended strongly on the method of

fixation for PCNA staining[190, 193]. Some authors described a correlation with grade or stage[142, 190, 192, 194-196], others did not[189, 191, 197-199]. Several authors described variable labeling indexes in different areas of a tumor.

Oomens *et al.*[189] found in patients with repeated biopsies that the Ki-67 labeling index decreased after the start of endocrine therapy. In an extension of this prospective study Santerse *et al.* (unpublished observation) found that Ki-67 labeling indexes started to increase already up to 6 months before PSA progression became apparent in these hormonally treated patients. Harper *et al.*[198] studied the pre-treatment TUR specimens of 86 hormonally treated patients and found a prognostic value of Ki-67 only in patients with metastatic disease (univariate analysis of cancer specific survival). Using a more or less identical patient sample she also found a weak prognostic value of PCNA[190]. In another study with only 17 hormonally treated patients the Ki-67 index showed only a prognostic trend (not statistically significant). In a study with 45 patients McLoughlin *et al.*[191] found no prognostic significance of Ki-67. However, in this study the prognostic impact was determined by comparing the Ki-67 labeling indexes of patients with and without progression, which is probably an inappropriate statistical method (in fact, this technique analyses the influence of prognosis on Ki-67 labeling!). Several studies with patients that were either untreated or hormonally treated found an independent prognostic value of PCNA[142, 192, 196]. Three larger studies investigated the prognostic value of PCNA[125] or MIB-1[195, 199] in radical prostatectomy specimens. The PCNA study found no prognostic value, the others found an independent prognostic value of the MIB-1 labeling index. In a study with a diverse patient sample (stage T_{1-4}, M_{0-1}, different treatments) the number of mitotic figures per high power field as simply counted in a routinely stained tissue slide was a significant prognostic marker at univariate analysis[181]. At multivariate analysis an independent value was found only for patients with $T_{1-2}M_0$ tumors. One study investigated the prognostic value of MIB-1 labeling in lymph node metastasis of 50 patients undergoing ^{125}I-implantation therapy[200]. A prognostic influence on the time to PSA progression was found at univariate analysis, but not at multivariate analysis (only DNA ploidy significant). The study may however have been biased because 32 out of 82 (39%) tissue sections contained less than 500 tumor cells and were excluded for immunohistochemistry.

Researchers clearly do not agree on the prognostic impact of proliferation rates in different patient groups. The prognostic value of proliferative activity in biopsies of patients undergoing radical prostatectomy has not been reported. In addition, the reported heterogeneity within areas of one tumor may potentially hamper the routine application.

Whether a study found a correlation with prognosis, tumor stage or grade seems not to depend on the method of determination of the proliferative activity. The expression of PCNA is not limited to progression through the cell cycle, as illustrated by the higher labeling indexes and marginal correlation with Ki-67 indexes. For this reason PCNA may be less desirable. Since PCNA is functionally related to p53 and p21[133], it might be useful to study these 3 antigens together. Ki-67 was initially thought

to be applicable only to frozen sections, but can also be applied to paraffin sections, although MIB-1 staining appears to be more sensitive[201]. More importantly, MIB-1 scores were about 2 times as high as Ki-67 scores in routinely processed tissues with a high linear correlation between the two antibodies[201]. The standard deviation of each individual assessment depends on the number of positive cells (binomial distribution, $SD=\sqrt{np(1-p)}$ with n=number of investigated cells, p=positive fraction). Thus, for a given number of investigated cells MIB-1 scores may statistically be more reliable which is of importance if the proliferative fraction is small as is the case in most prostate tumors.

During the last years it has been recognized that tumor growth is the net result of cell gain and loss. Study of cell proliferation in combination with cell death parameters may reveal more conclusive results in the near future. At this moment assessment of the proliferative activity of prostate cancer has no clear clinical benefit.

ANDROGEN RECEPTOR

The androgen receptor is a nuclear receptor belonging to the steroid receptor family and acts as a transcription factor that stimulates prostatic growth and secretion upon binding of androgen (testosterone or its more potent metabolite dihydro-testosterone)[67]. Normal prostatic tissue as well as most primary prostatic carcinomas depend on androgen for their maintenance and function. Androgen withdrawal leads to cell death by apoptosis in normal as well as malignant glands and this is the basis of endocrine treatment of prostate cancer.

Using a well defined monoclonal antibody directed against the amino-terminal part of the androgen receptor Ruizeveld de Winter and colleagues found strong expression in benign glands and stroma[202]. In primary tumors, staining intensity was in general less, but was not related to the Gleason grade[203]. Although not expected, it was found that most of the androgen independently growing tumors showed androgen receptor expression[204]. DNA analysis identified no mutations in the androgen receptor[203]. Studies from other institutions have identified androgen receptor gene mutations in a small fraction of hormonally escaped tumors, however[205, 206]. It therefore appears that androgen receptor mutations may play a role in androgen independent prostate cancer growth in a minority of cases.

It was hypothesized that the androgen receptor content of a tumor prior to the application of endocrine therapy would predict treatment response[207]. By measuring the androgen receptor content in the cytosol and nuclei of 23 patients with metastasized prostate cancer researchers from Johns Hopkins University showed that patients with low nuclear receptor levels (<110 fmol/mg protein) had a shorter response duration and a decreased survival[207]. Van Aubel *et al.*[208] studied the biopsy specimens obtained before the start of endocrine therapy of 37 patients with locally advanced or metastatic prostate cancer, but did not find a prognostic value of nuclear AR content. The

correlation between nuclear AR content and time to progression was used to evaluate the prognostic impact in the latter study. In a later study from Johns Hopkins a polycloncal antibody was used to quantify androgen receptor content[209]. A marked heterogeneity within and between samples was found, but AR content showed no prognostic value (in only 17 patients). In a more recent study, this heterogeneity was quantitated using an image analysis system[210]. Patients with tumors with a high variance of staining intensity did worse as defined by treatment response. Tilley *et al.*[211] also used image analysis to study two antibodies (to the carboxyl- and amino-terminal part of the protein, respectively) in biopsy specimens of 30 hormonally treated patients of all stages (T_1M_0 - T_xM_{1b-c}). The two antibodies showed quantitative differences, indicating that receptors may differ among tumors. The mean staining intensity was a prognostic marker in these patients. Other studies confirmed the prognostic value of androgen receptor content in hormonally treated patients at the univariate and multivariate level, with higher expression being related to a better prognosis[212-214].

Conflicting data have been published as to whether the androgen receptor level is of independent prognostic value in the pre-treatment tumor specimens of hormonally treated prostate cancer patients. It is however clear that AR determinations cannot be used to select patients who will and who will not benefit from endocrine treatment on an individual basis. Possibly, a combination with other markers (p53, neuroendocrine markers and DNA ploidy) could enhance the results. A potential problem is the use of a biopsy of the primary tumor to determine the androgen receptor content in a patient with metastatic disease. The androgen receptor content of the metastatic lesions might be different from the primary tumor. One study compared the expression level in 12 lymph node metastasis and matching radical prostatectomy specimens[215]. Only one metastasis showed no androgen receptor immunoreactivity in contrast to the radical prostatectomy specimen.

APOPTOSIS

Apoptosis or programmed cell death is a second mechanism of cell death besides necrosis[216]. Apoptosis is a very important mechanism which plays a role in a large number of physiologic processes (for example: embryonal development, selection of immuno-competent cells and tissue homeostasis). The histologic characterization of apoptotic cells has been described and is generally accepted. It was found that the apoptotic pathway is closely related to the cell cycle (reviewed by Meikrantz and Schlegel[217]). Actually, during progression from the G_1- to the S-phase of the cell cycle, the cell 'decides' whether to proceed with DNA replication and finally mitosis, or to activate the apoptotic pathway (the cell cycle check point)[217]. Since apoptosis is closely related to proliferation, it might play a role in malignancy as well.

It has been shown that physiological androgen levels inhibit apoptosis of prostatic epithelial cells[218]. In contrast, following androgen withdrawal prostate (cancer)

cells die by apoptosis[219]. At present there is still a debate going on as to whether p53 expression and cell cycle progression are required for prostatic epithelial cells to undergo apoptosis following androgen withdrawal[220, 221]. Probably both pathways are possible. The ability of prostatic tumor cells to undergo apoptosis might be related to the androgen sensitive phenotype.

Bcl-2 is a protein that is able to block the apoptotic pathway[222]. Expression of this protein may play a role in androgen independent prostate tumor growth. Indeed, transfection of bcl-2 in the androgen dependent prostatic tumor cell line LNCaP resulted in a cell line that grew androgen independently *in vitro* and *in vivo*[223]. If the cell cycle check point is not functioning due to an elevated bcl-2 level, the cells may survive with more and more genetic alterations leading to increasingly aberrant tumor behavior.

Bcl-2 labeling was found in the basal cell layer of normal prostatic glands[145, 224, 225]. Primary prostatic tumors expressed bcl-2 in 24%-62%[145, 152, 224-226]. In androgen independent tumors both the number of positive tumors and the intensity per tumor is increased[224, 225]. In a study with 325 heterogenous patients (with respect to tumor-stage and grade and treatment) the presence (yes or no) of apoptotic cells was correlated with tumor grade and was a prognostic indicator for tumor specific survival, but only at the univariate level[227]. Almost all researchers have found apoptotic cells in areas with proliferating cells (e.g., intestinal crypts, breast during weaning and tumors), although in tumors the numbers were variable[217]. Thus, a (semi)-quantitative determination seems preferable. Such a study has been performed on 28 radical prostatectomy specimens containing Gleason grade 6 prostate cancer[228]. The number of apoptotic cells was of prognostic value to predict the time to PSA progression. A multivariate analysis was not performed, since only Gleason grade 6 tumors were investigated.

The prognostic value of bcl-2 has only been studied in radical prostatectomy specimens[145, 152]. A prognostic value was found at univariate analysis, but not at multivariate analysis[145]. This study combined bcl-2, p53 and Ki-67 immunostaining. Bcl-2 expression was not related to Ki-67 staining, and a statistically not significant correlation was found with p53 expression. Interestingly, patients could be separated in 3 prognostically different groups. A group with low bcl-2 and Ki-67 (best prognosis), a group with high bcl-2 and Ki-67 (worst prognosis) and the remaining patients (intermediate prognosis). The other study found an independent prognostic value of bcl-2 expression on the time to PSA progression[152]. Interestingly, p53 expression was also investigated by these authors and the combination of both parameters better predicted clinical outcome.

Besides bcl-2 more proteins have been identified that either block (bcl-family) or promote (bax) apoptosis. The expression of bcl-2, bcl-x and mcl-1 (all apoptosis inhibitors) was shown to be related to prostatic tumor grade[226]. Bax was expressed by all tumors and the level was independent of tumor grade[226]. Westin *et al.*[229] reported on an interesting study with biopsy specimens of 18 prostate cancer patients obtained one day before and 7 days after the start of endocrine therapy. In 3 out of 18 no effect was seen.

In the other 15 the Ki-67 proliferation rate decreased and in 6 of these the number of apoptotic cells increased. This group showed in the initial biopsy well or moderately differentiated tumors without expression of bcl-2, p53 or c-myc. The 12 tumors of which the frequency of apoptotic cells was unaltered or even decreased, were moderately or poorly differentiated and expressed bcl-2, p53 or c-myc in the initial biopsy. These results suggest that the likelihood of remission can be predicted on the basis of biopsy specimens.

The role of apoptotic cells and bcl-2 expression in clinical tumors is at present not completely known. It is however clear, both from experimental studies and the clinical studies performed so far, that the study of apoptosis may become a clinical useful parameter. Prognostic studies on the expression level of other apoptosis related proteins should be performed (preferably combining the several proteins) on several patient groups. Because of the close relationship between apoptosis and proliferation, these studies should include proliferation markers as well.

OTHER MARKERS

TGF-ß1 inhibits epithelial cell growth (see under EGF), but it also stimulates angiogenesis and cell motility and suppresses immune reactions[230]. The expression of TGF-ß1 increases from benign to cancerous prostatic tissues[230, 231]. It can be found intra- and extra-cellularly. The level of extra-cellular TGF-ß1 increased with tumor grade[230]. Serum levels of TGF-ß1 were also increased in patients with prostate cancer compared to BPH controls and the level in tumors was related to tumor stage[232]. Some recent studies suggest that TGF-ß2 and the type-I and -II TGF-ß receptors are also important to study, but clinical studies are lacking as yet[233-235].

Tumor growth requires angiogenesis. The microvessel density has been studied in prostate cancer using antibodies to either factor-VIII (von Willebrand factor) or CD34[236-239]. Microvessel density was higher in tumors compared to adjacent benign tissue and was correlated with tumor-stage and grade. Microvessel density was a prognostic indicator in the TUR specimens of 25 patients treated with radiotherapy[238].

Several markers were the subject of only one or few studies. A prognostic value was found for (among others): tumor infiltrating lymphocytes (predictor of good prognosis in 325 heterogenous patients)[92], Sial Lewis-X antigen[240] and CD44 expression (chapter III)[241]. Several markers were related to tumor stage and/or grade: hyaluronidase activity (an extracellular matrix degrading enzyme)[242], high mobility group I protein (as identified by mRNA in-situ hybridization)[243], hepatocyte growth factor and its receptor c-met[244] and oncoantigen-519[245]. Negative findings were published on c-myc[141], Cathepsin-D[246] and Rb-gene product[247].

Some markers appear to be of interest, but clinical studies have not been performed yet. KAI-1 was identified as a metastasis suppressor gene in AT6.1 cells[248], and the gene product was absent in clinical prostate cancer metastasis[249]. Telomerase is

an enzyme that prevents chromosome shortening during repetitive replications and was found to be absent in benign prostatic tissues, but present in prostate cancer[250]. Telomerase activity could also be measured in biopsy specimens[251]. The expression of fibroblastic growth factor-8 (FGF-8) as well as its receptor (FGF-R type I) is upregulated by androgens and appears to be essential for androgen induced growth[252]. Normal adult rat tissues did not express FGF-8, whereas *in vitro* growing prostatic tumor cell lines did.

CONCLUSION

Although much effort has been put in the study of potential prognostic markers during the last years, presently no markers, apart from tumor-stage and grade can be used for clinical decision making, although it has been suggested that DNA ploidy can be used to select patients for endocrine therapy or watchful waiting. Based on the heterogeneity of prostate cancer it is to be expected that only combinations of markers will reveal sufficient prognostic power. Biopsy techniques may have to change substantially to improve sampling of heterogeneous tumors. Future studies should thus focus on combinations of markers that are derived from biological knowledge of the disease, for example a combination of cell-adhesion molecules to predict metastatic capacity. It will also be important to include patient derived factors like concomitant diseases and patient-age. Because basic prostate cancer research very probably leads to the identification of potential new prognostic markers, it will be necessary to develop clinical facilities (tissue-, serum- and data-banks) which allow the evaluation of candidate prognostic markers in a multifactorial setting. Tumor-stage and grade are strongly correlated with patient outcome. A multivariate analysis should be performed whenever possible and it should include those factors of which the clinical value already has been established. The general discussion on prognostic markers (chapter VIII) reviews the problems associated with the assessment of prostate cancer prognosis and provides recommendations for future prognostic marker research.

APPENDIX

The analysis of survival times requires specialized statistical techniques for two reasons. First, the event of interest (death, progression or something else) almost never occurs in all subjects. These subjects are generally referred to as censored subjects. Second, the duration of follow-up generally varies greatly between the subjects. In the studies described in this thesis two types of analysis were used: the Kaplan-Meier method[253] and Cox's proportional hazards model[254]. These are the two techniques that are most often being used to analyze survival data and will for a better understanding of the results briefly be described.

KAPLAN-MEIER METHOD

The Kaplan-Meier method[253] is a technique to graph survival functions. The survival period is divided in many small time periods and the probability of survival until a certain time point, $p(t)$, is calculated simply as the conditional likelihood of surviving all the preceding small time periods $p(t\text{-}1)$. If in such a time period no event occurred, the conditional probability of surviving that period is 1. Thus, the probabilities have only to be computed when one or more events occur (N_{events}). Censored subjects do not influence the survival curve, they only decrease the number of subjects at risk ($N_{at\ risk}$) as of the time-point they are censored. This can be represented mathematically by the following recursive formula:

$$p(t) = p(t\text{-}1) \times (N_{at\ risk} - N_{events}) / N_{at\ risk} \qquad (1)$$

The Kaplan-Meier method results in a step function and the curve should be drawn accordingly. If the subjects can be divided in two or more groups, for example based on a prognostic factor or treatment, the Kaplan-Meier curve can simply be constructed for the different groups separately. The median survival time can be estimated from the time point when the curve crosses the survival probability of 0.5. The tails of the curves become unstable due to the small number of subjects at risk and should therefore be interpreted with care.

The curves cannot be used to draw conclusions on the differences between the groups. This should be done by applying a suitable statistical test, usually the logrank test or the logrank test for trend in the case of more than two ordered groups. The logrank test tests the hypothesis that the number of events is equally divided over the groups based on the null hypothesis that the groups origin from one population. The logrank statistic gives no information about the magnitude of difference between the

groups. For this purpose, the hazard ratio (the observed over the expected number of events between the groups) with its 95% confidence interval can be computed.

COX'S PROPORTIONAL HAZARDS MODEL

The Kaplan-Meier method is not suitable to study the influence of several variables together on survival. For these purposes Cox's proportional hazards model was developed[254]. Fitting of the model results in a hazard function $H(t)$, the risk of dying in a very short time interval following time-point (t):

$$H(t) = H_0(t) \times \exp(B_1X_1 + B_2X_2 + \ldots + B_nX_n) \qquad (2)$$

For each variable entered the model computes a coefficient (B_1, B_2, ..., B_n) that fit the data best and tests if the coefficients are statistically significantly different from 0. If so, that variable is of independent value. No special distribution of the variables is assumed, the only assumption is that the variables have a constant influence over time (proportional hazards). This can and should be tested for. Several techniques can be used to obtain the most relevant model (comparable to ordinary multi-regression analysis).

X_1, X_2, ..., X_n represent the values of the variables in an individual. $H_0(t)$ represents the baseline hazard. It is calculated by taking all variables 0 ($\exp(0) = 1$). For each individual the outcome of $B_1X_1 + B_2X_2 + \ldots + B_nX_n$ can be computed and is often referred to as the prognostic index (PI). By defining PI ranges, patients can be separated into groups with different prognosis. It follows from formula (2) that a marker that is associated with an increased hazard will have a coefficient B_k that is greater than 0 and vice versa. It can thus easily be seen from the output of the computer program if a marker is associated with a good or a bad prognosis. The hazard difference of two individuals or groups with a different PI only depends on the PI of both subjects or groups and is, as a consequence, constant over time (proportional hazard):

$$H(t)_1 / H(t)_2 = \exp(PI_1 - PI_2) \qquad (3)$$

The coefficients as shown in the multivariate analysis of the chapters II-IV are depicted as hazard ratios, that is $\exp(B_k)$. A hazard ratio of >1 indicates a prognostic marker that is associated with an adverse prognosis and vice versa.

SCOPE OF THE THESIS

The need to identify reliable prognostic markers for prostate cancer has been highlighted in this chapter. Since no reliable markers that predict patient outcome on an individual basis are available at present, study of all markers that might be of value is warranted. Two of such markers were studied in radical prostatectomy specimens (chapter II and III) and two were studied in the pre-treatment TUR specimens of hormonally treated patients (chapter IV). All these studies made use of routinely formalin-fixed, paraffin-embedded prostate cancer specimens. The immunohistochemically defined expression level was correlated with Gleason grade, tumor-stage and prognosis. Multivariate analysis using Cox's proportional hazards model was performed to identify independent prognostic markers.

Chapter II describes the prognostic value of neuroendocrine cells in prostate cancer. The rationale for studying neuroendocrine cells in prostate cancer is described in full detail in chapter V. In brief, neuroendocrine cells are present in benign and malignant prostatic glands. These cells produce neuropeptides and biogenic amines some of which have growth factor activity. Moreover, prostatic neuroendocrine cells do not express the androgen receptor and are considered primarily androgen independent. Thus, neuroendocrine cells may influence prostate cancer growth and may be involved in the androgen independent phenotype.

Chapter III describes the prognostic value of CD44 expression in radically treated prostate cancer patients. CD44 forms a group of heavily glycosilated molecules formed by alternative splicing of a single mRNA[255]. Splice variants containing the v6-exon appear to play a causal role in the metastatic cascade[256]. Tumor related death of prostate cancer patients treated by a radical prostatectomy is mainly caused by metastasis and for this reason knowledge about the metastatic potential of a tumor is very relevant. CD44 expression in prostate cancer has only been studied in experimental tumor models; clinical data are lacking as yet.

The prognostic value of androgen receptor and bcl-2 expression in hormonally treated prostate cancer patients is discussed in chapter IV. Androgen effects are mediated through the nuclear androgen receptor, a member of the steroid receptor superfamily. It was hypothesized that decreased androgen receptor expression would predict a poor prognosis of hormonally treated patients and as described above (section on androgen receptor) this has indeed been reported by several authors, although some did not found a prognostic value of androgen receptor expression. Endocrine treatment of prostate cancer leads to tumor reduction by decreased proliferation and increased apoptosis. Bcl-2 is a molecule that inhibits the apoptotic pathway and thus, bcl-2 expression of prostatic tumor cells might predict a poor response on endocrine treatment. The prognostic value of bcl-2 expression in such patients has not been reported, however.

Chapter V reviews the concept of neuroendocrine differentiation in prostatic tissues with emphasis on experimental aspects and its influence on prostatic tumor cell biology. The experimental study of neuroendocrine cells in prostate cancer partially depends on the availability of experimental models containing these cells. An immunohistochemical survey was conducted to identify human prostatic xenograft models containing neuroendocrine cells and to characterize these cells. These studies are described in chapter VI.

Determination of proliferative activity of tumors by immunohistochemistry with antibodies against proliferation related antigens has been applied to a large number of tumors, among which prostate cancer (see above under proliferation). The most extensively studied proliferation marker is monoclonal antibody Ki-67. It was initially thought that this antibody is applicable to frozen tissue sections only, although recent studies showed that this is not the case. A new antibody against the Ki-67 antigen (MIB-1), applicable to routinely processed tissues, has been commercially available as of 1992. The prostatic cancer xenograft model PC-82 was used to compare the Ki-67 and MIB-1 defined proliferative indexes at several growth rates (chapter VII). Tumor growth was manipulated by maintaining different androgen concentrations in the tumor bearing mice and by castration of the mice.

The general discussion (chapter VIII) gives a brief discussion about the results of the studies described in chapter II-VII, but describes mainly potential new research directions for the study of prognostic markers and neuroendocrine differentiation in prostate cancer.

CHAPTER II

THE PROGNOSTIC INFLUENCE OF NEUROENDOCRINE CELLS IN PROSTATE CANCER: RESULTS OF A LONG-TERM FOLLOW-UP STUDY WITH PATIENTS TREATED BY RADICAL PROSTATECTOMY

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ABSTRACT

The distribution of immunohistochemically defined neuroendocrine (NE) cells in benign, pre-cancerous and neoplastic prostatic tissues and the prognostic value of these cells in prostate cancer were studied in the radical prostatectomy specimens of 90 patients from whom complete long-term follow-up data were available. The tissue blocks containing all the different Gleason patterns observed in a particular tumor were selected and immunostained. Since chromogranin B stained only a few cells compared to chromogranin A (CgA), NE cells were only defined by their reactivity with CgA. A semiquantificative CgA score was assessed for all distinct pathological areas. Cox's regression model was used to analyze the influence of final TNM classification (TNM, 1992), Gleason sum score (GSS), age and CgA score on the probability of progression and tumor specific death.

NE cells were demonstrated in all normal prostatic tissues and in most hyperplastic and intra-epithelial neoplastic lesions. CgA staining was seen in 78% of the tumors. CgA scores were not related with Gleason growth patterns, GSS or TNM classification and had no prognostic value. The independent prognostic variables in Cox's regression model were: GSS and pT stage for progression and GSS for tumor specific survival.

Theoretically, NE cells could influence tumor behavior and this discrepancy suggests the need for experimental studies to investigate the role of NE cells in the normal and neoplastic prostate.

INTRODUCTION

Prostate cancer has become the most prevalent tumor in males in the western world and its mortality in those countries is only surpassed by that of lung cancer[4]. A major part of prostate cancer research is directed towards the identification of prognostic factors, with the aim of better predicting individual patient outcome and optimizing treatment methods.

Neuroendocrine (NE) cells form a small, intrinsic component of normal prostatic epithelium and are also found in benign prostatic hyperplasia (BPH) and prostatic carcinoma[257, 258]. The role of prostatic NE cells is unknown but, according to their function in better studied organs (lungs, intestinal tract), probably concerns the maintenance of homeostasis and the regulation of secretion (reviewed by Noordzij *et*

al.[259]). NE cells secrete neuropeptides (serotonin, calcitonin and others) and specific neuroendocrine products (chromogranins, neuron specific enolase)[258, 260-262]. Prostatic NE cells are most often identified by immunohistochemical methods using specific antibodies for NE markers (e.g. chromogranin A, serotonin and neuron-specific enolase). It is important to realize that several histopathological categories of prostatic tumors with NE differentiation (i.e. containing NE cells) exist: small cell prostatic cancer, prostatic carcinoid, adenocarcinomas with focal NE differentiation and mixed tumors[257]. The most common pattern is an ordinary adenocarcinoma with focal NE differentiation.

Several authors have reported a prognostic value of NE differentiation in prostatic adenocarcinoma[263-268]. Others could not confirm these results, however[269, 270]. The results of these studies have not been subjected to multiple regression analysis and the follow-up periods in general were relatively short. In the present long-term follow-up study the prognostic influence of immunohistochemically defined NE cells present in radical prostatectomy specimens was studied.

MATERIAL AND METHODS

PATIENTS

During the period 1977-1987, 144 patients were underwent surgery in the Academic Hospital, Rotterdam for non-metastasized, clinically localized (T_1-T_2, TNM 1992) or locally advanced (T_3) prostate cancer. 43 patients had positive lymph nodes at operation (frozen sections of pelvic lymph nodes). None of these patients underwent radical prostatectomy, with the exception of 2 younger patients who had micrometastases. Thus, radical prostatectomy was performed on a total of 103 patients. The patients were followed regularly and, if necessary treated during follow-up for local recurrence or metastatic disease. The mean follow-up period was 86 months. All data concerning diagnosis, radical prostatectomy, pathology and follow-up were prospectively stored in a comprehensive database. Clinical progression was defined as histologically or cytologically proven local recurrence or the appearance of distant metastases. Tumor specific death was defined as death due to direct tumor effects or metastases or death due to tumor therapy.

TISSUE

All radical prostatectomy specimens were fixed in 10% buffered formalin, pH 7.4 and totally embedded in paraffin. All hematoxylin and eosin (H&E) slides were reviewed by one pathologist (THvdK) to identify the different Gleason growth patterns, to restage the tumors (TNM, 1992) and to obtain the Gleason sum scores. Only adenocarcinomas were included in the study. From each radical prostatectomy specimen the paraffin blocks containing all the different Gleason growth patterns observed in that particular tumor were selected (usually 2 to 3 blocks per tumor). If present, prostatic intra-epithelial neoplasia (PIN) was graded according to Bostwick and Brawer[271]. PIN-3 was termed high grade-PIN, whereas PIN-1 and PIN-2 were taken together as low-grade PIN. The histological material of 4 patients could not be identified and one specimen was of poor quality. Furthermore, in 5 specimens the previously diagnosed tumor could not be found (final stage pT0) and the remaining paraffin blocks of 3 patients no longer contained any tumor. All these 13 patients were excluded from analysis, leaving 90 evaluable patients.

IMMUNOHISTOCHEMISTRY

Tissue sections of 5μm thickness were cut and mounted on 3-amino-propyl-triethoxysilane (Sigma, United States) coated slides[272]. Following rehydration through xylene and a graded alcohol series, and blocking of the endogenous peroxidase activity with 3% hydrogen peroxide in 100% methanol, the slides were placed in a sequenza® incubator (Shandon, United Kingdom) and subsequently incubated with: normal goat serum (DAKO, Dakopatts, Denmark) diluted 1:10, mouse monoclonal anti-CgA (clone LK2H10, Organon technika, The Netherlands) diluted 1:60 or rabbit polyclonal anti-CgB (kind gift of Dr. R. Fischer-Colbrie, University of Innsbruck, Austria) diluted 1:750, biotinylated goat-anti-mouse antibody (DAKO) for visualization of CgA diluted 1:400, or biotinylated goat-anti-rabbit antibody (DAKO) for visualization of CgB diluted 1:400, and finally with peroxidase conjugated avidin-biotin complex (DAKO) diluted 1:1:200. All compounds were diluted in pH=7.4 phosphate buffered saline (PBS). The slides were rinsed with PBS in between each step except for the step following pre-incubation. The antibody-antigen binding was visualized with 0.075% 3,3'-diaminobenzidine-tetrahydrochloride (Fluka, Germany) with 0.08% hydrogen peroxide as substrate. The slides were lightly counterstained with hematoxylin, dehydrated and covered. Negative controls were included by replacing the primary antibody by PBS in a number of slides. Normal pancreas and normal prostatic epithelium which was present in most tissue sections served as positive controls.

QUANTIFICATION

Tissue slides adjacent to the immunohistochemically stained slides were routinely stained with H&E and the different Gleason patterns were identified and marked. If present, BPH areas and PIN lesions were also marked. The markings were copied on to the matching areas of the immunostained slides. A cell was identified as an NE cell if a brown, granular, cytoplasmic staining pattern was visible after immunostaining. The level of NE differentiation was assessed semi-quantificatively: -: no positive cells visible; ±: a few positive cells, widely scattered; +: some positive cells, more regularly distributed; ++: more numerous, regularly distributed positive cells or small clusters of positive cells; +++: numerous positive cells or larger clusters of positive cells. CgA scores were assessed in normal prostatic epithelium, BPH areas, low and high grade PIN lesions and in each Gleason pattern observed in a particular tumor. Normal, hyperplastic and PIN areas were also investigated in the specimens of the 5 patients with stage pT_0 disease. If more areas from the same pathological category were identified within one prostate, the highest score was taken as the CgA score for that category. The tumor CgA score was defined as the highest CgA score observed in all areas of that tumor.

STATISTICS

statistical analysis was performed with the statistical computer packages SPSS and STATA. Survival tables were constructed by the Kaplan-Meier method. The logrank test and logrank test for trend were used in the univariate analysis. Cox's regression model was used for multivariate survival analysis. Other statistical methods were the sign test, χ^2-test, Student's t-test and the Kruskal-Wallis test.

RESULTS

CLINICAL

The mean age of the 90 evaluable patients at operation was 62 years (range 47-74). The preoperative staging of all patients was $T_{1-3}N_0M_0$. Of these, 22 patients (24%) had a final stage of pT_2, while 66 (73%) and 2 patients (2%) had pT_3 and pT_4 disease, respectively. Of 7 patients (8%) with lymph node metastasis, 5 (6%) patients were in stage pN_1 and 2 (2%) were in stage pN_2. The 2 patients with positive lymph nodes at operation were

both staged pN_1. The bottom line of Table II.1 lists the distribution of the Gleason sum scores of the 90 tumors. The mean follow-up time was 86 months (range 1-203) and 15 patients were followed for more than 120 months. During follow-up, 37 patients (41%) showed disease progression and 14 (16%) died of their tumor.

TABLE II.1
Relationship between Gleason sum score and tumor CgA scores

Tumor CgA score	Gleason sum score							TOTAL
	4	5	6	7	8	9	10	
–	2	2	5	6	2	3	–	20
±	3	–	7	13	4	6	–	33
+	–	2	3	10	1	3	1	20
++	–	1	1	4	5	1	–	12
+++	–	–	–	3	1	–	1	5
TOTAL	5	5	16	36	13	13	2	90

IMMUNOHISTOCHEMISTRY

Immunohistochemically defined NE cells showed an intense granular cytoplasmic staining pattern (Figure II.1). However, the number of CgB positive cells was small compared to the number of CgA positive cells, whether normal, benign or malignant prostatic tissues were investigated. Therefore, it was decided to define NE cells only by their immunoreactivity for CgA. NE cells were found in all areas of normal prostatic epithelium (N=105, Figure II.1a). The CgA scores of the normal peri-urethral regions exceeded the CgA scores of the normal acinar regions if both were present in the CgA slides from one prostatectomy (N=18, $p<0.0001$, t-test, Table II.2). NE cells were also found in 19 of the 23 BPH nodules (83%) and in low- and high-grade PIN lesions (17 out of 25 (68%) and 38 out of 52 (73%), respectively, Figure II.1b), but the CgA scores of both categories were not statistically significantly different from the CgA scores in normal acinar epithelium (T-test, $p>.05$, Table II.2).

CgA staining was seen in 70 of the 90 prostatectomy specimens (78%, Figures II.1c and II.1d). A total of 249 Gleason patterns was examined for the presence of NE cells (2.7 per patient). The relationships between the CgA scores and both the primary Gleason growth patterns (sign test and χ^2-test, Table II.2) and the Gleason sum scores (Kruskal-Wallis test, Table II.1) were not statistically significant ($p>0.05$). Similarly, CgA scores did not correlate with pT or pN stage (χ^2-test, $p>0.05$).

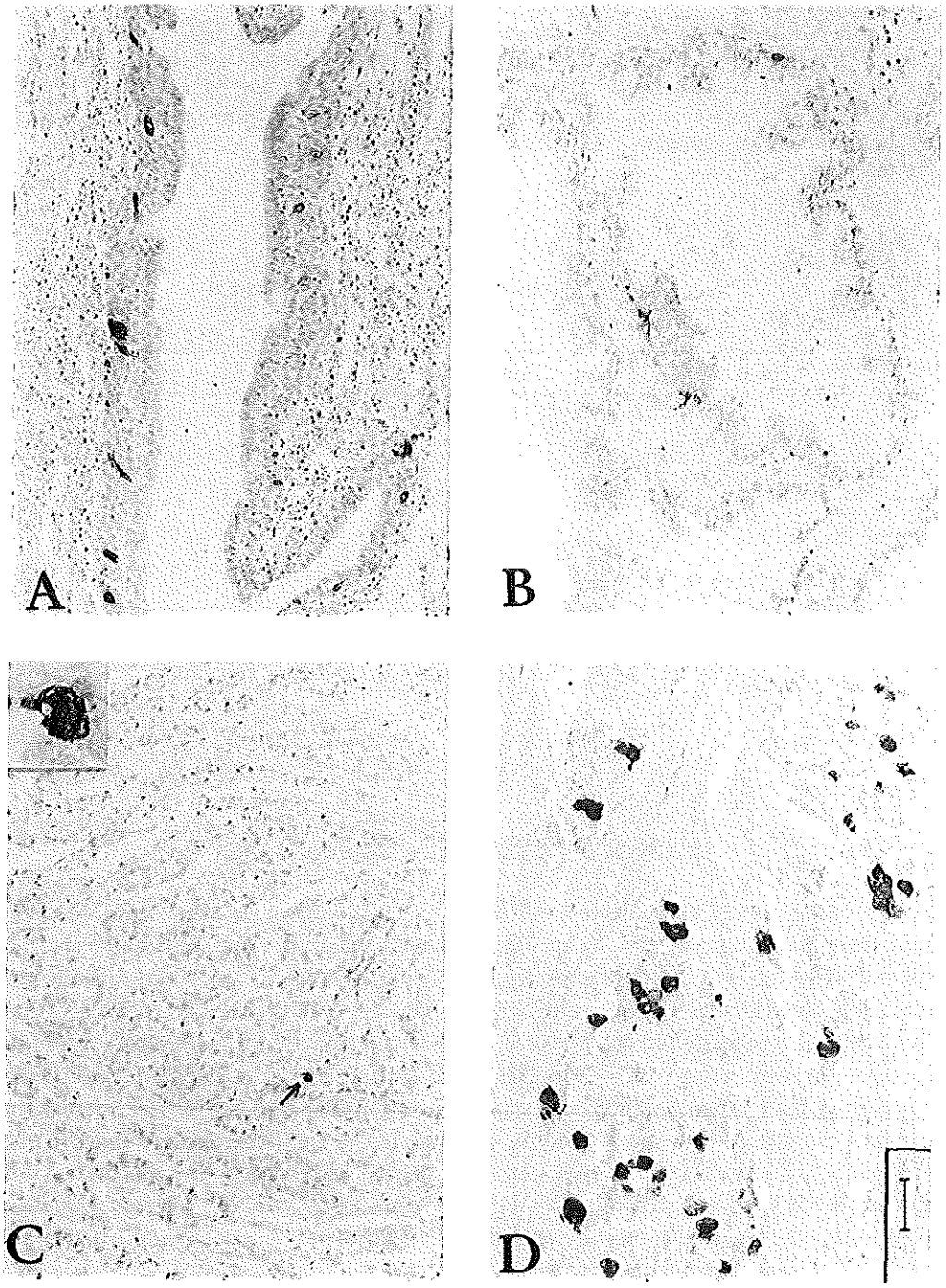
A
B
C
D

FIGURE II.1
CgA immunostaining. (A). Periurethral normal prostatic duct with NE cells located near the basal cell layer. (B). High-grade prostatic intra-epithelial neoplasia lesion with basally located NE cells. (C). Primary Gleason growth pattern 4 adenocarcinoma with one NE cell (arrow). Inset: positive cell at higher magnification (x880). (D). Primary Gleason growth pattern 4 adenocarcinoma with numerous NE cells. (x160).

TABLE II.2
Neuroendocrine cells in distinct pathological areas

CgA score	normal		BPH	PIN		Gleason growth patterns					
	centr.	gland.		low	high	1	2	3	4	5	total
–	–	–	4	8	14	1	16	25	13	9	64
±	–	10	8	8	18	1	5	23	24	3	56
+	1	72	7	6	15	1	–	8	11	6	26
++	17	4	3	3	4	–	–	4	8	1	13
+++	–	1	1	–	1	–	–	1	3	1	5
TOTAL	18	87	23	25	52	3	21	61	59	20	164

centr. = peri-urethral and ductal prostate - gland. = glandular prostate - low/high = low/high grade PIN

ANALYSIS OF PROGNOSTIC FACTORS

Kaplan Meier curves were constructed to investigate the influence of pT stage, Gleason sum score and tumor CgA score on the probability of progression and tumor specific survival (Figures II.2 and II.3, respectively). Univariate analysis using the logrank test for trend showed a prognostic value of pT stage ($p=0.001$) and Gleason sum score ($p<0.001$) for progression and only of the Gleason sum score ($p=0.001$) for tumor specific survival. The other variables tested showed no statistically significant correlation with prognosis (pN stage, age at operation and CgA score). The significant parameters from the univariate analysis were entered in Cox's regression model (Table II.3). Both pT stage and the Gleason sum score were independent prognosticators for the probability of progression. The Gleason sum score was the only independent prognosticator to predict tumor specific survival. Stepwise entering of the other variables did not improve the models.

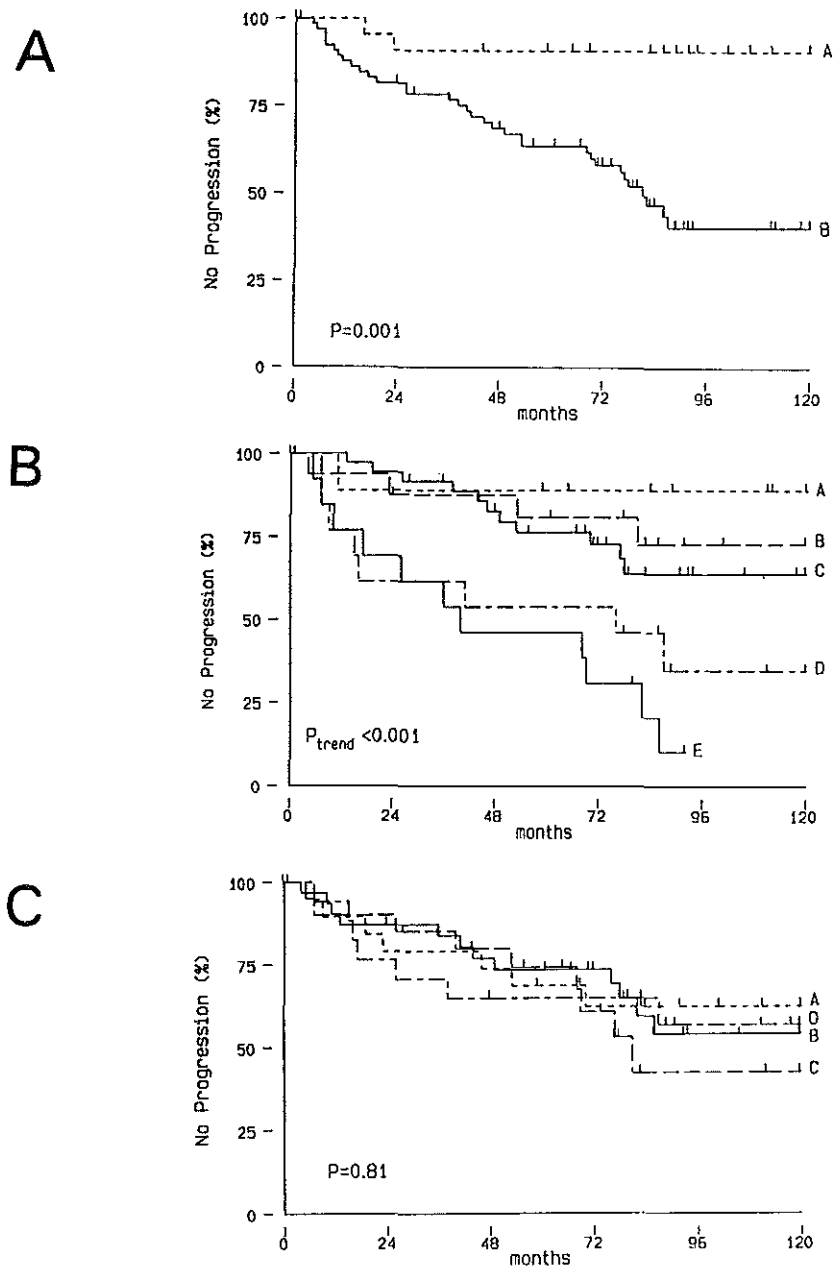

FIGURE II.2

Kaplan-Meier curves for clinical progression, curves truncated at 120 months. (A). Relation with pT-stage (A=pT_1 N=22, B=$pT_{3,4}$ N=68. (B). Relation with Gleason sum score (A=4-5 N=20, B=6 N=16, C=7 N=36, D=8 N=13, E=9-10 N=15). (C). Relation with tumor CgA score (A=– N=20, B=± N=33, C=+ N=20, D=++ - +++ N=17). Tick marks indicate censored patients.

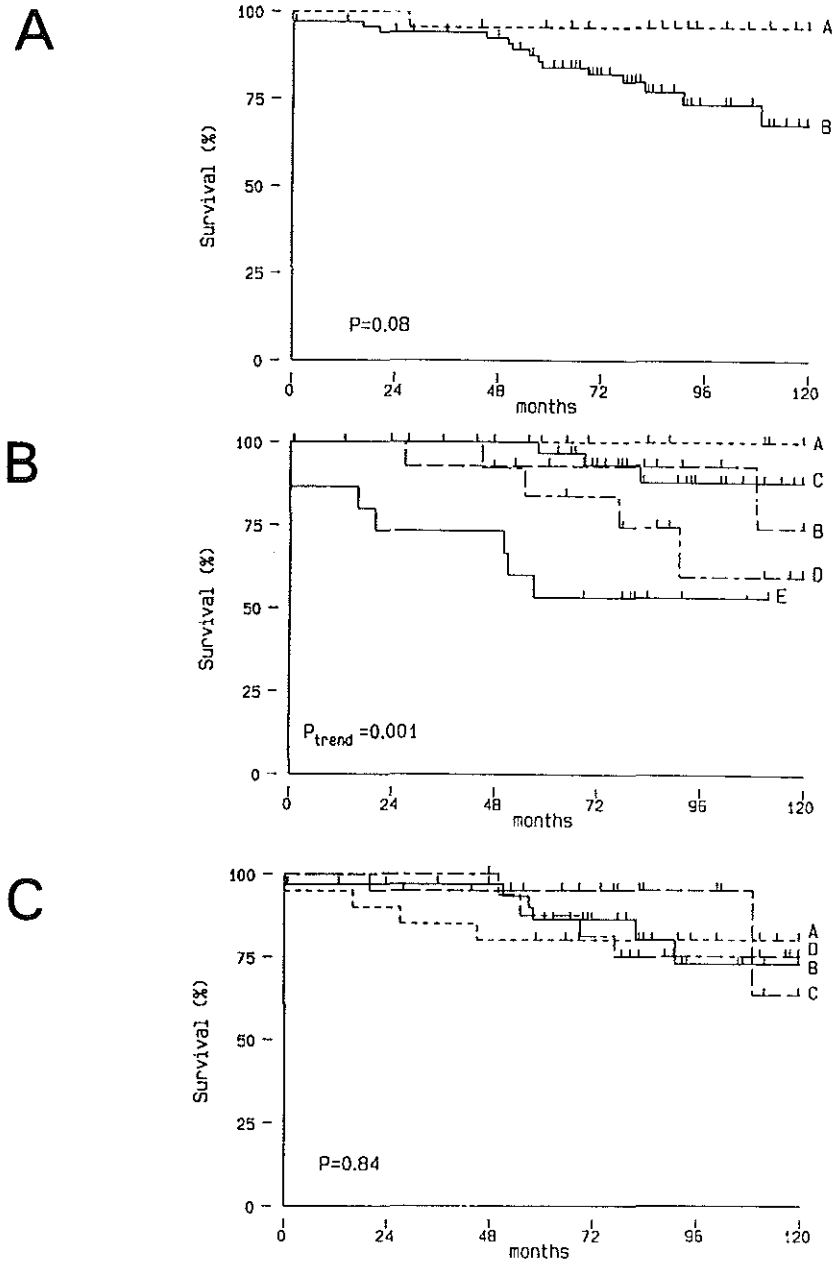

FIGURE II.3
Kaplan-Meier curves for tumor related death. See legend to figure II.2 for explanation.

TABLE II.3

Results of Cox's multi-regression analysis

Outcome parameter	variable	hazard ratio	95% confidence interval	*p*
clinical progression	GSS	1.60	1.16 - 2.21	0.004
	pT-stage	3.83	1.07 - 13.6	0.038
tumor specific survival	GSS	2.29	1.41 - 3.74	0.001

DISCUSSION

NE differentiation in prostatic adenocarcinoma has gained increasing attention during recent years. The present study was carried out to investigate whether the extent of NE differentiation is an independent prognostic factor in radical prostatectomy specimens. A substantial number of patients and a long-term follow-up made it possible to study more conclusively the relation between NE differentiation, tumor stage and grade and patient age with respect to the probability of disease progression and tumor specific death. Remarkably, the final pT stage of the tumors showed a relatively large proportion of pT_3 tumors. At operation, 29 patients had a clinically T_3 tumor and 32 of the clinically T_2 tumors were upstaged to stage pT_3. Since the treatment decision did not depend on biopsy tumor grade, the high frequency of tumors with Gleason sum scores 8-10 (N=28, 31%) can be explained by the observed relation between tumor stage and Gleason sum score ($p=0.018$, χ^2-test), as found by others[56].

Schmid *et al.*[261] found that in the more poorly differentiated prostatic tumors with NE cells CgA expression disappeared whereas CgB expression increased. These results indicate that defining prostatic NE cells only by their immunoreactivity for CgA could lead to an underestimation of the number of NE cells in certain prostatic tumors. In the present study a new polyclonal antibody (PE-11) directed against a purified CgB peptide was used. CgA and CgB staining both resulted in an intense granular cytoplasmic staining. However, the number of CgB positive cells was very small compared to the number of CgA positive cells, whether normal, hyperplastic or cancerous prostatic tissues were investigated. Moreover, CgB did not replace CgA expression in high grade (Gleason primary growth patterns 4 and 5) tumors. Control tissue (normal pancreas) revealed identical results for CgA and CgB. Attempts to increase CgB staining in prostatic tissues with pronase pre-treatment or with antigen retrieval in a microwave oven failed[273], and therefore, for the present study it was decided to define NE cells solely by their immunoreactivity for CgA. The number of

tumors with NE cells might be underestimated in the present study since CgA negative prostatic NE cells exist[258]. On the other hand, 78% of the tumors contained NE cells and it is questionable whether this figure would increase if another marker, for instance serotonin, were included.

CgA scores were assessed in pathologically distinct areas to investigate their putative relationship (Table II.2). If present, CgA scores were also assessed in normal prostatic epithelium. All normal prostatic epithelium areas (N = 105) contained NE cells, although the CgA scores were higher in the peri-urethral region than in the peripheral glandular region ($p < 0.001$, sign-test), an observation that was also made by R.J. Cohen *et al.*[274], but not by Aprikian *et al.*[258]. The fact that 17 of the 18 normal peri-urethral regions (94%) had a CgA score of + + and 74 of the 87 normal peripheral regions (85%) had a CgA score of + indicates that the inter-individual variation in the number of NE cells is rather limited as far as normal prostatic epithelium is concerned. This observation could serve as an internal control for the immunohistochemical procedure. NE cells were identified in 83% of the BPH areas and in 68% and 73% of the low- and high-grade PIN lesions, respectively. The distribution of the CgA scores in BPH and PIN lesions was statistically not significantly different from that seen in normal, glandular prostatic epithelium. No relationship was found between the size of the hyperplastic nodules and the CgA scores (data not shown), whereas others[275] even found an inverse relationship. It should be noted, however, that in the present study no detailed measurement of the size of the hyperplastic areas was performed.

No data concerning NE cells in PIN lesions have been published so far. The pattern of NE differentiation was identical in low- and high-grade PIN lesions, as was the distribution of CgA scores (Table II.2). NE cells in PIN lesions were in general basally located (Figure II.1b), which suggests that basal cells in these lesions show a multidirectional differentiation similar to that observed in the normal prostate. It is very likely, however, that the PIN-associated NE cells originate from non-neoplastic basal cells. Nevertheless, PIN-NE cells might play a role in the tumorigenesis of prostate cancer.

NE cells were found in 78% of the adenocarcinomas in the present study, a figure which is in line with the results of Aprikian *et al.*[258]. Other authors described smaller[263, 268, 270], or larger percentages[264]. Differences in tissue processing, types of tissue investigated and patient characteristics probably partially account for these differences. However, in one study[270] antigen retrieval was performed[273], a method which enhances the immunostaining of a number of antibodies including CgA clone LK2H10, and found NE cells in only about 30% of the specimens. The entrapment of benign NE cells in a tumor might also explain the high percentage of tumors with NE cells. However, in a tumor only cancerous NE cells were scored positive, which makes this explanation unlikely.

The outcome of the present long-term follow-up study indicates that in prostate cancer patients treated by radical prostatectomy, NE differentiation does not influence the probability of tumor progression or cancer related death. Even the extent of NE

differentiation, as defined by increasing tumor CgA scores (Table II.1), did not show a tendency towards a poor prognosis ($p > 0.05$, logrank test for trend). The distribution of the tumor CgA scores was statistically not significantly different from that of normal glandular epithelium CgA scores ($p > 0.05$, t-test). Some other recent reports also tempered initial enthusiasm regarding the prognostic influence of NE cells in prostate cancer[269, 270]. In the study of Aprikian *et al.*[269] this concerned the prognostic value of NE cells in metastatic lesions. M.K. Cohen *et al.*[270] found no prognostic influence of NE cells (CgA or neuron specific enolase positive cells) in 38 patients with clinically stage II (AJCC) prostate cancer treated by radical prostatectomy. The follow-up period in their study was relatively short (maximum 77 months) and the relationship between tumor grade and NE differentiation was not discussed in detail. In two studies, Cohen *et al.*[263, 268] described a striking prognostic influence of CgA positive cells in initial biopsies and TUR specimens with incidental prostate cancer. Abrahamsson *et al.*[264] studied the number of CgA positive cells in repeated biopsy specimens of 24 hormonally treated patients and generally found, with time, an increase in number of CgA positive cells which was paralleled by dedifferentiation of the tumor. The relation between tumor grade and NE differentiation in their study, which might have explained part or all of the prognostic value, was not confirmed by Aprikian *et al.*[258], and the data of the present study do not support this either. It should be stressed, however, that the study of Abrahamsson *et al.*[264] was based on hormonally treated patients. Since the NE cells in normal and neoplastic prostatic tissues do not contain androgen receptors[276], their presence in a prostatic tumor might be related to androgen independence. The studies of Abrahamsson *et al.*[264] and R.J. Cohen *et al.*[263, 268] may have been biased by the fact that only small tumor fragments were investigated (biopsies and TUR specimens containing small incidental carcinomas). Due to the widely scattered distribution of NE cells in most tumors, the likelihood of obtaining positively stained cells most probably correlates with both tumor volume and the size of the investigated tumor area. Since tumor volume is a predictor of progression[57], the prognostic value found in these studies may be partially explained by this relation. Furthermore, a substantial risk of obtaining false negative results exists, especially if only small numbers or small foci of positive cells are present. In the present study, Cox's regression identified both pT classification and Gleason sum score as independent prognosticators for progression, but only Gleason sum score for tumor related death. This can be explained by the above-mentioned relationship between pT classification and Gleason sum score combined with the small number of events in the survival analysis ($N = 14$, 16%).

As mentioned above, prostatic NE cells do not express the androgen receptor and therefore are considered to be primarily androgen independent[276]. In addition, prostatic NE cells secrete neuropeptides and some of these peptides show growth modulating activity (reviewed by Noordzij *et al.*[259]). If non-NE prostatic tumor cells contain receptors for these neuropeptides, tumor growth may be modulated by such peptides. Moreover, some neuropeptides were able to stimulate steroid receptors in a ligand independent fashion[277]. It is possible that the NE cells are related with the

androgen indepence of a tumor. On the other hand, the presence of NE cells in prostate cancer might be simply an epiphenomenon determined, for instance, by stromal-epithelial interactions. The function of NE cells in the normal and, especially, the neoplastic prostate remains largely unclear as yet and has to be clarified experimentally. Such studies, using well defined, newly established, prostatic tumor xenograft models with NE cells are in progress at our institution.

CHAPTER III

THE PROGNOSTIC VALUE OF CD44 ISOFORMS IN PROSTATE CANCER PATIENTS TREATED BY RADICAL PROSTATECTOMY

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ABSTRACT

CD44 forms a group of transmembranous glycoproteins formed by alternative splicing of a single mRNA. The expression of v6-exon containing variants correlates with metastasis and poor prognosis in a number of malignancies. The distribution and prognostic value of CD44s, CD44v5 and CD44v6 was studied immunohistochemically in the radical prostatectomy specimens of 97 patients with prostate cancer and in 12 lymph node metastasis. The mean follow-up period was 84 months. The percentage of CD44 immunoreactive cells was scored semiquantificatively. CD44 mRNA expression was studied in 9 prostate cancer and 8 benign prostatic hyperplasia (BPH) samples by RT-PCR.

Benign prostatic glands almost always expressed CD44s, CD44v6, and at a lower intensity CD44v5. CD44 scores decreased from low- to high-grade prostatic intraepithelial neoplasia. CD44s, CD44v5 and CD44v6 were expressed in 86%, 23% and 69% of the adenocarcinomas, respectively. Gleason sum score (GSS) and pT-stage were inversely correlated with CD44s and CD44v6 scores. CD44 was not found in the lymph node metastatic tumor cells. At the mRNA level 89% of the tumors and all BPH samples expressed CD44s. CD44v6-10 mRNA was present in 44% and 75% of the tumors and BPH samples, respectively. Loss of CD44s and CD44v6 predicted an adverse prognosis at univariate analysis. The independent prognosticators identified by multivariate analysis were: GSS, pT-stage and CD44s for clinical progression; GSS and CD44s for PSA progression; and GSS for tumor specific survival. Loss of CD44s expression in prostate adenocarcinoma predicts a poor prognosis, independent of stage and grade.

INTRODUCTION

Prostate cancer is the most prevalent non-skin tumor in western males and its mortality is second only to that of lung cancer[1]. The incidence and prevalence of the disease have been increasing dramatically over the last decade, which can partially be explained by the introduction of prostate specific antigen (PSA) as a serum tumor marker[2, 278]. On average, patients are now diagnosed at an earlier stage of the disease and often are candidates for curative treatment by means of a radical prostatectomy[10]. At present there are no tumor markers that are sufficiently able to divide patient groups in those

who will and those who will not benefit from radical surgery. A lot of prostate cancer research aims at the development and application of new prognostic tissue markers such as proliferation associated molecules (for example Ki-67[199]) and adhesion molecules (for example E-cadherin[177]).

CD44 is a transmembranous glycoprotein that was discovered in lymphocytes as a lymphocyte homing molecule (reviewed by Matsumura and Tarin[255]). Subsequently, it was found that numerous normal, and tumor tissues express CD44 as well[279, 280]. The human CD44 gene is composed of 19 exons, 9 of which are variably expressed due to alternative splicing of the mRNA[281]. Standard CD44 (CD44s) is composed of exons 1-5 and 15-19. (s1-s10) The variant forms contain one or more of the exons 6-14 (v2-v10; v1 is not expressed in humans) which are positioned between exon 5 and 15. The structure of the gene is depicted in Figure III.1. Variant CD44 molecules containing exon v6 appear to play a causal role in tumor metastasis[256]. The presence of CD44 molecules has been found to have prognostic value in several tumors[282-285].

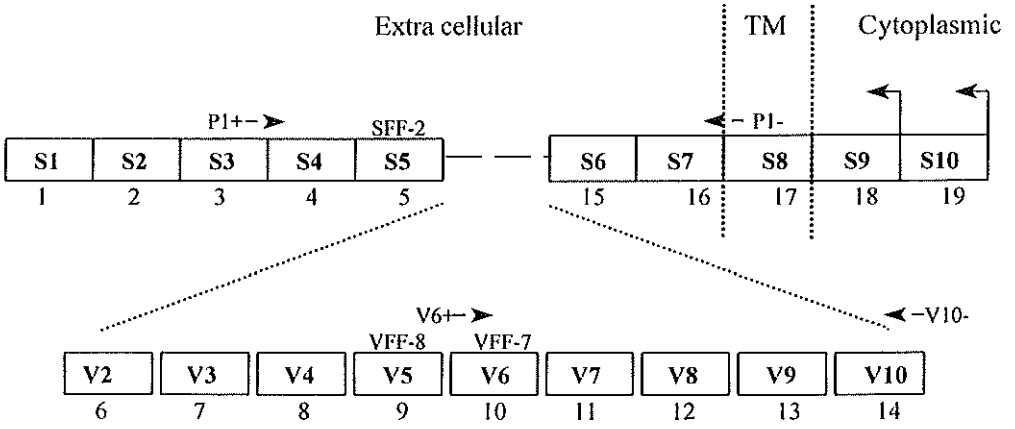


FIGURE III.1

Scheme of the human CD44 gene located on chromosome 11. Standard exons S1 - S5 and S6 - S10, variant exons V2 - V10. CD44 molecules are terminated either at exon 18 (short form) or 19 (long form). The location of the epitopes of the antibodies against CD44s (SFF-2), CD44v5 (VFF-8) and CD44v6 (VFF-7) is indicated. P1+, P1-, V6+ and V10- are the primers used for RT-PCR. TM = trans-membranous part of the molecule.

As yet, the expression of CD44 molecules in prostate cancer has only be investigated in cell lines. Preliminary immunohistochemical data from clinical prostate cancer indicated that tumor glands show heterogenous expression of CD44s and no CD44v6 expression[286]. In the present study the immunohistochemical expression pattern of CD44 was studied in prostate cancer tissues and the prognostic value was determined

in patients treated by radical prostatectomy. In addition, CD44 mRNA expression was checked by the reverse transcriptase polymerase chain-reaction (RT-PCR).

MATERIAL AND METHODS

PATIENTS

In the period 1980-1988, 159 patients were operated at the University Hospital, Rotterdam, The Netherlands for prostate cancer ($T_{1-3}N_xM_0$, TNM classification 1992[34]). In 17 of these the tumor was diagnosed in a trans-urethral resection (TUR) specimen performed for benign prostatic hyperplasia (BPH) i.e., stage T_{1a-b}. In 49 patients the pelvic lymph node dissection specimen contained metastatic tumor on examination of frozen sections. These patients did not undergo subsequent radical prostatectomy, except for two younger patients with micrometastasis, leaving 112 patients treated by radical prostatectomy. The patients were followed regularly and all data concerning diagnosis, treatment and follow-up were stored prospectively in a comprehensive database. Clinical progression was defined as cytologically or histologically proven local recurrence or the appearance of distant metastasis. Tumor death was defined as death due to direct tumor effects, metastasis or tumor therapy. PSA progression was defined as a PSA-level of ≥ 1.0 at two subsequent measurements, in which case the first elevated PSA-level was considered the date of failure. PSA progression was only considered in patients with a complete PSA history (N=29). If necessary patients were treated during follow-up for local recurrence or metastatic disease. One patient was lost to follow-up. Two patients died within one month following radical prostatectomy from myocardial infarction and pulmonary embolism, respectively.

TISSUES

All radical prostatectomy specimens were fixed in 10% buffered formalin and totally embedded in paraffin. The hematoxylin & eosin stained slides were reviewed by a single experienced genitourinary pathologist (THvdK) to obtain the Gleason sum score and to stage the tumors according to the TNM-1992 classification system. The material of 10 patients could not be retrieved and one tumor was a metastasis of a large bowel carcinoma. The previously diagnosed tumor was not found in the radical prostatectomy specimen of three patients with a T_{1a-b}-tumor (final stage pT_0). All these 14 patients were excluded from analysis, leaving 97 patients. Of each radical prostatectomy

specimen 2-3 tissue blocks were selected that contained at least all the Gleason growth patterns observed in that tumor and, if applicable, also contained normal prostatic glands, BPH and/or prostatic intraepithelial neoplasia (PIN). PIN lesions were graded according to Bostwick & Brawer[271]. PIN-I and PIN-II were taken together as low-grade PIN, PIN-III was termed high-grade PIN. To investigate the expression of CD44 in metastatic prostate cancer twelve paraffin-embedded parailiac lymph nodes containing a prostate cancer metastasis were also selected.

The expression of CD44 mRNA was studied in tumor samples from 9 radical prostatectomy specimens and 8 BPH samples derived from simple prostatectomies. These radical prostatectomy samples were not from the specimens that were used for the follow-up study because fresh frozen tissues have only been collected as of 1989. Directly after removal of the prostate macroscopical tumor lesions (or BPH tissue) were identified and a piece of tumor tissue was removed in a sterile fashion, snap-frozen in liquid nitrogen chilled isopentane (Merck, Darmstadt, Germany) and stored at -80°C until use.

IMMUNOHISTOCHEMISTRY

The selected tissue blocks of the radical prostatectomy specimens and the lymph nodes were immunostained with monoclonal antibodies against CD44s (clone SFF-2), CD44v5 (clone VFF-8) and CD44v6 (clone VFF-7), all from Bender MedSystems, Vienna, Austria[282, 283, 287-289]. CD44v5 has not been related to malignant processes, but was included for comparison with CD44s and CD44v6. For visualization of antigen-antibody binding an ultra-sensitive method (tyramide signal amplification system (TSA), DuPont NEN, Boston, MA), was used[290]. This method uses the activity of second antibody coupled horseradish peroxidase to catalyze the deposition of a large number of biotin molecules near the peroxidase molecule. The biotin molecules can be made visible by fluorochrome- or enzyme-labeled streptavidin. This procedure was used because preliminary results showed that the more well known avidin-biotin complex method[291] yielded false-negative results in a substantial number of cases.

Tissue sections were cut at 5μm and mounted on 3-aminopropyl-trietoxysilane (Sigma chemical Co., St. Louis, MO) coated glass slides[272]. After deparaffinization through xylene and ethanol endogenous peroxidase activity was blocked by rinsing the slides for 10 minutes in 3% hydrogen peroxide in ethanol. The slides were rinsed with distilled water and placed in a 10 mmol/l citrate buffer at pH=6.0. Antigen retrieval was performed in a microwave oven at 700W for 3x5 minutes[273]. After cooling and rinsing with PBS the slides were placed in a Sequenza immunostaining system (Shandon, Uncorn, UK). Following a 15 minute pre-incubation with 10% normal goat serum (DAKO, Glostrup, Denmark) in PBS, the slides were incubated for 90 minutes with either the primary antibody or PBS (negative control). The antibodies were diluted in PBS at 1:200 (SFF-2 and VFF-7) or 1:160 (VFF-8). Subsequently, the slides were

incubated for 30 minutes with a blocking buffer consisting of 0.15 mol/l TRIS-buffer (Gibco Brl, Breda, The Netherlands) at pH 7.5, 0.15 mol/l NaCl (Merck) and 0.5% blocking reagent (DuPont, TSA-kit). After a 30 minute incubation with a 1 to 50 diluted horseradish peroxidase-labeled goat anti mouse antibody (DAKO) an amplification solution consisting of biotinyl tyramide (DuPont) 1 to 50 diluted in 50% distilled water and 50% amplification diluent (DuPont) was applied. Finally, the slides were incubated with 1 to 400 diluted horseradish peroxidase-labeled streptavidin. All the components of the TSA-system were diluted in the blocking buffer. In between the incubations the slides were rinsed three times in a buffer consisting of 0.1 mol/l TRIS, 0.15 mol/l NaCl and 0.05% TWEEN-20 (Merck). The antigen-antibody binding was visualized with 0.75mg/ml PBS of diaminobenzidine hydrochloride (Fluka, Neu-Ulm, Germany) with 0.08% hydrogen peroxide as substrate. The slides were counterstained with Mayer's hematoxylin, dehydrated and covered.

The cellular localization of CD44 immunostaining was examined in detail in 12 randomly chosen sections using a confocal laserscan microscope system (Carl Zeis Jena, Germany). A fluorescent TSA method, which uses FITC-labeled streptavidin during the final incubation, was applied to these sections. The nuclei were counterstained with propidium iodide and the slides were covered with vectashield (Vector, Burlingame, CA).

QUANTIFICATION

The different Gleason growth patterns as well as areas of normal prostatic glands, BPH and PIN were identified and marked on the immunohistochemically stained tissue slides. The slides were studied at 25x magnification without knowledge of the clinical outcome of the patients. The percentage of CD44 positive cells (s, v5 and v6) in a particular area was scored semiquantificatively as: $<10\%$, 10-25%, 25-50% and $>50\%$. Scores were obtained for all pathological categories present in the tissue slides of one prostate. A tumor CD44 score was obtained by taking the lowest score observed in any of the primary Gleason growth patterns present in a tumor.

CD44 mRNA expression

Total RNA of the snap-frozen BPH and tumor samples was prepared by the guanidine isothiocyanate / cesium chloride centrifugation method using the RNAzol-B kit (Campro, Veenendaal, The Netherlands) as per the manufacturer's protocol. First strand cDNA was produced with 0.5μg of the isolated total RNA using random hexamer primers and M-MLV reverse transcriptase in the presence of RNase inhibitor and dNTPs (all from Perkin Elmer Cetus, Norwalk, CT) in a final volume of 10μl. The oligonucleotide primers were chosen upstream and downstream of the insertion point

of alternatively spliced mRNA[286], the positions are indicated in Figure III.1. The primers used were: (P1+) sense 5'-GACACTATTGCTTCAATGCTTCAGC and (P1-) antisense 5'-GATGCCAAGATGATCAGCCATTCTGCAAT. Thus, PCR of cDNA should amplify CD44s as well as all possible variant forms and should result in distinguishable PCR products. In addition, two exon specific primers were used to characterize the splice variants[292]: (V6+), sense 5'-TCCAGGCAACTCCTAGTAGT and (V10-), antisense, 5'-CTGATAAGGAACGATTGACA. The following combinations were used for PCR: P1+ with P1-, P1+ with V10-, and V6+ with P1-. PCR was performed with the total volume of cDNA using super Taq DNA polymerase (Sphaero, HT biotechnology, UK) in a thermal cycler (Perkin Elmer Cetus). The PCR-mix was heated to 94°C for 4 minutes followed by 40 cycles of 1 minute at 94°C, 1 minute at 60°C and 1 minute at 72°C. After the last cycle, the tubes were kept at 72°C for 10 minutes. The PCR products were separated on a 1% agarose gel in the presence of ethidium bromide and analyzed under UV-light.

STATISTICS

Statistical analysis was performed using the SPSS and STATA statistical computer packages. The sign-test, χ^2-test for trend and Spearman's rank correlation test were used to analyze the relation between several pathological variables and CD44 expression. For the analysis of survival data Kaplan-Meier curves were constructed and the logrank test for trend was performed. Multivariate survival analysis was performed using Cox's proportional hazards model.

RESULTS

CLINICAL

After exclusion of the patients that were not appropriate (n=1 lost to follow-up, n=10 radical prostatectomy specimens could not be retrieved, n=3 final stage pT_0 and n=1 tumor was not of prostatic origin), 97 patients were left for analysis. After clinical evaluation all patients were staged $T_{1-3}N_xM_0$. The T-stage distribution was: T_{1a-b} in 17, T_2 in 50 and T_3 in 30. The mean age at operation was 63 years (range 45-76). The final T-stage distribution was pT_2 in 25 patients; and pT_{3a-b}, pT_{3c} and pT_4 in 29, 41 and 2 patients, respectively. Lymph node metastasis were found in 7 patients (pN_1 in 5 and pN_2 in 2). The patients with positive lymph nodes at operation were both at stage pN_1.

The bottom line of Table III.1 lists the distribution of Gleason sum scores. The mean overall follow-up period was 84 months (range 0-178). Clinical progression occurred in 38 patients (39%) and 13 patients (13%) died from their tumor. At the end of the follow-up period 21 patients had died of other causes and 63 patients were still alive. Of the 29 patients that were evaluable for PSA progression 12 (41%) showed progression. The mean overall follow-up period for PSA progression was 81 months (range 21-109).

TABLE III.1

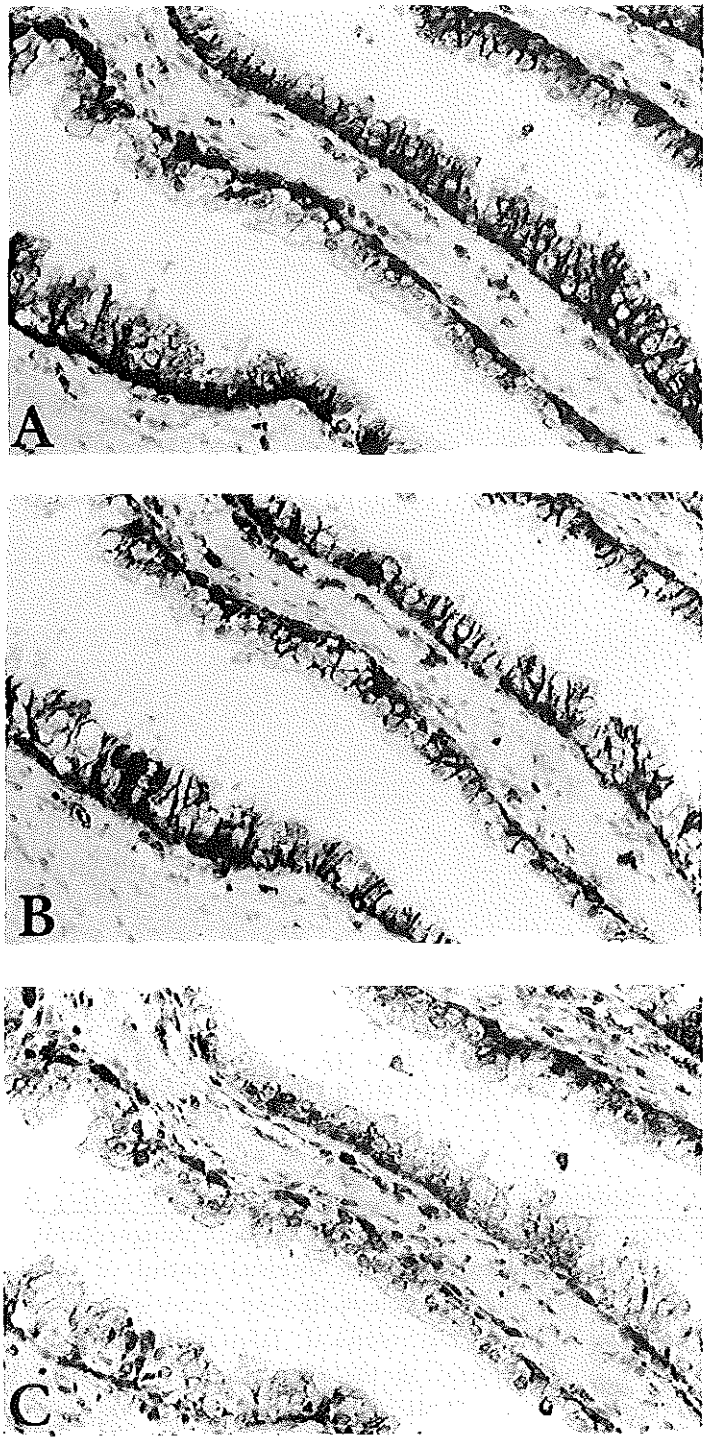
Relationship between CD44 expression and Gleason sum scores

		Gleason sum score							
marker	score	4	5	6	7	8	9	10	TOTAL
CD44s	<10%	–	1	4	8	9	4	2	29
	10-25%	–	–	4	12	–	6	–	22
	25-50%	–	1	7	13	3	4	–	28
	>50%	4	3	2	8	1	–	–	18
	R=−0.41, p<0.0001*								
CD44v5	<10%	2	4	13	40	11	14	2	84
	10-25%	2	1	3	1	–	–	–	7
	25-50%	–	–	–	1	2	–	–	3
	>50%	–	–	1	–	–	–	–	1
	R=−0.18, p=0.089*								
CD44v6	<10%	–	3	9	24	10	10	2	58
	10-25%	1	–	5	11	2	2	–	21
	25-50%	1	–	3	7	–	2	–	13
	>50%	2	2	–	–	1	–	–	5
	R=−0.34, p<0.0001*								
TOTAL		4	5	17	42	13	14	2	

*Spearman's rank correlation

CD44 EXPRESSION IN PROSTATIC TISSUES

CD44s and CD44v6 immunohistochemistry of benign prostatic glands showed a very intense membranous staining of almost all basal cells (Figure III.2). In addition, most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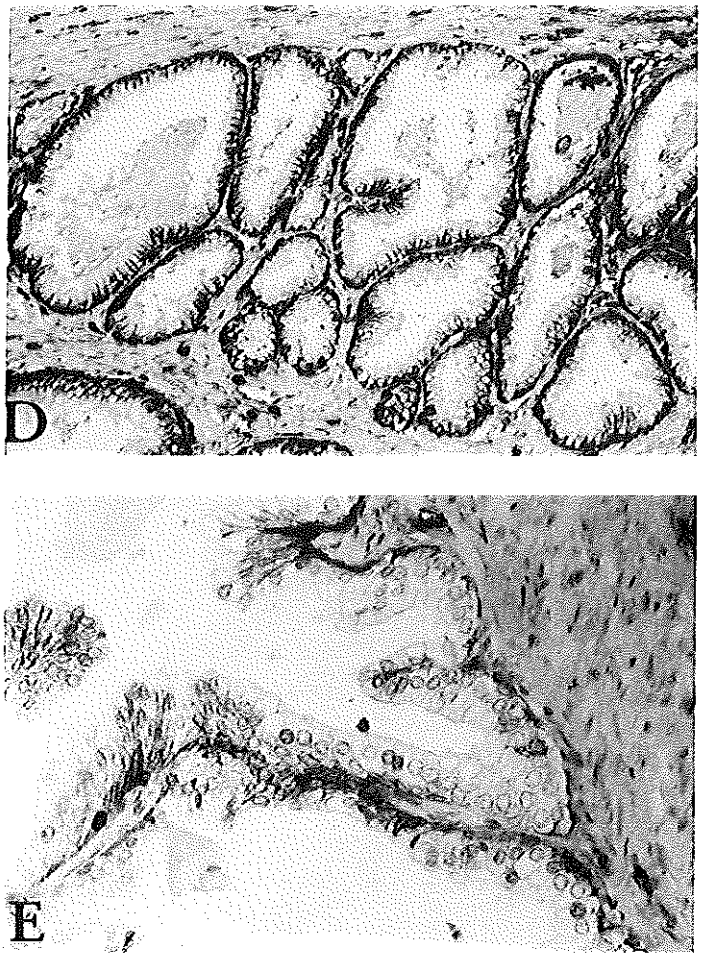
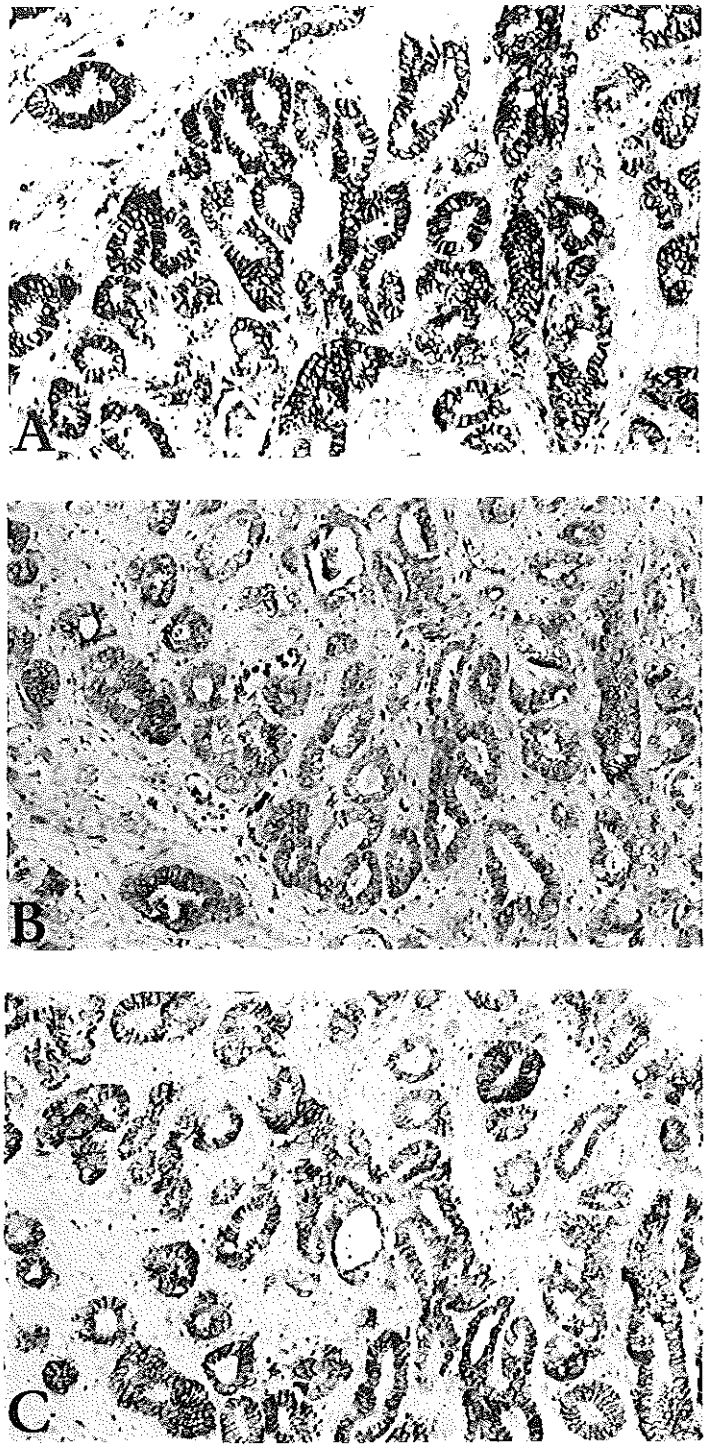


FIGURE III.2
CD44 immunohistochemistry of non-malignant prostatic tissues, counterstaining with hematoxylin. (A). CD44s, benign glands (x273). (B). CD44v5, benign glands (x273). (C). CD44v6, benign glands (x273). (D). CD44s, BPH (x138). (E). CD44s, high-grade PIN (x138).

luminal cells also expressed both antigens, although in general weaker. Identical staining patterns were found in BPH tissues (Figure III.2). Since benign glands were present in almost all slides, these could serve as an internal control of the immunostaining procedure. Areas with basal cell hyperplasia showed a very strong labeling of all basal cell layers. Detailed analysis by confocal laserscan microscopy showed that immunostaining was only present at the intercellular membranes, thus the basal membranes of the basal cells and the apical membranes of the luminal cells lacked immunoreactivity. In PIN lesions the majority of basal cells were labeled, but with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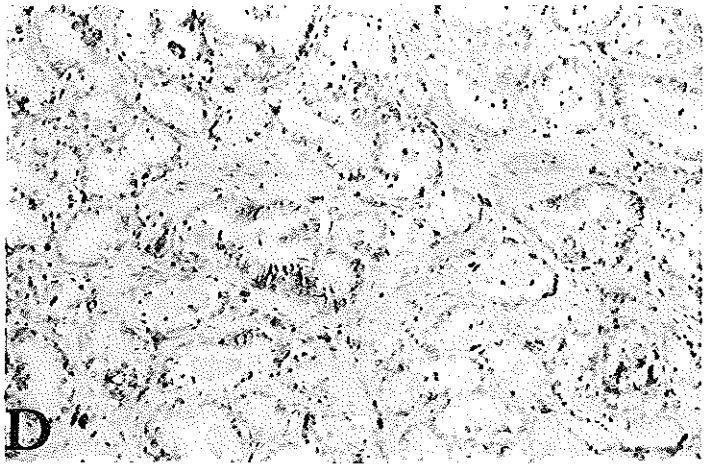
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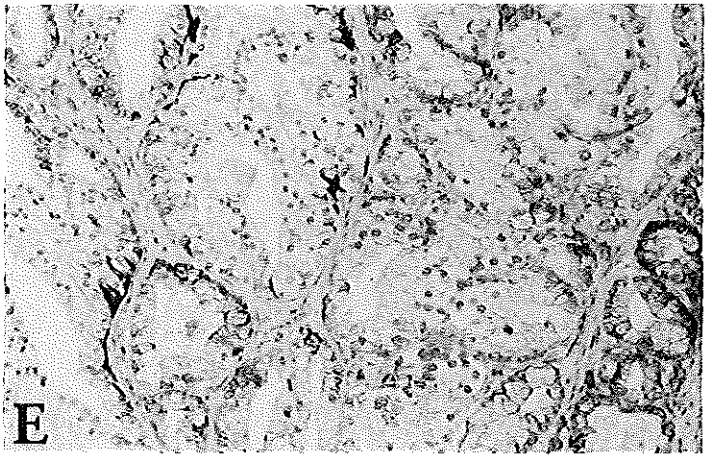
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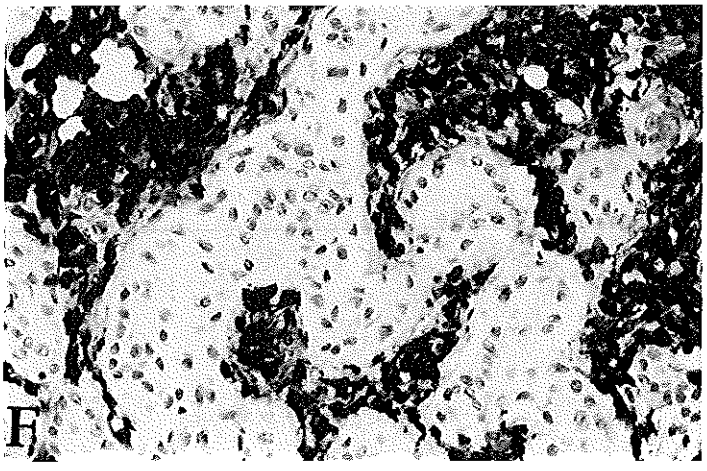
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FIGURE III.3
CD44 immunohistochemistry of prostatic cancer, counterstaining with hematoxylin. (A-C). Gleason growth pattern 3 adenocarcinoma with strong labeling of CD44s (A), CD44v5 (B) and CD44v6 (C) (x138). (D). Gleason growth pattern 3 tumor with reduced CD44s labeling (x138). (E). Gleason growth pattern 4 tumor with reduced CD44v6 labeling. Note basal localization of immunoreactive cells (x138). (F). Prostate cancer lymph node metastasis. Lymphocytes strongly immunoreactive with CD44s, metastatic tumor cells negative (x273).

TABLE III.2
Relationship between CD44 expression and Gleason growth patterns

		Gleason growth pattern					
marker	score	1	2	3	4	5	TOTAL
CD44s	<10%	–	2	9	20	14	45
	10-25%	–	1	14	14	7	36
	25-50%	1	5	17	24	2	49
	>50%	1	8	20	10	1	40
	p=0.041*						
CD44v5	<10%	–	11	53	58	22	142
	10-25%	–	5	4	4	–	13
	25-50%	–	–	1	5	–	6
	>50%	2	–	2	1	–	5
	p=0.41*						
CD44v6	<10%	–	4	26	34	18	82
	10-25%	–	2	15	15	3	35
	25-50%	1	2	11	10	3	27
	>50%	1	8	8	9	–	26
	p=0.25*						

*Sign-test

decreased intensity as compared to normal glands (Figure III.2). The luminal cell layer of PIN lesions showed reduced immunostaining of both CD44s and CD44v6. The scores of CD44s and CD44v6 were lower in high-grade PIN lesions than in low-grade PIN lesions ($p<0.05$, χ^2-test for trend). The patterns of CD44v5 labeling were comparable, but with a much lower intensity. In all areas the percentage of CD44v5

immunoreactive cells was smaller than that found with the other two antibodies ($p < 0.001$, sign-test). The lymphocytes that were present in most tissue sections showed strong immunoreactivity for CD44s. Occasionally, CD44v5 and CD44v6 immunoreactive lymphocytes were seen as well, especially in areas with prostatitis. Prostatic urothelium that was present in a number of tissue slides showed a strong immunolabeling of CD44s in all cell layers except for the luminal cell layer, and a comparable, but weaker labeling of CD44v5 and CD44v6. A more cytoplasmic staining pattern was present in seminal vesicles.

TABLE III.3

Relationship between CD44 expression and pT-stage

	CD44s		CD44v5		CD44v6	
score	pT_2	pT_{3-4}	pT_2	pT_{3-4}	pT_2	pT_{3-4}
<10%	1	28	18	68	9	49
10-25%	4	18	5	2	7	14
25-50%	9	19	1	2	4	9
>50%	11	7	1	–	5	–
	$p < 0.001$*		$p < 0.001$*		$p < 0.001$*	

*χ^2-test for trend

CD44 immunoreactive tumor cells were found in 83 adenocarcinomas (86%) for CD44s and in 22 (23%) and 67 (69%) for CD44v5 and CD44v6, respectively. Immunostaining was membranous and in most cases limited to the intercellular membranes (Figure III.3). This was identical to the pattern found in benign tissues. CD44v6 tumor scores were smaller than CD44s tumor scores ($p < 0.001$, sign-test). CD44v5 tumor scores were considerably smaller than CD44s and CD44v6 tumor scores ($p < 0.0001$, sign-test). The heterogeneity of immunostaining within one Gleason growth pattern was limited. The relationship between tumor CD44 scores, and primary Gleason growth patterns and Gleason sum scores is shown in Tables III.1 and III.2. The relationship between tumor CD44 scores and pT-stage is depicted in Table III.3. The expression of CD44 molecules was associated with favorable pathological factors such as a low Gleason sum score (Table III.1), pT_2-stage (Table III.3) and the absence of perineural invasion (data not shown). Twelve lymph nodes containing metastatic prostatic tumor cells were studied for the expression of CD44 molecules. Immunoreactive tumor cells could not be identified, whereas lymphocytes showed strong CD44s immunostaining and occasionally weak CD44v5 and CD44v6 immunostaining (Figure III.3).

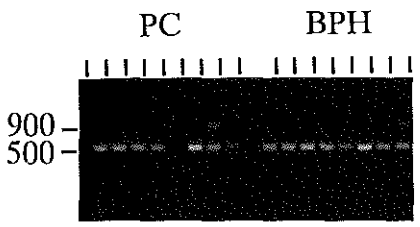

FIGURE III.4
Expression of CD44 mRNA in prostatic tissues (RT-PCR with primer set P1+ and P1-): lane 1 to 9 prostate cancer (PC), lane 10 to 17 BPH. Size indication in base pairs.

Figure III.4 shows the results of RT-PCR with a CD44 specific primer set (P1+ with P1-) of BPH and tumor mRNA. In 8 out of 9 tumors (89%) and in all 8 BPH samples a ≈500bp CD44 mRNA was found. In 4 of the tumors (44%) and 6 of the BPH samples (75%) an additional mRNA of ≈900bp was present. Sequence analysis showed that the ≈500bp fragment (actually 481bp) was identical to CD44s. RT-PCR with exon specific primers identified the ≈900bp fragment as CD44v8-10 (epithelial form, 877bp). In addition, all the samples that expressed CD44v8-10 also expressed CD44v6-10 (1138bp). The expression level of the latter mRNA was lower in all samples and was therefore probably not visible in Figure III.4.

TABLE III.4
Univariate analysis of prognostic markers

	outcome parameter					
	clinical progression		PSA progression		tumor specific survival	
variable	χ^2	p*	χ^2	p*	χ^2	p*
GSS	17.6	<0.0001	5.5	0.020	8.9	0.0029
pT-stage	18.7	<0.0001	9.0	0.0027	3.9	0.047
CD44s	19.6	<0.0001	9.6	0.0020	5.9	0.054
CD44v5	0.96	0.33	2.2	0.13	0.02	0.90
CD44v6	6.7	0.0097	2.2	0.14	2.8	0.094

*Logrank test for trend

PROGNOSTIC VALUE OF CD44 MOLECULES

For prognostic evaluation Gleason sum scores were grouped in 3 categories: 4-5, 6-7 and 8-10. pT-stage and pN-stage were grouped in 2 categories: pT_2 vs. pT_{3-4} and pN_0 vs. pN_{1-2}. The results of the univariate analysis of prognostic markers by means of a

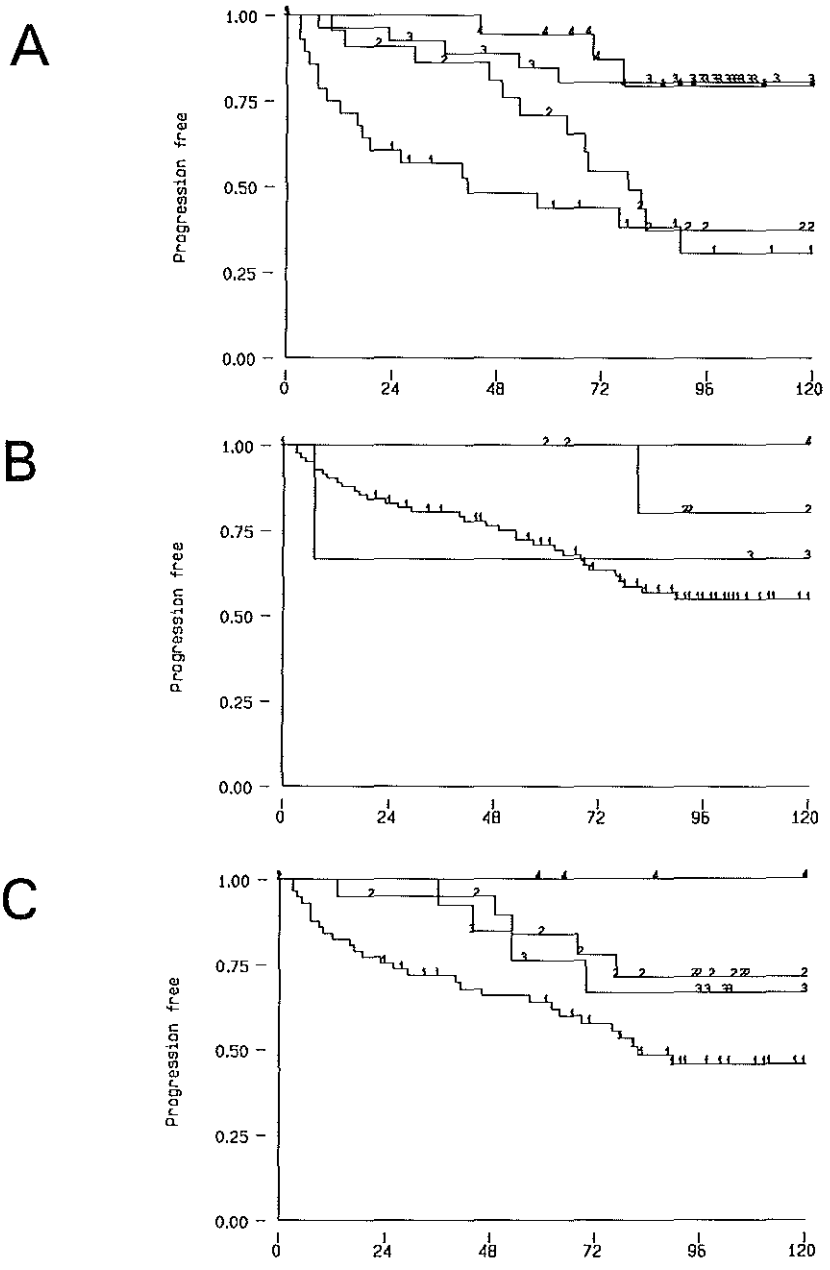

FIGURE III.5
Relationship between clinical progression and tumor scores of CD44s (A), CD44v5 (B) and CD44v6 (C), respectively. Kaplan-Meier curves, truncated at 120 months. Groups: 1 < 10%, 2 10-25%, 3 25-50%, 4 > 50%. Censored patients are indicated by a number along their line. Numbers of patients per group: (A). 1 N=29, 2 N=22, 3 N=28, 4 N=18; (B). 1 N=86, 2 N=7, 3 N=3, 4 N=1; (C). 1 N=58, 2 N=21, 3 N=13, 4 N=5.

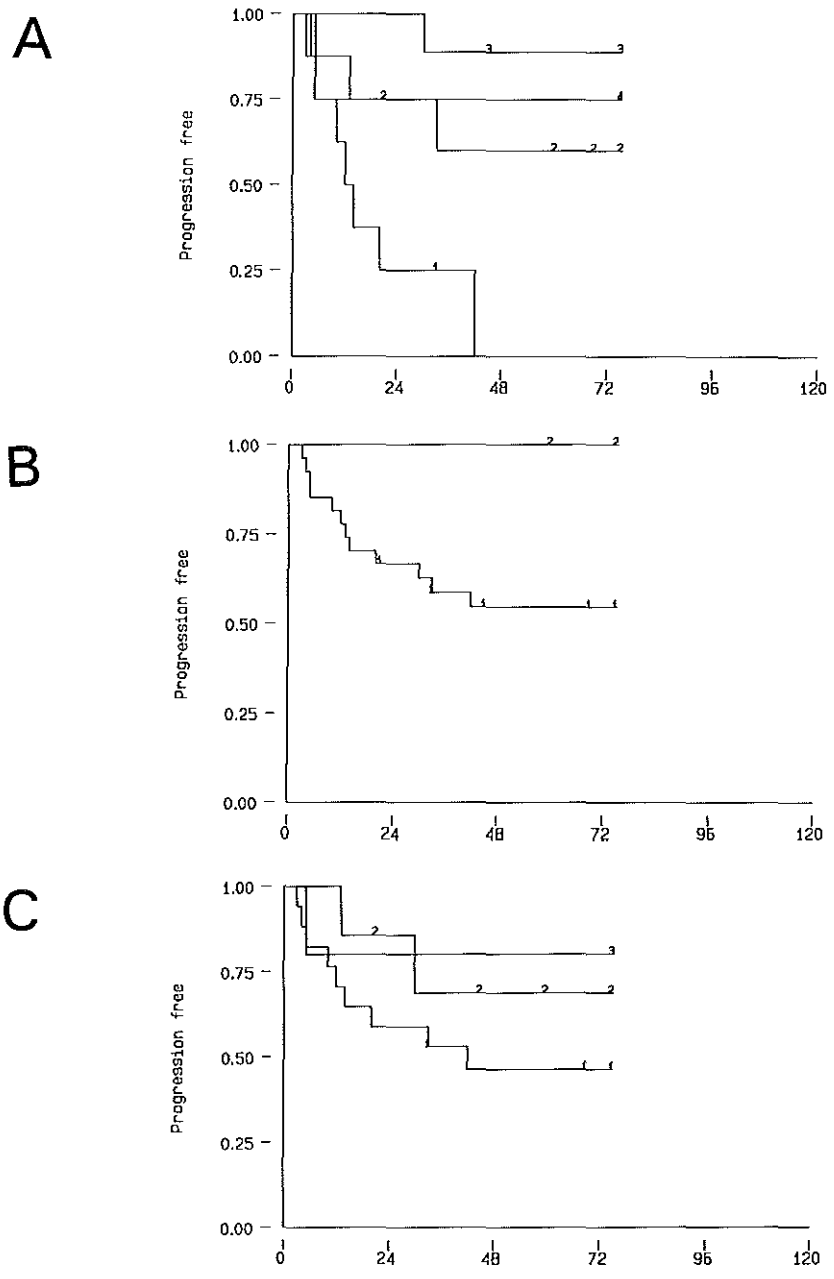

FIGURE III.6
Relationship between PSA progression and tumor scores of CD44s (A), CD44v5 (B) and CD44v6 (C), respectively. Kaplan-Meier curves, truncated at 75 months. Groups: 1 < 10%, 2 10-25%, 3 25-50%, 4 > 50%. Censored patients are indicated by a number along their line. Numbers of patients per group: (A). 1 N=8, 2 N=8, 3 N=9, 4 N=4; (B). 1 N=27, 2 N=2; (C). 1 N=17, 2 N=7, 3 N=5.

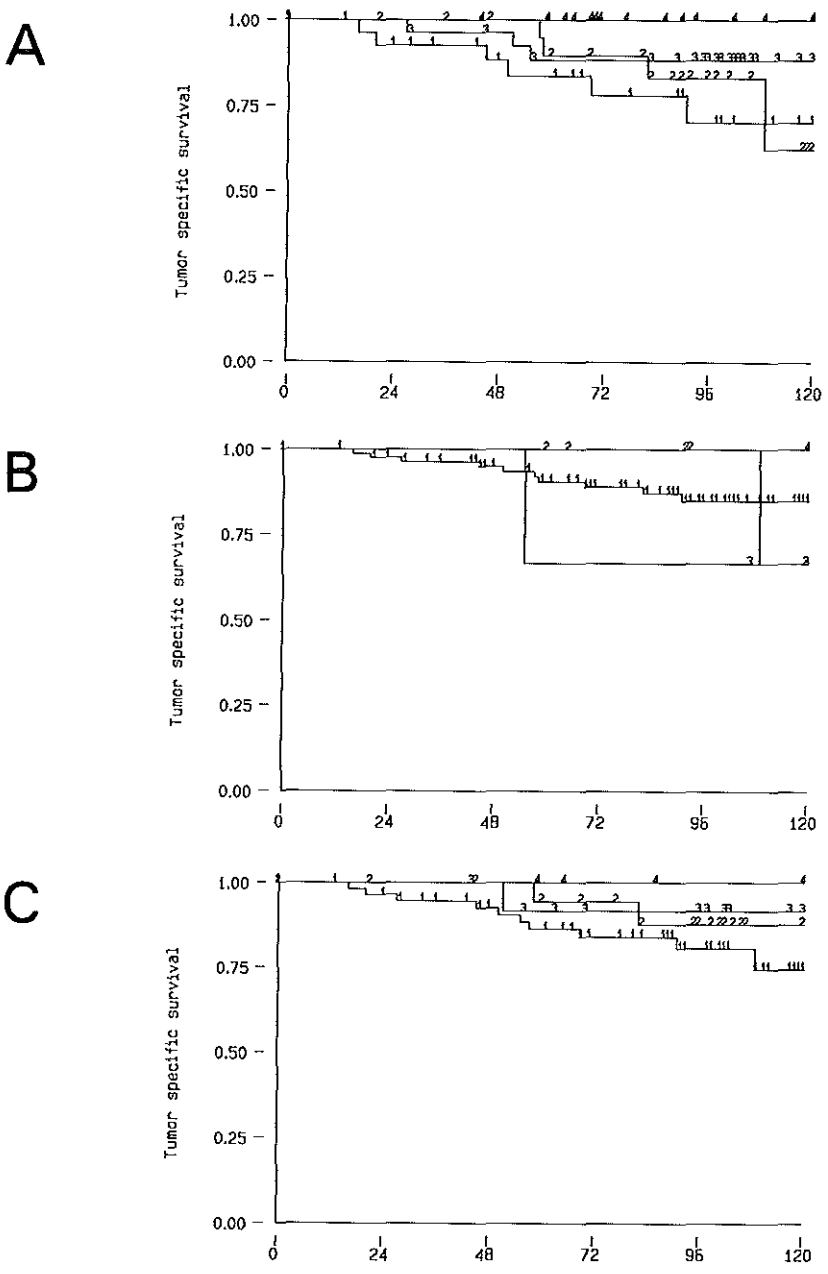

FIGURE III.7
Relationship between tumor related death and tumor scores of CD44s (A), CD44v5 (B) and CD44v6 (C), respectively. Kaplan-Meier curves, truncated at 120 months. Groups: 1 <10%, 2 10-25%, 3 25-50%, 4 >50%. Censored patients are indicated by a number along their line. Numbers of patients per group: (A). 1 N=29, 2 N=22, 3 N=28, 4 N=18; (B). 1 N=86, 2 N=7, 3 N=3, 4 N=1; (C). 1 N=58, 2 N=21, 3 N=13, 4 N=5.

logrank test for trend are shown in Table III.4. In addition to the well known prognostic value of the Gleason sum score and pT-stage, CD44s and, to a lesser extent, CD44v6 were also found to have prognostic value. For both CD44s and CD44v6 the prognosis was better if the percentage of immunoreactive cells was higher. pN-stage, age at operation ($p > 0.25$ in both cases) and CD44v5 expression (Table III.4) did not show prognostic value. The relationships between CD44 expression, and the several outcome parameters, are shown in the Kaplan-Meier curves of Figures III.5, III.6 and III.7. To investigate whether or not CD44 molecules also showed independent prognostic value, the variables were stepwise entered in Cox's proportional hazards model. Table III.5 shows the models that best fitted the data with all individual variables at $p < 0.05$. The expression of CD44s was of independent value in predicting clinical and PSA progression. In both cases the hazard ratio was <1, indicating a favorable prognosticator. The inclusion of CD44v6 expression did not improve the models. The Gleason sum score was the only independent variable to predict tumor specific survival. If only patients with pT_2 tumors were considered, identical results were found for the prediction of clinical progression and tumor related death (data not shown). Because CD44 expression has been shown to be associated with the metastatic process, the predictive value of CD44 was also investigated for the risk of local recurrence and metastasis separately. Both CD44s and CD44v6 expression showed a significant relationship, but at multivariate analysis only CD44s expression was of independent value, together with Gleason sum score.

TABLE III.5

Multivariate analysis of prognostic markers

outcome parameter	variable	hazard ratio	95% confidence interval	p
clincial progression	GSS	2.20	1.20 - 4.04	0.011
	pT-stage	4.89	1.11 - 21.5	0.036
	CD44s	0.674	0.467 - 0.974	0.036
PSA progression	GSS	2.90	1.09 - 7.73	0.042
	CD44s	0.381	0.179 - 0.974	0.012
tumor specific survival	GSS	4.43	1.55 - 12.7	0.006

Cox's proportional hazards model

DISCUSSION

A number of functions attributed to CD44 may play a role in tumor biology[293]. For this reason, studying the prognostic value of CD44 molecules in malignant processes is

warranted. The availability of a group of prostate cancer patients treated by radical prostatectomy and with long-term postoperative follow-up, allowed us to study the distribution of CD44 positive cells in benign and malignant prostatic tissues and to investigate the prognostic value of these cells in prostate cancer. It is important to recognize two factors that may hamper the interpretation of the results of the present study. First, the distributions of pT-stages and Gleason sum scores show a large proportion of locally extensive and/or poorly differentiated tumors. This can be explained by the fact that 30 (31%) of the patients were operated with a clinical T_3 tumor and that more than half of the clinical T_2 tumors were upstaged to pT_3 which is a well known fact[294]. The distributions of pT-stages and Gleason sum scores have changed over the past decade[10], which makes the patient sample from the present study difficult to compare with contemporary patients. Sub-group analysis of patients with pT_2 tumors, however, did not change the results significantly. Second, the PSA progression data are based on a small sample of patients and should therefore be interpreted with care.

For tissue markers whose presence indicates a poor prognosis the most intensely stained area of the tumor is generally used for grading or scoring based on the assumption that this part will determine the prognosis. It was initially thought that this also would apply to the expression of CD44 in prostate cancer. When the results were analyzed it became clear that the opposite was true. For this reason the tumor score was defined as the lowest score obtained in any area of a tumor (compare for example the expression of E-cadherin in prostate cancer[177]).

Immunohistochemistry of CD44s and CD44v6 in benign prostatic tissues was very much alike: intense immunolabeling was found in almost all basal cells and most luminal cells (Figure III.2). It was believed that variant CD44 molecules were expressed preferentially by malignant cells [255, 293], but several studies demonstrated high expression of these molecules in many normal organs among which the prostate[279, 280]. Staining of benign prostatic glands could be used as internal control for the immunohistochemical procedure. The staining pattern was membranous, and appeared to be limited to the intercellular membranes. This suggests that CD44 plays a role in the attachment of benign prostatic cells to each other rather than to the extracellular matrix.

PIN lesions showed reduced immunolabeling of CD44 (Figure III.3). In high-grade PIN CD44 scores were lower than in low-grade PIN suggesting that CD44 molecules are related to the maturation of prostatic cells. Since high-grade PIN is the putative precursor of prostate cancer, one would expect even lower CD44 scores in tumors. Indeed, if high-grade PIN and adenocarcinoma were present in one tissue slide, CD44s and CD44v6 scores tended to be lower in the tumor ($p < 0.01$, sign-test).

Most tumors expressed CD44s and CD44v6 (86% and 69%, respectively). In general tumor CD44 scores were inversely correlated with Gleason sum scores (Table III.1), Gleason growth patterns (Table III.2), pT-stage (Table III.3) and the presence of perineural invasion. Prostatic tumor cells metastatic to a lymph node did not show immnunolabeling of CD44 (Figure III.3). Although CD44v5 was present in only 23% of

the tumors, the patterns were comparable with CD44s and CD44v6. These results suggest that prostatic CD44 expression gradually decreases from benign, to pre-malignant, to low- grade tumor, to high-grade tumor and finally disappears in metastatic cancer.

CD44 was also detected at the mRNA level (Figure III.4), confirming that prostatic tissues indeed produce CD44 molecules. The percentage of tumors with CD44s (8/9, 89%) and CD44v6-10 (4/9, 44%) mRNA was comparable to the immunohistochemical findings. Since normal prostatic tissues express these molecules as well, it cannot be excluded that the mRNA was derived from contaminating benign cells.

The first experimental studies with prostate cancer cell lines showed expression of CD44s and splice variants, including v6 containing molecules at the mRNA and protein level[286, 295-297]. Lokeshwar *et al.*[295] showed that a neutralizing CD44 antibody inhibited cell proliferation and basement membrane invasion activity of PC-3 and TSU-Pr1 cells. Furthermore, it was shown that prostatic CD44 molecules interact with hyaluronic acid and the cytoskeleton[297]. These results indicate an active role of CD44 in the malignant behavior of prostate cancer. This is apparently at variance with the results from the present study. Since *in vitro* growing cell lines may have changed considerably during long-term culture, these experimental results may therefore not hold true for the tissue of origin.

CD44s and to a lesser extent CD44v6 were found to be of prognostic value in predicting clinical progression and PSA progression (Table III.4, Figures III.5, III.6 and III.7) with loss of expression being associated with an increased risk. As described above, CD44 expression was correlated with several pathological factors. This might explain part or all of the prognostic value of CD44. But, multivariate analysis showed that decreased expression of CD44s is an independent predictor for clinical progression and PSA-progression (Table III.5). Most studies on CD44 expression in clinical tumors have, however, found a positive correlation with pathological variables and an adverse prognosis[282-284, 289, 298-300]. In other studies such correlations were not found[301], or CD44 expression was even correlated with favorable prognostic markers or a better prognosis[285, 292, 302]. It appears therefore that the biological role of CD44 molecules is not identical in all organs and tumors. It could very well be that in tumors arising in epithelia that normally do not express CD44 (for example gastrointestinal epithelium[280]), acquired expression of CD44 is correlated with an adverse outcome[256, 282, 298], with CD44 acting as a growth and metastasis promoting molecule. Prostatic epithelium normally expresses CD44 and the presence of CD44 in prostatic tumors could be related to a less malignant tumor, with CD44 acting as an intercellular adhesion molecule that conserves tissue architecture and inhibits local spread and metastatic behavior.

In conclusion, CD44 immunohistochemistry can be performed reliably on formalin-fixed, paraffin embedded prostatic tissues with an internal control present, but requires an ultra-sensitive immunostaining protocol. CD44 molecules are expressed in normal prostatic epithelium as well as in prostatic adenocarcinoma. Loss of CD44s

expression is correlated with pathological variables and is an independent prognostic marker for clinical and PSA progression. Additional studies with more patients with locally confined tumors are needed. Intra-tumoral heterogeneity of CD44 expression is limited which makes it a candidate tumor marker applicable to biopsy specimens. Experimental studies on CD44 expression in prostate cancer have focussed on the adverse role of CD44; given the results of the present study, the opposite should be considered as well.

CHAPTER IV

THE PROGNOSTIC VALUE OF PRE-TREATMENT EXPRESSION OF ANDROGEN RECEPTOR AND BCL-2 IN HORMONALLY TREATED PROSTATE CANCER PATIENTS

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submitted for publication

ABSTRACT

Purpose. To determine the prognostic value of bcl-2 and androgen receptor (AR) expression in the pre-treatment trans-urethral resection (TUR) specimens of hormonally treated prostate cancer patients.
Material and methods. A total of 68 pre-treatment TUR specimens, 30 radical prostatectomy specimens and 21 palliative TUR specimens with androgen independent prostate cancer were stained with a monoclonal antibody against bcl-2. AR immunohistochemistry was performed on the pre-treatment TUR specimens only. The results were scored semiquantitatively and were correlated with tumor-stage and grade and with the occurrence of clinical progression or tumor related death.
Results. Bcl-2 expression by adenocarcinoma cells was found in 17%, 32% and 24% of the radical prostatectomy, pre-treatment TUR and palliative TUR specimens, respectively. The bcl-2 scores did not correlate with tumor-stage or grade. AR was expressed in 88% of the pre-treatment TUR specimens. AR scores were marginally related to tumor-grade, but not to tumor-stage. A prognostic value of bcl-2 or AR in the pre-treatment TUR specimens was not found. When a combined bcl-2/AR score was used, this parameter was an independent prognostic marker to predict clinical progression, together with Gleason grade and stage classification. Gleason grade was the only independent prognostic marker to predict tumor related death.
Conclusions. The expression of bcl-2 and AR in pre-treatment prostate cancer specimens is not related to the prognosis of hormonally treated prostate cancer. Bcl-2 expression is not increased in endocrine therapy resistant prostate cancer. Surprisingly, a combined bcl-2/AR score acts as an independent prognosticator for clinical progression.

INTRODUCTION

Endocrine treatment leads to tumor regression in about 40-50% of prostatic adenocarcinomas (complete and partial response), but with time almost all tumors will progress to an androgen independent state[29, 30]. Androgen action is mediated through the nuclear androgen receptor (AR), a ligand dependent transcription factor which is a member of the steroid receptor superfamily[303]. It was hypothesized that the expression level of AR protein in untreated prostate cancer would bear prognostic value for patients treated with hormonal therapy[207]. Several studies indeed showed some

prognostic value of AR expression in such patient groups[207, 212-214], although others failed to identify a relationship[209].

Androgen withdrawal leads to activation of the apoptotic cell death pathway in prostate cancer cells[219]. Bcl-2 is a molecule that inhibits the ability of cells to undergo apoptosis[222]. Bcl-2 is expressed in about 1 out of 4 primary prostate cancers[145, 152, 224, 226]. A higher figure of 60% was found in another study[225]. Increased expression was found in androgen independent prostate cancer[224, 225], implying that prostate cancers that express bcl-2 may be less responsive to endocrine treatment. The prognostic value of bcl-2 in hormonally treated prostate cancer patients has as yet not been reported. Because of the large variation in reported bcl-2 expression patterns in primary and endocrine therapy resistant prostatic tumors, we studied bcl-2 expression in radical prostatectomy specimens, trans-urethral resection (TUR) specimens obtained prior to endocrine treatment (pre-treatment TUR) and palliative TUR specimens containing endocrine therapy resistant prostate cancer. The prognostic value of bcl-2 and AR expression was investigated in the pre-treatment TUR specimens.

MATERIAL AND METHODS

patients

The pre-treatment TUR specimens of 68 hormonally treated patients obtained from 1987 to 1990 were used. In 12 of these (18%), prostate cancer was an incidental finding. Thirteen patients underwent a pelvic lymph node dissection and 10 of these contained metastatic prostate cancer. Metastatic disease was found in 34 patients (50%). The TNM 1992 stage-grouping classification was used. All but 5 of the patients received total androgen blockade by the anti-androgen flutamide combined with either bilateral orchiectomy or a gonadotropin agonist. Four patients were treated with a bilateral orchiectomy alone and one patient only received flutamide. The follow-up data of the patients was obtained retrospectively. The mean patient age was 76 (range 56-92). From 3 patients the clinical progression status was unknown and in 1 patient the cause of death could not be retrieved.

In addition to the pre-treatment TUR specimens, 30 radical prostatectomy specimens of patients with $T_{1-3}N_0M_0$ prostate cancer and palliative TUR specimens of 21 patients with endocrine therapy resistant prostate cancer were analyzed.

immunohistochemistry

All tissue specimens were routinely fixed in 4% buffered formalin at pH 7.4 and paraffin embedded. The Gleason grade of all tumors was assessed by one pathologist (THvdK). From all TUR specimens a tissue block containing sufficient tumor was selected. From each radical prostatectomy specimen 2 or 3 tissue blocks containing at least all the Gleason growth patterns observed in that tumor were selected.

Tissue sections were cut at 5μm, mounted on aminopropyl-trietoxy-silane (Sigma Chemical Co, St Louis, MO) coated glass slides and kept overnight at 60°C. After dewaxing in xylene, rehydration in a graded alcohol series and blocking of endogenous peroxidase activity with 10% H_2O_2 in methanol, the slides were placed in a 10 mmol/l citrate buffer at pH=6.0 and antigen retrieval was performed for 3x5 minutes in a microwave oven at 700W. The avidin-biotin complex method[291] was applied. In brief, following incubation with the primary antibody (anti bcl-2, clone 124, DAKO, Glostrup, Denmark, or anti AR, clone F39.4.1, Biogenex, Duiven, The Netherlands) the slides were subsequently incubated with biotinylated goat-anti mouse antibody (DAKO) and avidin-biotin complex (DAKO). All compounds were diluted in phosphate buffered saline (PBS). Antibody-antigen binding was visualized with diaminobenzidine/H_2O_2 (Fluka, Neu-Ulm, Germany). The nuclei were lightly counterstained and the slides were covered following dehydration. Negative controls were included by replacement of the primary antibody with PBS.

quantification

The different Gleason growth patterns were identified and a semiquantitative bcl-2 and AR score was assessed for all investigated tumors as follows: <5%; 5-25%; 25-50%; 50-75% or >75% of positive cells.

The STATA computer package was used for statistical analysis. To assess the relationship between the expression of bcl-2 and AR on the one side and tumor-stage and Gleason grade on the other side, Pearson's rank correlation was used. Differences between the patient groups were analyzed with Student's t-test. For the analysis of prognostic factors stage I tumors were excluded, because of an apparently much better prognosis (no clinical progression or tumor related deaths during the follow-up period). Kaplan-Meier curves were constructed and the logrank test or logrank test for trend was performed to investigate prognostic values at the univariate level. For multivariate analysis Cox's proportional hazards model was used.

TABLE IV.1

Tumor scores of bcl-2 and AR

score	bcl-2			AR
	radical prostatectomy	pre-treatment TUR	palliative TUR	pre-treatment TUR
<5%	25 (83%)	46 (68%)	16 (76%)	8 (12%)
5-25%	4 (13%)	6 (9%)	2 (10%)	3 (4%)
25-50%	1 (3%)	9 (13%)	2 (10%)	10 (15%)
50-75%	–	5 (7%)	–	14 (21%)
>75%	–	2 (3%)	1 (5%)	33 (49%)

data are presented as numbers, percentages between brackets

RESULTS

Immunoreactivity for bcl-2 was generally found in the basal cells of benign prostatic glands as well as in lymphocytes; one of these being present in almost all investigated tissue sections. This could be used as an internal positive control for the immunohistochemical procedure. Bcl-2 positive tumor cells were present in 5 of the radical prostatectomy specimens (17%), in 22 of the pre-treatment TUR specimens (32%) and in 5 of the palliative TUR specimens (24%). Overall, bcl-2 positive tumor cells (>5% positive per tumor) were found in 32 out of 119 (27%) investigated specimens. Table IV.1 shows the results in more detail. Bcl-2 scores were higher in the TUR samples (pre-treatment and palliative) compared to the radical prostatectomy specimens ($p<0.05$, t-test); there was no statistically significant difference between the pre-treatment and palliative TUR specimens.

The mean follow-up period of the patients that underwent TUR prior to endocrine treatment was 45 months (range 2-108). Eight patients (12%) were at stage I, 2 (3%), 10 (15%) and 48 (71%) were at stage II, III or IV, respectively. Clinical progression was observed in 32 patients (49%), 31 patients (46%) died of their prostate cancer, while 18 patients (26%) died of other causes. Only 16 tumors (24%) had a Gleason grade of less than 8. Gleason grade 8, 9 and 10 was found in 8 (12%), 21 (30%) and 25 (34%) tumors, respectively. Table IV.2 shows the lack of relationship between stage or Gleason grade and bcl-2 score in the pre-treatment TUR specimens. Figure IV.1 shows the Kaplan-Meier curves for the influence of bcl-2 scores on time to progression (Figure IV.1a) and time to tumor related death (Figure IV.1b). A prognostic value of bcl-2 scores was not found. Gleason grade ($p=0.0014$) and stage classification ($p=0.0091$)

were both prognostic markers for clinical progression at univariate analysis. Only Gleason grade was correlated with tumor related death (p=0.012)

TABLE IV.2
Relationship between Gleason grade, stage and bcl-2 scores

	Gleason grade							Stage classification			
score	4	5	6	7	8	9	10	I	II	III	IV
<5%	1	3	1	7	7	13	14	5	2	5	34
5-25%	–	–	1	–	1	1	3	–	–	2	4
25-50%	–	–	–	–	–	5	4	1	–	1	7
50-75%	–	–	1	1	–	1	2	1	–	2	2
>75%	–	–	–	1	–	1	–	1	–	–	1
Total	**1**	**3**	**3**	**9**	**8**	**21**	**23**	**8**	**2**	**10**	**48**
	p>0.05							p>0.05			

TABLE IV.3
Relationship between Gleason grade, stage and AR scores

	Gleason grade							Stage classification			
score	4	5	6	7	8	9	10	I	II	III	IV
<5%	–	–	–	1	–	3	4	2	–	–	6
5-25%	–	–	–	–	1	2	–	1	–	–	2
25-50%	–	–	1	3	2	4	–	1	–	2	7
50-75%	–	1	–	2	1	1	9	1	–	3	10
>75%	1	2	3	4	5	11	10	3	2	5	23
Total	**1**	**3**	**3**	**9**	**8**	**21**	**23**	**8**	**2**	**10**	**48**
	p>0.05							p>0.05			

AR expression was only investigated in the pre-treatment TUR specimens. Nuclear AR immunostaining was found in about 80-90% of the luminal epithelial cells in benign prostatic glands. These were present in most specimens and could be used as internal positive control. Half of the tumors showed nuclear AR immunoreactivity in over 75% of the tumor cells. The staining intensity in tumor cells varied significantly.

The overall AR scores are depicted in Table IV.1. Table IV.3 shows the relationships between Gleason grade, tumor stage and AR scores. A trend between AR expression and Gleason grade was noted, but did not reach statistical significance. The Kaplan-Meier curves of Figure IV.2 show the influence of AR scores on the time to clinical progression (Figure IV.2a) and tumor related death (Figure IV.2b); a prognostic value of AR was not found.

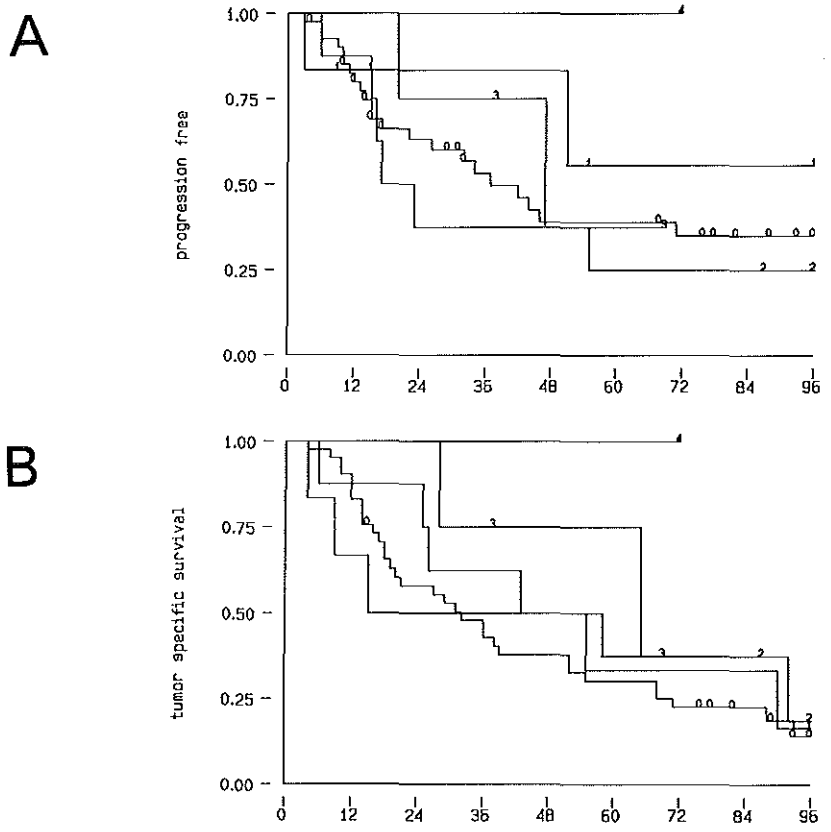

FIGURE IV.1
Prognostic value of bcl-2, Kaplan-Meier curves, truncated at 96 months. (A). Clinical progression. 0: <5% N=39; 1: 5-25% N=5; 2: 25-50% N=8; 3: 50-75% N=4; 4: >75% N=1; p=0.39, logrank test for trend. (B). Tumor related death. 0: <5% N=41; 1: 5-25% N=5; 2: 25-50% N=8; 3: 50-75% N=4; 4: >75% N=1; p=0.13, logrank test for trend.

Because bcl-2 expression is assumed to correlate with a worse prognosis, patients were divided in 2 groups: negative (<5%) vs. positive (>5%). In contrast, androgen receptor expression would be related to a good prognosis and for this reason, the patients were also divided in 2 groups: low (<75%) vs. high (>75%). Nevertheless,

division of the expression patterns in this way did not show a prognostic value of either bcl-2 or AR expression (logrank test). By combining the dichitomized bcl-2 and AR scores (bcl-2/AR score), 4 patient groups could be constructed. The Kaplan-Meier curve of Figure IV.3 shows the prognostic influence of bcl-2/AR score on the time to progression. Clearly two separate prognostic groups can be recognized: patients with low or high expression of both antigens (bcl-2 negative - AR low or bcl-2 positive - AR high, group 1 and 4) had a statistically significant better prognosis (p=0.016, logrank test) than the other patients (bcl-2 negative - AR high or bcl-2 positive - AR low, group 2 and 3). This remarkable difference was absent when tumor related death was considered (p=0.15, logrank test).

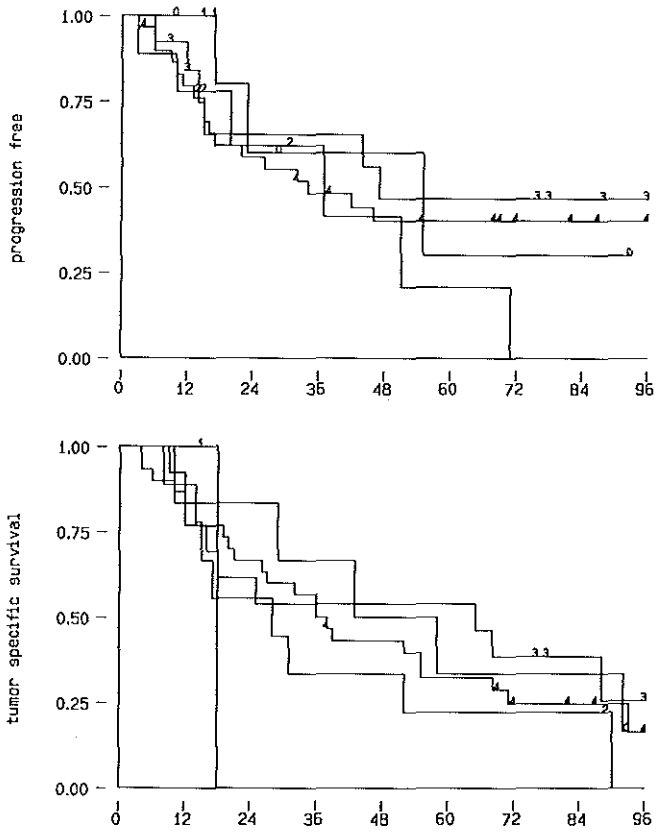

FIGURE IV.2
Prognostic value of AR, Kaplan-Meier curves, truncated at 96 months. (A). Clinical progression. 0: <5% N=6; 1: 5-25% N=2; 2: 25-50% N=9; 3: 50-75% N=12; 4: >75% N=28; p=0.67, logrank test for trend. (B). Tumor related death. 0: <5% N=6; 1: 5-25% N=2; 2: 25-50% N=9; 3: 50-75% N=12; 4: >75% N=30; p=1.00, logrank test for trend.

Multivariate analysis using Cox's proportional hazards model identified the combination of Gleason grade, stage classification and bcl-2/AR score as the model that best predicted clinical progression (Table IV.4). Only Gleason grade was an independent prognostic marker for tumor related death (Table IV.4).

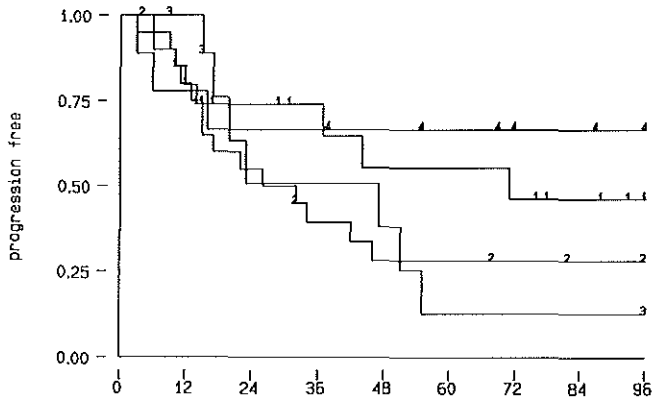

FIGURE IV.3
Relationship between bcl-2/AR score and progression free survival, Kaplan-Meier curves, truncated at 96 months. 1: bcl-2 <5% - AR <75%, N=20; 2: bcl-2 <5% - AR >75%, N=19; 3: bcl-2 >5% - AR <75%, N=9; 4: bcl-2 >5% - AR >75%, N=9; p=0.016, logrank test.

TABLE IV.4

Cox's proportional hazards model

outcome parameter	marker	hazard ratio	95% confidence interval	p
clinical progression	Gleason grade	1.5	1.1 - 2.0	0.006
	stage	3.5	1.1 - 11.2	0.035
	bcl-2/AR score	0.45	0.20 - 0.98	0.045
tumor related death	Gleason grade	1.3	1.0 - 1.6	0.020

DISCUSSION

Several authors have investigated prognostic tumor markers that will allow for the identification of those patients who will not benefit from endocrine treatment, for example, tumor DNA-ploidy[116] and expression of mutant p53[150]. Although bcl-2 expression in prostate cancer has been related to the androgen independent phenotype[224, 225], clinical studies on the prognostic value of hormonally treated patients have as yet not been published. The present study was performed to investigate the prognostic value of pre-treatment expression of bcl-2 and AR in the tumor specimens of such patients.

Bcl-2 expression was found in only a minority of untreated tumors (Table IV.1), a finding that is in line with most published series[152, 224, 226]. Bcl-2 scores were higher in the pre-treatment TUR specimens compared to the radical prostatectomy specimens, suggesting that the expression of bcl-2 is related to advanced disease[145, 225]. A relationship with Gleason grade or TNM stage classification could not be demonstrated in the pre-treatment TUR specimens, however (Table IV.2). The bcl-2 scores of androgen independent tumors (palliative TUR specimens) were not statistically significant different from the pre-treatment TUR specimens which is in contrast with the literature[224, 225]. McDonnell *et al.*[224] found bcl-2 expression more frequently in androgen independent tumors (10/13) compared to primary prostate cancers (6/19). Androgen independence was defined by these authors as a tumor that did not show an initial response on endocrine therapy. Moreover, 11 of these were derived from metastatic lesions and 5 were small cell carcinomas. Thus, the results are difficult to compare with those of the present study. Colombel *et al.*[225] applied the same antibody as used in the present study to frozen and paraffin-embedded prostate cancer sections. In their study bcl-2 expression was found in 62% of the primary tumors and in all hormonally treated tumors, which are extremely high figures compared to the literature and to those obtained in the present study[145, 152, 224, 226]. This is even more remarkable since the latter authors did not perform antigen retrieval on the paraffin-embedded sections, a procedure that is now recommended by the manufacturer and that was used in the present study. In the present study, the internal positive controls were positive in all cases and thus, we considered our results as valid.

Most nuclei (>80%) of the luminal cells in all benign prostatic glands displayed AR immunoreactivity. Likewise, most of the tumors in the pre-treatment TUR specimens expressed AR, as found by others as well[203, 209, 211-214]. In the present study a statistically non-significant trend between Gleason grade and AR scores was found (Table IV.3). Most studies found a weak correlation or no correlation at all between tumor-stage or grade and AR expression. In one study, AR expression was strongly correlated with Gleason grade[214]. The heterogeneity of AR immunostaining within tumors, is a consistent finding[203, 209, 211, 212, 214, 215].

Both markers tested in the present study showed no prognostic value. For bcl-2 it was expected that expression in pre-treatment TUR specimens would be correlated with a worse prognosis (Figure IV.1). Two studies with radical prostatectomy specimens demonstrated that bcl-2 expression was associated with an adverse prognosis at the univariate level[145] or multivariate level[152]. The prognosis of clinically localized prostate cancer is determined mainly by the presence of extensive disease at the time of operation, and thus, the results of the latter studies cannot simply be extended to the present study. In node positive breast cancer patients treated with adjuvant hormonal- or chemotherapy, bcl-2 expression was even correlated with a better prognosis[304]. These authors suggested that bcl-2 expression in tumors arising from epithelia with basal cell expression of bcl-2 (like breast and prostate epithelium), might predict a better prognosis due to a less aggressive mechanism of transformation. In addition, other mechanisms of androgen escape might be clinically more important. Actually, the results of the palliative TUR specimens support this. A recent experimental study with sublines of the Dunning R3327 rat prostate cancer model showed that bcl-2 expression is not an absolute requirement for androgen independent prostate cancer growth[305].

AR expression was also not correlated with patient outcome (Figure IV.2). Several studies did show such a prognostic value[212-214]. Others reported that not the percentage of positive cells but the degree of immunostaining heterogeneity[210] or the mean immunostaining intensity[211] would determine the prognosis. Both studies made use of computerized image analysis systems and in the latter study significant data could only be obtained when the results of two antibodies, against the C- and N-terminal parts of the AR, were combined. Possibly, analysis of the results from the present study in this way might reveal significant results as well. But, since evaluation of clinically suitable markers should be fast and simple, an easily applicable semiquantitative system was used.

Strikingly, a combined bcl-2/AR score was a prognostic marker for the prediction of clinical progression at the univariate and multivariate level (Figure IV.3, Table IV.4). Since endocrine treatment does not improve patient survival[306], the finding that only Gleason grade was an independent marker to predict tumor related death was not surprising. The dichitomized variables showed no individual prognostic value and if both were entered in Cox's proportional hazards model, also no independent prognostic value was found. Possibly, some interaction is present, because tumors that express bcl-2 (>5% of the tumor cells) had a favorable prognosis only if AR expression was high (>75%) and tumors without bcl-2 had a good prognosis only if AR expression was low (<75%). As yet a cell biological explanation for these findings is not apparent.

In conclusion, bcl-2 is expressed in a minority of prostate cancers, whether androgen dependent or independent and the expression level is not related to tumor-stage or grade. Bcl-2 and AR expression show no prognostic value in the pre-treatment specimens of hormonally treated prostate cancer patients. A combined bcl-2/AR score is of independent prognostic value to predict clinical progression, but not tumor related

death. Since this is the first study on the prognostic value of bcl-2 in hormonally treated prostate cancer, additional studies on bcl-2 and other related markers are needed.

CHAPTER V

NEUROENDOCRINE CELLS IN THE NORMAL, HYPERPLASTIC AND NEOPLASTIC PROSTATE

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ABSTRACT

Neuroendocrine cells can be demonstrated in normal, hyperplastic and neoplastic prostatic tissues. The products secreted by these cells can be used as tissue and/or serum markers but may also have biological effects. Neuroendocrine cells in prostate cancer most probably do not contain the androgen receptor and are therefore primarily androgen independent. Some of the neuropeptides secreted by the neuroendocrine cells may act as growth factor by activation of membrane receptors in an autocrine-paracrine fashion or by ligand independent activation of the androgen receptor in neighboring non-neuroendocrine cells. Evidence is accumulating from experiments with tumor models that neuropeptides indeed can influence the growth of prostatic tumor cells. Future research on neuroendocrine differentiation may answer some questions concerning the biological behavior of clinical prostatic tumors.

INTRODUCTION

In most developed countries prostate cancer has became the most prevalent tumor in men and in these countries its mortality is only being surpassed by lung cancer[4, 307]. A lot of research done in the field of prostate cancer is directed towards the identification of factors involved in tumor progression and the change from androgen dependency towards independency. Neuroendocrine differentiation is a possible factor which has received increasing attention during the last years[307].

Neuroendocrine (NE) cells are also known as APUD (amine precursor uptake and decarboxylation) cells or endocrine-paracrine cells[308]. The concept of NE/APUD cells was worked out by Pearse and co-workers from the diffuse endocrine epithelial organ concept of Feyrter[308, 309]. The APUD system consists of a group of apparently unrelated endocrine cells located in endocrine and non-endocrine tissues, which share a number of cytochemical, ultrastructural and functional characteristics[308]. In more detail, the endocrine/NE system consists of: the classical endocrine organs (e.g. adrenals, parathyroid gland), clusters of endocrine cells (e.g. pancreatic islets of Langerhans), dispersed epithelial endocrine cells (e.g. gastrointestinal NE cells), neurons and ganglia and the paraganglion system[310].

The clinical aspects of NE differentiation in prostatic carcinoma have been reviewed in more detail in a number of papers by Di Sant'Agnese[257, 311, 312]. This review

will therefore emphasize the experimental aspects: the role of NE cells in androgen insensitivity, tumor progression and tumor growth. Nevertheless, some clinical data will be provided to understand the concept and the questions that emerged from it.

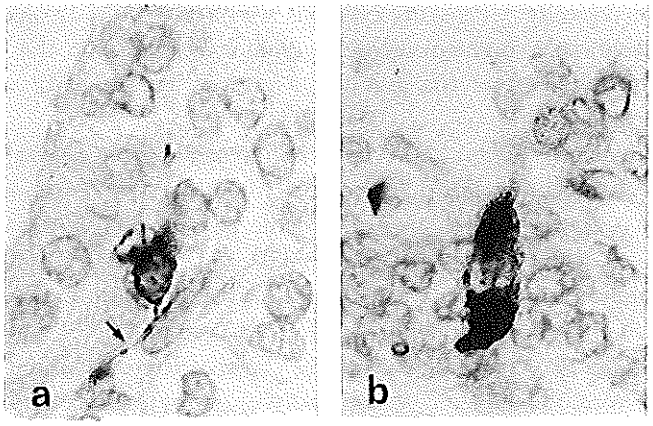

FIGURE V.1
Prostatic neuroendocrine cells, chromogranin A staining with immunoperoxidase technique, nuclear counterstaining with hematoxylin (900x). (A). Closed-type neuroendocrine cell with cytoplasmic process (arrow). (B). Open-type neuroendocrine cell.

NEUROENDOCRINE CELLS IN THE NORMAL PROSTATE

In normal prostatic epithelium, NE cells are found among the well known prostate specific antigen (PSA) producing exocrine cells and basal cells (Figure V.1)[313]. Such cells have been described for the first time by Pretl already in 1944 as the argentaffine basal cells[314]. In this study the argentaffine cells were identified by silver staining procedures as well as autofluorescence techniques[314]. Prostatic NE cells are located in the glandular and ductal epithelium and they form only a small part of the epithelial cell compartment. They clearly belong to the 'dispersed epithelial endocrine cell' type[310]. NE cells are found throughout the whole prostate although they are more abundant in the periurethral and ductal regions[274]. In the guinea pig prostate the number of NE cells increased with age[315], a phenomenon which was not described for humans[274]. NE cells in the glandular acini but not in the periurethral and ductal epithelium of the human prostate were found to disappear soon after birth and to reappear at puberty[274]. These

findings suggest a direct or indirect effect of circulating androgens on the acinar NE cells but not on periurethral and ductal NE cells.

Morphologically, two types of prostatic NE cells can be recognized: an open type reaching to the glandular lumen and a closed type separated by other cells from the lumen[260]. The latter type often has dendritic cytoplasmic processes interdigitating between neighboring cells[260]. Prostatic NE cells are ultrastructurally characterized by so-called dense core granules or neurosecretory granules[316]. Based on the variations in the ultrastructural morphology of the granules a greater heterogeneity of NE cells was suggested[316].

The heterogeneity of this cell type is also apparent from the variety of products (biogenic amines or peptide hormones) which they secrete. Chromogranin A and B (CgA and CgB), secretogranin II, neuron specific enolase (NSE) and serotonin (5-HT) are found in most, if not all prostatic NE cells[258, 260-262]. A small number of these cells also contains calcitonin (CT) and related peptides like calcitonin gene related peptide (CGRP) and katacalcin[317-321]. In addition, somatostatin (SMS)[321, 322], bombesin/gastrin related peptide (GRP)[317], midportion of the ß chain of thyroid stimulating hormone[323, 324], glycoprotein hormone α-chain[325] and parathyroid hormone related protein have been found[326].

The chromogranins are acidic glycoproteins which are widely expressed in NE cells[327]. Various biologically active peptides can be released from the CgA and CgB molecules by enzymatic action[327, 328]. A third chromogranin (chromogranin C) is now known as secretogranin II[327, 329]. Most of the immunohistochemical studies on NE differentiation have been performed with antibodies to CgA or 5-HT. NSE (γ-enolase, a subtype of the glycolytic enzyme enolase) was thought to be an exclusive marker of endocrine and NE cells, but it has been shown that NSE is secreted by a large variety of other cell types[330].

The heterogeneous morphology of the neurosecretory granules and the diversity of secreted products suggest that these cells exert a number of distinct functions, which are up to now virtually unknown. In parallel with functions of NE cells in more extensively studied systems (i.e. lung, pancreas and adrenals) a role of these cells in growth and differentiation, and in maintenance of homeostasis has been suggested[331-333]. Some of the neuropeptides share growth factor activity (reviewed in refs.[334-336]). Relatively high levels of CT, GRP/bombesin and SMS have been found in human semen[337-339]. Exposure to salmon-CT (sCT) decreased sperm motility *in vitro*[340]. Furthermore, sCT also increased the secretion of prostatic alkaline phosphatase by rat ventral prostate explants in a dose dependent manner[341]. These studies indicate that prostatic NE cells might also have some exocrine functions.

According to Feyrter's original concept of the diffuse endocrine organ, all NE cells throughout the body were thought to originate from the neural crest[342]. However, nowadays a local origin of the NE cells in most tissues is suggested and has been proven, for example, in colorectal epithelium[342]. A multidirectional differentiation has been postulated for normal tissues as well as for tumors arising in these tissues

(reviewed by DeLellis[343]). In a recent immunohistochemical study, some prostatic NE cells expressed basal cell specific cytokeratins and a few NE cells in hyperplastic glands displayed immunoreactivity for PSA[258, 344]. These observations suggest a common differentiation pathway of prostatic secretory, NE and basal cells and it is now generally accepted that prostatic NE cells indeed originate from the prostate. It may be hypothesized that the basal cell layer contains the prostatic stem cells and that these cells give rise to both the exocrine and NE phenotypes of the glandular epithelium.

NEUROENDOCRINE CELLS IN THE HYPERPLASTIC PROSTATE

The presence of NE cells in hyperplastic prostatic tissue has been demonstrated using silver staining techniques[345, 346]. In one study, up to 16 out of 20 hyperplastic nodules displayed NE differentiation[345]. However, in an other study it was found that hyperplastic nodules in general contained less argentaffin NE cells compared to the adjacent normal epithelium[346]. This was confirmed in a recent study using immunohistochemistry with a 5-HT antibody and chromatographic quantification of the 5-HT content of tissue homogenates[275]. On the other hand, Abrahamsson *et al.* found more NE cells in hyperplastic prostatic tissue than in normal glands as defined by immunoreactivity for 5-HT, thyroid stimulating hormone and CT[262]. Aprikian *et al.* found immunohistochemically defined NE cells in all investigated hyperplastic specimens, although the relation with the adjacent normal glands was not studied[258]. Whether or not neuroendocrine cells play a role in the pathogenesis of benign prostatic hyperplasia is not clear at the moment. The study of Cockett *et al.* gives some support to this idea since they found more NE cells in small hyperplastic nodules compared to normal prostate or large hyperplastic nodules[275]. This suggests that the growth of the more immature small hyperplastic nodules is stimulated by NE products. Alternatively, the presence of NE cells may simply reflect an enhanced proliferative activity of glandular epithelial cells.

NEUROENDOCRINE CELLS IN THE NEOPLASTIC PROSTATE

NE cells can be identified in prostate cancers (Figure V.2), although the percentage of tumors with NE cells varies in the literature from about 10% to almost 100%. This variation partially reflects the development of techniques used to identify NE cells. Two papers on the argentaffine cells in the hyperplastic and neoplastic prostate were published in the seventies[345, 346], but only recently, NE cells in prostate cancer have gained increasing attention.

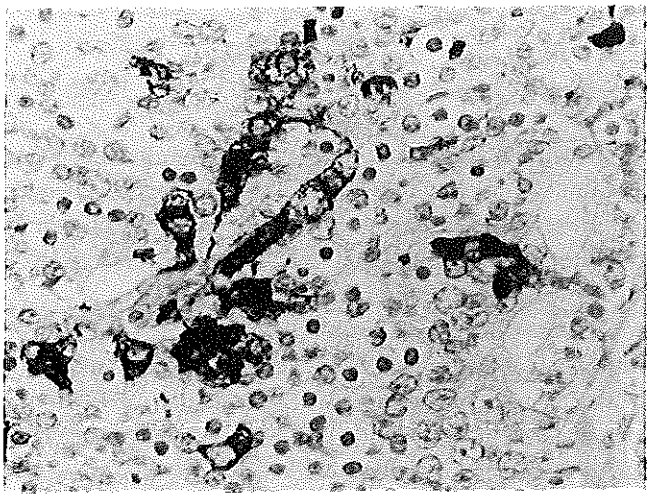

FIGURE V.2
Gleason growth pattern 4 prostatic adenocarcinoma with neuroendocrine cells. Chromogranin A staining with immunoperoxidase technique, nuclear counterstaining with hematoxylin (x175).

According to Di Sant'Agnese, the term NE differentiation in prostate cancer includes the pathological categories: small cell prostate cancer (SCPC), prostatic carcinoid, adenocarcinoma with scattered NE cells and mixed tumors of these three types[257]. A relatively small proportion of prostatic adenocarcinomas with NE differentiation contains NE cells with large eosinophilic granules. This phenomenon was termed Paneth cell like change[347]. Very recently it has been suggested to rename these cells 'NE cells with large eosinophilic granules'[347, 348]. Obviously, the tumors with this type of NE cells also fit within the category of prostate cancers with NE differentiation. From a clinical point of view, prostate cancers with evidence of eutopic or ectopic production of neuroendocrine hormones and/or markers and prostatic

malignancies associated with paraneoplastic syndromes linked to NE differentiation should also be included in the definition[257].

Undifferentiated small cell cancers, also referred to as NE carcinomas, occur in various organs, but most often in the lungs. SCPC is a relatively rare disease accounting for ± 1-5% of all prostate cancers, and has been described in a few larger studies[349-352]. It is a highly malignant disease with a mean survival time of 7-17 months[350, 351]. In about half of the patients the small cell component is preceded by a common adenocarcinoma suggesting that at least in a number of patients the small cell carcinoma may arise in a common adenocarcinoma. In line with this suggestion, a number of mixed tumors was found[350, 351]. Not all SCPC showed NE differentiation, however[349]. Prostatic carcinoid is a very rare entity. Only some case reports have been published as yet[353-358]. Carcinoids occur especially in the digestive system (appendix) and they are in general relatively benign[359]. It appears that prostatic carcinoid tumors behave more aggressively[257, 357], although detailed studies with follow-up data are lacking. Mixed carcinoid-adenocarcinoma tumors have been found and it has been suggested that carcinoid formation in a hormonally treated adenocarcinoma might be a selective effect of the treatment[357].

The most common pattern of NE differentiation in prostate cancer is a prostatic adenocarcinoma with scattered NE cells. The number of NE cells within an adenocarcinoma varies from patient to patient and their presence may have prognostic significance[263, 264, 266-268]. Some authors found a relation between the tumor grade and the number of NE cells[264, 323], but others did not[258]. NE cells were identified in about 50% of lymph node and bone metastases of prostatic adenocarcinomas[269]. This proves that NE cells are an intrinsic component of the adenocarcinoma and are not derived from preexistent benign glands. The presence of NE cells in metastatic lesions had no prognostic value[269]. Serum levels of CgA showed a 60% correlation with the immunohistochemical staining of this protein and the presence of metastatic disease was better predicted by serum CgA levels than by tissue immunoreactivity[360]. The possible correlation between tumor grade and the number of NE cells in a tumor may well account for part or all of the reported prognostic value of NE differentiation, which would indicate that the number of NE cells just reflects the level of dedifferentiation of a tumor. This possibility can be investigated with long term clinical follow-up studies using multiple regression analysis.

SECRETION PRODUCTS AND HORMONE SENSITIVITY

NE cells in prostatic tumors are most often recognized by their immunoreactivity for NE markers (CgA, NSE) or eutopic bioactive peptides (5-HT, CT, SMS and others). In addition to the eutopic peptide products, a number of ectopic peptides have been found, for example, adrenocorticotropic hormone (ACTH), leu-enkephalin and ß-endorphin[323]. Expression of these factors, especially ACTH, might cause a paraneoplastic syndrome as occasionally found in prostate cancer patients (reviewed by Matzkin and Braf[361]). In high grade tumors with marked NE differentiation CgB replaced CgA expression by NE cells[261]. This implicates that in immunohistochemical studies CgA as well as CgB immunoreactivity has to be assessed.

FIGURE V.3
Androgen receptor (AR) and chromogranin A (CgA) double labeling in a benign prostatic gland. Nuclear AR staining with immuno-fluorescence technique (FITC, originally green). CgA staining with alkaline phosphatase technique (originally red fluorescence signal). Most epithelial cells display a strong AR positivity (arrow-heads). The triangular-shaped closed-type neuroendocrine cell (arrow) is AR negative, (x250, see also Figures VI.4 b and d). Reprinted from Krijnen *et al.*, Histochemistry 1993;100:393-8, with permission of the publisher.

Kadmon *et al.* demonstrated elevated CgA serum levels in 12 out of 25 patients with metastatic, hormone insensitive prostate cancer[362]. Moreover, in 4 of these 12 patients PSA levels were in the normal range. Serum levels of NSE were found to be increased more often in patients with hormone refractory tumors (10/46) than in patients with hormone sensitive tumors (2/89)[363]. Abrahamsson *et al.* studied NE differentiation in repeated biopsy specimens of patients treated with hormonal therapy or with radiotherapy[264]. They found mostly an increasing number of NE cells during

follow-up paralleled by dedifferentiation and hormonal escape (i.e. progression) of the tumor. These results raise the question whether NE cells in prostate tumors are androgen sensitive or not. NE cells in benign and malignant prostatic tissues contained rarely, if at all androgen receptor immunoreactivity (Figure V.3)[276, 364]. In an other study, using a different antibody for the androgen receptor, prostatic NE cells generally expressed the androgen receptor[365]. Very recently the authors of the latter study confirmed the findings of the former two studies[348], leading to the conclusion that NE cells in the normal or neoplastic prostate do not contain the androgen receptor. It has been shown that the androgen receptor content of a prostatic tumor does not predict androgen (in)sensitivity[203, 204]. It is, however, unlikely that androgen receptor negative tumor cells will respond to androgen withdrawal. Therefore, NE cells in prostate cancer most probably form a primarily androgen insensitive tumor cell population.

GROWTH MODULATION BY NEOPLASTIC NEUROENDOCRINE CELLS

A number of peptides produced by NE prostatic tumor cells (5-HT, GRP/bombesin, CT, SMS) exhibit growth factor activities mediated by a membrane receptor. This may represent a way of paracrine or autocrine growth modulation[332, 335, 336]. Most of the prostatic adenocarcinoma cells surrounding NE cells contain the androgen receptor, even in androgen refractory carcinomas[276, 364]. It was found in COS cells transfected with steroid hormone receptors that DOPA activated several steroid hormone receptors (progesteron, estrogen, vitamin D and thyroid hormone-ß receptors) in a ligand-independent fashion[277]. In this way NE tumor cells might influence the growth of neighboring non NE tumor cells by androgen independent activation of the androgen receptor in a paracrine manner. This possibility warrants further investigation. It should be stressed that neuropeptides may enhance or inhibit growth, depending on the specific nature of the neuropeptide and properties of the target cell. Bonkhoff *et al.* found in an immunohistochemical study that proliferating cells in normal, hyperplastic and cancerous prostatic tissues were usually, but not necessarily located in proximity of clusters of NE cells[366]. This supports the concept of paracrine growth regulation by prostatic NE cells.

The following hypothesis can be postulated: NE cells in a prostatic adenocarcinoma form a subset of primarily androgen independent tumor cells which modulate the growth of neighboring non-NE tumor cells by the secretion of neuropeptides in a paracrine manner (Figure V.4). Granted this hypothesis, one would expect that following androgen withdrawal the proportion of NE cells will increase.

Abrahamsson *et al.* described in a group of 24 hormonally treated patients that the number of NE cells increased in time, although this was paralleled by dedifferentiation of the tumors (see above)[264]. Aprikian *et al.* were not able to confirm the observed increase in NE differentiation following short or long term hormonal therapy, however[258]. Larger follow-up studies on long-term androgen depleted tumors with assessments of absolute and relative numbers of NE cells should confirm the occurrence of this phenomenon. Even if the NE cell population does not increase following androgen withdrawal, the secreted neuropeptides still may stimulate the surrounding non-NE cells by acting as 'androgen substitutes'. The secreted neuropeptides may also have effects on stromal cells (epithelial-stromal interactions), a possibility which has not been studied as yet.

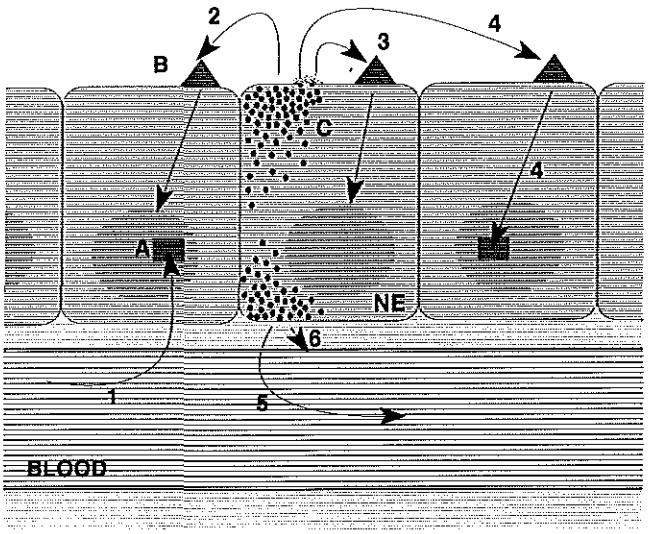

FIGURE V.4
Influence of a neuroendocrine (NE) cell on neighbouring non-NE prostatic tumor cells (working hypothesis). (1). Actions of androgen mediated by the nuclear androgen receptor (A). (2-4). Action of neuropeptides released from the neurosecretory vesicles (C) of the cell. (2). Paracrine stimulation of neighbouring non-NE cell by activation of neuropeptide receptor. (3). Autocrine stimulation. (4). Ligand-independent activation of the androgen receptor in non-NE cell. (5). Systemic effect of neuropeptides secreted into the blood (paraneoplastic symptoms). (6). Stromal effects of neuropeptides.

SPECIFIC NEUROPEPTIDES

SEROTONIN

Serotonin (5-HT) is well known as neurotransmitter and vasoactive peptide and some recent reports indicate that 5-HT also has growth factor activity[367, 368]. 5-HT stimulated DNA synthesis in hamster fibroblasts proved to be mediated by activation of the 5-HT_{1b} receptor[367]. This receptor is only expressed in rodents. Further experiments indicated that in humans 5-HT mediates the proliferation of smooth muscle cells mediated through activation of the 5-HT_{1d} receptor[368]. 5-HT might influence tumor growth indirectly by changing the local blood flow in a tumor due to its vasoactive action. It is very likely that 5-HT plays a role in the prostate since it is expressed by most if not all prostatic NE cells[258, 260, 262]. Unfortunately, it is as yet not known if human prostatic tumor cells contain 5-HT receptors and if so, which subtypes. As mentioned above, 5-HT may also exert its effect by ligand independent activation of the androgen receptor[277].

BOMBESIN/GASTRIN RELATED PEPTIDE

Gastrin related peptide (GRP) is the mammalian analogue of the amphibian peptide bombesin[369]. GRP stimulated the growth of cultured normal bronchial epithelial cells in a dose dependent manner[332]. *In vitro* studies with cultured pulmonary NE cells demonstrated that treatment with GRP increased the number of NE cells and stimulated their 5-HT expression while treatment with 5-HT did not[333]. It has also been shown that GRP stimulated growth of small cell lung cancer cells[370]. This effect could be blocked *in vivo* by an antibody against GRP and *in vitro* by GRP analogues which prevent binding of GRP to its receptor[370, 371]. *In vitro* studies with the androgen independent prostatic cancer cell line PC-3 similarly showed a growth stimulatory action of GRP which could be blocked by an anti-GRP antibody[372]. Saturable GRP binding sites were demonstrated on PC-3 cells, but no immunoreactivity for GRP was demonstrated in these cells, excluding an autocrine action of GRP[372]. The GRP antagonist RC-3095 was able to inhibit the growth of the androgen dependent human xenograft PC-82, the Dunning R-3327H rat prostate tumor and the androgen independent the DU-145 and PC-3 prostatic *in vitro* cell lines[373-376]. Saturable GRP binding sites were demonstrated on cells of all these models . Altogether, evidence exists that GRP has a potential role in the growth of (neoplastic) prostatic tissue.

CALCITONIN

The human (hCT) and salmon (sCT) subtypes of CT can be demonstrated in a subset of normal and neoplastic prostatic cells[258, 317-321, 323, 377]. In conditioned medium of cultures of prostate cancer cells immunoreactive CT was found in 4-fold higher concentration as compared to cultures of BPH cells[319]. *In vivo* administration of sCT to rats induced ornithine decarboxylase (a key enzyme associated with cell cycle progression and growth) in a number of organs[378]. It should be realized that CT also inhibits the pituitary secretion of luteinizing hormone which is an important hormone in the mediation of androgen secretion[379]. In addition, the secretion of prolactin, which influences the action of androgens on the prostate, was also found to be decreased by CT[380]. Therefore, a pituitary mediated growth inhibiting effect of CT on prostatic cancer growth is also expected. The direct growth modulating effects of CT have been studied in a few tumor systems. T-47D breast cancer cells contained high affinity receptors for CT and *in vitro* growth of these cells was dose dependently inhibited by CT[381, 382]. Comparable results were found in cells of the human gastric carcinoma cell line KATO III[383]. Following CT administration intracellular cAMP levels increased[381-383]. This increase was also found in four out of six renal adenocarcinoma cell lines and only these four cell lines were growth inhibited by CT administration[384]. In a panel of 13 small cell lung cancer cell lines two contained CT and only one was able to bind CT while no growth effect upon CT administration was found[385]. It has recently been shown that sCT dose dependently increased the cAMP concentration and the DNA synthesis in cells of the *in vitro* human prostatic cancer cell line LNCaP[386]. This growth stimulatory effect of CT is at variance with the results from other non prostatic tumor model systems[381-384]. Therefore, the effects of CT have to be confirmed in additional studies using other prostatic tumor models including *in vivo* models because CT also has systemic effects which may influence prostatic tumor growth.

SOMATOSTATIN

Abrahamsson *et al.* found somatostatin (SMS) immunoreactivity in 12 out of 40 prostatic adenocarcinomas[323], a result that has not been confirmed in by Aprikian *et al.*[258]. SMS receptors were found neither in 17 prostatic carcinomas nor in 2 BPH specimens[387]. On the other hand, binding sites for several SMS analogues were demonstrated in normal and neoplastic prostatic tissues[388]. A number of experimental studies concerning the growth modulating effects of SMS in prostate cancer have been published. Several SMS analogues (sandostatin, somatuline, RC-160, RC-121) decreased without exception the growth of prostatic tumor models *in vivo* (Dunning R-3327 and R-3327H, PC-82, DU-145, PC-3) and *in vitro* (LNCaP) [373, 374, 376, 389-395]. In the Dunning R-3327H rat tumor the SMS effect potentiated the castration induced growth inhibition even when the tumors became androgen independent[390], however, this was not

confirmed by others[389]. In experiments with heterotransplants of the androgen independent PC-3 cell line, SMS was able to inhibit growth only if the tumors were small (10 mm^3)[376]. Combination treatment of tumor models with an SMS analog and luteinizing hormone-releasing hormone (LH-RH) analog (D-TRP^6 LH-RH) resulted in a stronger growth inhibition than treatment with only one of the components[373, 391, 394]. SMS binding sites were demonstrated on cells of the PC-82[373], Du-145[374], PC-3[376] and Dunning R-3327H[391] models. SMS and prolactin binding sites were found to be down regulated in Dunning R-3327H tumors treated with analogues of SMS and LH-RH[391]. As noted above, prolactin may have a stimulating effect on prostate cancer and the down regulation of the prolactin receptor by SMS might partially explain the growth inhibiting effects of SMS. All these results strongly suggest a direct growth inhibiting effect of SMS (analogues) in prostate cancer.

The effects of some of the neuropeptides on prostate cancer growth have been investigated quite extensively. The relation with androgen levels and androgen receptor activity, the action of a combination of neuropeptides and the mechanisms of action are poorly understood at the moment. Furthermore, it is not clear whether the amounts of neuropeptides secreted by the NE cells are sufficient for biological activity on neighboring cells. For some of the neuropeptides, the presence of the corresponding receptor is not clear. Possibly other, as yet unknown, factors produced by prostatic NE cells may even be more important.

NEUROENDOCRINE DIFFERENTIATION IN PROSTATIC TUMOR MODELS

To enhance our knowledge of NE differentiation in prostate cancer, experimental models with NE cells are urgently needed. A heterotransplantable model of a small cell prostatic carcinoma has been established[396-398]. A SCPC is biologically different from an adenocarcinoma and therefore, this model is probably not useful for the study of the paracrine role of NE cells in prostatic adenocarcinoma. None of the available prostatic tumor models contains NE cells as defined by immunoreactivity for CgA, 5-HT, CT, SMS, NSE and thyroid stimulating hormone, although two heterotransplantable human tumor models which were recently established in this laboratory contain CgA positive cells (unpublished observation).

Possibly, NE differentiation can be induced in non-NE prostatic tumor models. Transfection of v-ras^H into DMS-53 small cell lung cancer cell line cells resulted in a cell

line with increased NE features[399]. Transfection of both c-raf-1 and c-myc oncogenes into SV-40 immortalized bronchial epithelial cells resulted in the generation of heterotransplantable large cell carcinoma cell lines with a NE phenotype[400, 401]. These studies indicate that NE differentiation can be induced and that it is associated with the expression of certain (proto) oncogenes. Future experiments have to show if a similar approach can be applied to prostate cancer.

CONCLUSION

Knowledge about the function of NE cells in the human prostate and prostate cancer is limited. Evidence is however accumulating that NE cells and tumors with NE cells are related to the androgen independent and poorly differentiated types of prostate cancer. However, at the moment it cannot be excluded that NE differentiation is only an epiphenomenon associated with dedifferentiation of a tumor. There is evidence that the secretion products of prostatic NE cells affect prostate cancer growth and possibly also affect tumor differentiation. Research directed towards identifying the role of NE cells in prostate cancer is likely to contribute to the understanding of the transition of androgen dependent to androgen independent prostate cancer.

CHAPTER VI

NEUROENDOCRINE DIFFERENTIATION IN HUMAN PROSTATIC TUMOR MODELS

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ABSTRACT

Neuroendocrine (NE) cells can be identified in benign and malignant prostatic epithelia. Factors regulating their presence and their functions are poorly understood, mainly due to a lack of suitable experimental models. A total of 15 *in vitro* and *in vivo* prostatic cancer tumor models, including a number of newly established *in vivo* models, were studied immunohistochemically for the presence of NE cells under different hormonal conditions.

None of the *in vitro* models (PC-3, DU 145, LNCaP, TSU) contained NE cells. Five of the 7 xenograft models established at this laboratory contained NE cells. In 3 of these, NE cells were found only in the initial mouse passages. In the other 2 (PC-295 and PC-310), the NE phenotype was stable. NE features were confirmed by transmission electron microscopy and by Western analysis of chromogranin A expression. Immunohistochemical double labeling experiments confirmed that NE cells in prostate cancer are post-mitotic (no Ki-67 expression) and do not express the androgen receptor. In the PC-295 and PC-310 models, short-term androgen withdrawal resulted in a rapidly increased number of NE cells. A time-course experiment with PC-295 bearing mice strongly suggests that this increase occurred by induction of NE differentiation rather than by rapid proliferation and subsequent differentiation or selective persistence. In conclusion, these models are suitable to resolve fundamental questions with regard to the presence and functions of NE cells in human prostate cancer.

INTRODUCTION

Neuroendocrine (NE) or endocrine-paracrine cells are characterized by a number of structural, cytochemical and functional properties[308]. The dispersed NE system consists of single or clustered NE cells located in the epithelium of several non-NE organs (lungs, gastro-intestinal tract)[310]. The normal prostate likewise contains epithelial NE cells belonging to this system. Prostatic NE cells secrete neuropeptides and biogenic amines (reviewed by Noordzij *et al.*[259]). In concordance with the functions of NE cells in other epithelial organs, it has been suggested, but not proven, that prostatic NE cells play a role in the maintenance of homeostasis and/or regulation of secretion of

prostatic glandular epithelium[331]. In addition, there is some evidence that these cells have exocrine functions as well[337, 402].

NE cells are present in the majority of prostate cancers[403]. It is of importance to distinguish three types of NE differentiation within prostate cancer. Small cell prostate cancer and prostatic carcinoid are relatively rare and are considered pure neuroendocrine tumors with a poor prognosis[257]. Conventional adenocarcinoma with scattered or clustered NE cells represents the most common type of prostate cancer with NE differentiation. The NE cells in both benign and malignant prostatic epithelium can be identified immunohistochemically with antibodies against products secreted by these cells or by routine transmission electron microscopy which visualizes the neurosecretory granules (also known as dense core granules or neuroendocrine granules)[262, 316, 404]. Chromogranin A (CgA) is a 68kDa acidic glycoprotein which is secreted by most, if not all, NE cells. It may play a role in the excretory pathway of neuropeptides and is most likely a precursor of several functional peptides (e.g., pancreastatin and chromostatin) which are formed by proteolytic cleavage of the CgA molecule[327, 405]. Antibodies against CgA are frequently being used as pan-NE markers.
During recent years it has become clear that some of the factors secreted by NE cells (e.g., serotonin and gastrin related peptide) display growth factor activity mediated by a corresponding membrane receptor[335, 336]. Bombesin (gastrin related peptide), for example, has been shown to stimulate the proliferation of the prostatic tumor cell line PC-3 which has saturable bombesin binding sites[372]. This stimulation could be inhibited by antibodies against the peptide. Prostatic NE cells in benign and malignant prostatic epithelium do not contain androgen receptor protein and are therefore considered to be androgen independent, at least for their existence[276]. All in all, this suggests that NE cells in a prostatic adenocarcinoma influence the growth of neighboring non-NE tumor cells in an androgen independent manner. Consequently, NE cells may play a role in the progression of prostate cancer towards androgen independence. The NE cells themselves, however, appear to be in a post-mitotic state[406]. For experimental studies on NE differentiation in prostate cancer, tumor models containing these cells are needed. In the present study, the occurrence of immunohistochemically defined NE cells was studied under different hormonal conditions in most of the commonly available *in vitro* prostatic tumor cell lines and in a number of *in vivo* human prostatic tumor xenograft models. Transmission electron microscopy and Western immunoblotting were performed to confirm the immunohistochemical data. To study the androgen receptor expression in prostatic NE cells and to assess their post-mitotic state, double labeling immunohistochemistry was performed.

MATERIALS AND METHODS

Tumor models

A panel of human prostatic cancer *in vitro* cell lines and *in vivo* xenograft models was used. This panel included the more well known, commonly available, *in vitro* cell lines (PC-3[407], DU 145[408], TSU[409] and LNCaP[410]) and *in vivo* xenograft models (PC-EW[411] and PC-82[412]) as well as a set of *in vivo* xenograft models more recently established at this laboratory (PC-133, PC-135, PC-295, PC-310, PC-324, PC-329, PC-339, PC-346 and PC-374). The latter models are considered to be representative for the different clinical manifestations of prostate cancer (androgen dependent vs. independent; primary tumor vs. metastatic lesion) and are described elsewhere in more detail [413]. Two sub-lines of the parental LNCaP-FGC cell line (lymph node carcinoma of the prostate, fast growing colony) i.e., LNO (lymph node origin) and R (resistant)[414] were also included. PC-346i is an androgen independent sub-line derived from the PC-346 xenograft model. Both the parental and PC-346i tumor models can be propagated *in vitro* as well as *in vivo*. The main characteristics of the investigated models are shown in Table VI.1.

Xenografts

Xenografts were serially transplanted in nude mice of the Balb/c or NMRI strain by subcutaneous implantation of small tumor fragments under ether anesthesia. The mice were obtained from the breeding facilities of the Erasmus University. Androgen dependent xenografts were transplanted in androgen supplemented male or female mice whereas androgen independent xenografts were generally transplanted in intact mice. Animals were supplemented with testosterone (Sigma, St. Louis, MO) containing custom-made Silastic implants[415]. Androgen withdrawal was achieved by removal of the Silastic implants and by castration of male mice under hypnorm anesthesia. The mice were sacrificed at different intervals following androgen withdrawal. After sacrifice of the animals the tumors were removed and divided in smaller fragments that were either snap frozen in liquid nitrogen and stored at -80°C or fixed in 4% buffered formalin and embedded in paraffin. In a number of cases small tumor fragments were fixed in glutardialdehyde for routine electron microscopy. The pancreas, adrenal and bowel tissues of some mice were removed, formalin-fixed and paraffin-embedded as well.

TABLE VI.1
Main characteristics of human prostatic tumor models

tumor model	origin	androgen sensitivity	growth	
			in vitro	in vivo
PC-3	bone	no	yes	yes
DU 145	brain	no	yes	yes
TSU	lymph node	no	yes	yes
LNCaP	lymph node			
. FGC		yes	yes	yes
. R		no	yes	no
. LNO		no	yes	no
PC-EW	lymph node	yes	no	yes
PC-82	RP	yes	no	yes
PC-133	bone	no	no	yes
PC-135	lymph node	no	no	yes
PC-295	lymph node	yes	no	yes
PC-310	RP	yes	no	yes
PC-324	TUR-P	no	no	yes
PC-329	RP	yes	no	yes
PC-339	TUR-P	no	no	yes
PC-346	TUR-P			
. PC-346		yes	yes	yes
. PC-346i		no	yes	yes
PC-374	skin	no	no	yes

RP = radical prostatectomy - TUR-P = trans-urethral resection of the prostate

Experimental design

To study the kinetics of NE cells following castration, 8 androgen supplemented PC-295 bearing female mice received BrdU, a thymidine analogue which is incorporated in DNA during the S-phase of the cell cycle. BrdU (30mg/ml, Sigma) was administered per osmotic pump (Alzet 1007D, Alza Corp., Palo Alto, CA) at a flow rate of 0.5μl/hr, thus the mice received 15μg BrdU per hour. The osmotic pumps were inserted subcutaneously 48 hours prior to castration under ether anesthesia. Together with removal of the androgen containing Silastic implants (i.e., castration), the osmotic

pumps were removed and 2 mice were sacrificed either directly (T_0) or after 3 (T_{-3}) or 6 (T_{-6}) days. In two mice androgens were re-substituted 3 days following androgen withdrawal and these mice were sacrificed after another period of 7 days (T_{-3+7}). This method ensured sufficient BrdU incorporation in cells that were cycling prior to castration. Antibodies against BrdU allow for the detection of cells in the S-phase[416]. Tumor volumes were calculated from two caliper measured perpendicular tumor diameters (D_1 and D_2) according to the formula:

$$V_{tumor} = \frac{\Pi}{6} \times (D_1 \times D_2)^{3/2} \qquad (1)$$

The tumors of these mice were processed as described above. In addition, bowel and skin tissues were embedded in paraffin to assess the efficacy of BrdU-incorporation. The microscopical (5x) pictures of routine H&E sections of the tumors were digitized. The total tumor-area (A_{tumor}) in these sections was determined using the KS-400 software (Kontron Elektrotechnik, Oberkochen, Germany). The number of NE cells was counted in the total tumor-area present in a particular section. Because prostatic NE cells are considered to be androgen independent, androgen withdrawal in this androgen dependent tumor model will lead to a selective decrease of non-NE tumor cells and thereby to an increased frequency of NE cells. Besides this, castration will have other effects like apoptosis, possibly some necrosis, murine stromal proliferation and swelling of the cells. All these factors will have their effect on total tumor volume and thus, an increased NE cell fraction observed following androgen withdrawal might in fact represent a concentration effect. To account for this potential concentration effect the number of NE cells (N_{NE}) per tumor area unit was adjusted to the decrease in tumor volume from the time of castration (T_0) to the time of sacrifice (T_{-x}) according to the formula:

$$corrected\ N_{NE} = \frac{N_{NE}}{A_{tumor}} \times \left(\frac{V_{tumor_{T_{-x}}}}{V_{tumor_{T_0}}} \right)^{2/3} \qquad (2)$$

Cell cultures

Cells were cultured in RPMI-1640 medium (Gibco BRL, Breda, The Netherlands) supplied with 7.5% fetal calf serum (Hyclone, Logan, Utah), 2mM glutamine (Gibco BRL), 50IU/ml penicillin (Gibco BRL) and 50μg/ml streptomycin (Gibco BRL) in 5% CO_2 at 37°C. For experiments, cells were cultured in quadriperm culture chambers (Nunc, Roskilde, Denmark) on 3-amino-propyl-trietoxy-silane (APES, Sigma) coated glass slides[272]. Experimental media contained dextran charcoal treated, i.e. androgen depleted, serum, either with or without 10^{-10}M of the not metabolizable synthetic androgen R1881 (17ß-hydroxy-17α-methyl-estra-4,9,11-trien-3-one, New England

Nuclear, Boston, MA). After four days the slides were rinsed with phosphate buffered saline (PBS, pH=7.4), air dried, wrapped in aluminum foil and stored at -20°C until use.

Immunohistochemistry

For the identification of NE cells, tissue sections and/or cell cultures of the several tumor models were immunostained with antibodies against CgA (clone LK2H10, Organon Technika, Oss, The Netherlands)[417] and 5-HT (rabbit polyclonal antibody, Eurodiagnostics, Apeldoorn, The Netherlands). If available, the paraffin-embedded patient tumor material from which a tumor model originated was also immunostained.

Frozen cell culture slides were brought to room temperature and were fixed in 4% buffered formalin for 10 minutes at room temperature. Subsequently, the slides were rinsed in PBS and processed for two minutes in 100% methanol and for 4 minutes in 100% acetone, both at -20°C. Paraffin embedded xenografts were cut at 5μm (2μm for double immunostaining) and the tissue sections were mounted on APES-coated glass slides. The slides were kept overnight at 60°C and were dehydrated through xylene and ethanol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 minutes. After rinsing with tap water, the slides were placed in a 10mM citrate buffer (pH 6.0) and antigen retrieval was performed in a microwave oven at 700W for 3x5 minutes[273]. The slides were allowed to cool down to room temperature and were rinsed with PBS. For immunohistochemical staining the tissue slides and air dried cell culture slides were placed in a Sequenza immunostaining system (Shandon, Uncorn, United Kingdom). The slides were pre-incubated with normal goat serum (DAKO, Glostrup, Denmark) diluted 1:10 for 15 minutes. The primary antibody was applied for 1 hour and subsequently the slides were incubated for 30 minutes with a 1:400 diluted biotinylated goat anti-mouse or goat anti-rabbit antibody (DAKO) for monoclonal and polyclonal primary antibodies, respectively. Finally, a horseradish-peroxidase labeled streptavidin-biotin complex (DAKO) was applied for 30 minutes. The complex was prepared at least 30 minutes prior to use at a dilution of 1:1:200. In between each step, except following the pre-incubation, the slides were rinsed 3x with PBS. All compounds were diluted in PBS. Immunostaining was visualized with 150mg diaminobenzidine hydrochloride (DAB, Fluka, Neu-Ulm, Germany) in 200ml PBS with 0.08% hydrogen peroxide as substrate. The slides were lightly counter stained with Mayer's hematoxylin, dehydrated and covered.

Double labeling immunohistochemistry was performed on tumors with CgA immuno-reactive cells using monoclonal antibodies against the androgen receptor (clone F39.4, kind gift of Dr. A.O. Brinkmann, Dept. of Endocrinology & Reproduction, Erasmus University)[202], Ki-67 antigen (clone MIB-1, Immunotech, Marseille, France)[418] or BrdU (clone IIB5, kind gift of Dr. B. Schutte, Dept. of Pathology, University of Maastricht, The Netherlands)[416] combined with anti-CgA (clone LK2H10) as the second primary antibody. BrdU/CgA-immunostaining was performed only in the PC-295 time-

course experiment. The Ki-67 antigen is only expressed in cycling cells (i.e., during the G1, S, G2 and M phases of the cell cycle) and the MIB-1 antibody can be used to identify cycling cells in, among others, prostate cancer[201]. The double labeling procedure consisted of two sequentially performed avidin-biotin complex methods as described above. The avidin-biotin complex reactive with the first primary antibody (i.e., MIB-1, anti-androgen receptor or anti-BrdU) was horseradish-peroxidase labeled, whereas the second complex (anti-CgA) was alkaline-phosphatase labeled. The alkaline-phosphatase reaction was visualized by a 30 minute incubation with AS-MX-phosphate (0.3mg/ml, Sigma) to which just prior to use new fuchsine (2.5μl/ml, Sigma), $NaNO_2$ (1.45mM, Sigma) and levamisole (0.5mg/ml, Sigma) were added. Levamisole inhibits the endogenous tissue type alkaline-phosphatase activity but does not block the alkaline-phosphatase linked to the immune-complex[419]. All these compounds were diluted in 0.2M (hydroxymethyl)-methylamine (TRIS-buffer, Gibco BRL) adjusted at pH=8.0. Prior to immunostaining of BrdU, but following the microwave procedure, the DNA of these tissue sections was uncoiled by incubation of the slides with 2N HCl for 30 minutes at 37°C and subsequently with 0.1M sodium-tetraborate buffer at pH 8.5 for neutralization[416]. In between the two immuno-labeling procedures the slides were rinsed with PBS for one hour and boiled in a microwave oven for 15 minutes in 10mM citrate buffer (PH=6.0) to diminish crossover reactivity. Pilot experiments showed that the peroxidase-DAB reaction product was resistant to the microwave step. During all immunohistochemical procedures negative controls were included by replacing the primary antibody/antibodies by PBS. Prostatic tissue obtained from a radical prostatectomy specimen served as positive control. Mouse pancreas, adrenal and bowel tissue sections were immunostained with anti-CgA to rule out cross-reactivity of the LK2H10 antibody with murine CgA. NE differentiation was defined as the presence of CgA and/or serotonin immuno-reactive cells in a particular tumor model. The frequency of immuno-positive cells was scored semi-quantitatively as - (0%), ± (0-1%), + (1-5%), ++ (5-10%) or +++ (>10%). The frequency of immuno-reactive cells in the PC-295 time-course experiment was quantified using the KS-400 software yielding a number of positive cells per tumor area unit which was adjusted according to formula (2).

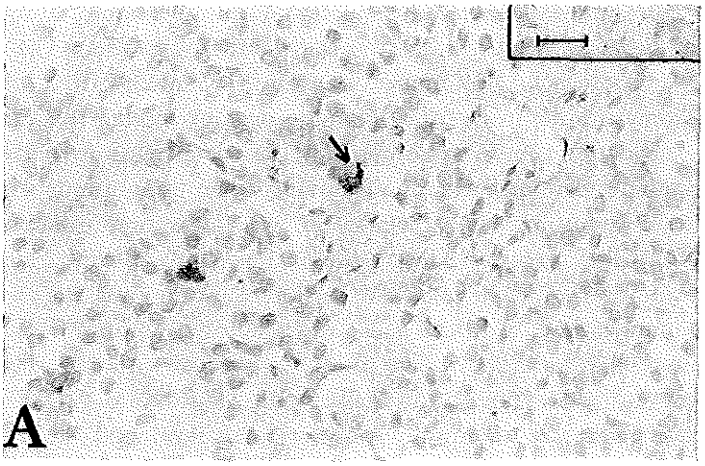
Western analysis

To further confirm the CgA immunohistochemical data Western analysis was performed with tissues from androgen stimulated as well as androgen deprived PC-295 and PC-310 tumors. Human pheochromocytoma tissue served as positive control. The frozen tissues were crushed in a liquid nitrogen chilled metal cylinder. The tissue homogenates were transferred to a lysis buffer consisting of 10mM TRIS (pH 7.4), 150mM NaCl (Sigma), 1% triton X100 (Merck, Darmstadt, Germany), 1% deoxycholate (Sigma), 0.1% sodium dodecyl sulphate (SDS, Gibco BRL), 5mM EDTA (Merck) and

protease inhibitors (1mM phenylmethyl-sulphonyl fluoride, 1mM aprotinin, 50mg/l leupeptin, 1mM benzamidine and 1mg/l pepstatin, all from Sigma). The samples were spun at 35,000 x G at 4°C for 10 minutes. The protein content of the supernatant was measured photometrically using the bio-rad protein assay (Bio-rad, München, Germany)[420]. Xenograft tumor tissue always contains murine (stroma) components which may cause considerable background staining when using a monoclonal antibody under sub-optimal conditions. For this reason, CgA was first immuno-precipitated. Goat anti-mouse labeled agarose (SIGMA) diluted 1:8 in PBS was incubated for 2 hours with the CgA antibody (clone LK2H10, 1:200). After washing with PBS and subsequent spinning at 2,500 x G, the pellet was incubated with the volume equivalent of 100μg sample protein for 2 hours. The pellet was washed with PBS, dissolved in 30μl Laemmli buffer, boiled 3 times and finally spun at 5,000 x G for 1 hour. The supernatant was transferred to an SDS-polyacrylamide gel and electrophoresis was performed. The gel was blotted to a 0.45μm cellulose nitrate membrane (Schleicher & Schuell, Dassel, Germany). Pre-stained markers were used as size standards (Novex, San Diego, CA). The immunoblot was blocked for 1 hour with TRIS/NaCl buffer (pH 7.7) to which 0.1% TWEEN-20 (Sigma), 5% dry milk and 2% bovine serum albumin (Sigma) were added. The CgA antibody was diluted 1:1000 in PBS and was applied overnight at 4°C. After rinsing with PBS the blot was incubated with a horseradish peroxidase labeled goat anti-mouse antibody (1:4000, DAKO) for 1 hour. Subsequently, a one minute incubation with a 1:1 mixture of luminol and oxidizing reagent (DuPont NEN, chemiluminescence kit, Boston, MA) was performed. Excess reagent was removed by placing the blot on a piece of Whatmann paper. Finally, the antibody was visualized by exposure of the blot to an X-ray film for 30 seconds.

RESULTS

The CgA antibody (clone LK2H10) did not cross-react with murine CgA (results not shown)[417]. Cell cultures of *in vitro* growing prostatic tumor models (PC-3, DU 145, TSU, LNCaP and PC-346) did not contain any immunohistochemically defined NE cells. This was not influenced by the androgen content of the medium (either with or without 10^{-10}M R-1881). Six out of 14 investigated prostatic xenograft models (PC-EW, PC-295, PC-310, PC-324, PC-346, PC-374) contained NE cells under various circumstances (Figure VI.1, Table VI.2). However, the NE phenotype appeared not to be stable in the PC-324, PC-346 and PC-374 models as NE cells could only be demonstrated up to the 3rd, 4th and 5th mouse passages of these models, respectively. PC-324 and PC-346 tumors showed NE differentiation irrespective of the hormonal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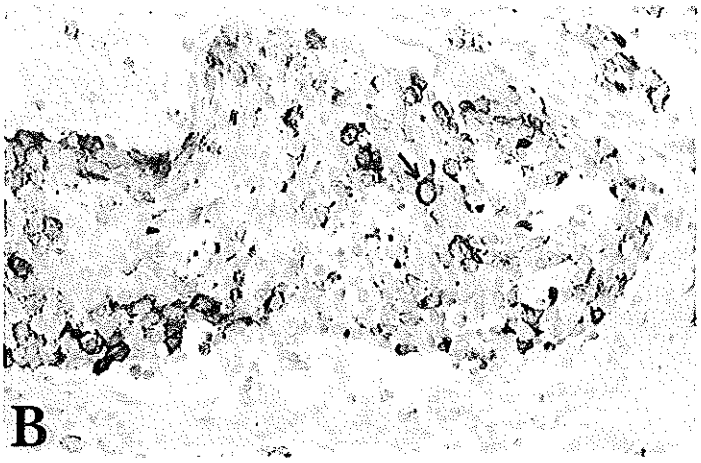
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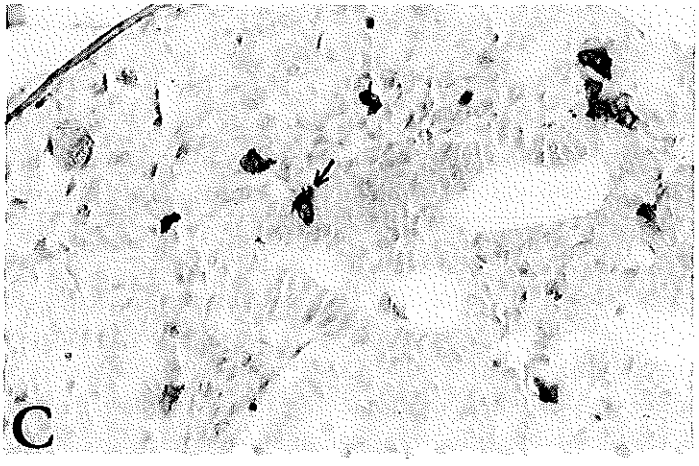
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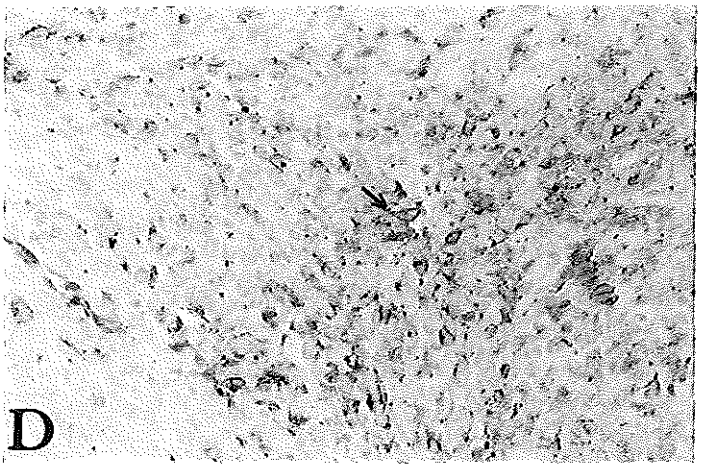
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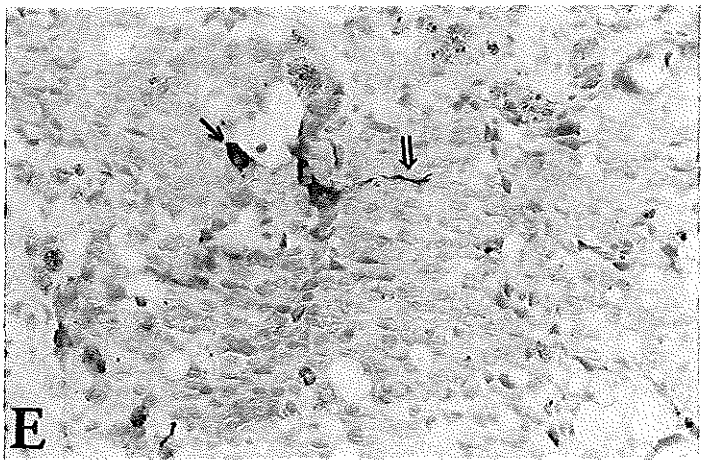
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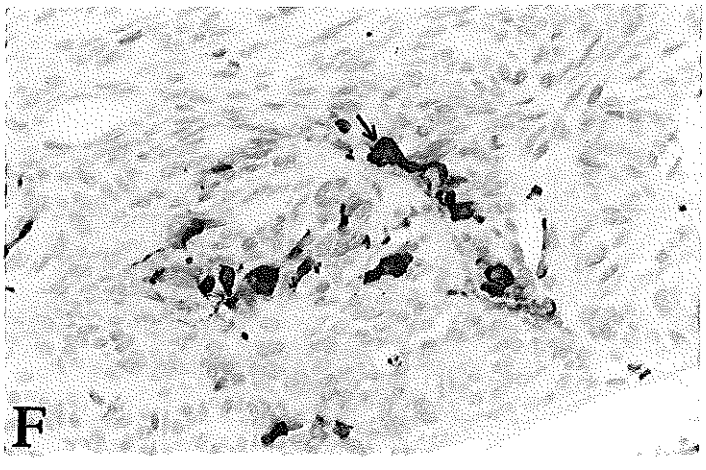

F

FIGURE VI.1
CgA immunohistochemistry in prostatic tumor models in vivo, counterstaining with hematoxylin. Arrows indicate some positive cells. (A). PC-EW, 15 days following castration. (B). PC-295, 6 days following castration. (C). PC-310, 44 days following castration. (D). PC-324, intact mouse. (E). PC-346, intact mouse. Note cytoplasmic process (open arrow). (F). PC-374, female mouse. (x273).

status of the tumor host (Figures VI.1d and VI.1e, Table VI.2). The number of NE cells in the PC-374 model was largest if tumors were grown in female mice that were not substituted with androgens (Figure VI.1f, Table VI.2).

TABLE VI.2
NE cells in prostatic tumor models in vivo

tumor model	NE cells patient tumor	experiments	NE cells
PC-3	NA	control (+ androgens):	
		. subcutaneous	–
		. intraprostatic	–
		castration	–
DU 145	NA	control	–
		castration	–
LNCaP	NA		
. FGC		control	–
PC-EW	+++	control	–
		castration	+
PC-82	++	control	–
		castration	–
PC-133	NA	control	–
		castration	–
PC-135	NA	control	–
		castration	–
PC-295	++	control	– - ±
		castration	+ - +++
PC-310	±	control	– - ++
		castration:	
		. 7 days	–
		. >11 days	++

continued

TABLE VI.2 *(continued)*

tumor model	NE cells patient tumor	experiments	NE cells
PC-324	+++	control:	
		. passage 1-3	- - +++
		. passage >3	-
		castration (passage >3)	-
PC-329	++	control	-
PC-339	-	control	-
		castration	-
PC-346	NA	control:	
		. passage 1-4	- - +
		. passage >4	-
		castration (passage <4)	- - +
. PC-346i		female	-
PC-374	-	control:	
		. passage 1-5	- - +
		. passage >5	-
		castration:	
		. passage 1-5	+
		. passage >5	-
		female (passage 4)	++

NA = patient tumor material not available - in vivo growing tumors were implanted subcutaneously unless stated otherwise

The three models in which the NE phenotype appeared to be stable (i.e. PC-EW, PC-295 and PC-310) are androgen dependent and in all these models androgen withdrawal resulted in an increased frequency of NE cells. PC-EW tumors showed massive necrosis, rather than apoptosis, following androgen withdrawal and could not be stimulated by androgens to grow again already after a short period of time[421] and therefore this model is not suitable for further experiments. The PC-295 and PC-310 models were derived from a lymph node metastasis and a radical prostatectomy specimen, respectively. These models are strictly androgen dependent and express both prostate specific antigen and the androgen receptor[413]. PC-295 tumors grown in androgen supplemented mice contained only very small numbers (<<1%) of NE cells. Following androgen withdrawal the fraction of NE cells started to increase after 2 days. This was paralleled by a fast decrease of total tumor volume. The percentage of NE

cells after 7 days of androgen withdrawal varied from 5 to 25%. Ultrastructurally, these cells contained largely uniformly sized neurosecretory granules with a mean diameter of 225 nm (Figure VI.2a). Most PC-310 tumors did not contain NE cells when grown in intact mice, but some of these tumors contained about 5% of NE cells. Following androgen withdrawal the frequency of NE cells in this model increased as well to about 10%, but this occurred after a period of at least 7 days. In this model the neurosecretory granules varied more in size and shape (Figure VI.2b); the mean diameter was 175 nm. The presence of NE cells in PC-295 and PC-310 tumors, as demonstrated immunohistochemically and ultrastructurally (Figures VI.1 and VI.2), was further confirmed by CgA Western immunoblotting (Figure VI.3). In both the PC-295 and PC-310 models the protein level of 68kDa CgA as well as smaller CgA derived peptides increased following androgen withdrawal.

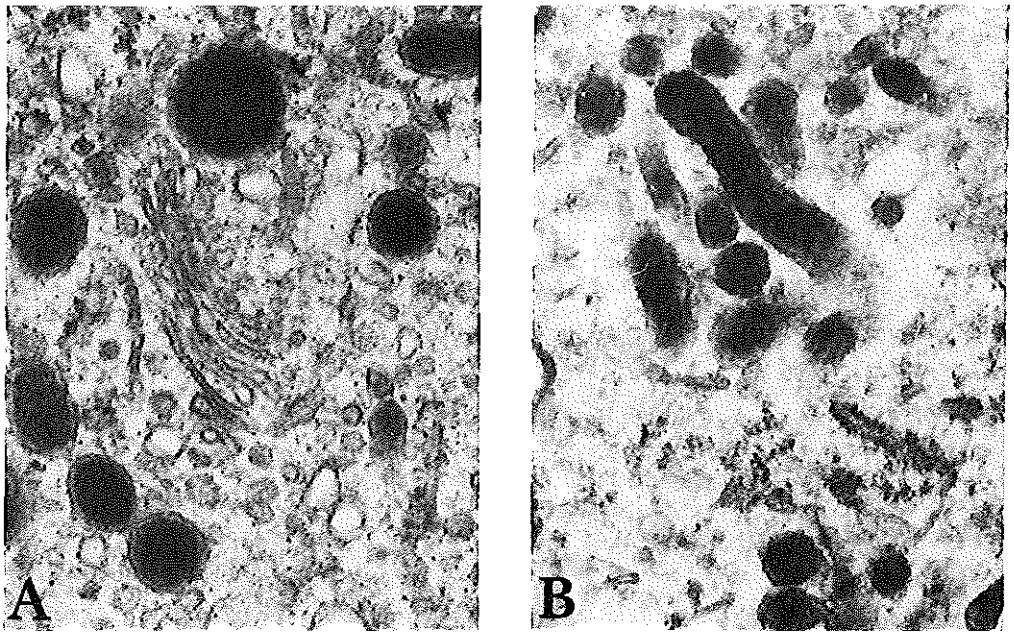


FIGURE VI.2
Transmission electron microscopy of neurosecretory granules. (A). PC-295, 6 days following castration. (B). PC-310, 12 days following castration. (x40,000).

The presence of NE cells in the original patient tumor material did not closely correlate with their presence in the resulting tumor model (Table VI.2). For example, the radical prostatectomy specimen from which the PC-82 xenograft model originated contained large numbers of NE cells, whereas the corresponding tumor model did not

contain any NE cells (results not shown). The same was found in the PC-329 model. By contrast, the skin metastasis from which the PC-374 model originated did not contain NE cells but the resulting model did so. Neither mouse strain (NMRI or Balb/c) nor gender of the tumor host was related to the presence and frequency of NE cells in a tumor model. Immunohistochemical double labeling with anti-androgen receptor or Ki-67 was studied in more than 1000 CgA immuno-reactive cells in both the PC-295 and PC-310 models. All the CgA immuno-reactive cells were androgen receptor protein and Ki-67 antigen negative (Figure VI.4).

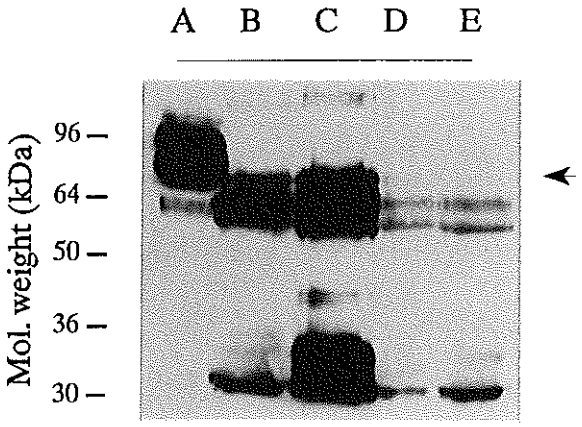
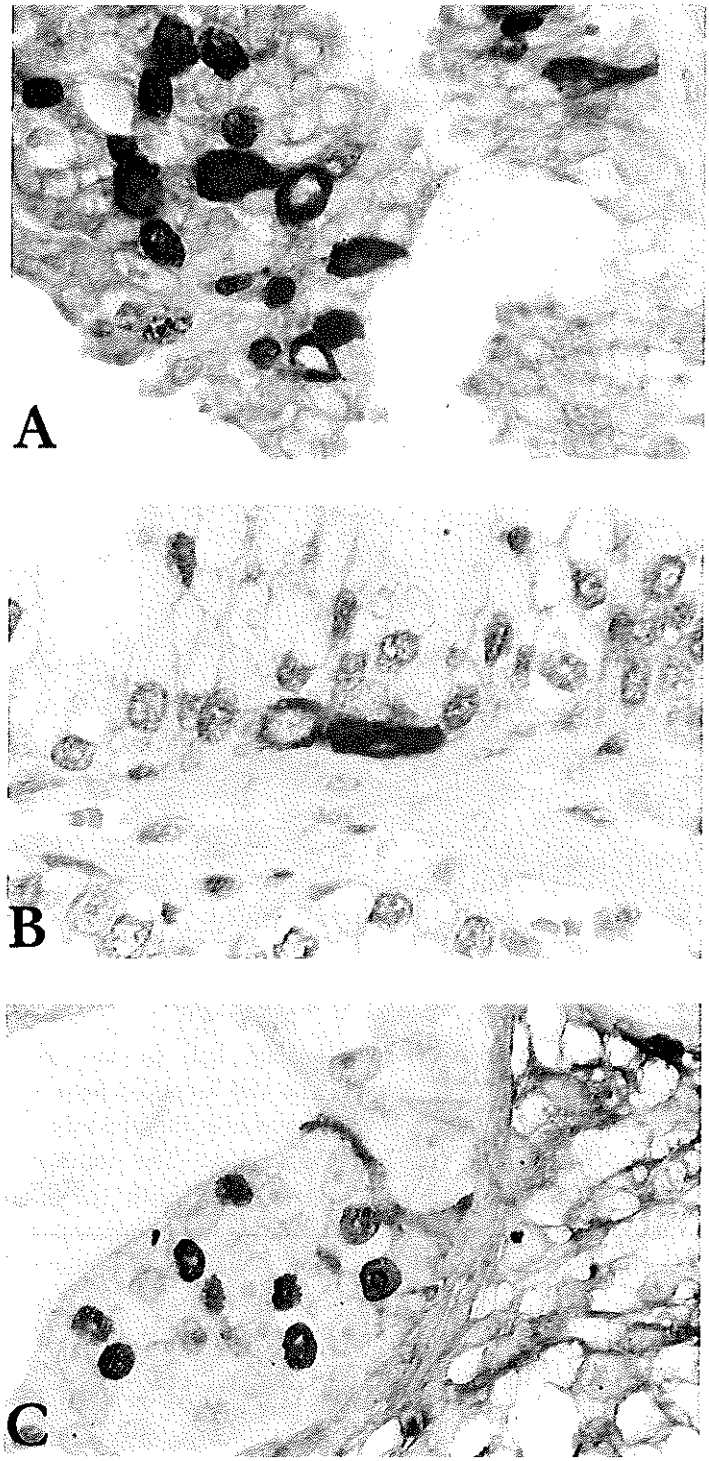


FIGURE VI.3
CgA Western immunoblot with approximate indication of size markers. Arrow at 68 kDa (intact CgA). (A). Human pheochromocytoma tissue. (B). PC-310, 12 days following castration (same tumor as figure VI.2b). (C). PC-295, 6 days following castration (same tumor as figure VI.2a). (D). PC-310, intact mouse. (E). PC-295, intact mouse.

Because androgen withdrawal reproducibly led to an increase in NE differentiation in PC-295 and PC-310 tumors, the cell kinetic aspects of this increase were investigated in PC-295 tumors which were grown in the presence of BrdU during 48 hours prior to castration of tumor bearing mice. The localization of BrdU incorporation in bowel and skin tissue correlated well with the time after BrdU administration (results not shown). Following androgen withdrawal tumor volumes decreased rapidly; this was paralleled by an increase in both the raw and the volume adjusted numbers (according to formula (2)) of NE cells per area unit (Table VI.3). BrdU/CgA double labeled cells could not be identified in over 1000 CgA immuno-reactive cells in any of these tumors (Figure VI.4).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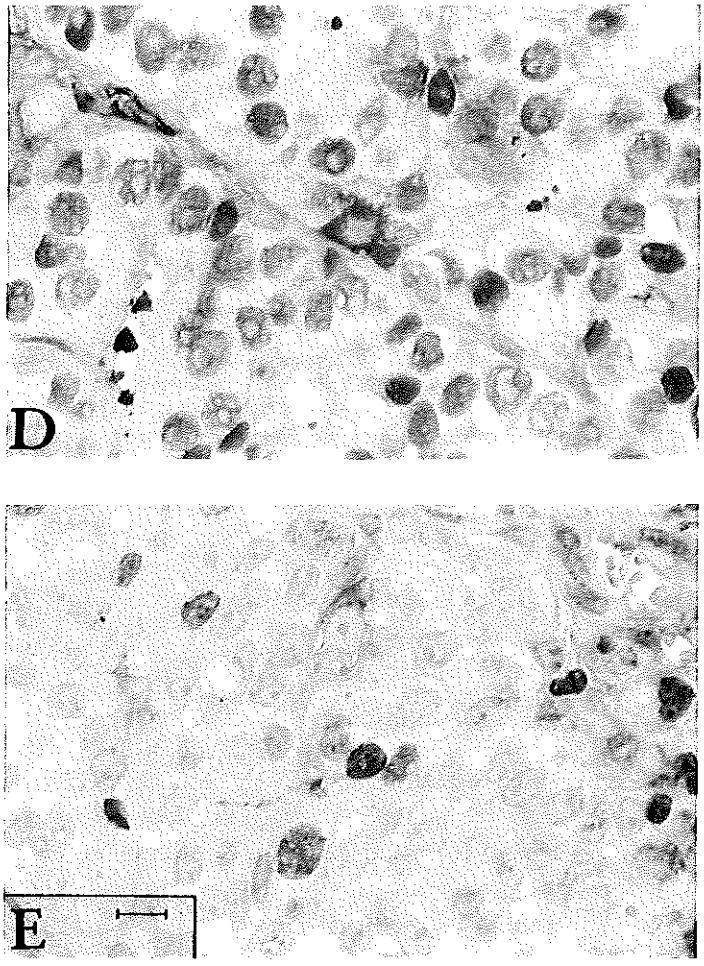

FIGURE VI.4
Immunohistochemical double labeling of NE cells, counterstaining with hematoxylin. (A). Radical prostatectomy, MIB-1 (brown) & CgA (red). (B). Radical prostatectomy, androgen receptor (brown) & CgA (red). (C-E). PC-295, intact mouse: (C). MIB-1 (brown) & CgA (red). (D). Androgen receptor (brown) & CgA (red). (E). BrdU (brown) & CgA (red). (x690, scale bar 7.7μm).

TABLE VI.3

Time-course experiment, PC-295

treatment	N	relative tumor volume	number of NE cells	
			not adjusted	volume adjusted
control	3	1	1 (0.31)	1 (0.31)
T_{-3}	4	0.80 (0.17)*	9.9 (6.0)	7.8 (4.4)*
T_{-6}	4	0.38 (0.078)*	20.3 (8.9)*	10.7 (4.7)*
T_{-3+7}	3	0.67 (0.28)*	3.3 (3.8)	4.2 (3.1)

Number of NE cells compared with control, data are presented as mean (standard deviation) - relative tumor volume = tumor volume at sacrifice divided by tumor volume at castration - volume adjustment according to formula (2) - * = $p<0.05$ compared to control (Student's T-test)

DISCUSSION

NE cells are present in most prostatic tumors[403], and the prognostic value of these cells has been investigated by several authors. Although the first papers reported a strong prognostic value[264, 267, 268], more recent papers tempered the initial enthusiasm[269, 270, 403]. Notwithstanding the debate about this matter and the eventual clinical relevance of these cells, NE cells can theoretically influence prostatic tumor growth. Moreover, this process might be related to the androgen responsiveness of a tumor. Hence, insight in the cell biology of prostatic tumor NE cells might answer questions specifically related to the progression and hormonal escape of prostate cancer. Functional aspects of NE cell derived peptides have been studied using well characterized prostatic tumor models without NE differentiation (e.g. the growth modulating activity[372, 386]). For the study of other, more mechanistical, aspects (e.g. the prostatic NE differentiation pathway, secretion products, paracrine activity, relation with androgen levels and androgen responsiveness), tumor models with NE differentiation are needed. This was the main reason to investigate the occurrence of NE differentiation in prostatic tumor models in the present study. A heterotransplantable small cell prostate cancer tumor model with NE features (UCRU-PR-2) has been described[396]. However, small cell cancer is biologically different from adenocarcinoma. Hence, this model is most probably not suited for the study of NE differentiation in prostatic adenocarcinoma and therefore, this model was not included in the present study.

Of the previously described prostatic tumor models selected for the present study (PC-3, DU 145, TSU, LNCaP, PC-EW and PC-82) only PC-EW contained NE cells

(Table VI.2). Although not extensively described, it was claimed that the LNCaP and PC-3 cell lines also would contain NE cells *in vitro*[422]. We were not able to confirm these results even not when the immunostaining protocol of these investigators was used. De Bruïne *et al.* have shown that NE cells were always present when a colon cancer cell line (NCI-H716) was grown in nude mice, but that NE cells could be detected *in vitro* only when the cells were cultured in matrigel or with certain extracellular matrix components[423]. This suggests that stromal-epithelial interactions are important for the induction or maintenance of NE differentiation. PC-3 and LNCaP tumors grown *in vivo*, either subcutaneously (LNCaP) or both subcutaneously and orthotopically (PC-3), did not contain NE cells in the present study (Table VI.2), substantiating the observation that these models indeed do not show NE differentiation. A number (5 out of 7) of human prostatic tumor xenograft models[413] contained NE cells under different hormonal circumstances. As reported[417], the employed LK2H10 antibody did not react with murine CgA in the present study (results not shown) and thus, all CgA immuno-reactive (i.e., NE) cells were assumed to be of human origin. Furthermore, CgA Western immunoblotting identified the presence of intact CgA (68 kDa) as well as proteolytic break down products in both the PC-295 and PC-310 models (Figure VI.3)[417]. Finally, the presence of NE cells was confirmed ultrastructurally by the identification of neurosecretory granules (Figure VI.2). These granules are considered conclusive evidence for NE cells[404]. The expression of several break down products of CgA varies among tissues[424], which may explain the differences between pheochromocytoma and prostatic tissues as shown in Figure VI.3. The 94 kDa band present in pheochromocytoma tissue may very well represent pro-CgA[327]. CgA expression has been demonstrated in non-NE cells e.g., in rat alveolar type II cells[425] and in the submandibular salivary glands of mice[426]. In the former study, it was hypothesized that CgA might play a role as a Ca^{2+}-binding protein, in the latter study the CgA immuno-reactive cells were not well characterized. It cannot be excluded that in some of the tumor models presently studied CgA was expressed in exocrine cells (for example the more diffuse staining pattern visible in the PC-324 tumor model). The NE phenotype of the more important PC-295 and PC-310 tumor models was confirmed by transmission electron microscopy (Figure VI.2).

As shown in Table VI.2, the NE phenotype is lost in ongoing mouse passages of the PC-324, PC-346 and PC-374 models. It is now generally assumed that prostatic NE cells are post-mitotic and are derived from locally differentiated non-NE cells[406]. Possibly, the NE cells from the original patient tumor were still present in the initial mouse passages of the PC-324 and PC-346 models, but gradually disappeared. In contrast, the patient tumor of the PC-374 model did not contain NE cells, whereas the initial mouse passages did, however. This suggests that environmental factors played a temporal role in the appearance of NE differentiation in this model. The selection of non-NE tumor cells with time may be explained by the presence of NE inhibitory factors or by the absence of essential NE induction or maintenance factors. This may be caused by the tumor itself, the host stroma or by a combination of these (stroma-

epithelial interactions). Another explanation might be that the tumor models loose the capacity of NE differentiation caused by de-differentiation due to long-term *in vivo* propagation. The fact that normal prostatic tissue always contains NE cells and that the majority of tumors, irrespective of their glandular differentiation pattern, do so as well[403], strongly suggests that differentiation into the NE phenotype is an intrinsic property of prostatic cells. This is also illustrated by the presence of NE cells in the PC-374 model, in contrast to the patient material. Likewise, Bang *et al.* were able to induce terminal NE differentiation in LNCaP and in a highly metastatic sub-line of PC-3 by treatment of cell cultures with cyclic-AMP analogs or with phospho-diesterase inhibitors[427]. It should be noted that, contrary to the findings of the present study, these investigators also found NE features in untreated cell cultures. The possibility that the PC-295 and PC-310 models (like PC-324, PC-346 and PC-374) will loose the NE phenotype in the future cannot be ruled out at this moment. Which factors are important for the induction, maintenance or disappearance of NE differentiation in prostatic tumor models will be the subject of future studies.

In the present study, some initial experiments were performed to compare the characteristics of the NE cells found in the tumor models with known characteristics of these cells in prostatic tissue. Because the NE phenotype was found to be stable in the PC-295 and PC-310 models, only these models were studied. Similar to the findings in clinical prostate cancer, the NE cells present in the tumor models (more than 1000 cells studied), whether grown in intact or androgen deprived mice, did express neither androgen receptor protein nor Ki-67 antigen (Figure VI.4). Androgen supplemented PC-295 and PC-310 tumors expressed the androgen receptor and Ki-67 in about 80% and 10% of the tumor cells, respectively. The probability that all the NE cells (albeit present in small numbers) in these tumors would be negative for both antigens by chance is extremely small ($P << 0.0001$). Analogous to the NE cells in benign and malignant prostatic tissues, these observations confirm the post-mitotic and androgen independent nature of prostatic NE cells. The observation by Bonkhoff *et al.*[366] that in human prostatic tumors with clusters of NE cells the Ki-67 immuno-reactive cells were located in close proximity to those clusters was not confirmed in the present study. However, proliferation decreased following castration in these androgen dependent models, at least at the short-term. Long-term castration experiments will show whether the NE cells can overcome the growth inhibiting effects of androgen withdrawal by paracrine growth stimulation and hence, play a role in androgen independent growth of prostate cancer.

Abrahamsson *et al.* studied the course of NE differentiation in hormonally treated patients and found in general that the number of NE cells increased when the tumor progressed to androgen independence[264]. Civantos *et al.* investigated the effects of neo-adjuvant androgen withdrawal therapy in patients treated by a radical prostatectomy and compared the data with non pre-treated prostatectomy specimens[428]. More prostatectomy specimens of pre-treated patients contained NE cells, although the results were not statistically significant. These findings are similar to our observations of

increased numbers of NE cells and increased CgA protein levels in short-term androgen deprived PC-EW, PC-295 and PC-310 tumors and implicates that either directly or indirectly androgen regulated genes play a role in the prostatic NE differentiation pathway. Interestingly, it has been shown that CgA expression is inversely correlated with estrogen levels in the female rat pituitary (estrogen receptor positive), but not in the adrenal (estrogen receptor negative)[429, 430]. Although pituitary and adrenal cells belong to the endocrine system, these findings also point to a relationship between steroids and NE differentiation.

Whether or not prostatic NE cells already are post-mitotic before the NE phenotype appears is not clear and therefore a time-course experiment with PC-295 tumor bearing mice, which had received BrdU for 48 hours, was performed. The raw and volume adjusted number of NE cells per area unit of tumor tissue increased following androgen withdrawal (Table VI.3). This strongly indicates that both a relative and an absolute increase in the number of NE cells is combined with a selective deletion of androgen dependent non-NE tumor cells (the major cause of the total tumor volume decrease). Thus, the selection of pre-existing NE cells after androgen withdrawal alone does not account for the observed increase in NE cells. Forty-eight hours of BrdU labeling ensured that all cells that were cycling prior to castration were labeled. The fact that no BrdU-labeling was found in over 1000 CgA immuno-reactive cells (Figure VI.4), combined with the fact that proliferation falls to a very low level shortly after castration, indicates that NE differentiation of prostatic tumor cells only takes place in cells that are in the G_0-phase already for a longer period of time. Since CgA is only expressed by fully NE differentiated cells, another post-mitotic cell type might exist, with a phenotype somewhere in between the exocrine and NE phenotypes which gives rise to the NE cells appearing following castration. Testing of such a hypothesis awaits the generation of immunohistochemical or molecular-biological markers for 'early' NE cells, prior to their expression of CgA.

In conclusion, NE cells can be identified in human prostatic tumor xenograft models, but the phenotype is not always stable. In the PC-295 and PC-310 tumor models NE differentiation can be induced reliably by castration of tumor bearing mice. The NE cells found in these models are most likely androgen independent for their persistence, do not express the androgen receptor and are post-mitotic. These findings are concordant with NE cells found in clinical prostate cancer. The cells that became NE differentiated following androgen withdrawal were most probably post-mitotic already before the NE phenotype was induced. These models are suitable to study fundamental issues concerning the presence and function of NE cells in prostate cancer.

CHAPTER VII

DETERMINATION OF THE KI-67 DEFINED GROWTH FRACTION BY THE MONOCLONAL ANTIBODY MIB-1 IN FORMALIN-FIXED, PARAFFIN EMBEDDED PROSTATIC CANCER TISSUES

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ABSTRACT

The applicability of MIB-1, a monoclonal antibody directed against the Ki-67 antigen was studied in the PC-82 and LNCaP prostatic tumor models at various levels of proliferative activity. Statistically significant correlations were found in LNCaP cultures between Ki-67 and MIB-1 scores ($r=0.84$, $p<0.001$) and in PC-82 tumors between MIB-1 scores and paraffin tissue Ki-67 (pKi-67),($r=0.90$, $p<0.001$), frozen tissue Ki-67 (fKi-67), ($r=0.86$, $p<0.001$), and BrdU uptake ($r=0.70$, $p<0.001$), respectively. pKi-67 scores doubled the fKi-67 scores which may be due to methodological differences. MIB-1 scores exceeded both the fKi-67 and pKi-67 scores. The affinity of MIB-1 for the antigen is much higher than the affinity of Ki-67 which may explain the differences. MIB-1 is a promising means of evaluating the presence of only minute amounts the Ki-67 antigen in paraffin embedded human tumor material, especially of relatively slowly growing tumors.

INTRODUCTION

Since its description in 1983 much attention has been paid to the mouse monoclonal antibody Ki-67[182]. Ki-67 is directed against a nuclear antigen expressed in human cells during the G1, S, G2 and M phases of the cell cycle, but not during the G0 phase[431]. Quiescent (G0) cells which enter the cell cycle do not express the antigen in the early G1 phase[431]. The proliferative activity of a number of human tumors including prostatic carcinoma has been studied by the application of Ki-67, as reviewed by Brown and Gatter[189, 432]. Routine studies were hampered by the fact that Ki-67 was thought to be applicable only to fresh frozen tissues. MIB-1 is a recently described mouse monoclonal antibody raised against recombinant parts of the Ki-67 antigen, which is applicable to routinely processed, paraffin embedded tissues following antigen retrieval[273, 418]. It has recently been shown that Ki-67 also reacts with paraffin tissue sections using this technique[433]. DNA replicating cells can be identified by a monoclonal antibody to BrdU (bromodeoxy-uridine), a thymidine analogue incorporated into DNA in the S phase of the cell cycle[416]. BrdU-uptake has been studied in human tumors[434-436]. The present study was performed to investigate the applicability of MIB-1 to routinely processed prostate cancer specimens.

Two human prostatic tumor models were used in the present study: the PC-82 xenograft model which is serially transplantable in athymic nude mice[412], and the *in*

vitro LNCaP cell line[437]. The growth rate of both hormone dependent tumor models can be manipulated by growing the tumors at various androgen concentrations[410, 438]. The potency of using the Ki-67 antibody for monitoring hormonal responses in the PC-82 tumor has been described previously[439].

In the present study MIB-1 scores were correlated with fKi-67 (frozen tissue Ki-67), pKi-67 (paraffin tissue Ki-67) and BrdU scores. This was achieved by application of the antibodies to frozen or paraffin tissue sections from the same PC-82 tumor or to slides with acetone fixed LNCaP cells. Hormonal manipulation of the PC-82 tumors and LNCaP cells resulted in tissues at various levels of proliferative activity. A number of radical prostatectomy specimens embedded 6 to 16 years ago was stained with MIB-1 in order to investigate the applicability of MIB-1 to archival clinical tissues.

MATERIAL AND METHODS

Experimental models

PC-82 tumor cells were subcutaneously implanted in nude mice of the Balb/c background. Tumor growth was manipulated by substitution of tumor bearing mice with various androgen levels and could be arrested by androgen withdrawal[438]. BrdU (10 mg/kg) was injected intraperitoneally one hour prior to sacrifice of the host animal. After sacrifice of the animal, tumor sections were either snap frozen in liquid nitrogen chilled isopentanyl 99% and stored at -80 °C or routinely fixed in 4% formalin in PBS (phosphate buffered saline, pH=7.4) and embedded in paraffin. Routinely processed prostatectomy specimens embedded 6 to 16 years ago were obtained from the department of pathology. To avoid the risk of detachment of cells or tissues from the glass slides, APES (3-aminopropyltriethoxysilane) coated slides were used in all experiments[272].

LNCaP-FGC (fast growing colony) cells were seeded on APES coated slides. The cells were grown in 7.5% fetal calf serum at standard conditions as described elsewhere[414]. The culture medium from exponentially growing cultures was replaced (after 2 days) by 5% DCC (dextran-coated charcoal) treated, i.e. androgen depleted, serum. Slides were fixed in acetone for 10 minutes at 0, 24, 48, 72 and 96 hours following androgen withdrawal. The LNCaP experiments were performed in triplicate.

Frozen-tissue-Ki-67 and BrdU staining

The Ki-67 and BrdU staining procedures have been described previously[189, 416]. Briefly, monoclonal antibody Ki-67 (DAKO, Denmark) and a monoclonal antibody specific for BrdU (Eurodiagnostics, The Netherlands) were applied to 5μm thick frozen and paraffin sections, respectively. Ki-67 was diluted 1:5 in PBS. The antibody-antigen binding was visualized using an indirect two-steps peroxidase method with DAB (3,3'-diaminobenzidine tetrahydrochloride) as chromogen. Prior to application of the BrdU antibody, these sections were subsequently placed in 2N HCl for 30 minutes to uncoil the DNA and in a borate buffer at pH 8.5 for neutralization[416].

Paraffin tissue Ki-67 (pKi-67) and MIB-1 staining

The procedure used for MIB-1 staining is the result of the optimization of the procedure recommended by the manufacturer. An identical procedure was used for pKi-67 staining except for the primary antibody. Paraffin PC-82 and prostatectomy sections of 5 μm thickness were cut and mounted on APES-coated slides[272]. The slides were kept overnight at 60 °C. After rehydration, the slides were placed in 10 mM citrate buffer adjusted at pH 6.0, whereafter antigen retrieval was performed in a microwave oven at 700W for 15 minutes[273]. Distilled water was regularly added to prevent drying of the slides due to evaporation. The slides were allowed to cool down to room temperature and were subsequently rinsed in PBS. Antigen retrieval was omitted for the acetone-fixed LNCaP cells. To avoid non-specific staining both tissue sections and LNCaP cultures were pre-incubated for 15 minutes at 37 °C with normal goat serum (DAKO, Denmark) diluted 1:10 in PBS. Overnight incubation with MIB-1 (Immunotech, France) diluted 1:200 in PBS or with Ki-67 (DAKO, Denmark) diluted 1:10 in PBS was carried out at 4 °C. After rinsing in PBS, the sections were incubated for 30 minutes at room temperature with a 1:400 dilution of biotinylated goat anti-mouse serum (DAKO) in PBS containing 3% normal goat and 3% normal human serum. After rinsing in PBS a final incubation with avidin biotin complex (ABC) consisting of streptavidin and biotinylated horseradish peroxidase diluted in PBS, as described by the manufacturer (DAKO) was performed for 30 minutes at room temperature. The staining was visualized with 0.075% DAB (Fluka, Germany) in PBS with 0.08% hydrogen peroxide as substrate for 7 minutes. Sections were rinsed in distilled water and counterstained with Mayer's hematoxylin. After dehydration, sections were mounted with malinol.

Quantification

Sections were examined at 400x magnification. The numbers of positive and negative tumor cell nuclei in the 4x4 central part of a 10x10 grid inserted in one of the oculars were counted. Adjacent microscopical fields were examined until 1000 or more cells were counted. Cells with an apparent brown staining of the nucleus and well demarcated nucleoli were judged positive. Scores are presented as percentages of cells counted. Spearmans' rank correlations were calculated since the data were not normally distributed. For statistical confirmation the Student-T test was performed using the software package SPSS 4.01 (statistical package for social sciences).

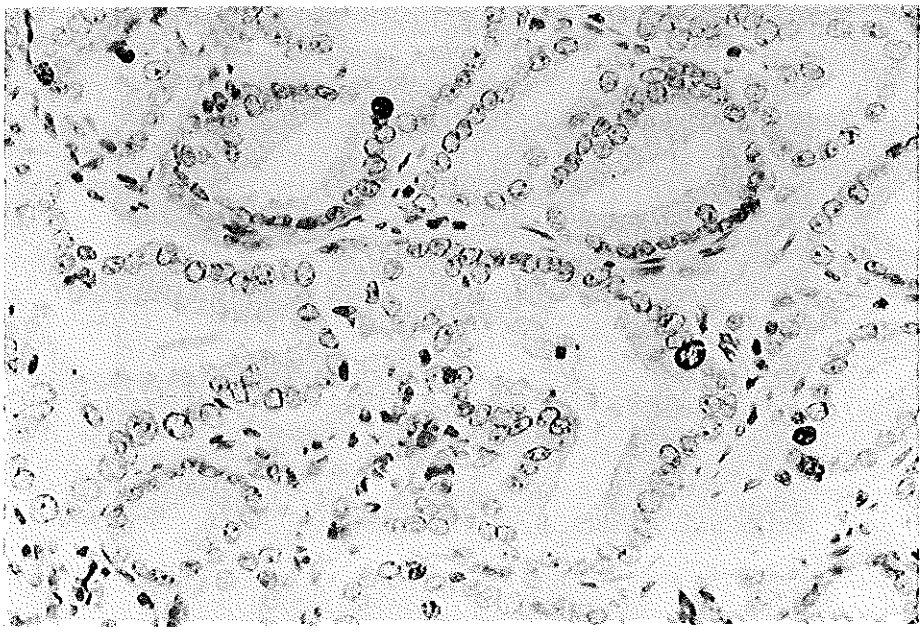
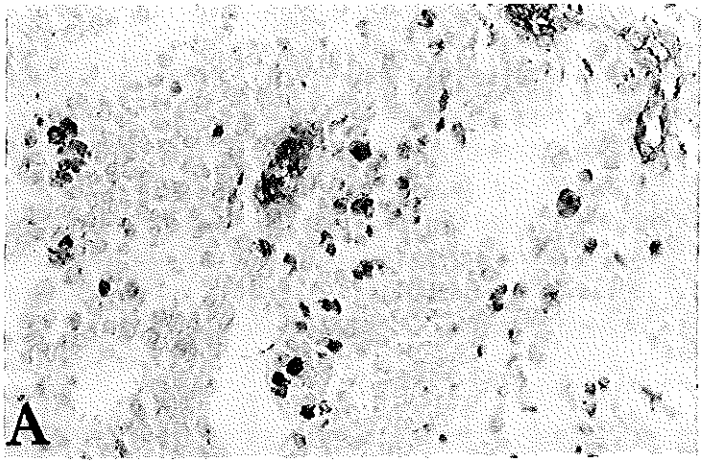


FIGURE VII.1
Gleason growth pattern 3 prostatic adenocarcinoma in a tissue section of a 16-year old radical prostatectomy specimen immunostained with MIB-1, (x270).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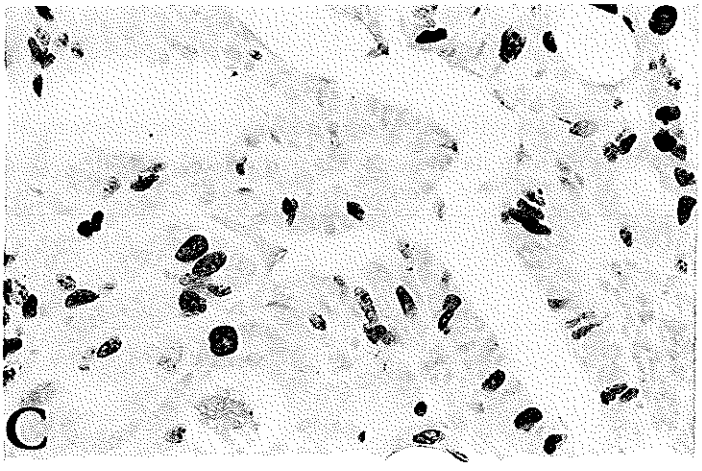
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FIGURE VII.2
Immunostaining of a PC-82 tumor sample grown in a hormonally untreated male mouse. (A). Fresh-frozen section, Ki-67 (fKi-67). (B). Paraffin-embedded section, Ki-67 (pKi-67). (C). Paraffin-embedded section, MIB-1, (x210).

RESULTS

Immunostaining of prostatectomy specimens

Figure VII.1 shows MIB-1 staining in a 16 years old prostatectomy specimen. Although variations in staining intensity were observed, positive and negative cells were generally easy to identify. Occasionally cytoplasmic staining was observed. Detachment of parts of the prostatectomy sections from the glass slide was seen, especially in sections containing the (fatty) periprostatic tissue. The risk of detachment decreased when the slides prior to antigen retrieval were kept overnight at 60°C. This procedure did not alter the immunoreactivity for MIB-1.

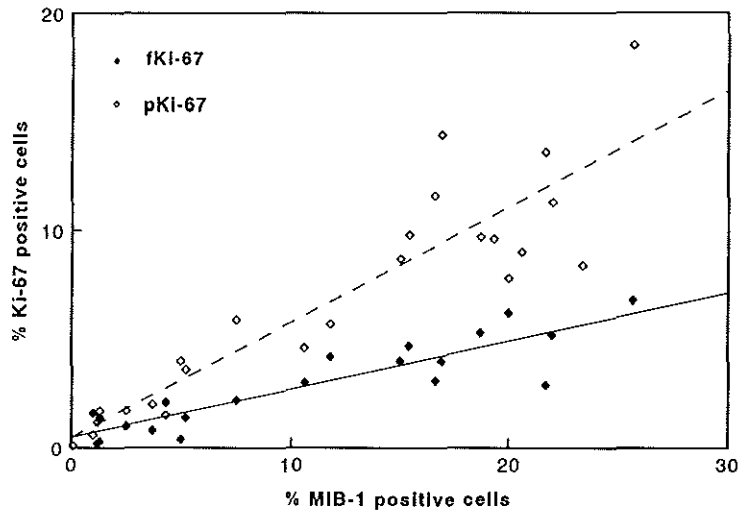

FIGURE VII.3
Correlations between MIB-1 scores and the respective fKi-67 (frozen-tissue based Ki-67) scores and pKi-67 (paraffin-tissue based Ki-67) scores, assessed on PC-82 tumor samples (N=21). Note the difference in scaling of the axes. Linear regression lines: fKi-67 (solid line) and pKi-67 (dotted line).

Immunostaining of prostatic cancer cell lines

Hormonal manipulation of the PC-82 tumor bearing mice resulted in tumors with various growth rates which were clearly reflected by the MIB-1, Frozen-Ki-67 (fKi-67), Paraffin-Ki-67 (pKi-67) and BrdU defined proliferative activities (data not shown). The intensity of MIB-1 staining was in general more pronounced than the intensity of fKi-67 and pKi-67 staining. Weak nuclear MIB-1 staining could still be observed when the antibody was diluted 1:5,000 (0.04 μg/ml). Ki-67 staining decreased at a concentration of 3.96 μg/ml (1:50 dilution). Only little intratumoral variation in the dispersion of positive nuclei was observed. MIB-1 scores ranged from 0.1 to 25.7% (mean 11.6%), fKi-67 scores ranged from 0.2 to 6.8% (mean 2.9%), pKi-67 scores ranged from 0.6 to 18.5% (mean 6.7%) and BrdU scores ranged from 0.0 to 7.5% (mean 2.4%). Figure VII.2 shows fKi-67 staining (2a), pKi-67 staining (2b) and MIB-1 staining (2c) in sections taken from one PC-82 tumor.

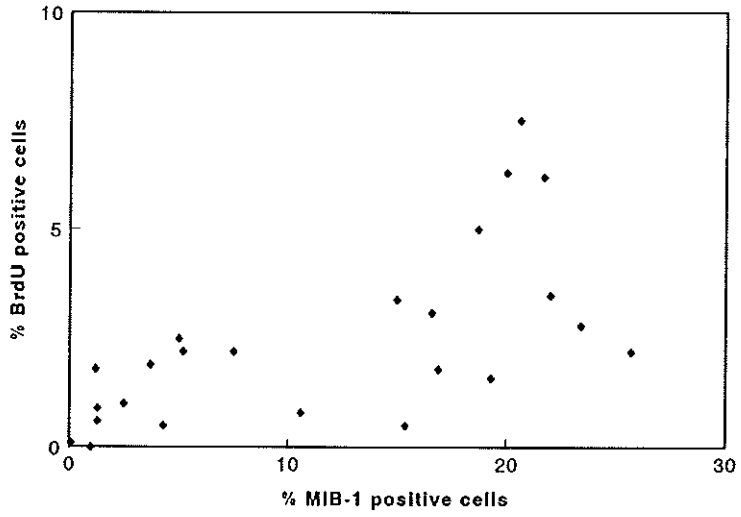

FIGURE VII.4
Correlation between MIB-1 scores and BrdU-uptake in 24 PC-82 tumors. Note the difference in scaling of the axes.

In PC-82 tissues a highly significant correlation was found between MIB-1 and fKi-67 scores ($r=0.86$, $p<0.001$, $n=21$) and between MIB-1 and pKi-67 scores ($r=0.90$, $p<0.001$, $n=25$), as depicted in Figure VII.3. Relatively weak correlations were found between MIB-1 scores and BrdU-uptake, shown in Figure VII.4, ($r=0.70$, $p<0.001$, $n=24$) and between fKi-67 scores and BrdU-uptake ($r=0.44$, $p=0.052$, $n=20$). The correlation between the fKi-67 and pKi-67 scores was 0.77 ($p<0.001$, $n=21$). MIB-1

scores exceeded both the fKi-67 scores (linear regression coefficient ß=3.67, 95% confidence interval (CI): 2.87-4.47) and the pKi-67 scores (ß=1.57, CI: 1.28-1.86). pKi-67 scores doubled the fKi-67 scores (ß=2.03, CI: 1.31-2.75).

The acetone fixed LNCaP cultures did not require antigen retrieval prior to the application of MIB-1. The growth inhibiting effect of androgen depletion on the LNCaP cells was clearly reflected by a loglinear decline of the Ki-67 and the MIB-1 scores, representing the exit of cells from the cell cycle (Figure VII.5). The MIB-1 and Ki-67 scores observed in LNCaP cultures were generally larger than those observed in PC-82 tissue sections and ranged from 16 to 70% (mean 39.6) for MIB-1 and from 7 to 41% (mean 19.8) for Ki-67. MIB-1 scores almost doubled the Ki-67 scores (ß=1.81, CI: 1.44-2.18), the correlation coefficient was 0.84 ($p<0.001$, $n=15$).

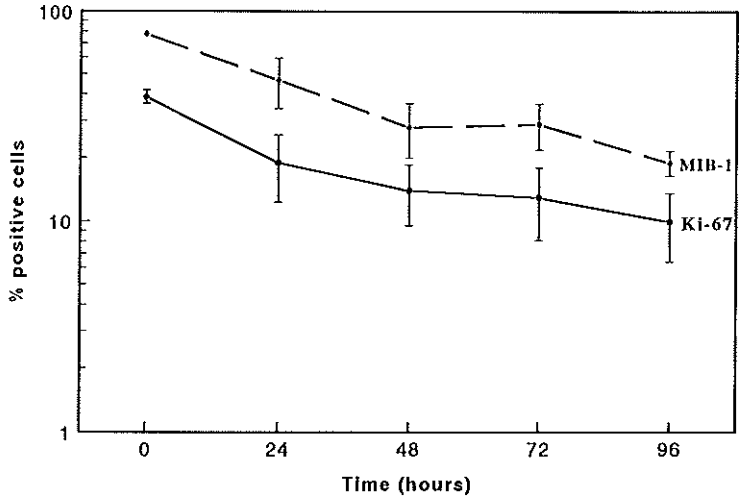

FIGURE VII.5
The relation of MIB-1 (dotted line) and Ki-67 scores (solid line) to time in a time-course experiment with hormone responsive LNCaP cells following androgen withdrawal at day 0, (mean ± standard deviation).

DISCUSSION

The present study was conducted to compare the novel proliferation marker MIB-1 with frozen-tissue based Ki-67 (fKi-67), paraffin-tissue based Ki-67 (pKi-67) and BrdU-uptake. This was achieved by immunostaining samples derived from hormonally manipulated PC-82 tumors and LNCaP cell cultures with the different antibodies[182, 416].

[418, 433]. In LNCaP cultures only Ki-67 and MIB-1 were studied. MIB-1 staining was also studied in archival radical prostatectomy specimens.

Antigen retrieval is essential for immunostaining with the MIB-1 or the Ki-67 (pKi-67) antibodies when applied to paraffin embedded tissue sections. As found by others[183], application of MIB-1 without antigen retrieval only stained mitotic cells (not shown). The mechanism of antigen retrieval remains unclear as yet, but it has been suggested that the formalin induced protein cross linkings are broken with this procedure, thus unmasking the epitopes of the various antigens[273]. In the present study MIB-1 staining could still be observed in a 16 years old prostatectomy specimen. Cattoretti *et al.* detected MIB-1 reactivity in a 60 years old Zenkers' fixed lymph node[183]. Cytoplasmic staining was occasionally observed in some of the prostatectomy specimens. A similar cytoplasmic staining has also been described in prostatic tissues stained by Ki-67, but appears not to be associated with proliferation[188]. fKi-67, pKi-67 and especially MIB-1 staining was not always equal in intensity, as Figure VII.2 shows. This may complicate the distinction of positive and negative cells. In the present study cells were scored positive only when both nuclear and nucleolar staining were visible. If cells showing only one of these patterns were included, scores would have been higher in a number of the sections.

Strong and statistically significant correlations were found between MIB-1 and fKi-67 scores ($r=0.86$, $p<0.001$) and between MIB-1 and pKi-67 scores ($r=0.90$, $p<0.001$) in PC-82 tumors (Figure VII.3) and between MIB-1 and Ki-67 scores ($r=0.84$, $p<0.001$) in LNCaP cultures (Figure VII.5). Surprisingly, MIB-1 scores exceeded both the fKi-67 scores ($ß=3.67$) and the pKi-67 scores ($ß=1.57$) in PC-82 sections. The methodological differences between the fKi-67 and MIB-1 staining procedures probably partially account for this discrepancy. However, the staining procedures were identical for Ki-67 and MIB-1 applied to paraffin PC-82 sections ($ß=1.57$) and LNCaP cultures ($ß=1.81$). Still a marked difference was observed in both cases as demonstrated by the regression coefficients. The described intensity difference between MIB-1 and Ki-67 labelling might partially account for this discrepancy. McCormick *et al.* have also shown a close relationship between MIB-1 and Ki-67 scores[440]. Although, based upon other types of tissue, they did not describe the discrepancy found in the present study. It has very recently been shown that the nuclear Ki-67-protein, i.e. the protein recognized by Ki-67, can exist free or associated with double stranded DNA[441]. This study demonstrated that, in contrast to Ki-67, MIB-1 recognized the free protein. Moreover, the affinity of MIB-1 for the DNA-Ki-67-protein complex largely exceeded the affinity of Ki-67 for the complex[441]. This indicates that binding of the Ki-67 protein to the DNA modulates its conformation which makes it an antigen for both Ki-67 and MIB-1. Since MIB-1 was raised against recombinant expressed Ki-67 protein, it was to be expected that MIB-1 also recognizes free Ki-67 protein. These results provide at least one plausible explanation for the differences between the Ki-67 and MIB-1 scores found in the present study. Furthermore, weak nuclear staining could still be observed when

MIB-1 was applied at a dilution of 1:5,000 (0.04 μg/ml), whereas nuclear Ki-67 staining decreased at a dilution of 1:50 (3.64 μg/ml).

Expectedly, BrdU pulse labeling scores are smaller than Ki-67 and MIB-1 scores[434, 442]. Van Dierendonck *et al.* have shown that Ki-67 scores cannot automatically be used to determine the growth fraction of a tumor[443]. Therefore, the relatively weak correlations found between Ki-67 and BrdU and between MIB-1 and BrdU are not surprising. Sasaki *et al.* described a correlation coefficient of 0.89 between Ki-67 and BrdU scores in 20 malignant human tumors[434]. The proliferation scores in their study (1.9%-37.5% for Ki-67 and 1.6%-23.4% for BrdU) were generally larger than the scores found in the present study. In a study of Van Weerden *et al.* using the PC-82 tumor model the correlation between Ki-67 and BrdU scores was comparable to that found in the present study[442]. The clinical use of BrdU-uptake is hampered as administration of BrdU to a patient may cause acute toxic reactions and/or chromosomal abnormalities[187, 444].

In conclusion, MIB-1 is a promising antibody and allows for the detection of very small amounts of the Ki-67 antigen in routinely processed, paraffin embedded archival tissues. MIB-1 might be of special value in tumors with relatively small growth fractions like prostatic carcinomas. At our institution a retrospective study to determine the prognostic value of MIB-1 in prostate cancer patients is currently in progress.

CHAPTER VIII

GENERAL DISCUSSION

PROGNOSTIC MARKERS

The main questions that have to be answered when a clinician is faced with a patient with prostate cancer are: is this patient to be treated and if so, how should he be treated? With the assessment of prognostic markers an attempt is being made to predict the biological behavior of the tumor in this specific patient, in order to improve patient care. It will be clear that the prognosis is not only based on tumor-derived factors, nevertheless the studies described in this thesis focus on tumor-based factors, and so will this discussion.

In patients who are to be treated with curative intent, the prognostic markers have to identify those patients in whom local progression or distant metastasis is already present at the time of diagnosis or treatment. In other words, in these patients the prognostic factors should give a reliable prediction of actual tumor-stage. In all other patients, for example expectively managed patients and hormonally treated patients, the prognostic factors have to predict the future biological behavior of a tissue that may change considerably with time. In addition, the prognosis of patients with advanced tumors is mainly determined by the metastatic lesions, whereas prognostic markers are usually determined in the primary tumor. Primary tumor cells are very probably not representative for metastatic tumor cells which form a highly selected cell population. It therefore appears that prediction of the prognosis of radically treated patients would be easier, but even for these patients it is at present not possible to reliably predict the clinical course for each individual.

Chapter I provides a review on most of the prognostic tissue markers that have been studied in prostate cancer. For almost all reviewed markers, except for tumor stage and tumor grade, conflicting results have been published or only one or a small number of reports have been published. As has been discussed in chapter I, a large number of studies are impaired by methodologic shortcomings like: small sample size, patient sample not well described, heterogenous patient sample with regard to stage distribution or treatment, short follow-up period, inappropriate end-point, inappropriate statistics or absence of multivariate analysis. These shortcomings probably influenced the results of several studies and may very well be responsible for part of the conflicting results. It is clear that the conclusion of the College of American Pathologists Conference on clinical relevance of prognostic markers in solid tumors[33], namely that no prognostic markers except for tumor-stage and Gleason score can be used clinically for decision making in prostate cancer patients, still holds true.

Another, more basic problem in the application of new prognostic tumor markers is inherent to the current prognostic system (TNM + grade)[445]. This is a mutually exclusive categorical classification system that for a number of reasons cannot be extended with new markers. The number of categories increases very rapidly with increasing numbers of variables ($T_{0-4}N_{0-3}M_{0-1}$; 5x4x2 = 40). If grade is incorporated the

number becomes 160 (M.D. Anderson or WHO grading, range 1-4, 4x40) or even 360 (Gleason grading, range 2-10, 9x40). With stage grouping the number of categories would be 20 or 45, respectively. The accuracy of the system is directly proportional to the number of patients per category. Thus, with new variables the accuracy may fall dramatically. In addition, the TNM categories are ordered by a worsening prognosis. The only way to add a new variable will be to study a large patient sample and create a new classification based on the results of all the variables tested. Finally, the TNM system is static, when there is a change in the data underlying the system, the system itself has to be adapted. For example, the introduction of the PSA RT-PCR assay may in the future classify patients that are now at stage I, II or III as stage IV. This would affect the overall prognosis of all classes and would make a new classification necessary. Despite these comments, the TNM/grade variables on itself are of major clinical value, and should therefore be part of each future prognostic system.

The chapters II-IV of this thesis describe studies on the application of prognostic tissue markers to prostate cancer. The extent of neuroendocrine differentiation as defined by expression of CgA did not show a prognostic value in patients treated with a radical prostatectomy (chapter II). This will be discussed below.

Neither the expression of the apoptosis inhibitor bcl-2 nor androgen receptor protein was of prognostic value in hormonally treated patients (chapter IV), whereas it was expected that the expression levels would be correlated with an adverse and good prognosis, respectively. Bcl-2 scores were higher in advanced tumors compared to radical prostatectomy specimens, but were not further increased in hormonally independent tumors. This suggests that bcl-2 expression is related to the presence of advanced tumor, and that bcl-2 independent pathways are more important for the acquisition of hormone independent prostatic tumor growth. These data are in contrast with published studies[224, 225]. Most authors described a prognostic value of androgen receptor expression[212-214]. Others found that only the heterogeneity of expression as determined by image analysis was of prognostic value[210, 211]. It would be of interest to analyze the androgen receptor immunostained tissue sections presented in chapter IV with image analysis as well, to investigate whether this would indeed result in a prognostic value. A combined bcl-2/androgen receptor score was of independent prognostic value to predict clinical progression, however.

Loss of expression of the cellular adhesion molecule CD44s was an independent prognostic marker to predict PSA progression and clinical progression in patients treated by radical prostatectomy (chapter III). Yet, it added only little to pT-stage and Gleason grade. The area under the receiver operating characteristic curve (ROC curve, a graph that displays sensitivity against 1 - specificity) increased from 0.76 to only 0.79 in the prediction of clinical progression if CD44s expression was included. This means, as an example, that at a sensitivity of 0.75 the specificity increased from 0.61 to 0.69. Since this was the first study on CD44 expression in clinical prostate cancer, it will be interesting to know the results of other studies. The observation that CD44 expression was completely absent in lymph node metastatic tumor cells deserves further

investigation in patients of whom primary as well as metastatic lesions (lymphatic or hematogenic) are available.

IMPROVEMENT OF PROSTATE CANCER PROGNOSTICATION

How can the assessment of the prognosis of prostate cancer patients be improved? First of all, as described above the interpretation of many studies on prognostic factors is impaired by methodologic shortcomings. Burke and Henson[445, 446] described a number of criteria that reports of clinical outcome studies on prognostic tumor markers should fulfill: a description of the sampling method, a description of the assay and, if applicable, assessments of different types of variability of the assay, a description of cut-off point criteria and whether the cut-off point was selected before the data were analyzed, a description of the subject inclusion criteria, characteristics of the subjects, number of subjects and events, the duration of follow-up and a description and justification of the multivariate model used. If these criteria are met, journal editors should be able to conclude on the scientific quality of a submitted paper. If feasible, it may be worthwhile to perform a meta-analysis on studies of promising markers on which conflicting results have been published (for example, nuclear DNA content). Promising markers should be studied prospectively according to consensus techniques in a multi-center setting. Second, with increasing knowledge about prostate cancer biology new markers that may predict patient outcome will become available and for this reason (and others) basic prostate cancer research is very important. Third, in patients who are not treated with curative intent, repeated marker determinations (response marker) may overcome the problem that the marker has to predict future tumor behavior. Since serum can be obtained very easily, these markers should preferably be serum-based. Fourth, it is essential that potential markers are being tested using multivariate analysis techniques since most markers are correlated with tumor-stage or tumor-grade (chapter I). In addition, pre-operative serum PSA levels, tumor-stage and grade are all strong prognostic indicators for patient outcome. Multivariate analysis techniques are able to identify independent prognostic markers, which implicates having an additional value over the other variables adjusted for.

If a prognostic study identifies a set of markers A,B,C and D as independent prognostic markers than the multi-regression model assumes that all these variables play a role in all patients (see appendix to chapter I). But, it may very well be that in one patient A and B cause progression whereas in another patient it is A and C while in a third patient only D is sufficient to cause tumor progression. It is also possible that a certain marker has only prognostic value in subgroups of patients. Modelling systems should be able to account for this and should thus be flexible. Some recent studies suggest that artificial network analysis could be a useful solution[447, 448]. A neural network system is a form of artificial intelligence which has been described as an electronic analogue of the nervous system[447]. It is too complex to discuss extensively in

this context, but basically, a neural network system 'learns' to distinguish different outcomes of events by receiving information of known events and their outcomes. Theoretically, neural network analysis of prognostic variables should allow the construction of survival probability curves for individual patients. Neural network analysis has several advantages: it is insensitive to missing values, new variables can be easily entered and tested, it is flexible, no specific distribution of data is assumed, proportional hazards are not required (which is a strong assumption of Cox's regression model) and it is model independent. Furthermore, it would allow the inclusion of the TNM/grade variables without the disadvantages of the TNM/grade system as described above. The disadvantages are that it is a complex technique (one of the AJCC criteria of prognostic systems is that they should be easily applicable) and that the underlying model is difficult to interpret. A pilot study has demonstrated that neural network analysis might be useful in prostate cancer screening as well as in predicting prostate cancer prognosis[449].

It is recommended that studies of prognostic tissue markers be performed according to the following scheme:

1. Identification of a potential marker
2. Development, testing and standardization of the assay
3. Application of the marker to easy accessible and well defined samples (e.g., radical prostatectomy specimens) to:
   - study the distribution in benign, pre-malignant and malignant tissues
   - study tissue heterogeneity
   - study the relationship with tumor-stage and tumor-grade.
4. Assessment of prognostic value in radically and hormonally treated patients at the univariate and multivariate level. If possible, including data on the ability of the marker to improve the sensitivity and specificity in predicting clinical progression or tumor death.
5. If an independent prognostic value is found by several independent researchers: assessment of the prognostic value in biopsy specimens using clinical tumor-stage and biopsy tumor-grade. Investigate whether the marker can be used as surrogate end-point marker or response marker.
6. If the independent prognostic value is confirmed: perform prospective multi-center studies using consensus techniques, quantification methods and cut-off values.
7. Perform clinical trials in which the patients are grouped according to the new marker.

In conclusion, potential markers should be identified by basic prostate cancer research and/or from the literature on other tumor types. Investigation of the prognostic value should be done on a trial and error basis according to the scheme described above. For multivariate analysis with a large number of variables flexible modelling systems should

be used. If a marker is to be used clinically, this should be properly studied by the conduction of randomized clinical trials.

NEUROENDOCRINE DIFFERENTIATION

Since the publication of the study described in chapter II (1995), a few additional clinical studies on the prognostic value of NE cells in prostate cancer patients treated by radical prostatectomy have been published, but did, unfortunately, not clarify the picture. Bubendorf *et al.*[199] found no prognostic value in 137 patients, whereas Weinstein *et al.*[450] found an independent prognostic value in 104 patients, but only if the analysis was restricted to the 59 Gleason grade 5 and 6 tumors in which case Gleason grade was of no prognostic value anymore. Both studies found no correlation between CgA immunostaining and tumor grade or stage. Berner *et al.*[143] studied immunohistochemically the NSE expression patterns in the TUR specimens of 80 patients who subsequently underwent pelvic lymph node dissection and either a radical prostatectomy ($T_{1-2}pN_0$) or radiotherapy ($T_{3-4}pN_0$) and did not find a correlation with tumor stage or grade or prognosis. Thus, for surgically treated patients it can still not be concluded whether the extent of NE differentiation is a prognostic factor. Nevertheless, it appears that prostatic NE cells do not have a strong influence on tumor growth or the metastatic cascade.

Two studies showed that 5-HT antagonists[451] and 5-HT uptake inhibitors[452] were able to dose dependently inhibit the growth of prostate cancer cell lines, substantiating the observation that 5-HT may play a role in prostate cancer growth (chapter V). Bombesin (GRP) has been shown to stimulate growth of PC-3 cells (chapter V). A recent study of Aprikian *et al.*[453] identified GRP receptors on PC-3, DU 145 and LNCaP cells. Treatment of DU 145 and PC-3 cells with GRP or GRP analogues resulted in an intracellular Ca^{2+} mobilization, but a growth influencing effect was not observed. A recent immunohistochemical study showed expression of the angiogenic peptides VEGF (vascular endothelial growth factor) and TGF-α[454] in NE cells of benign and malignant prostatic tissues, although the expression was not exclusively limited to the NE cells. Interestingly, a relationship between the presence of NE cells (CgA labeling) and neovascularization (endothelium labeling) of prostate cancer has been described[455]. In addition, VEGF can increase vascular permeability and may thus increase the metastatic ability of tumor cells[454]. Because (NE cell derived) TGF-α binds to the EGF-R which is expressed by prostatic tumor cells (chapter I), it may act as a mitogenic peptide in these cells. This study suggests that NE cells play an additional role in prostatic tumor biology that is distinct from the secretion of neuropeptides or

biogenic amines or from their putative role in androgen independent tumor growth. Expression of TGF-α has also been demonstrated in pure NE tumors (midgut carcinoid tumors, pheochromocytomas and medullary thyroid carcinomas)[456]. It will be interesting to study the prognostic value of VEGF and TGF-α in prostatic tumors.

For hormonally treated patients, NE differentiation appears to have a prognostic value. In addition to the studies already discussed in the chapters II and V, Krijnen *et al.* (accepted for publication) found an independent prognostic value of CgA immunostaining to predict hormonal escape in the pre-treatment TUR specimens of hormonally treated patients. Sheaff *et al.*[457] described a prognostic value of ß-human chorionic gonadotrophin immunostaining, a peptide that is expressed by a subset of prostatic NE cells (chapter V). No defenite conclusions can be drawn from this study since the patient selection and statistical methods were probably inappropriate. A number of studies have pointed towards a relationship between prostatic neuroendocrine cells and the androgen independent phenotype of prostate cancer (discussed in chapters V and VI) and the results of the prognostic studies with hormonally treated patients also support this.

It will be clear that experimental studies are needed in order to clarify the role of NE cells in prostatic (tumor) biology. The two xenograft models described in chapter VI (PC-295 and PC-310) will certainly be of value, especially because the NE phenotype could be induced readily and reproducibly by castration of the tumor bearing mice. In addition, it would be very useful if NE differentiation could also be induced in *in vitro* growing cell lines. Shen *et al.* were indeed able to induce a neuronal phenotype in the LNCaP cell line by culturing the cells without androgens[458]. Currently, attempts are being made in our laboratory to culture PC-295 cells *in vitro*.

The mechanism of the relationship between androgen withdrawal and the induction of NE differentiation is not known. A relationship between estrogen levels and expression of NE features has also been described in the rat pituitary[429, 430] and recently it was shown that estrogen upregulates the expression of 5-HT_{2a} receptors in rat female brain[459]. Thus, steroids appear to influence both the effector and affector parts of the NE system. Knowledge about the mechanism of induction of NE differentiation in prostatic (tumor) cells is important and may reveal essential clinical information.

An issue that has received little or no attention is the definition of the prostatic NE cell. Thus far, most researchers have used immunohistochemistry or Western blotting with antibodies against neuroendocrine cell specific products (CgA, NSE, 5-HT and others). By far, the most often used technique is CgA immunohistochemistry. In the studies described in chapters II and VI the NE cells were also defined by their expression of CgA. In addition, the data of chapter VI were confirmed by electron microscopy. Expression of the above mentioned immunologically defined markers is probably neither specific nor sensitive (G.J.M. Martens, personal communication). Other factors appear more specific and are essential for neuroendocrine function like prohormone convertases (PC1/PC3 and PC2)[460, 461], 7B2[462] and the enzyme peptidyl-

glycine α-amidating mono-oxygenase (PAM)[463]. PAM expression has been demonstrated at the mRNA level in normal rat prostate tissues[464]. A preliminary study with human benign and malignant prostatic tissues showed cytoplasmic expression of PAM at the protein (immunohistochemistry) and mRNA (in situ hybridization) level in a subset of epithelial cells (M.A. Noordzij and A. Treston, unpublished observations). Immunohistochemical double labeling experiments (PAM + CgA) with these tissues showed that PAM was not expressed by the NE cells, but by surrounding non-NE cells. Studies on the expression of the other markers are currently in progress at our institution. Thus, a fundamental discussion of how to define the prostatic NE cell seems warranted.

The questions that have to be studied in the near future are: what are the functions of NE cells in the normal prostate, if any? Do NE cells play a role in BPH? Do NE cells play a role in prostatic carcinogenesis? Is NE differentiation in prostate cancer of predictive value and should patients with large numbers of NE cells in their tumor be treated differently? Is the putative prognostic value of NE cells just related to the number of NE cells or restricted to the expression of one or more active peptides? Do neuroendocrine cells influence prostate cancer growth and if so, which peptides are responsible for this effect and can it be used to target therapy? Do the NE cells play a role in the transition of prostate cancer from androgen dependency to independence? What is the mechanism of increased NE differentiation following androgen withdrawal?

In conclusion, although a considerable number of studies have been published on NE differentiation in prostate cancer, its prognostic value in most patient categories is still controversial. Prostatic NE cells appear to influence tumor growth via several distinct mechanisms and may play a role in androgen independent tumor growth, but experimental data are sparse. Prostatic tumor models that contain these cells are now available and will allow more fundamental studies, but first of all, the prostatic NE cell should be satisfactorily defined.

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SUMMARY

The occurrence of prostate cancer has increased dramatically over the past decade. It is at present the second leading cause of cancer death in the western male population. On average, patients are now diagnosed at an earlier stage of the disease. Most patients with tumors that are clinically confined to the prostate are treated with curative intention (radical prostatectomy or radiotherapy). Patients with advanced disease are usually hormonally treated by medical or surgical castration which leads to a response in about 40-50% of the patients. Almost all these tumors will show progression with time, however, and the resulting hormone independently growing tumors are difficult to treat.

By the application of prognostic markers attempts are being made to predict patient outcome on the basis of tumor and patient characteristics. This should lead to a better selection of the optimal treatment and thereby to improved care. Tumor stage and, to a lesser extent, tumor grade are currently being used for clinical decision making. Nevertheless, it is very difficult to predict the clinical outcome on an individual basis. This thesis describes clinical studies on the application of additional prognostic tissue markers and experimental studies on neuroendocrine (NE) differentiation and tumor cell proliferation.

Chapter I reviews most of the prognostic tissue markers that have been studied in prostate cancer: tumor stage, tumor volume, tumor grade, tissue prostate specific antigen (PSA) levels, circulating PSA mRNA, nuclear DNA content, nuclear morphometry, p53, p21, epidermal growth factor and its receptor, C-erbB-2, E-cadherin, α-catenin, proliferation by counting mitotic figures and expression of proliferation associated proteins (PCNA, Ki-67 and MIB-1), androgen receptor (AR), apoptosis, bcl-2, transforming growth-factor ß1 and neovascularization. Of each marker the following characteristics are described (if applicable): differences between benign and malignant prostatic tissues, heterogeneity within tumors, the prognostic value in different patient groups and the applicability to biopsy specimens. Despite a large number of published studies, no prognostic marker except for tumor stage and grade can be used clinically. This is partially due to the heterogenous nature of prostate cancer.

NE cells are present in normal, hyperplastic and most of the malignant prostatic tissues. The products secreted by these cells may influence tumor growth. The prognostic value of the extent of NE differentiation was investigated in the radical prostatectomy specimens of 90 patients of whom long-term follow-up data were available (chapter II). The results were correlated with tumor stage, grade and the likelihood of clinical progression and tumor related death. NE cells were identified in 78% of the tumors. The extent of NE differentiation did not correlate with tumor stage, grade or prognosis.

The expression of sub-types of CD44 has been related to the metastatic potential of several human tumors. The expression patterns and prognostic value of CD44 were studied in the radical prostatectomy specimens of 97 patients with long-term post operative follow-up (chapter III). The expression was also studied in 12 prostate cancer lymph node metastasis specimens. CD44 expression decreased from benign to pre-malignant to malignant prostatic tissues, and was finally absent in lymph node metastatic tumor cells. Loss of expression of two types of CD44 (CD44s and CD44v6) was correlated with tumor stage, grade and prognosis. If the data were adjusted for differences in tumor grade and pathological stage, CD44s was still a prognostic marker for clinical and PSA based progression. CD44v6 was of no additional prognostic value.

The function of male sex hormones is mediated through the AR. A decreased expression of AR may predict a limited response of prostate cancer to hormonal treatment. This type of treatment induces programmed cell death in prostate cancer cells. This process can be inhibited in cells expressing bcl-2, or other related gene products. Thus, expression of bcl-2 may predict a poor response to hormonal therapy. The prognostic value of bcl-2 and AR expression was studied in tumor specimens of 68 prostate cancer patients that were obtained before the start of hormonal treatment (chapter IV). Neither AR nor bcl-2 expression was correlated with tumor stage, grade or prognosis. But, a combination of bcl-2 and AR was related to the likelihood of clinical progression, even if the data were adjusted for tumor stage and grade differences.

As described above NE cells may influence prostatic tumor growth. Besides, these cells may also play a role in hormone independent growth of prostate cancer. The concept of NE differentiation in normal, benign enlarged and malignant prostatic tissues is reviewed in chapter V with emphasis on experimental aspects. A model of how NE cells can influence prostate cancer biology is also presented.

For basic studies on NE cells in prostate cancer, experimental tumor models that contain these cells are needed. The presence of NE cells was investigated in a number of experimental human prostatic tumors growing *in vivo* (in immune deficient mice) or growing *in vitro* as cell cultures (chapter VI). NE cells were consistently found in two of the *in vivo* models (PC-295 and PC-310). Moreover, in both models the number of NE cells increased if the tumor bearing mouse was castrated. These models are now being used to further study the kinetics and mechanisms underlying NE differentiation.

Tumor growth is the net result of tumor cell divisions (proliferation) and cell death. Ki-67 is an antibody that can be used to microscopically visualize proliferating cells. Its applicability to clinical tumors was thought to be limited to fresh frozen tumor sections whereas routinely obtained tissues are almost always fixed in formalin and embedded in paraffin. The MIB-1 antibody recognizes the same protein as Ki-67, but can also be applied to routinely formalin-fixed and paraffin-embedded tissues. The percentages of proliferating cells as determined by Ki-67 and MIB-1 were compared in experimental prostate tumors (PC-82) that were growing at different growth rates due to hormonal manipulation of the tumor bearing mice (chapter VII). As expected a strong correlation was found between the two percentages, but, strikingly, MIB-1

percentages were about 4 times as high as Ki-67 percentages. In addition, it was found that Ki-67, like MIB-1, can be applied to routinely processed tissues using microwave pre-treatment. Ki-67 percentages determined in this way doubled the frozen tissue based percentages. Since prostate cancer is in general a slowly growing malignancy, the higher scores obtained with MIB-1 may reveal more reliable data.

The general discussion (chapter VIII) gives recommendations about future research on prognostic tissue markers in prostate cancer and addresses the statistical approaches that can be used in this type of research. Also an update of the clinical studies on NE differentiation in prostate cancer and possible future research directions in this field are given.

SAMENVATTING

Het aantal gevallen van prostaatkanker is de afgelopen 10 jaar sterk toegenomen. Het vormt op dit moment de tweede doodsoorzaak door kanker onder mannen in de westerse wereld, alhoewel de gemiddelde patiënt nu wordt gediagnostiseerd met een kleinere tumor dan vroeger. De meeste patiënten met een tumor die beperkt is tot de prostaat, worden behandeld met een in opzet genezende behandeling (radicale prostatectomie of bestraling). Patiënten met een grote of uitgezaaide tumor worden meestal behandeld met castratie door middel van operatie of medicijnen (hormonale therapie). Dit geeft een verbetering in ongeveer 40-50% van de patiënten, maar uiteindelijk gaan vrijwel alle tumoren toch weer groeien. Deze hormoon onafhankelijk groeiende tumoren zijn slecht te behandelen.

Door de toepassing van prognostische markers wordt geprobeerd een goede voorspelling te doen over het klinische beloop van prostaatkanker gebaseerd op patiënt- en tumor-factoren. Dit zou moeten leiden tot een betere selectie voor optimale therapie en daardoor tot een betere patiëntenzorg. De stagering (grootte) en gradering worden momenteel gebruikt bij de klinische besluitvorming. Niettemin is het zeer moeilijk om op individuele basis een goede voorspelling te geven. In dit proefschrift worden een aantal studies beschreven waarin aanvullende prognostische weefselmarkers worden bestudeerd in klinische prostaat tumoren. Verder bevat het experimentele studies naar neuroendocriene (NE) differentiatie en celdeling in prostaatkanker.

Hoofdstuk I geeft een overzicht van de meeste prognostische weefsel markers die zijn bestudeerd in prostaat kanker: stagering, tumor volume, gradering, weefsel prostaat specifiek antigeen (PSA), circulerend PSA mRNA, kern DNA inhoud, kern-vorm metingen, p53, p21, epidermale groeifactor en de receptor daarvoor, C-erbB-2, E-cadherine, α-catenine, celdeling zoals bekeken door het tellen van delende cellen en het aantonen van celdeling geassocieerde eiwitten (PCNA, Ki-67 en MIB-1), androgeen receptor (AR), apoptotische cellen, bcl-2, transformerende groeifactor-ß1 en vaatnieuwvorming. Van elke marker zijn, voor zover van toepassing, beschreven: verschillen tussen goedaardig en kwaadaardig prostaat weefsel, verschillen binnen de tumor, de prognostische waarde in verschillende patiënten-groepen en de toepasbaarheid op biopsieën. Ondanks het grote aantal gepubliceerde studies kan, buiten stagering en in mindere mate gradering niet één van deze markers klinisch toegepast worden. Dit is deels te wijten aan het heterogene karakter van prostaatkanker.

NE cellen zijn aanwezig in normale prostaat klierbuizen en in de meeste prostaat tumoren. De producten die door deze cellen worden uitgescheiden zouden de groei van de omliggende tumorcellen kunnen beïnvloeden. De prognostische waarde van de mate van NE differentiatie van de tumor werd bestudeerd in de radicale prostatectomie preparaten van 90 patiënten die na de operatie langdurig werden gevolgd (hoofdstuk II). De resultaten werden gerelateerd aan de stagering en gradering van de tumor en aan de

kans op tumor progressie of overlijden door de tumor. NE cellen werden gevonden in 78% van de tumoren. De mate van NE differentiatie correleerde niet met stagering of gradering van de tumor en ook niet met de prognose.

De expressie van sub-types van CD44 is gecorreleerd met het uitzaaiend vermogen van verschillende kwaadaardige tumoren. De expressie patronen en prognostische waarde van CD44 werd bestudeerd in de radicale prostatectomie preparaten van 97 patiënten die langdurig gevolgd werden na de operatie (hoofdstuk III). De expressie werd ook bestudeerd in 12 prostaat kanker lymfeklier uitzaaiingen. CD44 expressie nam geleidelijk af gaande van goedaardig naar prostaat intra-epitheliale neoplasie (verondersteld voorstadium van kanker) naar kanker en was uiteindelijk afwezig in de lymfeklier uitzaaiingen. Verminderde expressie van 2 types CD44 (CD44s en CD44v6) was gecorreleerd met stagering en gradering van de tumor en met de prognose. Wanneer de gegevens gecorrigeerd werden voor verschillen in stagering en gradering van de tumoren, bleef CD44s nog steeds van prognostische waarde voor het voorspellen van klinische progressie en PSA gebaseerde progressie.

Mannelijke geslachtshormonen oefenen hun functie uit via de AR. Een verminderde AR expressie zou kunnen betekenen dat hormonale therapie bij die patiënt minder succesvol is. Deze therapie induceert geprogrammeerde celdood in prostaat kankercellen. Dit proces kan geremd worden door de expressie van, onder andere, bcl-2. Een prostaat tumor die bcl-2 tot expressie brengt, zou dus minder gevoelig voor hormonale therapie zijn. De prognostische waarde van bcl-2 en AR expressie werd bestudeerd in tumor materiaal van 68 patiënten dat was verkregen voordat de hormonale therapie werd gestart. De expressie van bcl-2 noch van AR was gecorreleerd met stagering, gradering of met de prognose. Maar, een combinatie van bcl-2 en AR was gerelateerd aan het optreden van klinische progressie, zelfs wanneer de gegevens gecorrigeerd werden voor de stagering en gradering van de tumoren.

Zoals boven reeds werd beschreven, kunnen NE cellen de groei van prostaat tumoren beïnvloeden. Daarbuiten kunnen deze cellen ook een rol spelen in het hormoon onafhankelijk groeien van prostaatkanker. Hoofdstuk V bevat een literatuur overzicht over het concept van NE differentiatie in normaal, goedaardig vergroot en kwaadaardig prostaat weefsel, met nadruk op de experimentele aspecten. Tevens wordt in een model gepresenteerd op welke manieren NE cellen de biologie van prostaat kanker kunnen beïnvloeden.

Voor basale studies naar NE differentiatie in prostaatkanker zijn experimentele tumormodellen die deze cellen bevatten noodzakelijk. Het voorkomen van NE cellen werd bestudeerd in een aantal experimentele prostaat tumoren die groeien in muizen met een slecht functionerend immuun-systeem (*in vivo*), in een weefselkweek flesje (*in vitro*) of beiden (hoofdstuk VI). NE cellen werden aangetroffen in 2 van de *in vivo* modellen (PC-295 en PC-310) en in beide modellen nam het aantal NE cellen toe na castratie van de muis waarop de tumor groeide. Deze modellen worden momenteel gebruikt om experimentele aspecten van NE cellen in prostaatkanker te bestuderen.

Tumor groei is het netto resultaat van tumor celdelingen (proliferatie) en celdood. Ki-67 is een antilichaam dat gebruikt kan worden om delende cellen zichtbaar te maken. De toepassing van Ki-67 op klinische tumoren was beperkt doordat gedacht werd dat het alleen gebruikt kan worden op vers ingevroren weefsels, terwijl in de kliniek vrijwel alle weefsels worden gefixeerd in formaline en worden ingebed in paraffine. Het MIB-1 antilichaam herkent hetzelfde eiwit als Ki-67, maar kan ook toegepast worden op routinematig formaline gefixeerd, paraffine ingebed materiaal. In experimentele prostaat tumoren (PC-82) welke door hormonale manipulatie van de tumordragende muizen verschillende groeisnelheden hadden, werden de proliferatie percentages bepaald door middel van Ki-67 en MIB-1 en met elkaar gecorreleerd (hoofdstuk VII). Zoals verwacht werd een sterke correlatie tussen de percentages gevonden, maar MIB-1 percentages waren ongeveer 4 keer zo hoog als de Ki-67 percentages. Tevens bleek dat ook Ki-67, net als MIB-1, op routinematig verkregen materiaal kon worden toegepast na voorbehandeling in een magnetron. De op deze manier verkregen Ki-67 percentages waren 2 keer zo hoog als de op het ingevroren materiaal bepaalde percentages. Omdat prostaatkanker meestal langzaam groeit, zijn de hogere MIB-1 scores wellicht meer betrouwbaar.

In de algemene discussie (hoofdstuk VIII) worden aanbevelingen gedaan over toekomstig onderzoek naar prognostische markers voor prostaatkanker. Tevens wordt het overzicht van de klinische studies naar NE differentiatie in prostaatkanker geactualiseerd en worden mogelijke toekomstige onderzoeksrichtingen in dit gebied aangegeven.

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CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 6 augustus 1965 te Rotterdam, Pernis. Hij behaalde het VWO diploma aan de christelijke scholengemeenschap Maarten Luther te Rotterdam in 1985. In datzelfde jaar werd een aanvang gemaakt met de studie geneeskunde aan de Erasmus Universiteit te Rotterdam alwaar in 1990 het doctoraal examen en in 1992 het arts examen werden behaald. Van 1993 tot en met 1996 was hij werkzaam als AIO bij de afdeling urologie van de Erasmus Universiteit te Rotterdam onder begeleiding van prof. dr F.H. Schröder, dr G.J. van Steenbrugge en prof. dr T.H. van der Kwast. Gedurende deze periode is dit proefschrift tot stand gekomen. Momenteel werkt hij als arts-assistent algemene heelkunde in het Academisch Ziekenhuis Maastricht (opleider prof. dr G. Kootstra) in het kader van de opleiding urologie welke gedaan zal worden in het Academisch Ziekenhuis Rotterdam (opleider Prof. dr F.H. Schröder) en het Sint Franciscus Gasthuis Rotterdam (opleider dr J.H.M. Blom).

ADDITIONAL PUBLICATIONS

GH Mickisch, MA Noordzij, A van der Gaast, P Gebreamlack, KU Köhrmann, E Mogler-Drautz, H Kupper, FH Schröder: Dexverapamil to modulate vinblastine resistance in metastatic renal cell carcinoma. J Cancer Res Clin Oncol 1995;121suppl3:R11-6

MA Noordzij, GH Mickisch: Dexverapamil to overcome documented vinblastine resistance in metastasized renal cell cancer. In: G Staehler, S Pomer and H Rübben (eds): Basic and clinical urological oncology. Springer Verlag, Berlin, Germany, in press

P Koivistol, J Kononen, C Palmberg, T Tammela, E Hyytinen, J Isola, J Trapman, MA Noordzij, K Cleutjens, T Visakorpi, O-P Kallioniemil: Androgen receptor gene amplification: a possible molecular failure of androgen deprevation therapy in prostate cancer. Cancer Res, in press

VIDEOTAPES

GH Jordan, JLHR Bosch, MA Noordzij, FH Schröder: Operative treatment of Peyronie's disease: demonstration of two different surgical procedures. *EUR / AZR, 1994*

G Pazzerini, JM Nijman, MA Noordzij: Pediatric Urology. *EUR / AZR, 1995*

J DeKernion, GH Mickisch, MA Noordzij: Surgical treatment of renal cell cancer. In preparation