Functional Implications of Neuroendocrine Differentiated Cells in Prostate Cancer

Functionele implicaties van neuroendocrien gedifferentieerde cellen in prostaatkanker

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Abbreviations

ACTH	adreno corticotrophic hormone
AD	androgen dependent
AID	androgen independent
AR	androgen receptor
bFGF	basic fibrobalst growth factor
BrdU	bromodeoxyuridine
7B2	secretogranin V
CgA	chromogranin A
CT	calcitonin
DES	di-ethylstilbestrol diphosphate
DHT	di-hydrotestosterone
DHEA(S)	dehydroepiandrosterone (sulphate)
DRE	digital rectal examination
EGF	epidermal growth factor
ER	endoplasmatic reticulum
5-HT	serotonin/5-hydroxy-tryptamine
IGF-1	insulin-like growth factor
GRP	gastrin releasing peptide
LHRH	luteinizing hormone releasing hormone
MAPK	mitogen activated protein kinase
MoAb	monoclonal antibody
MSH	melanocyte stimulating hormone
NE	neuroendocrine differentiation
NEP	neutral endopeptidase
NSE	neurone specific enolase
PAM	peptidylglycine-alpha-amidating mono-oxygenase
PAMP	pro-adrenomedullin-N-terminal-peptide
PC-(295/310)	prostate cancer
PC1/2	prohormone convertase 1/2
POMC	pro-opio-melanocortin
PSA	prostate specific antigen
RSP	regulated secretory pathway
RT-PCR	reverse transcriptase polymerase chain reaction
SgIII	secretogranin III
SMS	somotostatin
SCLC	small cell lung cancer
SCPC	small cell prostate cancer
T	testosterone
TGF α/β	transfoming growth factor α/β
TNF	tumor necrosis factor
TRUS	transrectal ultrasonography
TURP	trans-urethral resection of prostate
VEGF	vascular endothelial growth factor
VIP	vasoactive intestinal peptide

SCOPE OF THE THESIS

This thesis focuses on NE differentiation in prostate cancer, especially in prostate cancer models. We studied the effects of androgen depletion on the NE differentiated status of in vivo and in vitro prostatic tumor models. Knowledge concerning the function of NE cells in the normal human prostate and in prostate cancer is limited. A number of groups have studied NE differentiation in different epithelial systems. NE cells were found to be present in most prostatic adenocarcinomas (Di-Sant'Agnese, 1994). There is accumulating evidence that NE cells and tumors with NE cells are related to the androgen independent and poorly differentiated types of prostate cancer. There are indications that at least some secretion products of prostatic NE cells, like GRP and 5-HT, affect both prostate cancer growth and possibly also tumor differentiation. However, the growth modulating effects of neuropeptides and their relation to circulating androgen levels and androgen sensitivity of prostate cancer need to be further assessed. Studies directed towards identifying the role of NE cells in hormonally treated prostate cancer may contribute to the understanding of the transition of androgen dependent to androgen independent prostate cancer. There are still unanswered questions that need to be answered: How is the process of neuroendocrine differentiation regulated and can this process be influenced? Are the NE cells necessary for the regulation of growth and differentiation of the prostate or do they play a role in the maintenance of prostatic homeostasis? Can these cells induce (androgen independent) growth of surrounding cells, for instance by ligand independent activation of the androgen receptor (AR) through neuropeptides secreted by NE cells?

The role of NE cells in the progression to androgen independent growth is still unclear and there are not many representative prostate cancer models with NE differentiation. Several groups have been developing prostate tumor models both in vitro cell lines (Romijn, 1996) and in vivo xenografts, like CWR-22 (Nagabhushan, 1996), LuCap (Liu, 1996) and the PC model systems developed at our instituton (van Weerden, 1996). The generally available prostate cancer cell lines, LNCaP, DU-145, PC-3 and TSU-Pr1 lack the potency to differentiate into NE cells. NE differentiation was studied in the panel of in vivo human prostate cancer xenografts available at our Institution. The androgen dependent PC-295 and PC-310 models constitutively have the NE phenotype and these models regress after androgen deprivation. Therefore, they are very suitable for studying the process of NE differentiation in prostate cancer and the role that NE cells may play in progression of prostate cancer. In both the PC-295 and PC-310 models, androgen deprivation induces an increase of the numbers of NE cells. The kinetics of NE differentiation was intensively studied after short-term hormone deprivation in both the PC-295 (Chapter 2) and the PC-310 model (Chapter 4). The process of NE differentiation of androgen dependent cells to NE cells and the consecutive maturation of these NE cells after long-term hormone deprivation in the PC-310 model are described in Chapter 5. In these studies, CgA, AR, PSA and MIB-1 were used as cellular differentiation and proliferation markers, with SgIII, PC-1, PAM and 7B2 as alternative NE markers associated with the regulated secretory pathway (RSP). In addition, the expression of different growth factors like 5-HT, GRP, VIP and VEGF were analyzed before and after androgen deprivation.

The production of a variety of secretory products of NE differentiated cells, such as GRP, CT, VIP, NGF, parathyroid hormone-related protein (PTHrP), neurotensin (NT), IGF-1, may result in paracrine and/or autocrine interactions between the NE and non-NE epithelial cells and the stromal cells in prostate cancer. Therapies that antagonize effects of autocrine or paracrine stimulations of tumor cell growth are currently being developed for small cell lung cancer patients. Such an approach may

also be relevant for the treatment of hormone refractory prostate cancer. A detailed study of the in vitro growth modulating effects of neuropeptides on four prostate cancer cell lines is described in Chapter 3, together with the development of an androgen independent subline of LNCaP cell line which shows an autocrine growth pattern after long-term androgen deprivation.

In Chapter 6, data are presented of clinical and experimental studies on the expression of pro-adrenomedullin N-terminal peptide (PAMP) and peptidyl-alpha amidating-mono-oxygenase (PAM) in prostate cancer. The expression patterns of these NE factors were correlated to patient tumor grade and clinical progression. The expression levels after induction by long-term hormone deprivation were studied in the human prostate cancer xenograft model PC-310.

In the general discussion (Chapter 7), the initial working hypothesis is evaluated that NE cells in a prostatic adenocarcinoma form a subset of primarily androgen independent cells which modulate the growth of neighbouring non-NE tumor cells by the secretion of neuropeptides. This hypothesis will be tested on the basis of our present data on NE differentiation in experimental hormone deprived prostate cancer. Recent publications by others as well as the current rate of developing hormone refractory prostate cancer were included. Finally, new directions and perspectives for future research in the field of NE differentiation and prostate cancer progression are presented.

CHAPTER 1

INTRODUCTION

1.1 The prostate

The normal prostate surrounds the proximal part of the human male urethra just below the urinary bladder and functions as a male accessory exocrine sex gland. The prostate is composed of an epithelial and a stromal part which together form branching secretory tubules and acini. The prostate secretes its products into the urethra. Prostatic development depends on an interaction between epithelial cells and the stroma in the presence of androgens (Cunha, 1994; Lee, 1996; Kooistra, 1997).Testosterone is the major circulating androgen, whereas its metabolite dihydrotestosteron (DHT) regulates prostatic growth and function and the production of seminal plasma. The withdrawal of androgens reduces the expression of prostate-specific genes and induces programmed cell death in exocrine cells which results in a decrease of prostate volume. The human male testes are not the only source of androgens, as the human adrenal glands produce low amounts of androgen precursors like dehydroepiandrosterone (DHEA). Less than 1% of male testosterone levels are derived from these adrenal androgens. The complex process of prostate growth regulation is not yet sufficiently understood. In the prostate several abnormalities are found, including benign prostatic hyperplasia (BPH) and prostatitis as benign prostatic diseases, whereas adenocarcinoma of the prostate, small cell prostatic carcinoma (SCPC), and the very rare prostatic carcinoid are malignant diseases.

1.2 Prostate cancer

1.2.1 Epidemiology and pathogenesis

In the western world, prostate cancer is the most commonly diagnosed malignant tumor and the second cause of cancer deaths in males (Schröder, 1995; Pienta, 1996; Schröder, 1996). The percentage of patients presenting with locally advanced or metastatic disease is still high, despite recent improvements in early diagnosis (Pienta, 1996; Hankey, 1999; Masuda, 1999). Refinements of diagnostic tools, such as plasma prostate specific antigen (PSA), and an increased number of screening protocols has lead to high increases in identification of prostate cancer in the early ninetees. This increase has levelled of to a still high number of new cases identified each year due to the use of PSA as diagnostic marker in an aging population. When prostate cancer is diagnosed earlier, the type of cancer is less advanced and is associated with better prognosis and survival rates for the patients.

According to Pienta *et al* , both molecular and environmental factors influence the course of each individual prostatic tumor. Genetic instability of prostatic tissues can be induced at higher age, through race differences and by differences in diet. Fat content, intake of vitamin A (Aboseif, 1997; Pienta, 1997; Olson, 1998; Smith, 1998) or lycopene, family history of cancer, and hormone levels (testosterone, DHT) have been identified to be related with prostate cancer oncogenesis (Montie, 1994; Pienta, 1996).

1.2.2 Diagnosis and prognostic markers

The early detection of prostate cancer is of high relevance for implementing curative therapeutical options. Given the variable biological behaviour of prostate cancer it has become pertinent to identify those cancers which can be potentially cured by radical surgery or radiotherapy. For this purpose diagnostic and prognostic markers are necessary as different combinations of tumor markers may better predict the final outcome of therapy for the patient (Gao, 1997). Relevant prognostic markers are considered clinically of significance when tested statistically significant in both univariate and multivariate analysis (Noordzij, thesis).

Nowadays, different grading systems are available of which the Gleason grading system is most commonly used as the prognostic marker for prostate cancer (Gleason, 1966; Gleason, 1992). Gleason grading is based on the main histological features of a tumor. There are five Gleason growth patterns ranging from poorly differentiated prostatic acini to undifferentiated, irregular and scattered growth of prostatic tumor cells. Most often, the Gleason Sum Score is used as prognostic marker, being the sum of the two most observed Gleason growth patterns. Other conventional prognostic markers are pathological stage and tumor volume (Epstein, 1993). The most commonly used diagnostic marker is PSA, and the most commonly used secondary tools are digital rectal examination (DRE) and transrectal ultrasonography (TRUS) (Gao, 1997; Gau, 1997; Polito, 1997). PSA has particular prognostic value as a follow-up marker, and together with DRE it is used for large screening protocols. Recently, several other tissue markers have been studied in prostate cancer. The Ki-67-based proliferative index (Bonkhoff, 1991; Bonkhoff, 1994b; Noordzij, 1995b; Glynne-Jones, 1996; Kyprianou, 1996; Bubendorf, 1998; Stapleton, 1998) in combination with i) apoptotic index, ii) tumor suppressor gene p53, iii) the relation with members of the anti-apoptotic bcl-2 family (Segal, 1994; Bubendorf, 1996; Krajewska, 1996; Bonkhoff, 1998) and iv) AR status (Bonkhoff, 1998) has been shown to be of prognostic significance to some extent for clinical progression of the disease. Molecular markers for prostate cancer metastasis are extensively studied like the cell adhesion molecules CD44 (Noordzij, 1997; Noordzij, 1999), E-cadherin (Umbas, 1992; Bussemakers, 1996) located on chromosome 11p and KAI 1 on chromosome 16q (Cheng, 1996; Pienta, 1996; Dong, 1997; Isaacs, 1997). The PTEN/MMAC1 tumor suppressor gene, located on chromosome 10, is of particular interest, because it is frequently lost in prostate cancer (Ittmann, 1996; Murakami, 1996; Vlietstra, 1998; Whang, 1998).

Other prognostic markers of which the relevance has not been assessed definitely could be the expression of epidermal growth factor receptor and its ligands (Glynne-Jones, 1996), altered expression of nuclear proteins like pp32 or NF-κβ (Keller, 1996; Epstein, 1997; Kadkol, 1998) and cell differentiation markers like prostate stem cell antigen (PSCA) (Isaacs, 1989; Mason, 1997; De Marzo, 1998; Reiter, 1998) and the cell cyle arrest marker, $p27^{kip1}$ (Yang, 1998). Chromogranin A, the marker for neuroendocrine differentiated cells has also been studied as a possible prognosticator (Noordzij, 1995a; Noordzij, 1995c; Krijnen, 1997; McWilliam, 1997; Abrahamsson, 1998; Deftos, 1998a; Deftos, 1998b). Finally, intratumoral vascularity or tumor

angiogenesis have been correlated to prostate tumor formation and progression (Fregene, 1993; Weidner, 1996; Lissbrant, 1997; Silberman, 1997). The vascular endothelial growth factor (VEGF) (Ferrara, 1996) is expressed in both prostate cancer patients (Harper, 1996; Jackson, 1997) and in prostate cancer models (Ferrer, 1997; Connolly, 1998; Ferrer, 1998; Muhkerjee, 1999). VEGF is the central growth factor for the formation of novel blood vessels, a process also known as the `angiogenic switch' during tumorigenesis (Hanahan, 1996a; Hanahan, 1996b; Hanahan, 1997).

1.2.3 Therapy of prostate cancer

Resection of organ-confined prostatic tumors by radical prostatectomy or cryo-surgery is the most commonly used strategy for prostate cancer patients, and pending the stage of disease, ` watchful waiting' is also a serious option. There are many potential targets for therapeutic intervention of androgen induced growth of the prostate (Coffey, 1987; Galbraith, 1997). Patients with obstructed urinary outflow due to non-organ-confined prostatic tumors are mostly surgically treated by trans-urethral resection of the prostate (TURP). Surgical castration, or orchidectomy, is often performed as therapy to directly influence the serum testoterone levels. The majority of the patients are chemically castrated by administration of luteinizing hormone releasing hormone (LHRH) agonists or by indirect hormone ablation therapy. This is performed by using anti-androgens like flutamide, zoladex and casodex. Oral estrogens (DES) are very rarely used for this purpose, because of their severe cardiovascular side effects. The human adrenal androgens dehydroepiandrosterone (DHEA) and dehydroepiandrosterone-sulphate (DHEAS) have been shown to activate mutant androgen receptors expressed in androgen dependent prostate cancer (Tan, 1997). Combinations of LHRH analogues and orchidectomy with an anti-androgen are considered to result in very low androgen levels by their blockade of both testosterone and the adrenal androgens, DHEA and DHEAS (Galbraith, 1997). Hormone therapy can be given prior to radiotherapy, TURP or radical prostatectomy to induce regression of the primary tumor.

The effects of androgen deprivation therapy on prostate cancer , i.e. preoperative hormonal therapy and anti-androgen (withdrawal) therapy have been widely studied in prostate cancer patients, using e.g bicalutamide (Casodex), flutamide or Estramustine-phosphate (EMP) (Van de Voorde, 1994; Civantos, 1995; Scher, 1996). Effects of androgen deprivation observed in clinical patients were induction of apoptosis, atrophy, basal cell prominence, and vacuolated luminal cells in the benign non-neoplastic parts of the prostate (Van de Voorde, 1994; Civantos, 1995; Scher, 1996). In the neoplastic prostate, small residual tumor glands surrounded by stroma with or without pyknosis and occasionally unaltered prostatic tumor areas were observed during androgen deprivation (Civantos, 1995). In addition, proliferative activity of the prostate cancers is reduced (Westin, 1995; van der Kwast, 1999).

Almost all clinical prostate cancer will eventually develop hormone therapy resistance which creates a major problem for urologists and scientists. Scher et al (Scher, 1996) described two variants of relapsed hormone refractory disease. These 2

groups include patients with either androgen independent, but hormone sensitive prostate cancer and those suffering from androgen independent, and hormone insensitive disease that is not responding to anti-androgen withdrawal therapy at all. In both *in vitro* cell lines (Limonta, 1992; Egawa, 1996; Esquenet, 1997; Myers, 1997) and *in vivo* models (Isaacs, 1981) androgen independent tumor growth was induced after treatment by androgen deprivation. Isaacs and Coffey stated that hormone refractory prostatic tumor growth was a result of tumor heterogeneity. Clonal expansion of androgen independent cells during hormone therapy will eventually lead to tumor progression (Isaacs, 1981; Coffey, 1987).

1.2.4 Hormone dependence and induction of apoptosis

The difference in the response to anti-androgen hormonal therapy is dependent on the possibility to induce apoptosis in hormone sensitive prostate cancer cells, whereas this is not induced in androgen insensitive cells (Scher, 1996). Apoptosis is a complex process of different cascades, e.g. p53 dependent, caspase activated, Fas/Fas-ligand dependent, etc. These pathways have a number of checkpoints where this process is either induced, blocked or reversed. Some examples of proteins that inhibit induction of apoptosis are high expression levels of the anti-apoptotic Bcl-2 protein (Raffo, 1995) or overexpression of the mutated p53 protein which can determine the status of androgen dependence (Isaacs, 1991; Martikainen, 1991; Denmeade, 1996; Liu, 1996; Lu, 1996; Dorai, 1997; McDonnel, 1997; Rokhlin, 1997; Saeed, 1997). Another observation done after hormone deprivation involves the down regulation of VEGF expression and rapid reduction of blood flow in prostate cancer tissue (Joseph, 1997; Levine, 1998; Shabsigh, 1998). However, this is not a uniform response, as in the Dunning rat R3327 prostate cancer progression model, the levels of VEGF mRNA were elevated after androgen withdrawal (Haggström, 1998).

1.3 Prostate cancer progression

In spite of initial succesful hormonal treatment, many if not all prostatic cancers eventually progress to a hormone therapy-refractory stage after prolonged treatment. Various molecular mechanisms responsible for this escape after treatment of the disease are under extensive study. Effective therapies for these hormone refractory patients are not yet available. With respect to AR involvement in androgen independent growth of advanced prostate cancer several mechanisms may be involved (figure 1).

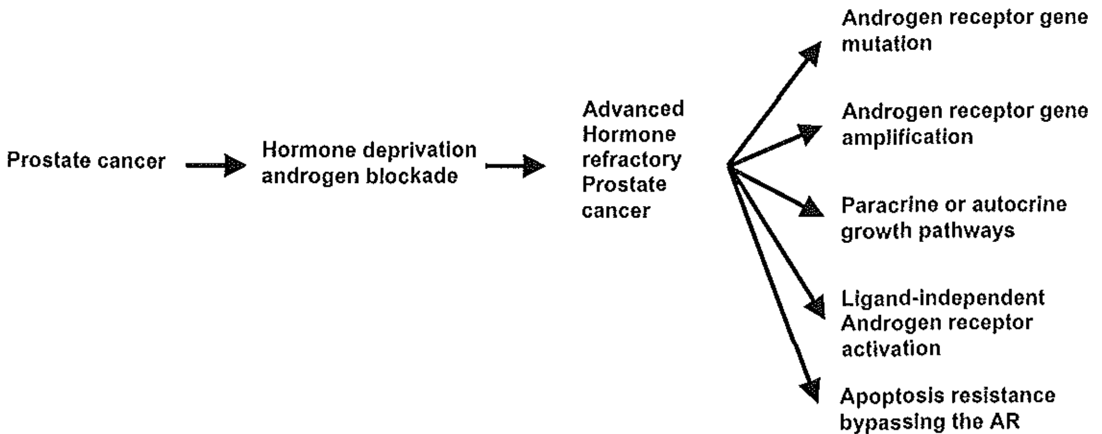

Figure 1: Schematic presentation of different ways of advanced prostate cancer formation after hormonal therapy.

Altered preferences or cross reactivity with other growth factors due to either AR mutations or AR gene amplification, paracrine growth factor induced or ligand-independent activation of the androgen receptor are possible escape mechanisms of hormone refractory prostatic tumors (Ruizeveld de Winter, 1994; Culig, 1998; Koivisto, 1998). Likewise, the inability to induce apoptosis during anti-androgen treatment, i.e. a form of apoptosis resistance, could be a possible escape mechanism. Androgen receptor expression in prostatic carcinoma is modulated by androgen blockade or hormone deprivation (van der Kwast, 1991; Ruizeveld de Winter, 1992; Van der Kwast, 1996). The expression levels of the androgen receptor in advanced hormone refractory tumors differs from patient to patient as the expression levels can be heterogeneous, depending on initial tumor stage, length of therapy and the occurrence of AR gene mutations or amplification (van der Kwast, 1991; de Vere White, 1997; Stanford, 1997; Gil-Diez de Medina, 1998; Gregory, 1998). One study in the PC-82 human prostate xenograft model clearly showed down-regulation of nuclear AR expression shortly after androgen deprivation (Ruizeveld de Winter, 1992) and re-expression after androgen re-supplementation. In the PC-310 human xenograft model spontaneous re-expression of the AR occurs after initial down-regulation following androgen deprivation (Chapter 4 & 5). This temporal down regulation of AR expression in prostate cancer could imply a way to circumvent androgen deprivation induced apoptosis. The cells expressing the AR in high amounts might be more prone to undergo apoptosis than cells that express relatively low levels of the AR. AR expression is probably down-regulated because no more DHT is formed to stabilize the protein, due to the rapidly decreasing serum testosterone levels after androgen deprivation.

The proliferation of prostatic cells has been shown to be regulated by a number of growth factors, mitogens, which are probably produced by the prostatic epithelium itself, e.g. neuroendocrine cells, or by the surrounding stromal tissue (Coffey, 1987; Byrne, 1996; Culig, 1996; Peehl, 1996a; Scher, 1996; Culig, 1997a). Androgen

independent growth of clinical prostate cancer is found by detection of elevated PSA levels (Polito, 1997; Smith, 1998). Androgen independent PSA expression can be induced through autocrine or paracrine mechanisms (Hsieh, 1993; Gkonos, 1996; Walls, 1996). Regulation of prostate cell growth has been reported for epidermal growth factor (EGF) and transforming growth factors TGF-α or-ß (Carruba, 1996; Jones, 1997), basic fibroblast growth factor (bFGF), heparin-binding growth factors, vitamins A and D and insulin-like growth factors (IGF). TGF-ß is a growth factor which can both stimulate and inhibit proliferation (Martikainen, 1990; Desruisseau, 1996; Lamm, 1998). Enhanced expression of TGF-ß or decreased expression of its receptors may therefore be potentially involved in prostate cancer progression (Culig, 1996). Insuline-like growth factors have gained interest in prostate cancer research as high plasma IGF-1 levels have been shown to be present in clinically advanced prostatic carcinoma, whereas IGF is normally found at very low plasma levels (Boudon, 1996; Peehl, 1996b; Quinn, 1996; Chan, 1998; Cohen, 1998; Wolk, 1998). Prostatic carcinogenesis in the Noble rat seems to be dependent on functional IGF and VEGF (Wang, 1998). Other factors that are important in IGF action, like the IGF binding proteins (IGFBP) have also been shown to be produced by androgen independent prostatic cell lines and its effects could be enhanced by LHRH agonists (Srinivasan, 1996; Marelli, 1999).

1.3.2 Paracrine growth modulation in prostate cancer

Different cytokines (Sokoloff, 1996) and neuropeptide growth factors have been implicated in paracrine growth modulation of prostate cancer. Interleukin-6 is an example of a cytokine which has been found in plasma during prostate cancer progression and is produced by the prostatic cancer cell lines, LNCaP, DU-145 and PC-3 (Siegall, 1990; Borsellino, 1995; Twillie, 1995; Hobisch, 1998). Other cytokines are tumor necrosis factor-α (TNF-α), interferon-α and -γ (IFN-α and -γ), interleukin-2 (IL-2) (Sokoloff, 1996), and leukemia-inhibitory factor (Kellokumpu-Lehtinen, 1996). Furthermore, several researchers have shown the growth modulating capacities of T-lymphocyte conditioned medium (Hsieh, 1995) and the bone conditioned medium both of which contain many cytokines (Lang, 1995; Hullinger, 1998; Lang, 1998). Examples of neuropeptide growth modulatory factors are bombesin/gastrin releasing peptide, nerve growth factor, and somatostatin which can have both growth stimulating as well as inhibiting effects (Bogden, 1990; Milovanovic, 1992; Brevini, 1993; Geldof, 1997). The activity of these proteins is strongly dependent on the presence of bioactivating enzymes, like the amidating enzyme peptidyl-glycine-alpha-amidating-mono-oxygenase (PAM) (Treston, 1993; Vos, 1995; Saldise, 1996; Vos, 1996; Prigge, 1997) (Chapter 6).

Directly related to these growth factors is neutral endopeptidase 24.11 (NEP, CD10), a peptidase that can abrogate the growth modulatory effect of neuropeptide growth factors (Cohen, 1996; Krongrad, 1997; Papandreou, 1998) by processing these peptides to smaller inactive peptides.

Testosterone independent activation of androgen receptor can be achieved in different pathways. LHRH is able to synergistically activate the androgen receptor in the presence of low androgen concentrations (Culig, 1997b). IL-6 can activate androgen

receptor mediated prostate specific antigen expression in prostatic carcinoma either ligand-independent or synergistically with the synthetic androgen methyltrienolone (R1881) (Hobisch, 1998). More recently, the possible involvement of the mitogen-activated protein (MAP) kinase pathway in steroid independent activation of the androgen receptor, e.g. through mitogens like EGF or IGF is being studied in advanced hormone refractory prostate cancer (Nazareth, 1996; Zhu, 1997; Chen, 1999; Jenster, 1999; Putz, 1999).

1.4 Prostate cancer models

1.4.1 Model studies

To study factors involved in development and progression of prostate cancer, the establishment and characterization of experimental human tumor model systems is necessary. For years the Dunning and Noble rat prostate cancer models have been used for numerous in vivo studies (Isaacs, 1981; Kadar, 1988; Wang, 1998). Human prostate cancer tissue culturing (Stone, 1978; Kaighn, 1979; Horoszewicz, 1983; Brower, 1986; Lizumi, 1987; Limon, 1990; Bright, 1997) as well as heterotransplantation (Pretlow, 1993; van Steenbrugge, 1994) of human prostate cancer into athymic nude mice, have an important place in prostatic cancer research. These models are applied to study various aspects of human prostatic cancer, such as androgen-regulated cell proliferation and cell death, the role of growth factors in growth and progression, molecular and cytogenetics, tumor metastasis, multidrug resistance, and more. Furthermore, prostate cancer model research focuses at molecular-biological studies on androgen regulated gene expression, DNA in situ hybridization studies to identify chromosomal aberrations in prostatic carcinoma tissues and clinicopathological studies on the phenotypic changes of human prostatic carcinomas during their progression to androgen independence.

The induction of prostate specific tumor models in mice has gained recent interest since the discovery of Cre-mediated recombination dependent conditional knockout models (Akagi, 1997). The principle of this model is cloning of two loxP sites in a gene of interest, e.g. PTEN, used in combination with prostate specific expression of Cre under control of the PSA promotor which might then lead to initiation of murine prostatic tumors. This technique gives many possibilities to investigate the involvemnet of different tumor suppressor genes in the oncogenesis of prostate cancer.

1.4.2 In vitro prostate cancer cell lines

The four most commonly used human prostatic cancer cell lines are LNCaP (Horoszewicz, 1983), PC-3 (Kaighn, 1979), DU-145 (Stone, 1978) and TSU-Pr1 (Lizumi, 1987) which have also been used for the development of orthotopic and xenograft in vivo models; (Rembrink, 1997; Sato, 1997). Likewise, Baley et al used ras and myc transformed murine cell lines, RM-9, RM-1 and RM-2 to set up subsequent orthotopic in vivo models (Baley, 1995). More recently, Romijn et al (Romijn, 1996) established a new cell line, PC-346C from the PC-346 xenograft model.

1.4.3 Human prostate cancer xenograft models

For many years our laboratory has put much effort into the establishment of in vivo human prostatic xenograft tumor models (van Steenbrugge, 1994). Clinical specimens of human prostate cancer were transplanted subcutaneously in athymic nude mice. Subsequent transplantation has finally resulted in 11 serially transplantable prostatic tumor models in athymic nude mice (Noordzij, 1996; van Weerden, 1996). The whole panel is of interest because of the great variety in phenotype and origin of both the AD and AID models. The 10 presently available androgen dependent (AD) and independent (AID) tumor models and their origin are listed below (Table I). Three models have been established over 10 years ago and have been studied extensively. In particular, the PC-82 model is a well known model for studying androgen dependent prostate cancer (Hoehn, 1980; Ruizeveld de Winter, 1992; van Weerden, 1992). More recent research focuses on the AD models, PC-346, PC-295 and PC-310 which are interesting for studying different treatment strategies, e.g androgen deprivation or medical castration with Casodex or flutamide (5-FU) to observe the effects of androgen deprivation on prostatic tumors. The PC-346 model is of special interest for a number of reasons which are the existence of AID sublines, in vitro cultures and the spontaneous incidence of androgen independent tumors. The AID models PC-324 and PC-339 are of interest as progressive models because of the patient status, i.e. hormone refractory under hormonal treatment.

The AD tumors are grown with supplementation of testosterone (T) via silastic implants. The models have different doubling times and lag phases and they have been characterized as reported in detail (van Weerden, 1996). The models represent prostatic tumors with moderately to poorly differentiated and very poorly differentiated growth patterns.

Table I: Overview of the human prostate cancer xenograft models

Model	**Established**	**AD/AID**	**Origin**
PC-82	1977	AD	primary tumor
PC-133	1981	AID	soft tissue metastasis
PC-135	1982	AID	primary tumor
PC-295	1990	AD	lymph node
PC-310	1990	AD	primary tumor
PC-324	1991	AID	palliative TUR-P
PC-329	1991	AD	primary tumor
PC-339	1991	AID	palliative TUR-P
PC-346	1991	AD	palliativeTUR-P (1 week androcur)
PC-374	1992	AID	skin metastasis

TUR-P = trans urethral resection of the prostate

The xenograft models are homogeneous in their hormone responsiveness and they are therefore easy to manipulate, e.g.by removal of T implants or by transplantation of AD tumors to female mice. Tumor volume measurements can easily be performed and blood and tumor tissue can be easily sampled. These data give the researcher potential information about prostatic tumor behavior during androgen deprivation.

More in vivo models have been developed by other groups representing both xenograft models, like UCRU-PR-2 (Haaften-Day, 1987; Jelbart, 1988; Jelbart, 1989), CWR22 and its sublines (Pretlow, 1993; Wainstein, 1994; Nagabhushan, 1996; Agus, 1999) as well as transgenic mice models, like CR2-T-Ag (Garabedian, 1998) and Gγ/T or ßh1/T (Perez-Stable, 1996; Perez-Stable, 1997).

Primary cultures from xenograft tissues (Limon, 1990), makes it possible to study effects both *in vivo* as well as *in vitro*. The in vitro cultures have the advantages that they are easier to manipulate and that a lot of cells can be generated without too much burden to living animals. Furthermore, the comparison with the in vivo situation is very well possible as we will show in Chapter 4.

1.5. Neuroendocrine differentiation

1.5.1 Neuroendocrine cells: characteristics and origin

Most glandular epithelial systems, like the gastro-intestinal tract, bronchi and the prostate are constituted of a basal cell layer, which separates the luminal epithelial cells from the stromal cells. In the glands, the luminal cells are divided in two types, exocrine luminal cells and neuroendocrine (NE) cells, which both secrete into the lumen. Epithelial systems are initiated from a stem cell compartment (Mason, 1997; De Marzo, 1998; Reiter, 1998). The stem cells have several unique characteristics, like expression of Bcl-2, specific cytokeratins and the cell cyle arrest marker $p27^{kip1}$. Xue et al studied neuroendocrine differentiated cells in the prostatic epithelium with respect to cytokeratins and Bcl-2 (Xue, 1997; Xue, 1998b). They found that there were different subgroups of serotonin (5-HT) positive NE cells that showed expression of cytokeratins specific for either stem cells, cq basal cells or luminal cells. This meant that prostatic NE cells may either differentiate directly from prostatic stem cells or after several cell divisions from luminal prostatic cells (Xue, 1998a).

NE cells appear in two types, namely an open type reaching out to the lumen and a closed type which is captured in between the basal and exocrine luminal cells, but does not secrete into the lumen. NE cells can be identified by immunohistochemistry (Pearse, 1969; Azzopardi, 1971) through their affinity for silver (Argentaffin) or with specific antibodies against secreted NE products like serotonin (5-HT) (Xue, 1997) and gastrin-releasing protein (GRP) or against secretion-associated granins, like Chromogranin A (CgA) (O'Connor, 1986; Abrahamsson, 1989; Schmid, 1994; Hendy, 1995), which is the commonly used marker for NE differentiation.

Most studies on human NE differentiation have been performed in NE differentiated systems, like lung, pancreas and intestine. In a few studies NE differentiation was induced by several methods (Mabry, 1989; Pfeiffer, 1989; de Bruine,

1993; Bang, 1994). In a colorectal cancer model (NCI-H716) showing NE differentiation in vivo, NE differentiation could be induced by culturing the non-NE tumor cells in the presence of native extracellular matrix, fibroblast layers or in a defined medium with basic fibroblast growth factor (de Bruine, 1993). Transfection of v-ras^{H} into DMS-53 small cell lung cancer cells resulted in a cell line with increased NE features (Mabry, 1989). Transfection of both c-raf-1 and c-myc oncogenes into SV-40 immortalized bronchial epithelial cells revealed a large cell carcinoma with a NE phenotype (Pfeiffer, 1989). Subsequently, these investigators were able to establish heterotransplantable xenografts from these carcinoma cell lines with a comparable phenotype (Pfeiffer, 1991). Thus, on the one hand stromal-epithelial interactions are essential and on the other hand expression of certain (proto) oncogenes are associated with the induced level of NE differentiation. These experiments might be important in the study of prostate cancer since no prostate cancer models are available at the moment which show NE differentiation *in vitro*.

1.5.2 Neuroendocrine cells: the regulated secretory pathway

In principle, the NE cells synthesize, store and release peptide signaling molecules in accordance with the physiological demands of an organism. For this purpose the NE cells have a unique regulated secretory pathway (RSP) (Holthuis, 1995; Huttner, 1995; Holthuis, 1996b; Shennan, 1996; Kaether, 1997). Many features of this pathway are still poorly understood, but the RSP has been carefully characterized in the South-African clawed toads, *Xenopus laevis* which were used for their capacity to adapt their skin colour to a black background (Holthuis, 1995; Van Horssen, 1995; Braks, 1996; Holthuis, 1996b). This has led to a better understanding of the process of regulated secretion in NE cells. The central protein produced by the RSP in *Xenopus laevis* is called proopiomelanocortin (POMC) which also plays an important role in the hypothalamo-pituitary-adrenal axis. The POMC peptide hormone production starts at the level of the endoplasmatic reticulum (ER), where the protein is translocated, *N*-glycosylated, phophorylated and folded. The proteins are transported from the ER to the Golgi complex, where they will traverse the cis-, medial-, and trans-cisternae. Post-translational modifications, a.o. the remodelling of *N*-linked oligosaccharide side chains and the stepwise biosynthesis of *O*-linked glycans. Furthermore, protein sulphation at tyrosine residues takes place at the trans-Golgi network. The trans-Golgi network is involved in the correct distribution of all secretable proteins over the different pathways. For the RSP, the peptide precursor proteins are sorted and packaged into secretory granules which is followed by subsequent proteolytic maturation. Next to CgA, the secretory granules can be identified by different granins, like Secretogranin III (SgIII) and Secretogranin V (7B2) (Martens, 1989; Sigafoos, 1993; Braks, 1996). Enzymes involved in this maturation are the prohormone convertases PC1 and PC2, carboxypeptidase H and the amidating enzyme PAM. Final secretion of bioactive peptides is dependent on an as yet unknown extracellular stimulus. During the whole RSP procedure, POMC is clipped C-terminally to form endorphins, melanocyte stimulating hormone (α-, ß-MSH) and adrenocorticotropic hormone (ACTH). α-MSH is responsible for initiating the *Xenopus laevis* skin colour

transformation. Evaluation of markers of the RSP is ongoing in *X. laevis*, human lung and prostate cancer (Huttner, 1995; Eib, 1996). Markers of NE differentiation, a.o. those of the RSP or the PAM marker are currently being evaluated for additional prognostic value in prostate cancer.

1.5.3 Neuroendocrine cells: neuropeptides

A number of neurosecretory products of NE prostatic tumor cells, like serotonin (5-HT), gastrin releasing peptide/bombesin (GRP), calcitonin (CT), vasoactive intestinal peptide (VIP) (Mack, 1997) and somatostatin (SMS) exhibit growth factor activity mediated by a membrane receptor. This may represent a way of paracrine growth modulation (Willey, 1984; Dalsgaard, 1989; Seuwen, 1990). Most of the prostatic adenocarcinoma cells surrounding NE cells contain the AR. It was found in COS cells transfected with steroid hormone receptors that dopamine activated several steroid hormone receptors (progesteron, estrogen, vitamin D and thyroid hormone-ß receptors) in a ligand-independent fashion (Power, 1991). In this way NE tumor cells might stimulate the growth of neighbouring non NE tumor cells by androgen independent activation of the AR in a paracrine manner. Bonkhoff *et al.* studied the relation between proliferation and NE differentiation at the immunohistochemical level and found in normal, hyperplastic and cancerous prostatic tissues that proliferating cells were usually, but not necessarily, located in proximity of NE cells (Bonkhoff, 1991) suggesting paracrine growth regulation (Hoosein, 1996). This is supported by results of Aprikian et al, who think that NE cells may promote progression and androgen independence of prostate cancer through the action of secreted gastrin releasing peptide (GRP) (Aprikian, 1996; Aprikian, 1997; Han, 1997; Aprikian, 1998).

Serotonin (5-HT)

Serotonin is well known as neurotransmitter and vasoactive substance. Some recent reports indicate that serotonin has growth factor activity (Seuwen, 1988; Abdul, 1994; Abdul, 1995). Serotonin can stimulate DNA synthesis in hamster fibroblasts which is mediated by activation of the 5-HT_{1b} receptor (Seuwen, 1988). This receptor is only expressed in rodents. Growth experiments with antagonists or uptake inhibitors of serotonin showed direct inhibition of the growth of prostatic tumor cells lines (Abdul, 1994; Abdul, 1995; Hoosein, 1996). Serotonin might also influence tumor growth indirectly by changing the local blood flow in a tumor due to its vasoactive action. The fact that serotonin is expressed and secreted by most if not all prostatic NE cells makes it an interesting neuropeptide to study in prostatic cancer NE cells, although it is as yet not known if non-NE prostatic tumor cells contain 5-HT receptors.

Gastrin releasing peptide (GRP)

Gastrin releasing peptide (GRP) or bombesin stimulated the growth of cultured gastric carcinoma cells (Bold, 1994) and normal bronchial epithelial cells (Willey, 1984) and *in vivo* small cell lung cancer (SCLC) models (Alexander, 1988; Chu, 1996; Rogers, 1997) in a dose dependent manner. *In vitro* studies with cultured pulmonary NE cells

demonstrated that treatment with GRP increased the number of NE cells and stimulated their serotonin expression, while treatment with serotonin did not (Speirs, 1993). This effect could be blocked by antibodies against GRP. It has also been shown that GRP stimulated growth of small cell lung cancer cells (Cuttitta, 1985). This effect could be blocked *in vivo* by monoclonal antibody 2A11 (Moab 2A11), an antibody against GRP (Avis, 1991; Kelley, 1997) and *in vitro* by GRP analogues which prevented binding of GRP to its receptor (Cuttitta, 1985; Mahmoud, 1991). *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 (Bologna, 1989). Saturable GRP binding sites were demonstrated on PC-3 cell membranes, but in this case no immunoreactivity against GRP was demonstrated (Bologna, 1989). However, GRP immunoreactivity was demonstrated in PC-3 cells in other studies (Kaighn, 1979; Hoosein, 1993). The bombesin/GRP antagonist RC-3095 was able to inhibit the growth of the androgen dependent PC-82 *in vivo* and the androgen independent DU-145 *in vitro* or *in vivo* as xenograft (Milovanovic, 1992; Pinski, 1993). In both models, saturable GRP binding sites were demonstrated (Milovanovic, 1992; Pinski, 1993). Furthermore, the signal transducing mechanism of the interaction of GRP and GRP-like peptides with prostatic cancer cells has well been studied (Zachary, 1993; Aprikian, 1996; Aprikian, 1997; Han, 1997; Wasilenko, 1997). This has resulted in either a Ca^{2+} or a tyrosine phophorylation dependent signal transduction pathway leading to differences in cell motility (Aprikian, 1997). All together, evidence exists that GRP can play a potential role in androgen independent growth of prostate cancer (Siegfried, 1994; Aprikian, 1998).

Calcitonin (CT)

Calcitonin was demonstrated in a subset of normal and neoplastic prostatic cells (Abrahamsson, 1987; Davis, 1989; di-Sant'Agnese, 1989; Aprikian, 1993; Deftos, 1998a). In conditioned medium of cultures of prostate cancer cells immunoreactive CT was found in 4-fold concentration compared to cultures of BPH cells (Shah, 1992). *In vivo* administration of salmon CT to rats induced ornithine decarboxylase (a key enzyme associated with cell cycle progression and growth) in a number of organs (Nakhla, 1987). It should be realized that serum CT also decreases the pituitary secretion of luteinizing hormone which is an important mediatior of androgen secretion (Leicht, 1974). In addition, the secretion of prolactin, which is thought to mediate the action of androgens on the prostate, was also found to be decreased by CT (Bloom, 1979). Therefore, pituitary mediated growth inhibitory effect of CT on prostate cancer growth might be possible. The direct growth modulating effects of CT have been studied in a few tumor systems. In vitro growth of T 47D breast cancer cells was dose dependently inhibited by CT (Iwasaki, 1983; Ng, 1983) by inducing increased intracellular cAMP levels. Comparable results were found in KATO III cells, a human gastric carcinoma cell line (Nakamura, 1992). In a study with six human renal adenocarcinoma cell lines four showed increased cAMP levels upon CT administration (Kinoshita, 1985). These four lines were also growth inhibited by CT, while the two lines that did not show a cAMP response displayed no growth inhibition. In a study on a panel of small cell lung cancer

cell lines, two out of 13 expressed CT and one other cell line was able to bind CT (Bepler, 1988). In this study no growth effect of CT was observed. Recently, a few experimental studies on CT in prostate cancer showed that CT was able to stimulate growth of prostate cancer cell lines through cAMP signal tranduction and induced Ca^{2+} levels (Shah, 1994; Ritchie, 1997).

Somatostatin (SMS)

Abrahamsson et al found somatostatin immunoreactivity in 12 out of 40 prostatic adenocarcinomas (Abrahamsson, 1987), a result that could not be confirmed in another study, however (Aprikian, 1993). In a study of Reubi *etal*, neither in 17 prostatic carcinomas, nor in 2 BPH specimens SMS receptors were found (Reubi, 1987). A number of experimental studies concerning the growth modulating effects of SMS in prostate cancer have been published. The androgen sensitive *in vivo* Dunning R-3327 rat prostate cancer model or its subline R-3327H were studied for the effects of SMS analogues. In two studies two different SMS analogues (sandostatin and somatuline) were injected in R-3327H tumor bearing mice resulting in growth inhibiting effects of both SMS analogues (Siegel, 1988; Bogden, 1990). In one of these studies this potentiated the castration induced growth inhibition even after the tumors became androgen independent (Bogden, 1990). In another study this extra growth inhibiting effect was not confirmed, however (Siegel, 1988). Treatment of R-3327H bearing mice with the SMS analogue, RC-160, a luteinizing hormone-releasing hormone (LH-RH) analogue (D-TRP^6 LH-RH) or a combination of both resulted in growth inhibition, with the strongest inhibition found in the combined treatment (Kadar, 1988). This was associated with down-regulation of both the SMS and prolactin receptors, the strongest down-regulation being found after the RC-160/D-TRP^6 LH-RH combined treatment. As prolactin probably has a stimulating effect on prostate cancer, down regulation of the prolactin receptor by SMS might explain the growth inhibiting effects. In the PC-82 xenograft model the combination of SMS analogues (RC-121, RC-160) with an LH-RH analogue (D-TRP^6) gave a stronger growth inhibition than treatment with a single analogue (Milovanovic, 1992). In addition, PSA, insulin like growth factor I, and growth hormone serum levels were greatly reduced in the PC-82 tumors following treatment. SMS receptor positive prostatic adenocarcinoma were succesfully treated with ^{188}Re-RC-160, a directly-radiolabeled somatostatin analogue (Zamora, 1996). *In vivo* experiments with the SMS receptor positive DU-145 (Stone, 1978) tumor also demonstrated a growth inhibiting effect of SMS analogue RC-160 (Pinski, 1993). Following treatment, serum levels of growth hormone and gastrin decreased and the epidermal growth factor receptor was down regulated. *In vitro* experiments with the androgen dependent LNCaP (Horoszewicz, 1983) demonstrated a direct proliferation and protein synthesis inhibiting effect of SMS (Brevini, 1993). This effect was dose dependent and reversible. All these results strongly suggest a growth modulating effect of somatostatin (analogues) in prostate cancer.

1.6 Neuroendocrine differentiation in the prostate

1.6.1 Neuroendocrine differentiation in normal and neoplastic prostate

NE cells are identified in normal prostate and prostate cancers (Azzopardi, 1971; Kazzaz, 1974), although the reported percentages of prostatic tumors with NE cells vary from 30% to almost 100% (Bono, 1985; Abrahamsson, 1987; Abrahamsson, 1989; Cohen, 1990; Aprikian, 1993). NE cells have been localized in nearly all clinical prostatic adenocarcinomas (Abrahamsson, 1986; di-Sant'Agnese, 1992b; Aprikian, 1993; Bonkhoff, 1995; di-Sant'Agnese, 1995; Noordzij, 1995a; Noordzij, 1995c). NE cells have also been identified in neoplasia of many other epithelial organs like breast, lung, stomach, and pancreas (Grube, 1986; Gould, 1988; Helman, 1988; Lehrer, 1988; Linnoila, 1988; Nesland, 1988; Blumenfeld, 1996; Linnoila, 1996).

According to di-Sant'Agnese, NE differentiation in prostate cancer may be found in adenocarcinoma, small cell prostate cancer (SCPC), the rarely found prostatic carcinoid, mixed tumors of these 3 types. It may also be evident in prostate cancer with ectopic or eutopic production of neuroendocrine hormones and in prostatic malignancies associated with paraneoplastic syndromes (di-Sant'Agnese, 1992a). Undifferentiated small cell cancers, or NE carcinomas, mostly occur in the lung. SCPC is a relatively rare disease accounting for ± 5% of all prostate cancers (Schron, 1984; Ro, 1987; Tetu, 1987; Oesterling, 1992). It is a highly malignant disease with a very short survival time of 7-17 months. In about half of the SCPC patients the small cell component is preceded by a common adenocarcinoma and also mixed tumors were found (Tetu, 1987; Oesterling, 1992). Not all SCPC showed the NE phenotype, however (Ro, 1987).

NE cells in prostatic tumors are most often recognized by their immunoreactivity for NE markers (CgA, NSE) or eutopically expressed bioactive substances (serotonin, gastrin releasing peptide or bombesin, calcitonin, and somatostatin). In addition to the eutopic neurosecretory products, a number of ectopic peptides have been found, e.g. adrenocorticotropic hormone (ACTH), leu-enkephalin and ß-endorphin (Abrahamsson, 1987). Expression of these factors, especially ACTH, can cause Cushing's syndrome as found in some prostate cancer patients (Matzkin, 1987).

The most common expression pattern of NE differentiation found in prostate cancer is prostatic adenocarcinoma with scattered NE cells. The number of NE cells within a tumor varies from patient to patient and the prognostic significance of their presence is still a matter of debate. In some studies a relation between the tumor grade and the number of NE cells was found (Abrahamsson, 1987; Abrahamsson, 1989; Cohen, 1991; Schmid, 1994; Cussenot, 1998). In other studies this could not be confirmed, however (Aprikian, 1993; Noordzij, 1995a; Noordzij, 1995c). NE cells were also identified in lymph node and bone metastases of prostatic adenocarcinomas (Aprikian, 1993; Aprikian, 1994).

The possible correlation between tumor grade and the number of NE cells found in individual tumors may well account for part or all of the reported prognostic value of NE differentiation in prostate cancer (di-Sant'Agnese, 1998a; di-Sant'Agnese, 1998b). Markers that show prognostic value in univariate statistical analysis also have

to be tested in multivariate analysis in order to be proven as an independent marker for prostate cancer (Noordzij, 1995a; Noordzij, 1995c). Clinical follow up studies have been performed that deal with the correlation using this multiple regression analysis. Most of these studies on the prognostic value of CgA for NE differentiation did not show a correlation between CgA expressing NE cells in prostatic adenocarcinomas and bad prognosis or clinical progression of prostate cancer (di-Sant'Agnese, 1992b; Aprikian, 1993; Cohen, 1994; di-Sant'Agnese, 1995; Noordzij, 1995a; Noordzij, 1995c; Casella, 1998). One study showed significant correlation of NE differentiation of prostatic tumors before treatment with progressive behavior in hormonally treated patients (Krijnen, 1997).

Kadmon et al demonstrated elevated CgA serum levels in 12 out of 25 patients with metastatic, hormone insensitive prostate cancer (Kadmon, 1991), although PSA levels were in the normal range in 4 of these 12 patients. Others found comparable results with respect to elevated CgA levels in serum of hormonally treated patients (Cussenot, 1996a; Theodorescu, 1997; Cussenot, 1998; Wu, 1998b) or in NE tumors (Kimura, 1997). Serum levels of another NE marker, neuron specific enolase (NSE), were found to be increased more often in patients with hormone refractory tumors than in patients with hormone sensitive tumors (Tarle, 1991).

Abrahamsson et al studied NE differentiation in repeated biopsy specimens of patients treated with hormonal therapy or with radiotherapy (Abrahamsson, 1989; Abrahamsson, 1996). They generally observed increasing numbers of NE, i.e. CgA positive, cells during follow-up paralleled by cellular dedifferentiation and hormonal escape, i.e. progression, of the tumor. Other studies showed that androgen deprivation in clinical prostate cancer induced both NE differentiation and increased expression of the proliferative marker Ki-67 (Van de Voorde, 1994; Guate, 1997; Jiborn, 1998; Pruneri, 1998). One study showed growth arrested tumors with differential expression of the androgen receptor (AR), but no induction of NE differentiation was found upon androgen deprivation therapy (Reuter, 1997). These results raise the question whether NE cells in prostate tumors are androgen sensitive or not.

Immunohistochemically defined NE cells in both normal and hyperplastic prostatic tissue did not show immunoreactivity against the AR (Bonkhoff, 1993; Krijnen, 1993). One of these studies reported that prostatic NE tumor cells very rarely contained the AR (Bonkhoff, 1993) while in the other study all NE tumor cells were AR negative (Krijnen, 1993). It is generally believed that NE cells in normal or neoplastic prostate do not contain the AR. However, in one study prostatic NE cells as well as tumoral NE cells usually showed immunoreactivity against the AR (Nakada, 1993). In this latter study, as compared to the two former studies, a different antibody against the AR was applied. Although it has been shown that the AR content of a prostatic tumor does not predict androgen (in)sensitivity (van der Kwast, 1991; Van der Kwast, 1996), it is unlikely that AR negative tumor cells will respond to androgen withdrawal.

Neuroendocrine (NE) cells are considered to be non-proliferating cells which do not express the androgen receptor (Krijnen, 1993) and therefore are assumed to be unaffected by androgen deprivation. NE cells show a heterogeneous cytokeratin

expression pattern as there are basal, luminal and intermediate NE cell types (Bonkhoff, 1994a; Bonkhoff, 1994b; Xue, 1997; Xue, 1998a). Xue et al recently showed no co-expression of 5-HT with the anti-apoptotic oncogene Bcl-2 (Xue, 1997), thus showing that NE cells are non-proliferating, androgen independent cells which do not have an active Bcl-2 pathway. In addition, NE cells are often found near Bcl-2 positive prostate cancer cells (Colombel, 1992; Cohen, 1994).

1.6.2 NE differentiation in human prostatic xenograft models

Previously, the NE differentiation status of the human prostate cancer xenograft models and the initial patient material was studied for CgA by Noordzij et al (Noordzij, 1996) (Table II). The number of NE cells found in the PC-295 and PC-310 xenograft models closely resembles the expression pattern of NE differentiation found in clinical prostatic tumors. The PC-295 prostate cancer xenograft model showed induced NE differentiation only after androgen deprivation. PC-295 showed fast regression and restimulation of growth by androgens failed already within 3 weeks after androgen withdrawal. The PC-310 model showed a similar pattern of induction of NE differentiation after androgen deprivation, but in contrast to PC-295 this tumor shows slower regression and formation of tumor residues, which can be restimulated by androgens even after longer periods of androgen deprivation (Noordzij, 1996; van Weerden, 1996). In PC-346, PC-374 and PC-324, NE differentiation had been found in the patient material and the first passages of the xenografted tissue in nude mice. No NE differentiation was observed in PC-82, PC-133, PC-135 PC-329 and PC-339. Evaluation of the complete panel of available xenografts with a more sensitive staining protocol, enabled us to detect CgA positivity in recent passages of PC-324, PC339, PC329, and only small numbers in PC-82 tumor tissue of both androgen supplemented as well as the androgen deprived status. We think that the PC-324 model is a small cell prostate cancer (SCPC) tumor model. Pathologically, the tumors showed features of SCPC, like poor differentiation, Gleason sum scores of 9-10 and expression of CgA. Furthermore, the tumor cells overexpresses p53 and Bcl-2, which are typical characteristics for SCPC. RT-PCR analysis for CgA, SgIII and PC-1 mRNA confirmed that PC-324 is indeed a neuroendocrine tumor (unpublished results).

Table II: Human prostate cancer xenograft models

Model	Year	Origin	AD	Neuroendocrine Patient	Differentiation Xenograft
PC-82	1977	Primary tumor	+	–	–
PC-EW	1981	Lymph node	+	+++	+
PC-133	1981	Bone	–	–	–
PC-135	1982	Primary tumor	–	–	–
PC-295	1990	Lymph node	+	++	+
PC-310	1990	Primary tumor	+	+	+
PC-324	1991	TUR-P	–	+++	– (LP +++)
PC-329	1991	Primary tumor	+	–	–
PC-339	1991	TUR-P	–	–	–
PC-346	1991	TUR-P	+	∀	– (LP ∀)
PC-374	1992	Skin meta	–	–	– (LP + or ∀)

Neuroendocrine Differentiation (= immunoreactivity against Chromogranin A)
TUR-P = trans urethral resection of the prostate, meta = metastasis,
AD = androgen dependence, LP = low passage on nude NMRI mice

CHAPTER 2

KINETICS OF NEUROENDOCRINE DIFFERENTIATION IN AN ANDROGEN DEPENDENT HUMAN PROSTATE XENOGRAFT MODEL

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ABSTRACT

It was previously shown in the PC-295 xenograft that the number of chromogranin A (CgA) positive neuroendocrine (NE) cells increased after androgen withdrawal. NE cells did not proliferate and differentiated from G_0-phase arrested cells. Here we further characterized NE differentiation, androgen receptor status and apoptosis-associated Bcl-2 expression in the PC-295 model after androgen withdrawal to assess the origin of NE cells.

PC-295 tumor volumes decreased by 50% in 4 days. Intraperitoneal BrdU incorporation and MIB-1 labeling decreased to 0% and the apoptosis was maximal at day 4. Androgen receptor expression and PSA serum levels decreased rapidly within 2 days. The number of NE cells increased six-fold at day 4 and thirty-fold at day 7. Five and ten percent of the CgA positive cells were BrdU positive after continuous BrdU labeling for 2 and 4 days, respectively. However, no MIB-1 expression was observed in CgA positive cells. NE cells expressed the regulated secretory pathway marker secretogranin III, but were negative for androgen receptor and Bcl-2. Bcl-2 expression did increase in the non-NE tumor cells.

In conclusion, androgen withdrawal leads to a rapid PC-295 tumor regression and a proliferation independent induction of NE differentiation. The strictly androgen independent NE cells that were still present after 21 days differentiated mainly from G_0-phase arrested cells.

INTRODUCTION

Neuroendocrine (NE) cells form an androgen independent subfraction of the prostate(Abrahamsson, 1996). NE cells have been localized in nearly all clinical prostatic adenocarcinoma with different expression patterns(Abrahamsson, 1986; di-Sant'Agnese, 1992b; Aprikian, 1993; Bonkhoff, 1995; di-Sant'Agnese, 1995; Noordzij, 1995a; Noordzij, 1995c). These cells produce various growth modulating neuropeptides in a paracrine or autocrine way(Seuwen, 1990; Iwamura, 1994b; Abdul, 1995; Aprikian, 1996; Solano, 1996; Zia, 1996; Mack, 1997).

Possible roles for NE cells in the prostate may be regulation of homeostasis and secretion of prostatic fluid, either actively or passively. NE cells can be identified by routine electron microscopy (dense core granulas) or by immunohistochemistry with specific antibodies against secreted products for example serotonin(Xue, 1997) or secretion associated proteins, like Chromogranin A (CgA)(Abrahamsson, 1989; Schmid, 1994; Hendy, 1995), which is a marker for neuroendocrine differentiation. NE cells are considered to be non-proliferating cells and do not express the androgen receptor(Krijnen, 1993) and are therefore probably unaffected by androgen deprivation. Consequently, they will not undergo apoptosis under such circumstances. Therefore, it is relevant to assess whether or not CgA positive cells co-express the anti-apoptotic oncogene Bcl-2(Cohen, 1995). Moreover, NE cells show a heterogeneous cytokeratin expression pattern as there are basal, luminal and intermediate NE cell types(Bonkhoff, 1994b; Bonkhoff, 1994a; Xue, 1997) and are often found near Bcl-2 positive prostate cancer cells(Colombel, 1992; Cohen, 1994).

The single expression of CgA is not the only requisite for the determination of NE differentiated cells as there exists a regulated secretory pathway (RSP) in NE cells(Holthuis, 1996b) next to the lysosomal and an exocrine constitutive pathway. Along the RSP pathway secretion and processing of bioactive neuropeptides and growth hormones, like insulin and glucagon in the pancreas(Smeekens, 1992; Tucker, 1996), are regulated. The RSP consists of a sequence of processes linked from transcription/translation of various factors to final secretion of neuropeptides at the plasma membrane from secretory granules(Holthuis, 1995). Different markers can be identified, such as granular markers Secretogranin III (SGIII) and Secretogranin V (7B2)(Martens, 1989; Sigafoos, 1993; Braks, 1996), peptidylglycine α-amidating monooxygenase (PAM)(Vos, 1995; Vos, 1996), the processing enzymes prohomone convertase 1 and 2 (PC 1 & 2), carboxy peptidase E. Evaluation of these specific markers of the RSP is ongoing in X. Laevis, lung and prostate cancer(Huttner, 1995; Eib, 1996).

The number of prostate cancer cases still increases and the search for a curative therapy for metastasized cancer continues. NE cells have been found in most prostatic adenocarcinomas and the role of NE cells in the progression to androgen independent growth is still unclear. Fundamental questions related to a possible role for NE differentiation in the progression of prostate cancer can only be solved by using representative prostate cancer models with NE differentiation. Several groups have recently been developing tumor models both in vitro cell lines(Romijn, 1996) and in vivo xenografts(Liu, 1996; Nagabhushan, 1996; van Weerden, 1996). NE differentiation has been studied in a panel of 11 in vivo human prostate cancer xenograft models. Some models do not express the NE phenotype and PC-324 and PC-346 lost their NE phenotype after a few passages in nude mice(Noordzij, 1996). The PC-295 and PC-310 models are androgen dependent models of which a part of the cells constitutively show the NE phenotype. Therefore, these two models are very suitable for studying NE differentiation in prostate cancer and the role that NE cells may play in the progression

of prostate cancer. In both the PC-295 and PC-310 models androgen deprivation leads to an increased number of NE cells. In a previous study with the androgen dependent PC-295 model(Noordzij, 1996), tumors continuously labeled with the S-phase marker BrdU for two days prior to castration showed no double labeling of NE cells after castration with BrdU. This shows that the increased NE phenotype is not induced prior to castration. Absence of BrdU and another proliferation marker, MIB-1, shows that NE cells are non proliferating and thus G_0 phase arrested cells prior to androgen withdrawal. However, it can not totally be excluded that proliferation occurring after androgen withdrawal is responsible for the increase in NE differentiation in the PC-295 model.

In this study, we further looked for the origin of NE cells by focusing on the kinetics of the process of NE differentiation. Furthermore, we characterized changes in apoptosis and subpopulations of prostatic epithelial cells as marked by their expression of CgA, SgIII, growth modulating neuropeptides, androgen receptor and Bcl-2 after androgen withdrawal in the PC-295 model both shortly after castration and after prolonged androgen deprivation.

MATERIALS AND METHODS

PC-295 xenograft model

The nude mouse human prostate cancer xenograft model PC-295 was established from a pelvic lymph node metastasis(van Steenbrugge, 1994; van Weerden, 1996). The tumors usually grow with a doubling time of about 14 days and a lag phase of 2-3 months. The model represents a strictly androgen dependent and moderately differentiated tumor, histologically organized in solid sheets and microacini. In short, PC-295 tumors were implanted subcutanuously at both shoulders of intact nude NMRI males, obtained from the breeding colony of the Erasmus University Center for Animal Research. Optimal growth conditions were reached by supplementation of PC-295 transplanted mice with testosterone implants, as previously described (van Steenbrugge, 1984). The subcutaneous tumors developed within 2-3 months and were grown up to a maximum volume of 2000 mm^3. Tumor volume changes were followed weekly by two perpendicular diameter measurements (D1 and D2) after which the volume was calculated from the formula: $V=(\Pi/6)(D1 \times D2)^{3/2}$.

Castration experiments with the PC-295 human prostate cancer xenograft model

Two consecutive castration experiments were performed with 39 testosterone-supplemented PC-295 bearing male NMRI mice in total. Androgen withdrawal was performed by castrating the mice under hypnorm anaesthesia (Janssen Pharmaceuticals, Oxford, UK) and by removing the silastic implant. The mice received bromodeoxyuridine (BrdU) to check the effect of androgen withdrawal on the proliferative activities in the PC-295 tumor tissue. One group of mice received BrdU (1mg/ml) intraperitoneally (IP) as standard labeling for one hour before sacrifice. The second group received BrdU (30mg/ml) via subcutanuously inserted osmotic Alzet micropumps (Alzet 1007D, Alza Corp., Palo Alto, CA) having a flow rate of 0.5 µl/hour as of the moment of castration until sacrifice. Three mice were sacrificed at each time point after castration. For the first experimental set-up, these time points were 0, 0.5, 1, 1.5, 2, 4, 7, 14 and 21 days, whereas in the second set-up, mice received BrdU for 0, 1.5, 2, 4 and 7 days after castration.

After blood samples were taken for determining serum prostate specific antigen levels, mice were sacrificed. Tumor volumes were measured and tumors were

removed. The tumors were cut into small pieces which were either fixed in 4% buffered formalin and paraffin embedded for immunohistochemical analysis or snap frozen in liquid nitrogen and stored at - 80°C for biochemical analysis. The paraffin embedded material was processed routinely for hematoxylin and eosin staining.

Immunohistochemistry

To identify the fraction of cells expressing the NE phenotype, paraffin embedded tissue sections of the PC-295 xenografts were stained immunohistochemically with antibodies against CgA (clone LK2H10; ICN Pharmaceuticals, Aurora Ohio) and SGIII (rabbit polyclonal antibody; Department of Animal physiology, University of Nijmegen) (Holthuis, 1996a). For identification of the proliferative capacity, tissue sections were stained with antibodies against BrdU (clone IIB5, Eurodiagnostics, Apeldoorn, The Netherlands) and against the proliferation associated Ki-67 antigen (MIB-1, Immunotech, Marseille, France). In addition, apoptotic cells were identified by the TUNEL-method (TdT-Kit, Boehringer Technologies Mannheim, Germany). Other antibodies used were directed against the androgen receptor (clone F39.4, kindly provided by Dr. A.O. Brinkmann, Department of Endocrinology and Reproduction, Erasmus University); the cytoplasmatic Bcl-2 antigen (clone 124, Dako, Glostrup, DK) and against the neuropeptides bombesin (MoAb 2A11; kindly provided by Dr F. Cuttita, NCI, Bethesda, USA), serotonin (5HT; rabbit polyclonal antibody), vasoactive intestinal polypeptide (VIP, rabbit polyclonal) and Calcitonin (rabbit polyclonal).

Paraffin embedded xenograft tissues were cut at 4 µm sections for single immunostaining and 2 µm for double immunostaining. The sections were mounted on 3-amino-propyl-triethoxysilane coated glass slides and incubated overnight at 60°C. The slides were deparaffinized through xylene and 100 % ethanol and endogenous peroxidase activity was blocked with 3.3% hydrogen peroxide (H_2O_2) in methanol for 10 minutes. After rinsing with tap water and demi, the slides were placed in 10 mmol/L citrate buffer (pH 6.0). Antigen retrieval was then performed in a microwave at 700 watt for an initial 12.5 minutes and a subsequent 5.5 minutes(Shi, 1991). The slides were allowed to cool down to room temperature and then rinsed with phosphate buffered saline (PBS). The tissue slides were then put into the sequenza immunostaining system (Shandon, Uncorn, UK). All slides were preincubated with normal goat serum (DAKO, Glostrup, DK) diluted 1:10 in PBS, which yields for all compounds used hereafter, for 15 minutes. The primary antibody was incubated at the appropriate concentration for 2 hours at room temperature or overnight at 4°C. The secondary antibody was incubated for 30 minutes, being either horseradishperoxidase conjugated goatαmouse or goatαrabbit (1:50), or biotynilated goatαmouse and goatαrabbit (1:400) for monoclonal and polyclonal antibodies, respectively. In case of biotinylated goatαmouse and goatαrabbit, a horseradishperoxidase streptavidin biotin complex diluted 1:1:200 in PBS, prepared at least 30 minutes prior to use, was incubated for a subsequent 30 minutes. Between the subsequent steps, the slides were rinsed four times with PBS. The bound horseradishperoxidase was visualized in 10 minutes with diaminobenzidine (Fluka, Neu-Ulm, Germany) in PBS containing 0.075% H_2O_2 as substrate. Slides were rinsed extensively in tap water and finally counterstained in Mayer's Haematoxylin, dehydrated through a series of alcohol and embedded in malinol.

To assess the properties of the NE cells, double staining of CgA respectively with BrdU, MIB-1, androgen receptor and Bcl2 was performed on the PC-295 xenograft tumors. The double staining consists of two consecutive stainings with two primary antibodies. The first staining was always an horseradishperoxidase-related stable diaminobenzidine complex, whereas the second staining was performed with an alkaline phosphatase conjugated goatαmouse secondary antibody. The alkaline

phosphatase was visualized in 30 minutes incubation of the slides with AS-MX-phosphate (0.3mg/ml; Sigma) mixed with new fuchsin (2.5μl/ml; Sigma), $NaNO_2$ (1.45 mmol/L; Sigma) and levamisole (0.5 mg/ml; Sigma) in the dark. All compounds were diluted in 0.2 mol/L TRIS buffer (Gibco) adjusted at pH 8.0. In between both stainings, the slides were rinsed with PBS for one hour and again boiled in a microwave in 10 mmol/L citrate buffer (pH 6.0) for 10 minutes. For immunostaining of incorporated BrdU, the slides were treated with 2N HCl at 37°C for 30 minutes to uncoil DNA and subsequently neutralized with a 0.1 mmol/L sodium tetra borate buffer (pH 8.5). As negative control, PBS replaced the primary antibody in all stainings. Radical prostatectomies, containing normal prostatic tissue were used as positive control for CgA, SgII, androgen receptor, Bcl2 and Ki-67 expression.

For all markers, except androgen receptor, the number of positive cells were determined by quantitative counts of all cells in tumor squares at 310x magnification from which the number of positive cells per square mm was calculated. In total, positive cells were scored as percentage of the total cell number, in $\geq$ 25 squares. For the androgen receptor, the level of immunostaining was assessed semi-quantitatively.

Western Analysis

We further confirmed the expression patterns of immunohistochemically determined CgA, SGIII and androgen receptor in our castration series of PC-295 by Western immunoblotting. As positive controls, we used material of human pheochromocytoma for CgA, of rat pituitary for SGIII and of the human in vitro cell line LNCaP for androgen receptor expression. The procedure of protein extraction was used as previously described(Noordzij, 1996). Frozen tissues of the PC-295 xenografts were crushed in a liquid-nitrogen-chilled metal cylinder. The tissue homogenates were transferred into a lysis buffer (10mM TRIS (pH 7.4), 150 mM NaCl (Sigma), 1 % Triton X-100 (Merck, Germany), 1 % deoxycholate (Sigma), 0.1 SDS (Gibco), 5 mM EDTA (Merck) and protease inhibitors (1mM phenylmethylsulfonyl fluoride, 1mM aprotinin, 50 mg/L leupeptin, 1 mM benzamidine and 1 mg/L pepstatin; all from Sigma). After centrifugation of the mixture at 100,000 rpm at 4°C for 10 minutes, the protein content of the supernatants was measured by the Bradford method (Bio-Rad protein assay, München, Germany).

20 μg of each sample was transferred to an SDS polyacrylamide gel and gel electrophoresis was performed with prestained markers as size standards (Novex, San Diego, CA). The gels were blotted to a 0.45μm cellulose nitrate membrane (Schleicher & Schuell, Germany). The immunoblot was blocked with PBS (pH 7.7) containing 0.1% Tween-20 (Sigma) and 5% dry milk for one hour. The CgA, androgen receptor or SGIII antibodies were added in their optimal concentration and incubated overnight on an orbital shaker at 4°C. After rinsing four times 15 minutes with PBS, incubation for one hour was performed with the secondary horseradishperoxidase-conjugated antibodies and goatαmouse for mouse monoclonal, goatαrabbit for rabbit polyclonal antibodies, respectively. Subsequently, a short incubation with a mixture of 10 ml luminol and 100 μl oxidizing agent (BM chemiluminescense kit, Boehringer Mannheim GmbH, Germany) followed, after washing for four times 15 minutes with PBS. Excess reagent was removed and antibodies were visualized by exposure of the blots to an X-ray film.

Reverse Transcriptase-polymerase chain reaction

RNA was isolated by using the single step RNAzol™ B method (Campro, The Netherlands(Chomczynski, 1987). Frozen tissue (100mg) was homogenized in 1 ml of RNAzol™ . Chloroform (0.1ml) was added and the mixture was vortexed for 15 seconds followed by 5 minutes incubation on ice. The homogenate was then centrifuged at 4 °C

at 12,000 g for 15 minutes. The upper water-phase containing the RNA was removed and mixed with an equal volume of isopropanol. This mixture was then kept at 4 °C for 15 minutes and after that centrifuged at 4°C at 12,000 g for 15 minutes. The supernatant was removed and the RNA pellet was washed twice with 75 % ethanol by vortexing and centrifugation at 4°C at 12000 g. The pellet was then dried and resuspended in sterile H_2O. The concentration was determined at OD 260 and solutions of 1µg/µl were prepared for further use in Reverse Transcriptase-polymerase chain reaction (RT-PCR). The quality of the isolated RNA was checked by determining the 260/280 ratio and by formaldehyde gel-electrophoresis to check the ribosomal (28 S and 18S) bands.

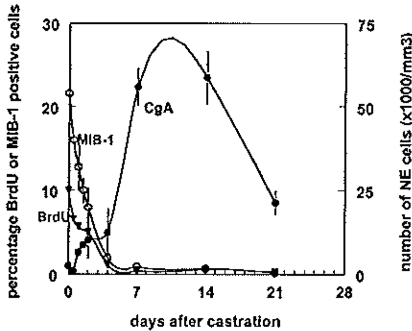
RT-PCR was performed for CgA, SGIII, PAM, PC1 and 2 and ß2-microglobulin (ß2MG) with a standard protocol. Reverse transcriptase reaction was performed with a mastermix containing 5 mM $MgCl_2$, PCR buffer, 10 mM dNTP's, RNAse inhibitor (10 units), Reverse transcriptase (25 units), 2.5 mM random hexamer primers and 0.5 µg RNA in a total volume of 10 µl covered with 50 µl of mineral oil. The mastermix was then processed at 42°C for 60 minutes followed by a 15 minutes incubation at 99°C and the reaction stopped at 4°C for five minutes. The cDNA mix that was formed was then used totally with the mastermix of the polymerase chain reaction (PCR) protocol. In this protocol, the mastermix contained reaction buffer, Goldstar Red DNA polymerase (Eurogentec, Seraing, Belgium; 1 unit), 15 µM sense and antisense primer in a total volume of 40 µl. All samples were first denaturated at 94°C for 10' and then amplification was performed for 35 cycles of 1' 94°C, 1' 60°C and 1' 72°C and a final extension at 72°C for 10'. The PCR product was checked on an 1% agarose gel and, if necessary, followed by Southern blotting.

RESULTS

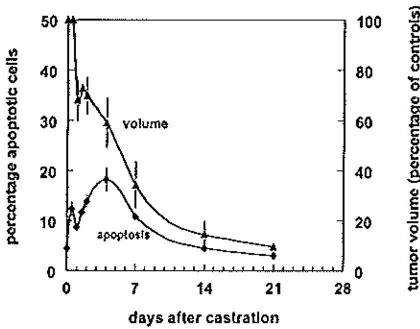
A decline in proliferative activity (***Figure 1A***), a rapid and persistent loss of androgen receptor expression (***Figure 1C***), as previously shown for the PC-82 xenograft model(Ruizeveld de Winter, 1992), and an increase of apoptosis in the PC-295 human prostate cancer xenograft is associated with a rapid tumor volume decrease (***Figure 1B***) after androgen withdrawal. The percentage of BrdU incorporating cells after one hour IP administration decreased rapidly from a basal percentage of 10 in the controls to nearly zero at day four after castration. Accordingly, the MIB-1 expression decreased from an initial percentage of 20 in the controls to near zero at day four after castration. Within a period of two days, the expression of androgen receptor in about 90 % of PC-295 cells disappeared. The percentage of apoptotic cells increased from the moment of castration to a maximum at day four, when about 20 % of the tumor cells were apoptotic. The prostate specific antigen serum levels paralleled the dramatic tumor volume decrease, and decreased rapidly after castration to zero after 7 days (***Figure 1C***).

Androgen withdrawal also leads to an immediate increase in the proportion of NE cells. The number of NE cells, as marked by the expression of CgA, increased from an initial 1,000 cells per square mm to 60,000 NE cells per square mm at day seven after castration (***Figure 1A***). The course of CgA expression in time is given in ***Figure 2*** in which the immunostainings of the different time points are shown. The typical granular staining for CgA increased clearly and positive cells were still observed 21 days post-castration. The NE cell was the main tumor cell type found already from day 7 post-castration on, as determined by immunohistochemistry for both CgA and SgIII.

1A : proliferation and NE differentiation



1B : apoptosis and tumor volume



1C : AR expression and PSA level

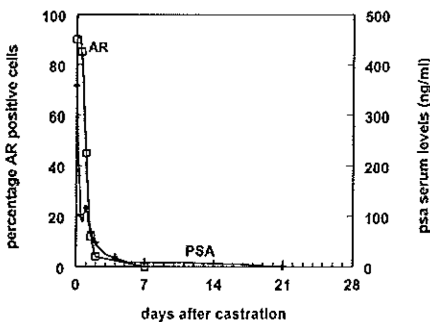


Figure 1: Monitoring of proliferation, apoptosis, NE differentiation and tumor volume changes in the PC-295 model after androgen withdrawal.
A) Results of the immunohistochemical staining for proliferation (BrdU, ↯(Mice received BrdU IP one hour prior to sacrifice.); MIB-1, **O**) and neuroendocrine differentiation (CgA,●). Proliferation is shown in mean percentage positive cells scored per 1000 cells (n=3), whereas the NE differentiation is shown as the number of CgA positive cells per mm^3. One-way ANOVA showed that both BrdU and MIB-1 decreased in time (K-W, $p<0.05$). At each time point mean values are given (n=3).
B) Results from volume measurements (▲) after castration and the immunohistochemical staining for apoptosis by TUNEL (◆) Apoptosis is shown as percentage of positive cells scored per 1000 cells. Tumor volume decrease was significantly different from controls (t-test, $p < 0.05$). At each time point mean values are given (n=3).
C) Results from prostate specific antigen serum measurements (PSA,□) and immunohistochemical staining for the androgen receptor (AR, +; scored as pecentage/1000 tumor cells) for the different time points post-castration.

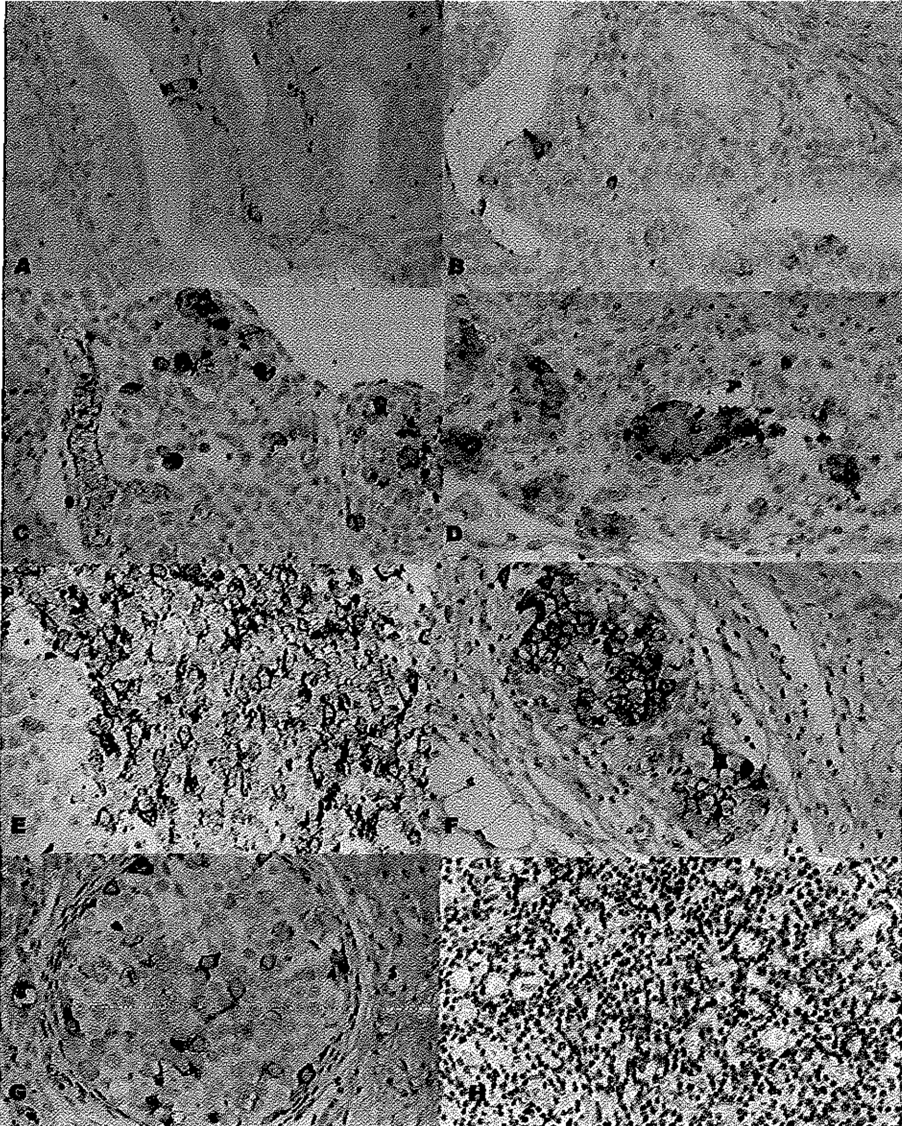
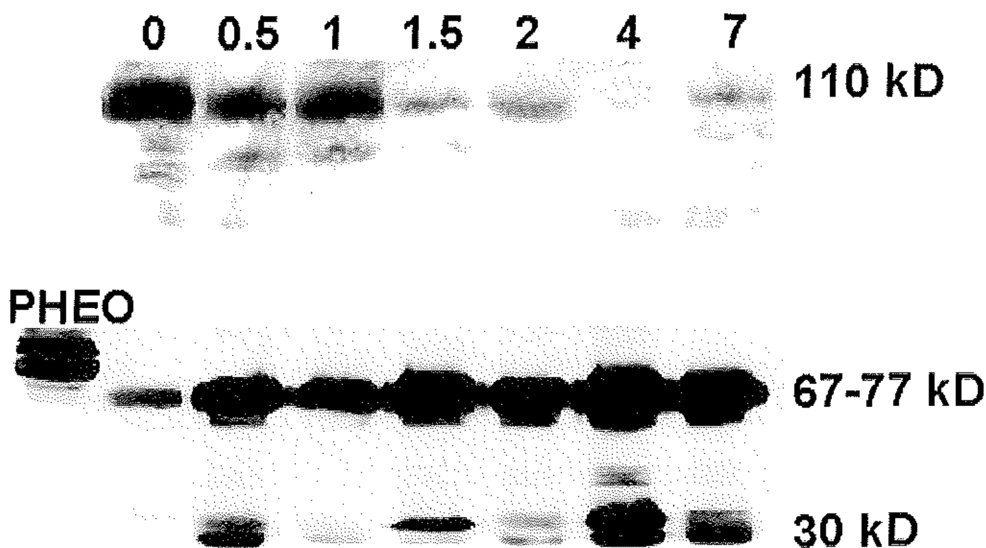


Figure 2: Immunohistochemical staining for CgA in the PC-295 model at different time points post-castration.
A) Control tissue of a poorly differentiated PC-295 tumor with one NE cell; B) PC-295 tumor 1 day after castration, with one NE cell (open type); C) PC-295 tumor 2 days after castration, with dispersed NE cells; D) PC-295 tumor 4 days after castration, with a cluster of NE cells; E) PC-295 tumor 7 days after castration, with a large number of NE cells F) PC-295 14 days after castration; G) PC-295 tumor 21 days after castration; and H) H & E staining of the PC-295 tumor in the control situation

It was clear that the characteristic cribriform growth pattern of untreated PC-295 tumors disappeared around four days post-castration, finally resulting in a solid tumor largely composed of islets of CgA positive cells surrounded by murine stromal cells. Increased expression of the neuropeptides serotonin and bombesin was detected in part of the CgA positive cells at 7, 14 and 21 days post-castration (Data not shown). Increased expression of somatostatin receptors was seen 4 and 7 days post-castration, whereas expression of calcitonin or VIP was not detected at all. The PC-295 tumor cells showed increased expression of Bcl-2 from 4 days post-castration on. The Bcl-2 expression was found in non-NE cells at day 14 and 21 post-castration in predominantly CgA and SgIII immunostained tumor fields.

Western blot analysis confirmed the immunohistochemical results, showing the castration-induced increase of CgA expression associated with a decreased expression of the androgen receptor (***Figure 3***). The specific CgA 68 kD signal and the amount of smaller 30 kD CgA derived peptide clearly increased after androgen withdrawal, whereas 112 kD AR expression was clearly lost two days after castration. An increased expression of 63/61 kD SGIII (***Figure 4***) was observed from 7 days post-castration on The amounts of the processed forms of SG III, as indicated by the 48, 28 and 20 kD fragments, decreased after androgen withdrawal.



FIGUUR 3: Expression of the androgen receptor (AR) and Chromogranin A (CgA) in the PC-295 model following androgen withdrawal analyzed by Western blotting.
Both panels show the time points T_0, $T_{-0.5}$, T_{-1}, $T_{-1.5}$, T_{-2}, T_{-4} and T_{-7}. In the upper panel the decreasing signal of androgen receptor expression is found at 110-112 kD. The CgA signal can be found between 66 and 75 kD in the lower panel, which are the lowest bands in the control Pheochromocytoma (Pheo). Both panels also show the processed proteins or degradation products.

Immunohistochemical double labeling of PC-295 tumors clearly showed absence of any expression of MIB-1 in CgA positive cells. For BrdU labeling however, a different result was observed. Tumors of mice injected with BrdU one hour prior to sacrifice did not show BrdU labeled CgA positive NE cells. Continuously BrdU labeled tumors respectively showed 5% and 10% double labeled NE cells at day two and four post-castration, while 40% and 70% of the whole cell population was BrdU positive,respectively.

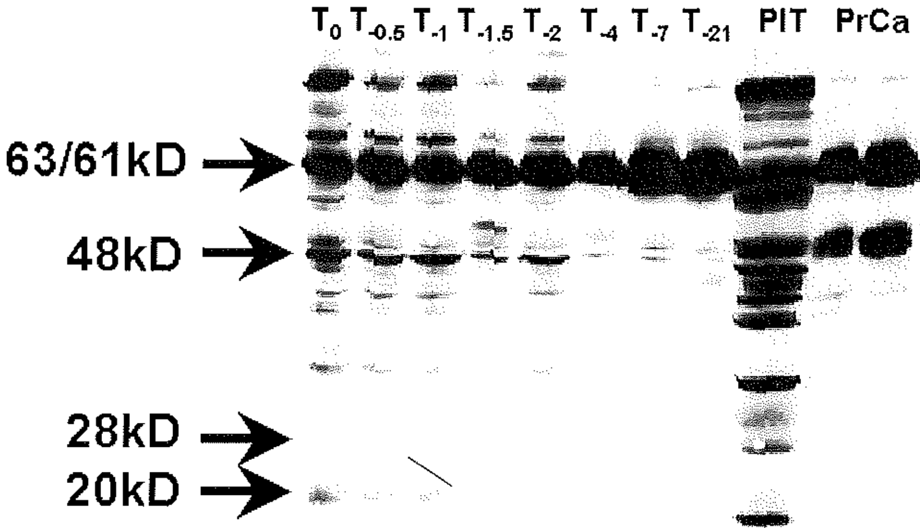


FIGURE 4: Expression of Secretogranin III in the PC-295 model following androgen withdrawal analyzed by Western blotting.
Secretogranin III is expressed as 63/61 kD (upper arrow) double band in prostate carcinoma (PrCa), pituitary (Rat) and in the PC-295 models at all indicated time points. The processed forms of the protein are also visible at the lower bands of 48 kD, 28 kD and 20 kD (compare the pituitary lane)

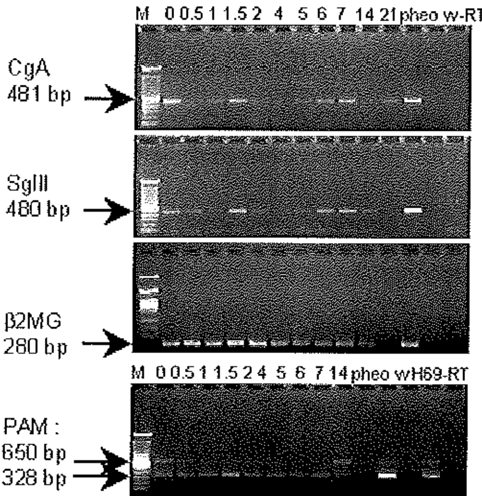


Figure 5: Results of RT-PCR for NE markers in the PC-295 model postcastration
Expression patterns of CgA, SGIII, β2MG and PAM are shown for the different time points in the tumors post-castration. The lengths of the PCR products are given. H69 is a small cell lung cancer cell line, and -RT and w are controls for reverse transcriptase and water, respectively.

RT-PCR with human specific CgA and SGIII primers demonstrated a clear increase of the NE phenotype in PC-295 after four days of androgen withdrawal by using β2MG expression as internal human control (***Figure 5***). No significant variation in PAM expression was noted after castration, as indicated by two alternatively spliced mRNA variants.

DISCUSSION

In this report, we extend the previous observations done in the PC-295 human prostate cancer xenograft model and describe in more detail the characteristics of NE differentiation in prostate cancer under hormonal regulation. The growth and regression of the PC-295 human prostate cancer xenograft in the presently described castration experiment was comparable to the result of earlier studies performed with this model(van Weerden, 1996). The tumor doubling time was 13 days and the half-life time of tumors after castration was 4.3 days, which was within the same range as found in earlier experiments. After 21 days of androgen withdrawal only 10 % of the initial tumors (200 in stead of 2000 mm^3) was left, so the PC-295 tumor model regressed rapidly after androgen withdrawal. Both Western blot analysis and immunohistochemistry showed a rapid decrease in androgen receptor expression in the PC-295 tumors(Noordzij, 1996), comparable to the behavior of the fully androgen dependent human PC-82 xenograft after castration(Ruizeveld de Winter, 1992). This was parallelled by a clear and rapid decrease of the prostate specific antigen serum levels, clearly demonstrating the androgen dependent character of the PC-295 model.

The immunohistochemical results showed a rapid decrease of BrdU and MIB-1 positive cells from 10% and 20% in the controls to about 0% in tumors at day 4 and 7, respectively. Both nuclear proliferation markers showed a 50% decrease two days post-castration. The TUNEL staining for apoptosis showed a maximum of apoptotic cells at four days after castration. The NE phenotype was clearly and rapidly induced at 4 days after androgen withdrawal at which time point there were only a few proliferating cells left.

Analysis for CgA by Western and RT-PCR confirmed the increased expression of the CgA protein and RNA in time from a basal expression level in the controls to a maximum at 7 and 14 days post-castration. There is considerable variation in the basal level of NE cells in the androgen supplemented situation, which could be explained by an inter-tumoral variation between different mice, which seems to be associated with environmental, e.g. host-derived factors, rather than that it is due to an intrinsic tissue heterogeneity. Expression levels of the NE phenotype in the different tumors were confirmed by comparing the immunohistochemical picture with the Western and RT-PCR data at the different time points. In spite of inter-tumoral differences in CgA expression levels at the moment of castration, a clear increase in the mean value of NE differentiation was observed 4, 7 and 14 days post-castration. RT-PCR analysis showed that expression of CgA mRNA initially decreased following androgen withdrawal where after it increased again after day 4 when the tumor cells did not proliferate. The difference between immunohistochemistry and RT-PCR from day 0 to day 4 is most likely caused by inter-tumoral variation in basal CgA expression levels. Another explanation is that the levels of CgA mRNA are higher in NE cells of the controls compared to NE cells directly after androgen withdrawal.

The induction of NE differentiation after androgen withdrawal has now clearly been shown. To further characterize NE cells and the process of NE differentiation we are testing other and possible early markers of NE differentiation. In the PC-295 model, we detected increased expression of SGIII at both protein level by Western blotting and

immunohistochemistry and at mRNA level as determined by RT-PCR analysis. This clearly indicates that the regulated secretory pathway (RSP) is active in these prostatic NE cells. Holthuis et al were able to clone the various components of the genetic route of the RSP in Xenopus laevis(Holthuis, 1995), and found several NE markers that are co-expressed with SgIII, like Ac45 and X7365 for the RSP. The expression of CgA is not a requisite for regulated secretion of bioactive neuropeptides, as CgA is just a general marker for NE cells and not functionally involved. It will be relevant to study RSP related proteins, like SgIII and PC1 which are currently being studied, to establish whether neuropeptides can be actively secreted by NE cells in the PC-295 model.

No changes in the PC-295 model were found in PAM-mRNA expression levels as determined by RT-PCR. PAM was expressed as three splice variants which was similar to the expression pattern of PAM in lung cancer cell lines(Vos, 1995). PAM is not necessarily expressed in NE cells, as it is a protein that activates neuropeptides by amidation after their secretion from NE cells. It is therefore not surprising that the expression of PAM did not increase in the PC-295 model after castration.

Serotonin and bombesin, but not but VIP and calcitonin were expressed in the PC-295 model. Using somatostatin autoradiography by [^{125}I-TYR^{0}]-SS-28, which binds with high affinity to the five known somatostatin-receptor subtypes, specific binding was demonstrated for ss-receptors type sst1 and sst2 in PC-295 tumor tissue (L. Hofland, Department of Internal Medicine, unpublished results). These results indicate that a number of growth modulating neuropeptides and some of their receptors can play a role in the PC-295 model.

From the double stainings of CgA with BrdU and MIB-1, it was concluded that the greater part of the NE cells was in the G_0-phase of the cell cycle which was in agreement with other studies on prostate cancer(Bonkhoff, 1994a; Bonkhoff, 1994b). From the double labeling studies with MIB-1 and CgA it was seen that the few proliferating cells at 7 days post-castration were surrounding the predominantly CgA positive islets of the PC-295 human prostate cancer tissue. Subsequently, comparing the Western blot results for CgA and androgen receptor clearly showed that the NE cells did not express the androgen receptor. NE cells thus seem to be an androgen independent, non-proliferating part of the prostatic epithelium.

Bonkhoff et al predominantly found in benign prostatic glands that proliferation was restricted to the prostatic basal cell layer and that basal cells did not express the androgen receptor. In prostate cancer specimens, androgen receptor negative NE cells expressed cytokeratins 18 and 5, and these cells were found in the proximity of proliferating cells. In the PC-295 experimental tumor model, clustered NE cells are thus also found in the proximity of proliferating cells, indicating a possible interaction between NE cells or their products and neighbouring proliferating cells. The fact that basal, proliferating cells and CgA positive NE cells are the only cells surviving androgen deprivation suggests that the absence of androgen receptor expression plays a key role in their survival. Another factor for cellular survival is Bcl-2 that protects cells from undergoing apoptosis. This is also shown in our model as Bcl-2 expression increases after androgen withdrawal. Post-castration, Bcl-2 is not expressed in NE cells but in a proportion of non-NE putative androgen independent cells in the PC-295 xenograft. In a recent publication(Xue, 1997), Xue et al showed a specific keratin expression pattern for most NE cells in prostate cancer patient material by using serotonin as NE marker combined with the luminal epithelial cell marker (Cytokeratin 18) and a typical basal cell marker (cytokeratin 5;RCK 103). A minority of the prostatic NE cells expressed only cytokeratins for basal cells or luminal cells. NE cells thus form a heterogeneous population. However, no expression of Bcl-2 in NE cells in patient material was found, suggesting that Bcl-2 expression is lost during NE differentiation of Bcl-2 positive basal

cells(Xue, 1997). In the in vivo model LuCaP, cell lineage markers prostate specific antigen, neuron-specific enolase (NSE) and Bcl-2, were used to mark the different epithelial cell types found in prostate cancer tissue(Liu, 1996). They proposed a model in which Bcl-2 positive proliferating epithelial cells differentiated into either a lineage of NSE expressing NE cells or a lineage of prostate specific antigen expressing exocrine secretory cells. In this latter population, the expression of NSE is heterogeneous. These amphicrine, i.e. both NSE and prostate specific antigen positive, cells, may be induced to express Bcl-2, which might result in tumor cells surviving hormonal manipulation. These results partly support the stem cell model for benign prostatic hyperplasia proposed by Isaacs and Coffey(Isaacs, 1989). They propose a model of prostatic stem cells, which divide into a limited number of amplifying, basal cells followed by a limited number of cell divisions to finally form transit cells that differentiate into the different epithelial cell subpopulations. In prostate cancer, most probably the amplifying step in this model is not limiting, resulting in numerous types of exocrine and endocrine cells expressing different combinations of cytokeratins.

In the PC-295 model, we found BrdU positive NE cells. As we did not find MIB-1 expression in NE cells, the NE cells apparantly did not proliferate, rather than that a fraction of CgA positive cells entered S-phase shortly post-castration. Such cells are supposed of being arrested before mitosis. Another more plausible explanation is that the BrdU labeled cells were amplified post-castration and differentiated into NE cells in the time that followed between BrdU labeling and sacrifice at day 2 or 4 after castration. Considering Xue et al and the present results, there are possibly two routes of NE differentiation in androgen dependent epithelia: a regular route from proliferating cells via an intermediate cell type to NE cells and an alternative route of induced NE differentiation from exocrine luminal cells by androgen withdrawal.

The CgA and SgIII stainings, the increased apoptosis and the non-proliferative status of most of the NE cells seen at the controls (T_0) and at 7 days post-castration in the currently described PC-295 xenograft, clearly show the process of neuroendocrine differentiation as being induced by androgen withdrawal. From the histological picture it is also clear that the changes in NE expression are not due to a selection of NE cells. The BrdU positive NE cells are probably the best indicators of the post-castration amplified cells that differentiate into a NE cell. The greater part of the NE cells found after 4 days of androgen withdrawal differentiated from G_0-phase arrested luminal or intermediate cells. Clearly, the induction of the NE phenotype is a proliferation independent process in the PC-295 model. PC-295 tumors did not grow without androgens neither in female mice nor after long-term castration of PC-295 tumor bearing mice (data not shown). This may be explained by the rapid decrease in proliferation and the rapid tumor regression of the PC-295 tissue following castration. Probably, the decreased tumor vascularity in PC-295, observed after prolonged androgen withdrawal, caused the death of surviving non-proliferating cells. All together, neither the induction of the NE phenotype, nor Bcl-2 expression of non-NE cells post-castration in PC-295 did not lead to androgen independent growth or maintenance of the tumor vascularity.

As the prognostic value of CgA expression or occurrence of NE differentiation in prostate cancer is still under debate, further testing of SgIII and PAM as possible better NE-related prognostic markers for the progression of prostate cancer is still relevant. Possible changes in expression of components of the regulated secretory pathway or PAM might have effects on, for example, overproduction of bioactive growth stimulating neuropeptides.

The PC-295 in vivo model is a potential model for further studies of the induction of NE differentiation by androgen withdrawal, the regulated secretory pathway in

particular. In addition, PC-310, another androgen dependent, NE differentiated prostate cancer model is currently under investigation and might provide us with additional knowledge on NE differentiation. Differentiation of NE cells from basal or luminal cells and the role of NE cells in progression of prostate cancer are important aspects to be studied as well. SGIII, PAM, or other markers for NE differentiation are to be tested retrospectively for their prognostic value in the survival of patients undergoing radical prostatectomies. Human prostate cancer xenograft models with NE differentiation will be used to answer fundamental questions on prostate cancer.

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CHAPTER 3

ANDROGEN INDEPENDENT GROWTH IS SPECIFICALLY INDUCED BY NEUROPEPTIDES IN HUMAN PROSTATE CANCER CELL LINES

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Abstract

BACKGROUND: Androgen independent growth leads to progressive prostate cancer after androgen ablation therapy. This may be caused by altered specificity of the androgen receptor (AR), by ligand independent stimulation of the AR, or by paracrine growth modulation by neuropeptides secreted by neuroendocrine cells.

METHODS: We established and characterized the androgen independent FGC-DCC from the androgen dependent LNCaP cell line. The androgen independent DU-145, LNO, FGC-DCC and PC-3 and the androgen dependent LNCaP and PC-346C cell lines were used to study growth modulation of gastrin releasing peptide (GRP), calcitonin (CT), serotonin (5-HT) and vasoactive intestinal peptide (VIP) by ^{3}H-thymidine incorporation. Specificity of the growth-modulating effects was tested with the anti-GRP monoclonal antibody 2A11 and induction of cAMP by neuropeptides.

RESULTS: Androgen independent growth stimulation by neuropeptides was shown in DU-145 and PC-346C. 2A11 inhibited GRP-induced ^{3}H-thymidine incorporation in DU-145 and PC-346C and inhibited proliferation of the FGC-DCC and PC-3 cell lines. With some exceptions, cAMP induction paralleled growth stimulation. Dideoxyadenosine (DDA) inhibited the GRP-induced growth effect in DU-145 and PC-346C, whereas oxadiazoloquinoxaline-1-one (ODQ) had no effect on ^{3}H-thymidine incorporation. None of the neuropeptides stimulated growth of LNCaP, LNO, FGC-DCC and PC-3.

CONCLUSIONS: GRP-induced growth of DU-145 and PC-346C was specific and cAMP mediated. Androgen independent growth of FGC-DCC cells was mainly due to an induction of Bcl-2 expression and possibly through the activation of an autocrine and neuroendocrine-like pathway as has been shown for the PC-3 cell line as well. Growth induction of non-NE cells by neuropeptides could be a possible role for neuroendocrine cells in clinical prostate cancer.

Introduction

Prostate cancer is rapidly becoming the main cause of death for elderly men. Initially, most cases of prostate cancer can be successfully treated by an approach of androgen suppression or androgen receptor blockade. However, a major problem in the treatment of metastasized prostate cancer is the development of androgen independent disease. This androgen independent growth can be caused by at least four different mechanisms. First, mutations in the androgen receptor could lead to unrepressable growth activation of prostatic cancer cells. Second, the androgen receptor may interact with growth hormones other than testosterone or di-hydrotestosterone, e.g. glucocorticoids like LHRH (Culig, 1997b). Third, androgen receptor activation could be induced by a ligand independent activation through different types of growth factors, like Insulin-like growth factor(Peehl, 1996a; Chan, 1998; Lamm, 1998) or biogenic amines (Zhu, 1997). A fourth mechanism is receptor specific paracrine or autocrine growth modulation of human prostatic cancer cells by neuropeptides secreted by neuroendocrine (NE) cells.

NE cells are present in the normal and neoplastic prostate(Noordzij, 1995c). Neuropeptides are growth factors either eutopically or ectopically produced by these NE cells. Serotonin (5-HT)(Abrahamsson, 1987; Seuwen, 1990; Abdul, 1994), bombesin or gastrin releasing peptide (GRP)(Cuttitta, 1985; Bologna, 1989; Moody, 1993; Speirs, 1993; Bold, 1994; Aprikian, 1997), vasoactive intestinal peptide (VIP)(Gkonos, 1996; Solano, 1996), calcitonin (CT)(Iwasaki, 1983; di-Sant'Agnese, 1986; Shah, 1994; Ritchie, 1997), parathyroid hormone-related protein (PTHRP)(Iwamura, 1994a; Cramer, 1996; Peehl, 1997; Wu, 1998a), and somatostatin (SMS)(Brevini, 1993) are all examples of growth modulating neuropeptides in neoplasia of lung, intestine, pancreas, prostate and other epithelia(Tutton, 1987; Dalsgaard, 1989). Most of the known neuropeptides act via cAMP induction through stimulation of adenyl cyclase(Gkonos, 1995; Gysbers, 1996; Ichikawa, 1997) and Ca^{2+} mobilization(Bunn, 1990; Sethi, 1991; Han, 1997; Wasilenko, 1997) or via cGMP-coupled receptor proteins(Sinnet-Smith, 1990). The different mechanistic actions of these neuropeptides can be studied by using specific blockers of adenyl cyclase or guanylyl cyclase(Reid, 1990; Wessels-Reiker, 1993; Cellek, 1996; Gysbers, 1996).

The role of NE cells and their secreted bioactive neuropeptides in the progression of human prostate cancer from an androgen dependent to an androgen independent state is still unclarified. Previously, we showed that androgen suppression in the NE differentiated human xenograft model PC-295 lead to a proliferation independent induction of NE differentiation(Jongsma, 1998). We also showed expression of different components of the regulated secretory pathway, indicating that NE cells in prostate cancer are capable to actively secrete neuropeptides and other growth factors. Observations in another NE differentiated model, PC-310, showed a similar induction of NE differentiation after androgen suppression (unpublished results). The question remains why this induction takes place and whether these NE cells have the potential to induce androgen independent cell growth. Other groups investigated NE

differentiation in the prostate(Abrahamsson, 1996; Noordzij, 1996; Krijnen, 1997; Qiu, 1998) and growth modulating effects of different neuronal-associated peptides like neurotensin (NT)(Seethalakshmi, 1996; Seethalakshmi, 1997), nerve growth factor ß (NGF-ß)(Paul, 1996; Geldof, 1997; Angelsen, 1998) and neuropeptides like prolactin(Nevalainen, 1997), and adrenomedullin(Miller, 1996) on prostate cancer cell lines. Extensive studies on neuropeptides and growth factors have been done in small cell lung cancer (SCLC)(Bepler, 1988) cell lines, which are well known for their autocrine growth pattern. One of these investigations resulted in a potential therapy for SCLC, i.e. the use of the anti-GRP monoclonal antibody 2A11 as possible intervention treatment for SCLC patients(Avis, 1991).

A study with a steroid dependent non-SCLC cell line showed that these cells adapted to serum free and growth factor free medium by producing GRP in an autocrine way associated with the appearance of NE features like neurite outgrowth and expression of NE specific markers(Siegfried, 1994). Prostate cancer cell lines showing the NE phenotype have not been described so far. However, it was shown that the androgen dependent LNCaP cell line adapted to androgen depletion by androgen independent cell proliferation(Pousette, 1997), expression of the anti-apoptotic protein Bcl-2(Raffo, 1995; Dorai, 1997; Saeed, 1997), and strong neurite outgrowth. One group reported induced expression of both Bcl-2 and neuron specific enolase (NSE) after androgen suppression of the LNCaP cell line(Shen, 1996).

In this study, we investigated the growth modulating properties of GRP, CT, VIP, and 5-HT on six human prostate cancer cell lines comprising both androgen dependent and independent cell lines, including an androgen independent variant of the LNCaP cell line that we established ourselves, with the aim to assess whether both types of prostatic cells can be stimulated to proliferate under influence of different neuropeptides in an androgen deprived situation. This effect of neuropeptides was assessed by ^{3}H-thymidine incorporation for proliferation and a radioimmunoassay (RIA) for cAMP induction. Specific antibodies and direct blockers of signaling transduction pathways were used to investigate the mechanism of growth activation by GRP in these prostatic cancer cell lines. Furthermore, we characterized the changes in expression of NE markers, chromogranin A (CgA), secretogranin III (SgIII), neuron specific enolase (NSE), expression of androgen receptor, PSA, and the anti-apoptotic marker Bcl-2 during the establishment of the androgen independent variant of the androgen dependent LNCaP cell line. Using the two variants of LNCaP, we showed that androgen deprivation induced changes in gene expression and the ability to proliferate under influence of neuropeptides and androgens in an androgen deprived situation.

MATERIAL AND METHODS

Culture of cell lines

The androgen dependent prostatic cancer cell line LNCaP (kindly provided by Dr. Julius Horoszewicz) is basically the same cell line that is made available through ATCC(Horoszewicz, 1983). The androgen independent PC-3(Kaighn, 1979) and DU-145(Stone, 1978) were obtained from the American Type Tissue Collection (Rockville,

MD). These three cell lines were maintained in RPMI 1640 (Gibco BRL, Life Technologies, Scotland) supplemented with 7.5% fetal calf serum (FCS). All culture media were supplemented with penicillin 100U/ml, streptomycin 100 μg/ml, and glutamine 0.1 mg/ml. The androgen independent LNO cell line(van Steenbrugge, 1991) was grown in RPMI 1640 supplemented with androgen depleted, i.e. 5% dextran charcoal stripped FCS (DCC).

The androgen dependent PC-346C cell line(Romijn, 1996), was established recently at our laboratory from a human prostate cancer xenograft model in nude mice(van Weerden, 1996), and was found to be androgen dependent. These cells were grown in Dulbeco's Modified Eagle's Medium (DMEM)/F12 (Gibco) medium supplemented with various growth factors, 2% FCS and 10^{-10}M of the synthetic androgen R1881 as described before(Romijn, 1996). This culture medium was designated as Swedish Culture Medium (SCM). The mouse fibroblast cell line Swiss 3T3(Todaro, 1963) was maintained in RPMI1640 with 7.5% FCS. The human breast cancer cell line ZR-75.1 was maintained in DMEM supplemented with 10% FCS and 10^{-9}M Estradiol (E2).

Establishment of FGC-DCC from LNCaP

A seventy percent confluent culture of LNCaP (passage 37) was deprived of androgens and subsequently cultured in RPMI 1640 supplemented with 5% DCC. From this point onward the cultured cells were named FGC-DCC. At each subsequent passage, confluent flasks were frozen at -80°C for protein or mRNA extraction. The first passages grew relatively slow with a clear neurite outgrowth pattern known from neuronal cells. After about 10 passages the FGC-DCC cells started to grow with a growth rate exceeding that of the parental LNCaP cell line.

Proliferation assays with ^{3}H-thymidine incorporation

We evaluated the growth modulating properties of the neuropeptides GRP, VIP, CT (Sigma) and 5-HT (RBI, Natick, MD, USA) by incubation of the different cell lines in flat bottomed 96 well plates (Co-star) with different concentrations of the neuropeptides. The cell lines LNCaP and PC-346C were plated at 5000 cells/well, LNO, FGC-DCC, PC-3 and DU-145 were plated at 2500 cells/well and the control Swiss 3T3 was plated at 3000 cells/well. All experiments, except for PC-346C, were performed in standard RPMI 1640 supplemented with 5% DCC. PC-346C experiments were performed in DMEM/F12 supplemented with insulin, selenite, transferrin, bovine serum albumin, epidermal growth factor, and 2% DCC (SCM-A). Testing of neuropeptide activity with these cell lines was performed under the condition of androgen depletion in DCC. This resulted in variable basal levels of ^{3}H-thymidine incorporation as some cell lines grew slowly without androgens (LNCaP, LNO and PC-346C) and others grew relatively fast (FGC-DCC, DU-145 and PC-3). The PC-3 and DU-145 cell lines grew even faster in 5% DCC than in 7.5% FCS. Determination of the appropriate conditions for all cell lines led to the following concept. Cells needed to adjust 48 hours to 5% DCC in 96 wells plates before starting the incubation with neuropeptides. After 48 hours of culture, neuropeptides were added to the wells to a final volume of 250 μl at concentrations ranging from 10^{-12} to 10^{-7} moles/liter. After 32 hrs 0.5 μCi of ^{3}H-thymidine (5 Ci mmol,

Amersham, Inc, UK) was added for 16 hrs. The plates were then frozen at -20°C until final measurement. Cells were harvested with a Skatron cell harvester on ß-plate filters (*Wallac Oy*, Finland) and were subsequently counted in a 1205 betaplate liquid scintillation counter (LKB, Wallac, Turku, Finland). Data were expressed as means of 8 duplicates per neuropeptide concentration.

Specificity of the growth modulating effects of GRP in Du-145, 3T3 and PC-346c was analyzed in the ^{3}H-thymidine incorporation assay with the anti-GRP MoAb 2A11(Cuttitta, 1985) (kindly provided by Dr. F. Cuttitta, NCI, Rockville, MD). The MoAb 2A11 was added at 1-10 µg/ml prior to incubation of these cell lines with GRP. Inhibition of signaling transduction pathways activated by GRP was investigated in the ^{3}H-thymidine incorporation assay by adding the specific adenyl cyclase blocker, dideoxyadenosine (DDA) or guanylyl cyclase blocker, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline (ODQ) at inhibiting concentrations just before exogenous GRP was added to the cells.

Proliferative activity of the androgen dependent LNCaP, PC-346C and the androgen independence of FGC-DCC was tested through addition of R1881 at concentrations ranging from 10^{-13} to 10^{-8} M.

Radioimmunoassay (RIA) for c-AMP induction by neuropeptides

LNCaP-FGC, PC-346c, and ZR-75.1 cells were plated on 24 well plates at $2.5x10^5$ cells/well. Swiss 3T3 was plated at $2.0x10^5$ cells/well and PC-3 and DU-145 were plated at $1.5x10^5$ cells/well. After 3 days of incubation cells were washed with PBS and subsequently incubated with 0.1 mM Isobutylmethylxanthine for 30 minutes (IBMX, Sigma) in their experimental media. The cells were then incubated with different neuropeptides, GRP, VIP, CT and 5-HT for 10 minutes to induce intracellular c-AMP levels. Culture medium was removed and the cells were lysed with cold 0.1 N HCl. The 24 well plates or the contents of each well were stored at -80°C until final measurement with a RIA for ^{125}I-succinyl-c-AMP (scintillation proximity assay, Amersham).

Western Blot analysis

We investigated the expression patterns of the NE markers CgA, SgIII and NSE as well as Bcl-2, and androgen receptor (AR) in the established FGC-DCC cell line by Western blotting. For expression of CgA and SGIII human pheochromocytoma was used as positive control and the LNCaP cell line for AR expression. The procedure of protein extraction was as previously described(Noordzij, 1996).

20 µg of each protein sample was transferred to an SDS (Gibco) polyacrylamide gel and gel electrophoresis was performed with prestained markers as size standards (Novex, San Diego, CA). The gels were blotted to a 0.45µm cellulose nitrate membrane (Schleicher & Schuell, Germany). The immunoblot was blocked with PBS (pH 7.7) containing 0.1% Tween-20 (Sigma) and 5% dry milk for one hour. The CgA, androgen receptor, NSE, Bcl-2 and SGIII antibodies were added in their optimal concentration. The blot was incubated overnight on an orbital shaker at 4°C. After rinsing the blot four times 15 minutes with PBS, incubation for one hour was performed with the secondary HRP-conjugated to GoatαMouse and GoatαRabbit antibodies, for mouse monoclonal

and rabbit polyclonal primary antibodies, respectively. Subsequently, a short incubation with a mixture of 10 ml luminol and 100 µl oxidizing agent (BM chemiluminescense kit, Boehringer Mannheim GmbH, Germany) followed, after washing for four times 15 minutes with PBS. Excess reagent was removed and antibodies were visualized by exposure of the blots to an X-ray film.

Reverse transcriptase polymerase chain reaction (RT-PCR) analysis

RNA was isolated by using the single step RNAzol^{TM} B method (Campro, The Netherlands(Chomczynski, 1987). Cell suspension was added to 1 ml of RNAzol^{TM}, whereafter chloroform (0.1ml) was added and the mixture was vortexed for 15 sec. followed by 5 minutes incubation on ice. The homogenate was then centrifuged at 4 °C at 12,000 g for 15 minutes. The upper, RNA containing, water-phase was removed and mixed with an equal volume of isopropanol. This mixture was then kept at 4 °C for 15 minutes whereafter it was centrifuged at 4°C at 12,000 g for 15 minutes. The supernatant was removed and the RNA pellet was washed twice with 75 % ethanol by vortexing and centrifugation at 4°C at 12000 g. The pellet was then dried and resuspended in sterile H_2O. Concentration was determined at OD 260 and solutions of 1µg/µl were prepared for further use in Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).

RT-PCR was performed with human specific primers for CgA, SGIII, androgen receptor, Bcl-2 and ß2Microglobulin (ß2MG) for mRNA quality control. RT reaction was performed with a mastermix containing 5 mM $MgCl_2$, PCR buffer, 10 mM dNTP's, RNAse inhibitor (10 units), RT (25 units), 2.5 mM random hexamer primers and 0.5 µg RNA in a total volume of 10 µl covered with 50 µl of mineral oil. The mastermix was then processed at 42°C for 60 minutes followed by a 15 minutes incubation at 99°C and the reaction stopped at 4°C for five minutes. The resulting cDNA mix was used in total with the mastermix of the PCR protocol. This mastermix contained reaction buffer, supertaq DNA polymerase (HT Biotechnology Ltd., UK), 15 µM of both sense and antisense primers in a total volume of 40 µl. All samples were first denaturated at 94°C for 10' and then amplification was performed for 35 cycles of 1' 94°C, 1' 60°C and 1' 72°C and a final extension at 72°C for 10'. The PCR product was checked on an 1% agarose gel.

RESULTS

Table I lists the data of the ^{3}H-thymidine incorporation assays and the cAMP measurements following exposure of the cell lines to various neuroppetides. The growth modulating effects of the four neuropeptides, GRP, CT, 5-HT and VIP varied among the prostatic cancer cell lines. In control cell lines, incubation with the neuropeptides resulted in increased ^{3}H-thymidine incorporation in response to GRP, CT and 5-HT (Swiss 3T3) or induced increased intracellular cAMP level in response to VIP (ZR-75-1). The androgen dependent PC-346C cells responded significantly to all four neuropeptides as is shown in *figure 1*. Growth stimulation varied from 30% by 5-HT at high concentrations

Table 1: Growth modulating effects of neuropeptides on human prostate cancer cell lines under androgen deprived culture conditions

Cell line	FCS	Assay	GRP	CT	5-HT	VIP	R1881
FGC	AD	^{3}H-T cAMP	+ 35%(10^{-7}) -	- 40% -	- 0% -	- 30% NA	++ 200% -
FGC-DCC	AID	^{3}H-T cAMP	+ 5% NA	- 15% NA	- 10% NA	- 50% NA	-40% NA
PC-346C	AD	^{3}H-T cAMP	+ 60% -	+ 80% -	+ 30%(10^{-7}) -	+ 100% 2-3 x	+100% -
DU145	AID	^{3}H-T cAMP	+ 45% 2-3 x	+ 60% 3 x	+ 15% -	- 0% -	NA
PC-3	AID	^{3}H-T cAMP	- 6% 1.5 x	- 10% -	- 7% 1.7 x	- 0% NA	NA
Swiss 3T3	AID	^{3}H-T cAMP	+ 25% 2 x	+ 30% 1.3 x	+ 30% 1.5 x	+ 20% -	NA
ZR-75-1	AID	^{3}H-T cAMP	NA	NA	NA	NA 7.5-9 x	NA

Table 1:Growth modulating effects of the neuropeptides on human prostate cancer cell lines LNCaP, FGC-DCC, PC-346C, DU-145, PC-3 and control cell lines Swiss 3T3 and ZR-75-1 under androgen deprived culture conditions. Tritium thymidine incorporation (^{3}H-T) values indicated are means (n=8) of percentage growth stimulation or inhibition compared to the control situation over the whole range of neuropeptide concentrations that were tested. If the effect is seen only at one concentration, this concentration is indicated in moles between brackets. The values of cAMP induction by the four neuropeptides are the mean induction ratios of two separate experiments (n=2). If marked with -, no significant changes in cAMP were induced by that specific neuropeptide. NA = not analyzed, AD= androgen dependent, and AID= androgen independent.

to 100% by VIP. Significant induction of cAMP could be measured after incubation of PC-346C with VIP, but not in response to GRP, CT or 5-HT. In the LNCaP cell line, no or little growth stimulation was observed with any of the neuropeptides, whereas VIP and CT even inhibited ^{3}H-thymidine incorporation. No significant induction of intracellular cAMP could be measured. Similarly, no significant growth stimulation of the androgen independent cell line FGC-DCC was observed after incubation with the neuropeptides GRP, CT, VIP and 5-HT and VIP decreased ^{3}H-thymidine incorporation by 50%. The synthetic androgen, R1881, served as a positive control for proliferation of the androgen dependent cell lines. The dose-response relationship of R1881 in the LNCaP parental cell line and the androgen independent FGC-DCC are shown in *figure* 2. In contrast to LNCaP showing a biphasic growth pattern in response to R1881, FGC-DCC did not show any increase of ^{3}H-thymidine incorporation in response to R1881.

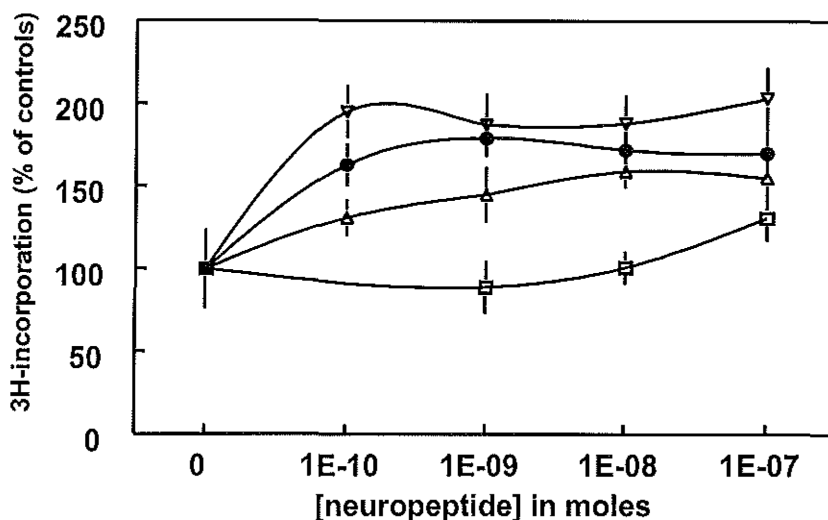

Figure 1: The growth modulating effects of neuropeptides on PC-346C
The tritium thymidine incorporation as percentage of control levels are given for PC-346C cells after exposure to CT (•), VIP (∇), GRP (△) and 5-HT ( ). Values shown are means of 8 duplicates and standard deviations are shown as error bars at each neuropeptide concentration. Significant stimulation was observed with VIP, CT and GRP (Student T-test, $p > 0.01$).

The androgen independent DU-145 cells showed a significant increase in ^{3}H-thymidine incorporation in response to CT, GRP and to a lesser extent 5-HT. This was accompanied by significant increases in intracellular cAMP levels for CT and GRP. None of the neuropeptides tested did stimulate ^{3}H-thymidine incorporation in the PC-3 cell line, but the neuropeptides GRP and 5-HT significantly induced intracellular cAMP levels.

The growth modulating effects of GRP in DU-145, PC-346C and Swiss 3T3 are shown in *figure 3a-c*. The specificity of the stimulatory effect of GRP with these cell lines is shown by parallel incubations with the anti-GRP MoAb 2A11. Clearly, MoAb 2A11 blocked GRP-induced increase of ^{3}H-thymidine incorporation in all three cell lines in a concentration dependent manner by intercepting the exogenously added GRP before it can reach GRP receptors on the cell surface. In absence of exogenously added GRP, ^{3}H-thymidine incorporation in PC-3 cells decreased by 50% after incubation with MoAb 2A11 when the cells were grown under standard culture conditions (data not shown). For DU-145 and PC-346C, the GRP-induced ^{3}H-thymidine incorporation was further studied after incubation with the adenyl and guanylyl cyclase blockers, DDA and ODQ. DDA significantly blocked increased ^{3}H-thymidine incorporation at 10μM and 0.1μM, whereas ODQ at 2μM and 20 nM had no effect on the before mentioned growth effects of GRP in PC-346C and DU-145.

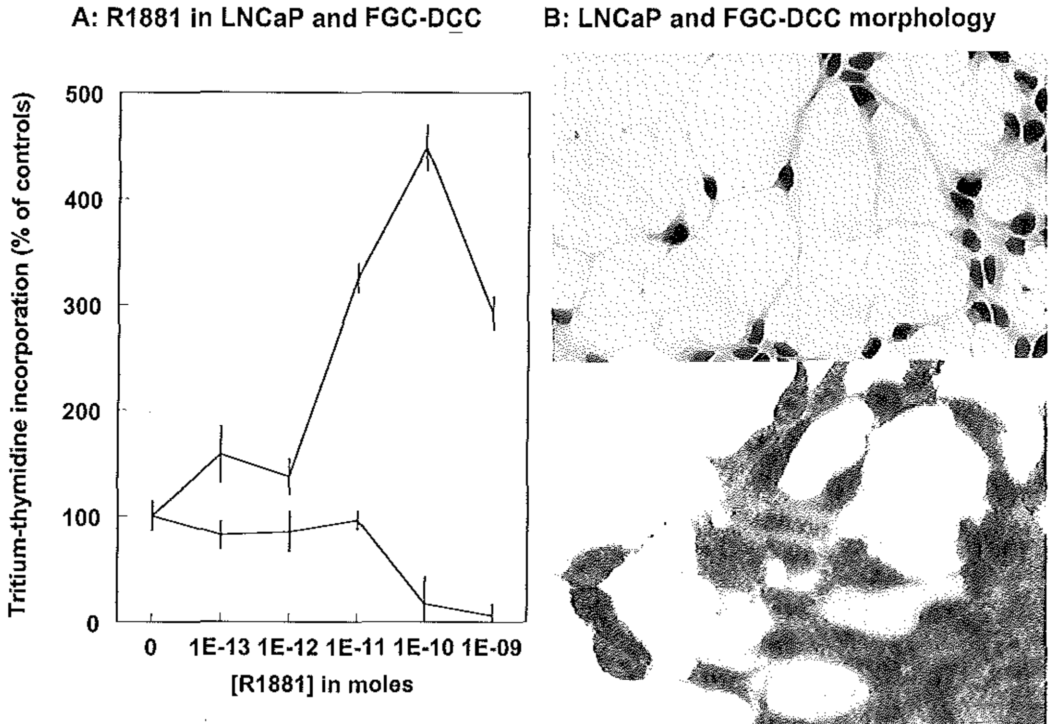

Figure 2: The effect of androgen suppression on the androgen dependent LNCaP cell line

A) The dose-response relationship of R1881 in LNCaP and FGC-DCC under androgen suppression. Tritium thymidine incorporation as percentage of control levels shows clearly the biphasic growth pattern of LNCaP (∇), whereas FGC-DCC ( ) shows no growth in response to R1881 and even a decrease in proliferation at higher concentrations

B) The changes in morphology of FGC-DCC after androgen suppression of the LNCaP parental. Clearly, neurite outgrowth is visible in the FGC-DCC cells which is absent in the LNCaP before androgen depletion (magnification 310 X).

Characterization of the FGC-DCC cell line, showed induction of Bcl-2 expression from passage 3 on and an increased expression of SGIII and NSE after hormonal depletion at both protein level (Western blot, *figure 4*) and mRNA level (data not shown). Clearly, the 26 kD Bcl-2 protein is expressed in FGC-DCC and not in the parental LNCaP. The expression of the 61/63 kD double band of SgIII and expression of the 28 kD processed form can be clearly seen in FGC-DCC, whereas these are almost absent in LNCaP. The 44 kD NSE protein is expressed in both LNCaP and FGC-DCC. Androgen receptor was expressed from the second passage of FGC-DCC on, whereas PSA levels in FGC-DCC culture medium dropped to zero after hormone withdrawal (data not shown).

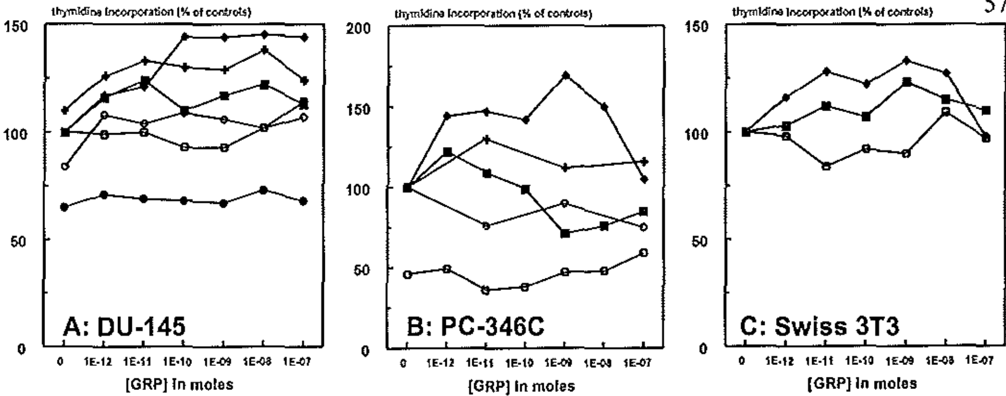

Figure 3: Effects of MoAB 2A11, DDA and ODQ on the specificity of the GRP induced growth in DU-145, Swiss 3T3 and PC-346C
The GRP induced increase (◆) in tritium thymidine incorporation in DU-145 (A), Swiss 3T3 (B) and PC-346C (C) is inhibited partly by 1 μg 2A11/ml (■) and completely by 10 μg 2A11/ml ( ). DDA blocks the GRP action in DU-145 (A) and PC-346C (C) at 0.1 μM (O) and in DU-145 (A) at 10 μM (●), whereas 20 nM ODQ (+) has no effect on the GRP induced growth effect in DU-145 (A) and PC-346C (C).

DISCUSSION

We have demonstrated that growth of prostatic cancer cell lines under the condition of androgen depletion can be modulated by neuropeptides which are known to be produced by neuroendocrine cells. The action of these neuropeptides differed from one cell line to the other irrespective of their androgen dependent status. Furthermore, we described the establishment and properties of FGC-DCC, an androgen independent derivative of LNCaP,.

Significant growth effects of VIP, GRP, CT and 5-HT were measured in the androgen dependent PC-346C cell line and in response to GRP, 5-HT and CT in the androgen independent DU-145. The effect of 5-HT that we observed in DU-145 was comparable to the results of Hoosein et al(Abdul, 1994; Hoosein, 1996), who observed an increased proliferation in response to relatively high concentrations of 5-HT (0.1μM and higher), as we observed increased ^{3}H-thymidine incorporation already at 0.01 and 0.1μM 5-HT in DU-145. Thus, we observed a higher sensitivity of DU-145 cells for 5-HT. The growth stimulation of DU-145 by CT was not found by other investigators(Shah, 1994; Ritchie, 1997), who tested the action of neuropeptides under different culture conditions, i.e. total serum depletion instead of 5% as used in the present study. This may be also an explanation for the differences in growth effects in response to neuropeptides observed by other investigators in PC-3 and LNCaP. We observed no significant growth effects of any of the tested neuropeptides in the LNCaP, LNO and FGC-DCC cell lines. This also applied to the PC-3 cell line, which in contrast to other studies, did not react to exogenously added GRP. Various studies have shown binding of ^{125}I-Tyr^{4}-bombesin to prostatic cell lines and that PC-3 possessed far more binding sites for GRP than DU-145 or LNCaP(Reile, 1994; Aprikian, 1996). We only observed significant growth stimulation of PC-3 by exogenously added GRP under the culture

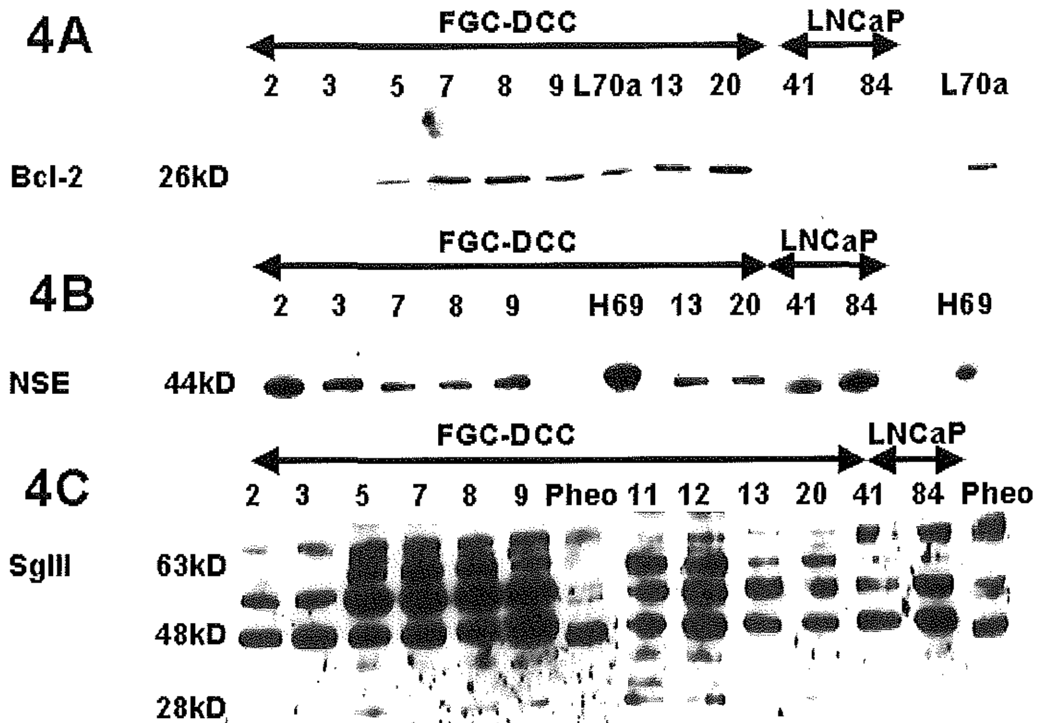

Figure 4: Characterization of FGC-DCC establishment by Bcl-2, NSE and SgIII expression

A) Expression of 26 kD Bcl-2 protein in FGC-DCC (marked by different passage numbers) and LNCaP (low and high passage number) on western blot. L70a is a Bcl-2 expressing subline of LNCaP and served as positive control.

B) Expression of NSE as a 44 kD band on western blot in FGC-DCC (marked by different passage numbers) and LNCaP (low and high passage number). H-69 is a human small cell lung cancer (SCLC) cell line, which served as a positive control.

C) Expression of SgIII in FGC-DCC (marked by different passage numbers) and LNCaP (low and high passage number) on western blot. Human pheochromocytoma served as a positive control. SgII is clearly visible as a 63/61 kD double band together with the 48 kD and 28 kD processed forms of SgIII.

condition of medium with 1 and 2.5% DCC, but not with 5% DCC. However, the basal ^{3}H-thymidine incorporation rate of PC-3 cells was much lower with 1% DCC than with 5% DCC in the experimental medium, indicating changes in cell growth by serum depletion. Furthermore, the growth induction by GRP that Bologna et al observed in PC-3(Bologna, 1989) was relatively at minor level when compared to the GRP-induced growth that we observed in DU-145. Their results with PC-3 in response to GRP seem to be similar to our observations, which showed that exogenously added GRP did not induce significant growth stimulation. Following the incubation with the anti-GRP antibody 2A11 in PC-3 cells, further substantiated that PC-3 proliferation in standard culture medium is strongly dependent on GRP as ^{3}H-thymidine incorporation dropped by 50%. Probably, GRP is endogenously produced at high concentrations by PC-3. This

endogenous GRP production is partly inhibited by culturing the PC-3 cells at lower DCC serum concentrations, which explains the additional growth stimulation by exogenously added GRP with 1 and 2.5% DCC in the experimental medium.

In six occasions of increased ^{3}H-thymidine incorporation levels (Table 1), neuropeptide exposure was associated with increased intracellular cAMP levels. By contrast, intracellular cAMP levels increased upon exogenously added GRP and 5-HT in PC-3 cells, without affecting cell proliferation. This observation is consistent with the possibility that PC-3 cells produce GRP and 5-HT, as stated above. As PC-3 cells do have receptors for both GRP and 5-HT, PC-3 cells can be stimulated to grow in an autocrine way by these neuropeptides. We can not offer an explanation for the fact that we did not observe intracellular cAMP induction in PC346C in response to GRP and CT, whereas these peptides did increase cAMP in DU-145, Swiss 3T3 and PC-3. Possibly, the cAMP containing medium used for culturing the PC-346C cell line abrogates measurement of cAMP changes following exposure to GRP and CT. To speculate about the observed cAMP induction by VIP in the PC-346C cells, we think that the cAMP induction in PC-346C cells after exposure to VIP is stronger than that after exposure to GRP and CT. The major part of our data on intracellular cAMP measurements seem to match with those of Gkonos et al(Gkonos, 1995). However, it was striking to see that their induced levels of intracellular cAMP were about ten-fold higher then ours, e.g. salmon CT induced a three-fold increase of cAMP in DU-145 whereas Gkonos et al observed a thirty-fold induced increase of cAMP by human CT in DU-145.

The increased ^{3}H-thymidine incorporation observed in GRP-exposed DU-145, PC-346C and control cells of Swiss 3T3 could specifically be blocked by the anti-GRP MoAb 2A11 (at 10μg/ml and 1μg/ml) when added prior to administration of the neuropeptide GRP. As discussed above, the anti-GRP MoAb 2A11 also appeared to compete with endogenously produced GRP in PC-3 cells. Thus, for prostatic carcinoma the MoAb 2A11 has shown to be a potent inhibitor of GRP-induced proliferation and of GRP producing cell lines, as was shown before for SCLC cell lines(Cuttitta, 1985; Moody, 1993; Speirs, 1993; Halmos, 1997).

Adenyl cyclase inhibitor DDA blocked the GRP-induced increase of ^{3}H-thymidine incorporation in DU-145 completely or by 50% at 10 μM and 0.1 μM DDA, respectively. On the other hand, guanylyl cyclase inhibitor ODQ did not affect the GRP-induced growth modulation in DU-145. Thus, GRP specifically induces intracellular cAMP in DU-145 as measured by RIA for cAMP. The same results with DDA were obtained for GRP exposed PC-346C cells, whereas ODQ partly blocked the GRP-induced growth stimulation. The results of DDA and ODQ in DU-145 and PC-346C have to be interpreted with caution as in contrast to MoAb 2A11, these cyclase inhibitors do not interfere directly, but which might at high concentrations(Reid, 1990; Wessels-Reiker, 1993; Gonzalez, 1997)have an indirect effect on cell cycle progression through the MAPK signaling pathway. In conclusion, GRP is a potent NE growth factor in prostate cancer cell lines that acts specifically under androgen suppression via induction of the

second messenger cAMP in both androgen independent (DU-145, PC-3) and androgen dependent (PC-346C) cells.

The LNCaP and FGC-DCC cell lines showed a clear difference in their androgen dependent status as LNCaP showed its normal biphasic growth pattern with an optimum growth at 10^{-10} M R1881 as published before(van Steenbrugge, 1991), whereas FGC-DCC could not be stimulated by androgens at all, indicating that FGC-DCC is growing androgen independently. As expected, the synthetic steroid did not influence intracellular cAMP levels in the parental LNCaP. No major changes were observed between LNCaP and FGC-DCC with respect to their response to the four neuropeptides. VIP induced a 50% decrease in ^{3}H-thymidine incorporation at 10 to 100 nM. However, the anti-GRP MoAb 2A11 decreased the basal ^{3}H-thymidine incorporation of FGC-DCC cells by 40% at 10 µg/ml whereas the LNCaP cells did not respond to MoAb 2A11. Like the PC-3 cells, FGC-DCC cells after androgen suppression possibly have adjusted to an autocrine growth pattern, by producing growth factors like GRP endogenously.

As expected, AR expression did not disappear totally at protein or at mRNA level in FGC-DCC but it was low in comparison to the parental LNCaP. PSA levels decreased rapidly to zero after androgen suppression and continued to be low in FGC-DCC after subculturing. The neuronal appearance of the FGC-DCC cells after androgen suppression was prominent, as was also seen by others shortly after androgen withdrawal(Raffo, 1995; Shen, 1996; Saeed, 1997). The expression of the anti-apoptotic protein Bcl-2 was induced gradually during passage 2 and were at high level of expression from passage 3 on for at least 18 passages. Likewise, others found an increase in Bcl-2 expression(Raffo, 1995; Shen, 1996) or a decrease in Bcl-2 expression because of initiation of the apoptotic program(Saeed, 1997). Our results clearly show an induction of Bcl-2 expression during LNcaP prostatic cancer cells progression from an androgen dependent to an androgen independent state. The expression pattern for the NE markers SGIII and NSE were different to that of Bcl-2 as there was already a basal but low expression of both markers in the parental LNCaP. No expression of the NE marker CgA was observed in LNCaP and FGC-DCC at protein level or at mRNA level. To our knowledge, no further reports are available about NE differentiation in prostatic cell lines except for that of Shen *et al* (Shen, 1996). They found an induction of NSE expression at protein level after 10 and 20 days of androgen suppression of the LNCaP cell line. In our hands, however NSE was already expressed in the parental LNCaP cells when cultured under standard conditions, whereas expression increased slightly after subsequent passaging. In the in vivo model LuCaP, three cell lineage markers PSA, neuron-specific enolase (NSE) and Bcl-2, were used to mark different epithelial cell types found in prostate cancer tissue(Liu, 1996). They proposed a model in which Bcl-2 expressing proliferating epithelial cells could differentiate into either a lineage of NSE expressing NE cells or a lineage of PSA expressing exocrine secretory cells. In this latter population, the expression of NSE is heterogeneous. These amphicrine, i.e. both NSE and PSA positive, cells, can be induced to express Bcl-2, which might result in tumor cells escaping hormonal

suppression. In the presently studied androgen dependent LNCaP cell line both PSA and NSE, but not Bcl-2, are expressed,. Clearly, Bcl-2 expression is induced in FGC-DCC and PSA expression is down-regulated. We conclude that FGC-DCC is not a NE-differentiated cell line although the cells show a characteristic neuronal morphology. We found no significant increase in the expression of NE markers, as CgA was not expressed at all and the other markers were present in both the parental LNCaP and the FGC-DCC cell lines. Expression of the androgen receptor in FGC-DCC cells further supports this, as androgen receptor expressing NE cells have not been demonstrated in prostate cancer sofar(Krijnen, 1993). Instead the concept of Liu *et al*(Liu, 1996) is applicable here, namely that the FGC-DCC subline of LNCaP is an example of a neoplastic, androgen independent, NSE, SgIII, Bcl-2 and AR expressing, but PSA negative prostatic epithelial cell that could possibly metastasize. This cell type could be committed to cell division and not to differentiation, which might be the reason why NE markers as well as the androgen receptor are expressed.

We have shown in this study the induction of androgen independent growth both by adding growth modulating neuropeptides to prostatic cancer cell lines and by androgen suppression of an androgen dependent cell line, LNCaP leading to an androgen independently growing cell line, FGC-DCC. The growth modulating effect of GRP on DU-145 and PC-346C was specifically induced as shown with MoAb 2A11 and the adenyl and guanylyl cyclase inhibitors. The growth of the metastatic PC-3 cell line is inhibited by MoAb 2A11 and strongly dependent on serum concentration which indicates an autocrine growth pattern for GRP. The FGC-DCC cells were also inhibited by MoAb 2A11, which is indicative of an androgen suppression-induced activation of an autocrine pathway in these cells. In vitro, we have shown that induction of Bcl-2 expression is one way to bypass androgen withdrawal which possibly leads to progression of prostate cancer. In vivo, there are some studies that support the idea of a role for NE differentiation in the progression of prostate cancer(Krijnen, 1997; Jiborn, 1998). Induction of NE differentiation is a result of androgen withdrawal as we showed in two in vivo human prostate cancer xenograft models(Jongsma, 1998). Krijnen et al(Krijnen, 1997) showed in hormonally treated patients that clinical progression of prostate cancer correlated well with increased expression of NE, i.e. CgA positive, cells. By measuring the effects of neuropeptides in vitro, we provide evidence for androgen independent growth of non-NE cells induced by growth factors that are known to be produced by NE cells. In vivo, the expression of markers from the regulated secretory pathway are being studied, comprising the regulated secretion of bioactive neuropeptides from prostatic NE cells. Studying the progression of a NE differentiated in vivo model, assessing growth modulatory effects of neuropeptides in vivo and studying the process of induction of NE differentiation in vivo and in vitro after androgen withdrawal contributes to assess the role for NE differentiation in clinical prostate cancer.

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CHAPTER 4

ANDROGEN DEPRIVATION OF THE PC-310 HUMAN PROSTATE CANCER MODEL SYSTEM INDUCES NEUROENDOCRINE DIFFERENTIATION.

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ABSTRACT

Neuroendocrine (NE) cells are androgen independent cells and secrete growth-modulating neuropeptides via a regulated secretory pathway (RSP). We studied NE differentiation following androgen withdrawal in the androgen dependent prostate cancer xenograft PC-310. Expression patterns of Chromogranin A (CgA), Secretogranin III (SgIII) and prohormone convertase-1 (PC1) were analyzed to mark the kinetics of NE differentiation both in vivo and in vitro. PC-310 tumor-bearing nude mice were sacrificed at 0, 2, 5, 7, 14 and 21 days post-castration. PC-310C cultures initiated from collagenase-treated tumor tissue could be maintained up to four passages, and androgen deprivation experiments were performed similarly. PC-310 tumor volumes decreased by 50% in 10 days post-castration. Proliferative activity and prostate specific antigen (PSA) serum levels decreased to zero post-castration. In vivo, androgen receptor (AR) expression decreased initially but returned to control level from 5 days post-castration on. PSA levels in PC-310C culture media first decreased but increased after 5 days. CgA, SgIII and 7B2 expression increased in vivo from 5 days post-castration on. Subsequently, PC1 and peptidyl α-amidating monooxygenase, as well as the vascular endothelial growth factor were expressed from 7 days post-castration on and finally growth factors like gastrin releasing peptide and serotonin were expressed in a small part of the NE cells 21 days post-castration.
Like in the PC-310 xenograft, NE differentiation was induced and AR expression relapsed after prolonged androgen suppression in PC-310C. For PC-310C cells this relapse was associated with secretion of PSA. PC-310C is the first culture of human prostatic cancer cells having the NE phenotype. The PC-310 model system is a potential androgen dependent model for studying the role of NE cells in the progression of clinical prostate cancer. Androgen deprivation of NE differentiated prostate cancer may induce the formation of NE and AR^+ tumor residues which are capable of actively producing growth factors via a RSP, possibly leading to hormone refractory disease.

INTRODUCTION

Neuroendocrine (NE) differentiated cells form an androgen independent subpopulation of the prostatic glandular cells (Abrahamsson, 1996). NE cells are considered to be non-proliferating cells which do not express the androgen receptor (Krijnen, 1993) and therefore are assumed to be unaffected by androgen deprivation. NE cells have been localized in nearly all clinical prostatic adenocarcinomas in different frequencies (Abrahamsson, 1986; di-Sant'Agnese, 1992b; Aprikian, 1993; Bonkhoff, 1995; di-Sant'Agnese, 1995; Noordzij, 1995a; Noordzij, 1995c).

Prostatic NE cells may regulate homeostasis and secretion of prostatic fluid, either actively or passively. NE cells can be identified by immunohistochemistry (IHC) with specific antibodies against secreted products for example serotonin (5-HT) (Xue, 1997) and gastrin-releasing protein (GRP) or secretion-associated proteins, like Chromogranin A (CgA) (O'Connor, 1986; Abrahamsson, 1989; Schmid, 1994; Hendy, 1995), which is the commonly used marker for neuroendocrine differentiation. NE cells show a heterogeneous cytokeratin expression pattern as there are basal, luminal and intermediate NE cell types (Bonkhoff, 1994b; Bonkhoff, 1994a; Xue, 1997) and are often found near Bcl-2 positive prostate cancer cells (Colombel, 1992; Cohen, 1994). Xue et al showed no co-expression of 5-HT with the anti-apoptotic oncogene Bcl-2 (Xue, 1997). Likewise, we previously showed that CgA positive cells did not co-express Bcl-2 (Jongsma, 1998) and that there was a proliferation independent induction of NE differentiation after androgen withdrawal. This induction did not allow progression of tumor growth of non-NE tumor cells after prolonged androgen suppression. Other studies revealed that androgen deprivation in clinical prostate cancer induced NE differentiation (Jiborn, 1998) or showed correlation of NE differentiation with progressive behavior (Krijnen, 1997).

Most of the studies on the prognostic value of CgA for NE differentiation did not show a correlation between CgA expressing NE cells and poor prognosis or progression of prostate cancer (Abrahamsson, 1986; di-Sant'Agnese, 1992b; Aprikian, 1993; di-Sant'Agnese, 1995; Noordzij, 1995a; Noordzij, 1995c). There are more markers of the NE phenotype next to CgA as NE differentiated cells have an activated regulated secretory pathway (RSP) (Holthuis, 1996b) next to the lysosomal and an exocrine constitutive pathway. Along the RSP pathway secretion and processing of bioactive neuropeptides and growth hormones, like insulin and glucagon in the pancreas (Smeekens, 1992; Tucker, 1996), are regulated. The RSP consists of a sequence of processes linked from transcription/translation of various factors to final secretion of neuropeptides at the plasma membrane from secretory granules (Holthuis, 1995). Different markers can be identified, such as granular markers Secretogranin III (SgIII) and Secretogranin V (7B2) (Martens, 1989; Sigafoos, 1993; Braks, 1996), carboxy peptidase E and the processing enzymes prohormone convertase 1 and 2 (PC1 and 2). The enzyme peptidylglycine α-amidating monooxygenase (PAM) (Vos, 1995; Vos, 1996) is expressed in or in the near vicinity of NE cells. Evaluation of these markers of the RSP is ongoing in X. Laevis, human lung and prostate cancer (Huttner, 1995; Eib, 1996). It may be relevant to evaluate the prognostic value of the markers of the RSP and the PAM marker in clinical specimens with long term follow up data or evaluate the expression in prostate cancer models.

The role of NE cells in the progression to androgen independent growth is still unclear and there are not many representative prostate cancer models with NE differentiation. Several groups have recently been developing prostate tumor models both in vitro cell lines (Romijn, 1996) and in vivo xenografts (Liu, 1996; Nagabhushan, 1996; van Weerden, 1996). The presently available prostate cancer cell lines lack the

potency to differentiate into NE cells. NE differentiation was studied in the panel of in vivo human prostate cancer xenograft models developed at our Institution. Some did not express the NE phenotype at all, whereas some lost their NE phenotype after a few passages in nude mice (Noordzij, 1996). It appeared that in the androgen dependent PC-295 and PC-310 models part of the cells constitutively has the NE phenotype. These two models are very suitable for studying NE differentiation in prostate cancer and the role that NE cells may play in the progression of prostate cancer. In both the PC-295 and PC-310 models, androgen deprivation induces increased numbers of NE cells. The kinetics of NE differentiation have been intensively studied in the PC-295 model (Jongsma, 1998). In this completely androgen dependent model, the tumor rapidly regressed and like the non-NE cells also the NE cells eventually died.

The PC-310 xenograft does not regress completely after androgen withdrawal. Therefore, we considered this tumor model more suitable for studying the process of NE differentiation of androgen dependent cells to NE cells and the consecutive maturation of these NE cells in the long-term. We first characterized the kinetics of NE differentiation after androgen depletion in the PC-310 model by using CgA, MIB-1, AR and PSA as cellular markers and analyzed the expression of SgIII, PC-1, PAM and 7B2 as the different markers of the RSP, and the expression of different growth factors like serotonin, gastrin releasing peptide, vasoactive intestinal peptide and VEGF after androgen suppression. Among the limited number of available in vitro cell lines of human prostate cancer, none showed the NE phenotype (Noordzij, 1996). Attempts were made to set up in vitro cultures of the PC-310 xenograft and the effect of androgen deprivation was evaluated whether the in vitro PC-310 culture had the potency of NE differentiation and behaved similarly to the xenograft in vivo in an androgen depleted environment.

MATERIALS AND METHODS

PC-310 xenograft model

The nude mouse human prostate cancer xenograft model PC-310 was established from a primary prostatic tumor after radical prostatectomy of a previously untreated patient (van Weerden, 1996). The tumors usually grow with a doubling time of about 16 days and a lag phase of 2-3 months. The model represents a strictly androgen dependent and histologically moderately differentiated tumor, organized in solid sheets and microacini. In short, PC-310 tumors were implanted subcutanously at both shoulders of intact NMRI male nude mice (Harlan, Horst, The Netherlands). Optimal growth conditions were reached by supplementation of PC-310 transplanted mice with testosterone implants, as previously described (van Steenbrugge, 1984). Subcutanously growing tumors developed within 2-3 months and were grown up to a maximum volume of 2000 mm^3. Tumor volume changes were followed weekly by two perpendicular diameter measurements (D1 and D2) after which the volume was calculated from the formula: $V=(\Pi/6)(D1 \times D2)^{3/2}$.

Castration experiment with the PC-310 human prostate cancer xenograft model

Castration experiments were performed with 24 testosterone-supplemented PC-310 bearing male NMRI mice (Harlan, Horst, The Netherlands) at the Erasmus Center for Animal Research (Project nr. 102.98.02). Androgen withdrawal was performed by castrating the mice under hypnorm anesthesia (Janssen Pharmaceuticals, Oxford, UK) and by removing the silastic testosterone implant. Mice were sacrificed at 0, 2, 5, 7, 14 and 21 days after castration, four mice per time point.

After blood samples were taken for determining serum prostate specific antigen levels, mice were sacrificed. Tumor volumes were measured and tumor nodules were removed. The tumors were cut into small pieces which were either fixed in 4% buffered formalin and paraffin embedded for immunohistochemical analysis or snap frozen in liquid nitrogen and stored at - 80°C for biochemical analysis. The paraffin embedded material was processed routinely for hematoxylin and eosin staining.

In vitro initiation of primary culture from PC-310 xenograft tissue

Initiation of primary cultures from xenograft material was performed according to Limon *et al* (Limon, 1990). Tumors were mechanically disaggregated after washing the tumor tissue in RPMI1640 + P/S, and necrotic tissue and blood clots were removed. The tissue was minced into 1- to 2-mm^3 pieces which were put into RPMI 1640 + P/S.

After sedimentation for 10-15 minutes the supernatant was separated from the pellet. The supernatant was centrifuged at 175g for 10 minutes and the resulting pellet was resuspended in 3 ml medium and transferred into a culture flask. 10 ml medium containing collagenase A (final concentration 200 U/ml) was added to the pellet obtained after sedimentation and this suspension was incubated at 37 °C for 2-3 hours. The cell suspension was transferred into centrifuge tubes, RPMI1640 + P/S was added and the cells were centrifuged at 175g for 10 minutes.

The resulting pellet was resuspended in 10 ml Swedish culture medium, i.e. in Dulbeco's modified Eagles medium (DMEM F12) supplemented with various growth factors, 2% FCS, 10^{-10}M R1881 and P/S as described before (Romijn, 1996). After sedimentation for 10-15 minutes, the ensuing supernatant was centrifuged, resuspended in medium and transferred into culture flasks. Subsequent supernatant cultures were established by resuspending the sedimented pellet and repeated sedimentation for another 10-15 minutes. The different supernatant fractions were incubated in different culture flasks. The PC-310 xenograft was initially grown as a mixed culture of mouse fibroblasts and PC-310C human epithelial cells. After passaging of the cultures and subsequent treatment with Amphotheracin B, which could effectively kill mouse fibroblasts, pure cultures of PC-310C cells were obtained.

Androgen deprivation of PC-310C cultures

Series of confluent flasks with PC-310C were rinsed with PBS and subsequently cultured in medium with dextran charcoal (DCC) treated FCS for 0, 2, 5, 7, 14 and 21 days. At each time-point, culture medium was sampled and cells were harvested and frozen at -80°C for RNA and protein extraction. Likewise, small petri dishes were plated with PC-310C cells (n=2) and cells were grown in DCC containing medium. At the different time-points, the level of proliferation was assessed as the percentage cells with blue mitotic figures and the level of apoptosis was assessed as the percentage cells containing blue and pink apoptotic bodies by quantitative immunofluorescence microscopy of Hoechst 33342 and propidium iodine (PI) staining of the PC-310C cultures.

The PC-310C cells were also grown on glass slides under various conditions and were used for immunohistochemical staining for NE-associated proteins.

Immunohistochemistry

To identify the fraction of cells expressing the NE phenotype, paraffin embedded tissue sections of the PC-310 xenografts were stained with antibodies against CgA

(monoclonal clone LK2H10; ICN Pharmaceuticals, Aurora Ohio or polyclonal rabbit anti human CgA, DAKO), SgIII (rabbit polyclonal antibody; provided by the Department of Animal physiology, University of Nijmegen) (Holthuis, 1996a) and PC1 and PC2 (Alexis Biochemicals, 10P's, Breda, The Netherlands). For identification of the proliferative capacity, tissue sections were stained with the antibody against the proliferation associated Ki-67 antigen (MIB-1, Immunotech, Marseille, France). In addition, apoptotic cells were identified by counting the apoptotic bodies in the hematoxylin and eosin staining of the tissues. Other antibodies used were directed against the androgen receptor (clone F39.4, kindly provided by Dr. A.O. Brinkmann, Department of Endocrinology and Reproduction, Erasmus University); the cytoplasmic Bcl-2 antigen (clone 124, Dako, Glostrup, DK), the growth factor VEGF (rabbit polyclonal, DAKO) and against the neuropeptides bombesin (rabbit polyclonal GRP, DAKO; and anti-GRP MoAb 2A11; kindly provided by Dr F. Cuttitta, NCI, Bethesda, USA), serotonin (5-HT; rabbit polyclonal antibody), vasoactive intestinal polypeptide (VIP, rabbit polyclonal) and Calcitonin (rabbit polyclonal).

Paraffin embedded xenograft tissues were cut at 4 µm sections for single immunostaining and 2 µm for double immunostaining. The sections were mounted on 3-amino-propyl-triethoxysilane coated glass slides and incubated overnight at 60°C. The slides were deparaffinized and endogenous peroxidase activity was blocked with 3.3% hydrogen peroxide (H_2O_2) in methanol for 10 minutes. The following steps were also performed for the PC-310C glass slides after a 10-minute fixation in 4% formaldehyde. Antigen retrieval was then performed in 10 mmol/L citrate buffer (pH 6.0) in a microwave at 700 watt for an initial 10 minutes and a subsequent 5 minutes(Shi, 1991). The slides were allowed to cool down to room temperature and then put into the sequenza immunostaining system (Shandon, Uncorn, UK) and rinsed with phosphate buffered saline (PBS). All slides were pre-incubated with normal goat serum (DAKO, Glostrup, DK) diluted 1:10 in PBS, which yields for all compounds used hereafter, for 15 minutes. The primary antibody was incubated at the appropriate concentration for 2 hours at room temperature or overnight at 4°C. The secondary antibody was incubated for 30 minutes, being either horseradish-peroxidase conjugated goatαmouse or goatαrabbit (1:50), or biotinylated goatαmouse and goatαrabbit (1:400) for monoclonal and polyclonal antibodies, respectively. In case of biotinylated goatαmouse and goatαrabbit, a horseradish-peroxidase streptavidin biotin complex diluted 1:1:200 in PBS, prepared at least 30 minutes prior to use, was incubated for a subsequent 30 minutes. Between the subsequent steps, the slides were rinsed four times with PBS. The bound horseradish-peroxidase was visualized in 10 minutes with diaminobenzidine (DAB, Fluka, Neu-Ulm, Germany) in PBS containing 0.075% H_2O_2 as substrate. Slides were rinsed extensively in tap water and finally counterstained in Mayer's Hematoxylin, dehydrated through a series of alcohol and embedded in malinol.

To assess the properties of the NE cells, double staining of CgA respectively with MIB-1, androgen receptor and Bcl2 was performed on the PC-310 xenograft tumors. The double staining procedure consisted of two consecutive stainings with two primary antibodies. The first being always a horseradish-peroxidase-related stable diaminobenzidine complex, whereas the second staining was performed with an alkaline phosphatase conjugated goatαmouse secondary antibody. In between both stainings, the slides were rinsed with PBS for one hour and again boiled in a microwave in 10 mmol/L citrate buffer (pH 6.0) for 10 minutes. As negative control, PBS replaced the primary antibody in all stainings. Radical prostatectomies, containing normal prostatic tissue were used as positive control for CgA, SgIII, PC1, PC2, androgen receptor, GRP, 5-HT, VEGF, Bcl2 and MIB-1 expression.

For most markers, the number of positive cells was determined by quantitative counts of all cells in tumor squares at 310x magnification from which the number of positive cells per square mm was calculated. In total, positive cells were scored as percentage of the total cell number, in ≥ 10 squares. For androgen receptor, VEGF, GRP, 5-HT, PC1, and PC2 the level of immunostaining was assessed semi-quantitatively.

Western blot analysis

We further confirmed the expression patterns of CgA, SgIII, Bcl-2, 7B2 and androgen receptor in our castration series of the PC-310 tumor by Western blotting. As positive controls, we used material of human pheochromocytoma for CgA, of rat pituitary for SgIII and of the human in vitro cell line LNCaP for androgen receptor expression. The procedure of protein extraction was as previously described (Noordzij, 1996). Frozen tissues of the PC-310 xenografts were crushed in a liquid-nitrogen-chilled metal cylinder. The tissue homogenates or PC-310C cell culture pellets were transferred into a lysis buffer (10mM TRIS (pH 7.4), 150 mM NaCl (Sigma), 1 % Triton X-100 (Merck, Germany), 1 % deoxycholate (Sigma), 0.1 SDS (Gibco), 5 mM EDTA (Merck) and protease inhibitors (1mM phenylmethylsulfonyl fluoride, 1mM aprotinin, 50 mg/L leupeptin, 1 mM benzamidine and 1 mg/L pepstatin; all from Sigma). After centrifugation of the mixture at 100,000 rpm at 4°C for 10 minutes, the protein content of the supernatants was measured by the Bradford method (Bio-Rad protein assay, München, Germany).

20 µg of each sample was transferred to a SDS polyacrylamide gel and gel electrophoresis was performed with pre-stained markers as size standards (Novex, San Diego, CA). The gels were blotted to a 0.45µm cellulose nitrate membrane (Schleicher & Schuell, Germany). The immunoblot was blocked with PBS (pH 7.7) containing 0.1% Tween-20 (Sigma) and 5% dry milk for one hour. The CgA, 7B2 (Mon 102/144, kindly provided by Prof. Dr WM van der Ven, Leuven, Belgium), Bcl-2, androgen receptor or SgIII antibodies were added in their optimal concentration and incubated overnight on an orbital shaker at 4°C. After rinsing four times 15 minutes with PBS, incubation for one hour was performed with the secondary horseradish-peroxidase-conjugated antibodies and goatαmouse for mouse monoclonal, goatαrabbit for rabbit polyclonal antibodies, respectively. Subsequently, a short incubation with a mixture of 10 ml luminol and 100 µl oxidizing agent (BM chemiluminescense kit, Boehringer Mannheim GmbH, Germany) followed, after washing for four times 15 minutes with PBS. Excess reagent was removed and antibodies were visualized by exposure of the blots to an X-ray film.

Reverse Transcriptase-polymerase chain reaction

RNA was isolated by using the single step RNAzol™ B method (Campro, The Netherlands (Chomczynski, 1987). Frozen tissue (100mg) or cell culture pellets were homogenized in 1 ml of RNAzol™. Chloroform (0.1ml) was added and the mixture was vortexed for 15 seconds followed by 5 minutes incubation on ice. The homogenate was then centrifuged at 4 °C at 12,000 g for 15 minutes. The upper water-phase containing the RNA was removed and mixed with an equal volume of iso-propanol. This mixture was then kept at 4 °C for 15 minutes and after that centrifuged at 4°C at 12,000 g for 15 minutes. The supernatant was removed and the RNA pellet was washed twice with 75 % ethanol by vortexing and centrifugation at 4°C at 12000 g. The pellet was then dried and resuspended in sterile H_2O. The concentration was determined at OD 260 and

solutions of 1µg/µl were prepared for further use in Reverse Transcriptase-polymerase chain reaction (RT-PCR) or Northern blot analysis. The quality of the isolated RNA was checked by determining the 260/280 ratio and by formaldehyde gel-electrophoresis to check the ribosomal (28 S and 18S) bands.

RT-PCR was performed for CgA, SgIII, PAM, PC1 and 2 and ß2-microglobulin (ß2MG) with a standard protocol. Reverse transcriptase reaction was performed with a mastermix containing 5 mM $MgCl_2$, PCR buffer, 10 mM dNTP's, RNAse inhibitor (10 units), Reverse transcriptase (25 units), 2.5 mM random hexamer primers and 0.5 µg RNA in a total volume of 10 µl covered with 50 µl of mineral oil. The mastermix was then processed at 42°C for 60 minutes followed by 15 minutes incubation at 99°C and the reaction was stopped at 4°C for five minutes. The cDNA mix that was formed was then used totally with the mastermix of the polymerase chain reaction (PCR) protocol. In this protocol, the mastermix contained reaction buffer, Supertaq polymerase (HT Biotechnology Ltd., Cambridge, UK; 1 unit), 15 µM sense and antisense primer in a total volume of 40 µl. All samples were first denatured at 94°C for 10' and then amplification was performed for 35 cycles of 1' 94°C, 1' 60°C and 1' 72°C and a final extension at 72°C for 10'. The PCR product was checked on a 1% agarose gel and, if necessary, followed by Southern blotting. The internal control of human ß2MG clearly showed the equal amounts of mRNA that were used for each time-point.

Northern blot analysis

Northern blot analysis was performed by running a formaldehyde gel in MOPS buffer containing 20 µg of each sample including the controls. The RNA content of the gel was consequently blotted overnight onto a hybond-N^+ filter in 10 x Standard saline citrate (10xSSC; 1.5 M sodium chloride and 0.15 M sodium citrate). The filter is checked for RNA and ribosomal bands are marked. After rinsing the filter in 2xSSC, the blot is cross-linked in a GS gene linker™ UV chamber (Bio-RAD, München, Germany) and ready for (pre)hybridization with different probes against CgA, SgIII, PAM and B2-microglobulin as control.

RESULTS

Androgen withdrawal in the PC-310 xenograft showed a decrease in volume directly after castration. The PC-310 tumor doubling time was 16 days before castration. After castration, the PC-310 tumors had regressed with 50% within 9 days. Between 14 and 21 days post-castration tumor volume was maintained at about 30-40 % of the initial tumors. (Figure 1a). The decline in tumor volume was associated by a rapid decrease of proliferating, MIB-1 expressing, cells from 20 % in the controls to zero at 7 days post-castration (Figure 1a). Apoptotic counts increased after 2, 5, and 7 days post-castration, and returned to control levels after 14 and 21 days (data not shown). The serum PSA level dropped rapidly post-castration and was maintained around zero after 14 days (figure 1b). The expression of the 110-112 kD androgen receptor protein decreased directly after castration but a markedly increased expression was observed on Western blot after 5 to 7 days post-castration (figure 2a).

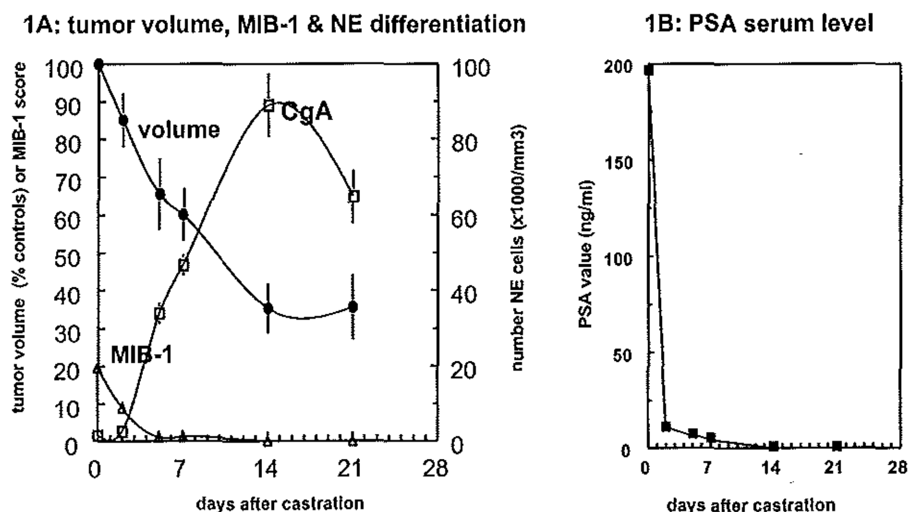

Figure 1: Castration experiment of PC-310 xenograft. Effect on tumor volume, MIB-1 score, the number NE cells and PSA value (ng/ml)
A) The effects of androgen withdrawal on tumor volume (●), MIB-1 expression (△) and the number of NE cells marked by CgA (□) at different time points post-castration (n=3).
B) PSA (■) serum levels in the nude mice PC-310 model at different points in time post-castration (n=3).

Expression of the NE marker CgA increased rapidly after castration up to an approximately 40-fold increase after 14 days of androgen withdrawal (figure 1a). From the histological picture it is clear that changes in NE differentiation are not due to a selection of NE cells as a 40-fold increase in the number of NE cells is seen, when there is only a 60-70 % volume decrease at 14 and 21 days post-castration. On Western blot, the increased expression of CgA at 78 and 68 kD could clearly be visualized as could also the increased processing of the protein into 49, 31 and 18 kD proteins after 14 and 21 days of androgen withdrawal (figure 2b). Likewise, increased mRNA levels for CgA were found in Northern blot analysis (figure 3). RNA of the same series of tissues was reverse transcribed and the resulting cDNA was used in subsequent PCR reactions analyzing the expression of CgA and other markers of NE differentiation. Expression of markers of the regulated secretory pathway (RSP) were found to be increased after androgen deprivation of PC-310 in vivo. Induction of SgIII and 7B2 were found to be comparable to the increased CgA expression at 5 or 7 days after androgen withdrawal (figure 4). Temporal induction of mRNA expression of PC1 and PC2 was found at day 5 and 7, respectively. PAM-mRNA expression was temporarily induced at day 7 post-castration, when the expression of the largest of the three PAM splice variants(Vos, 1995) was mainly increased. Increased expression of the RSP markers was confirmed by immunohistochemistry (IHC) and Western blotting (table 1). From this table, it is clear that the expression of SgIII and 7B2 is already apparent at day 5 and 7 post-castration, whereas the processing enzymes PC1, PC2 and PAM are expressed later in the NE cells at day 7 and becomes more apparent at day 14 and 21 post-castration. The anti-apoptotic protein Bcl-2 was expressed at low levels in the androgen supplemented mice, but decreased at both mRNA and protein level after androgen withdrawal (result not shown). Furthermore, expression of the

endothelial growth factor, VEGF, was clearly increased at 14 and 21 days post-castration. Interestingly, also GRP and 5-HT expression could clearly be detected as a relatively late event of NE differentiation from 14 days post-castration on. Expression of vasoactive intestinal peptide or calcitonin was not found during the 21 day period studied. From IHC double labeling studies, we found that about 50 % of the epithelial tumor cells expressed the androgen receptor (CgA^-, AR^+), and the other 50 % were NE cells (CgA^+, AR^-).

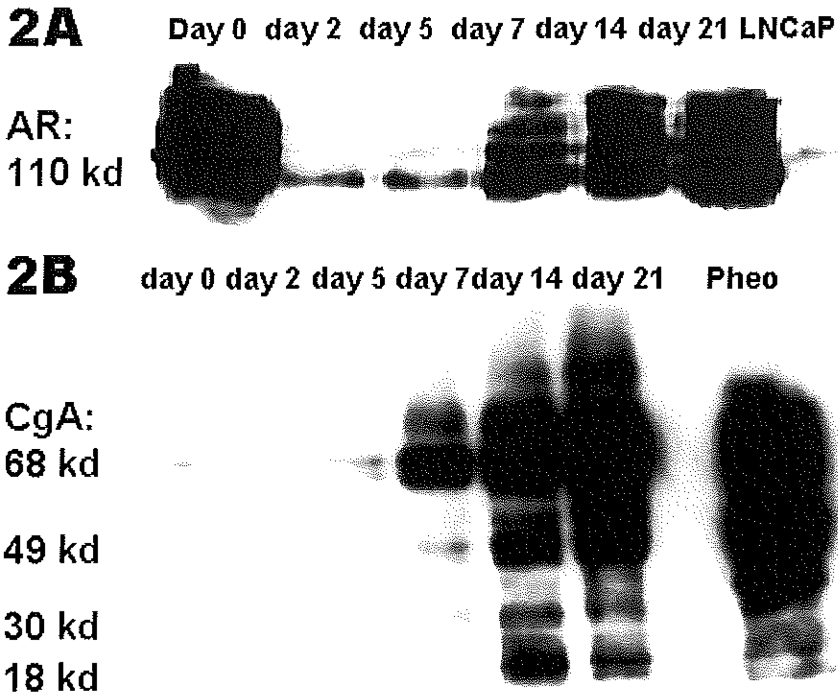

Figure 2: Expression of the androgen receptor (AR) and Chromogranin A (CgA) following castration in the PC-310 xenograft model. Both panels show the time points T_0, T_{-2}, T_{-5}, T_{-7}, T_{-14} and T_{-21} after androgen withdrawal. In the upper panel (2A) the signal of AR expression is found at 110-112 kD which first rapidly decreased and is subsequently reexpressed in the PC-310 model. The increased CgA signal can be found between 68 and 78 kD in the lower panel (2B), which is the highest band in the control pheochromocytoma (Pheo). After 7 days clear processing of the protein is shown by smaller fragments at ± 49 kD and at ± 30 kD.

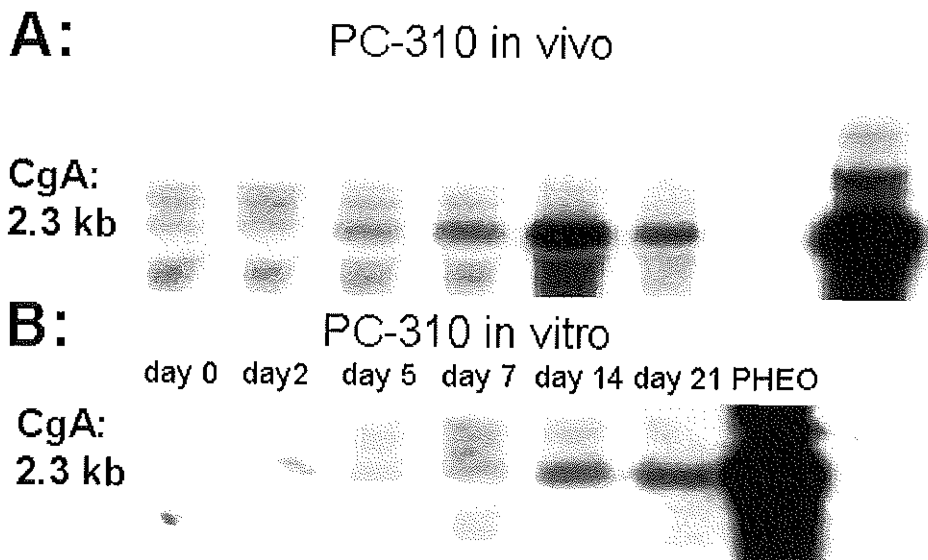

Figure 3: Expression of NE marker CgA on Northern blot in the PC-310 model system after androgen withdrawal. CgA mRNA expression at 2,3 kB clearly increased post-castration in the PC-310 xenograft model (3A) and in the in vitro cell line.

Table 1: Expression of NE markers, neuropeptides and growth factors in PC-310 in vivo after androgen suppression as determined by immunohistochemistry and Western blot analysis (7B2).

Time (days)	CgA	SgIII (Sg3)	7B2	PC1	PAM	GRP	VEGF	5-HT
Control	+	±	±	-	-	-	-	-
2	+	±	±	-	-	-	-	-
5	++	+	+	-	-	-	-	-
7	+++	++	++	±	+	±	+	-
14	++++	+++	+++	++	++	+	++	±
21	+++++	++++	+++	++++	+++	+	++++	++

CgA, SgIII, PC1, PAM, GRP, VEGF, 5-HT were all determined by IHC, whereas 7B2 was determined from Western blot. Expression was scored as following: –, no expression, ±, sporadic expression, +, focal expression, ++, few cells positive per optical field, +++, clustered cells positive per optical field, ++++, more clusters of positive cells per optical field, +++++, 50% of tumor positive. The expression of 7B2 was determined from weak expression (±) to high expression (+++).

Table 2: Effects of androgen withdrawal on PC-310C in vitro. Proliferation, apoptosis and culture PSA levels after androgen withdrawal

Time (days)	Apoptosis (%) (total)	Mitotic cells (%)	PSA culture levels (ng/ml)
Control	2.1 ± 0.5	2.8 ± 1.3	278 ± 89
2	5.7 ± 1.3	2.3 ± 0.9	610 ± 140
5	7.8 ±1.5	0.9 ± 0.9	943 ± 178
7	10.0 ± 2.7	2.1 ± 1.6	948 ± 195
14	6.3 ± 2.3	1.2 ± 0.6	186 ± 90
21	5.9 ± 1.6	0.4 ± 0.6	505 ± 159

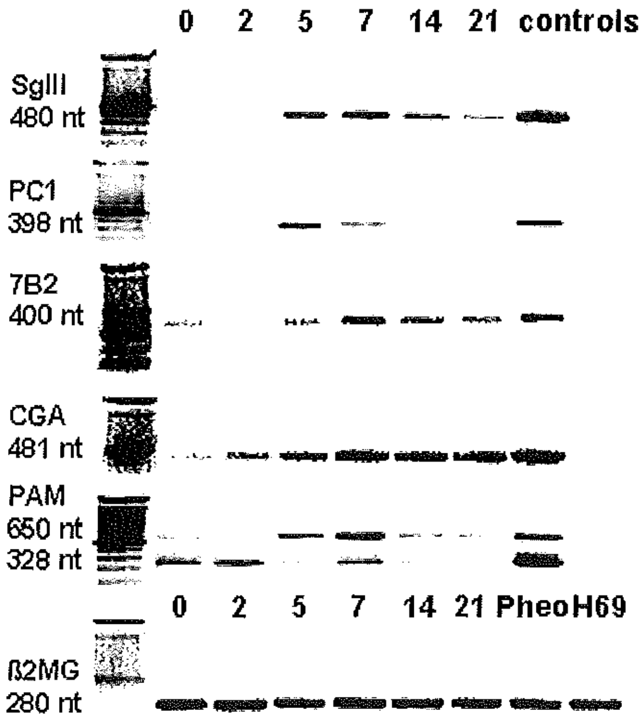

Figure 4: NE differentiation and regulated secretory pathway in PC-310. RT-PCR was performed for expression of SG3, PC1, 7B2, CgA, PAM and ß2MG in PC-310 xenograft after castration. H69 was used to check PC1, 7B2 and PAM analysis and Pheo for SG3 and CgA. The products of the primer sets are indicated as the length in nucleotides of the mRNA that is specifically amplified.

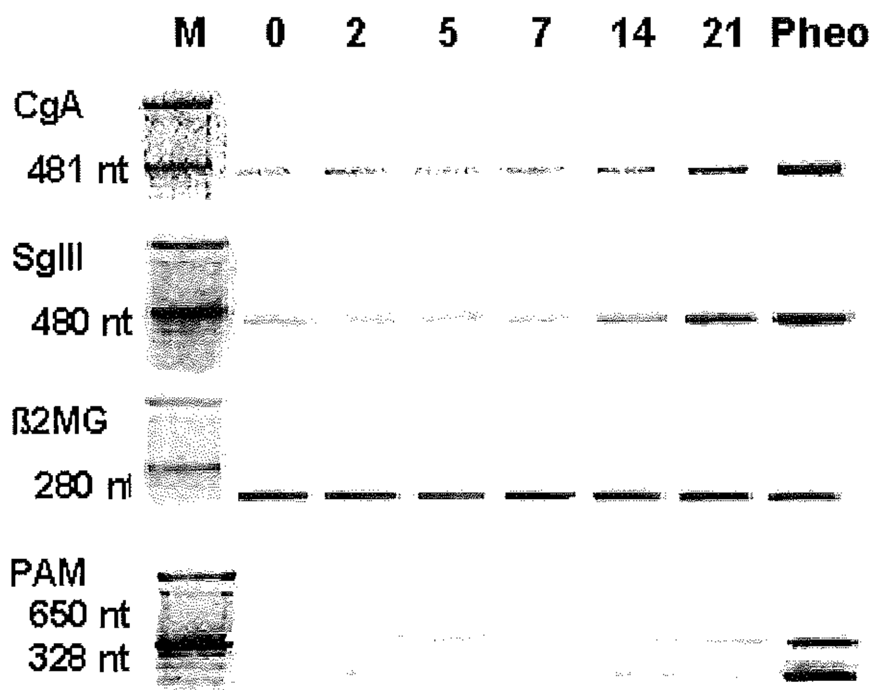

Figure 5: Expression of CgA, SG3, PAM and ß2MG in PC-310C. The products of the primer sets are indicated as the length in nucleotides of the mRNA that is specifically amplified. Pheochromocytoma was used as a control for CgA and Sg3 expression and H69 was used for PAM expression.

Androgen depletion of PC-310C cultures led to increased numbers of apoptotic cells and a decreased level of proliferation as determined by Hoechst 33342 and PI staining (table 2). The total amount of apoptotic cells was counted as the increased number of Hoechst 33342 or Hoechst 33342/ PI staining nuclei containing fragmented DNA and/or apoptotic bodies, whereas the number of blue, Hoechst 33342 stained, mitotic figures decreased in time after androgen suppression. PSA levels of the culture medium initially decreased but without refreshing the culture medium PSA levels increased in time. Some proliferating cells were still present after 21 days of androgen withdrawal and PSA levels in the medium were high. Androgen receptor expression initially decreased to a limited extent after androgen withdrawal but after five days of androgen depletion an increased level of the 110-112 kD androgen receptor was found. Similarly to the in vivo grown PC-310 tumor, in PC-310C cultures the expression of the NE markers CgA and SgIII increased after 14 days of androgen withdrawal at both mRNA level (figure 3b, 5) and protein level (figure 6b). The mRNA levels of PC1 and PC2 did not increase and a slight increase in the levels of PAM-mRNA was found (figure 5).

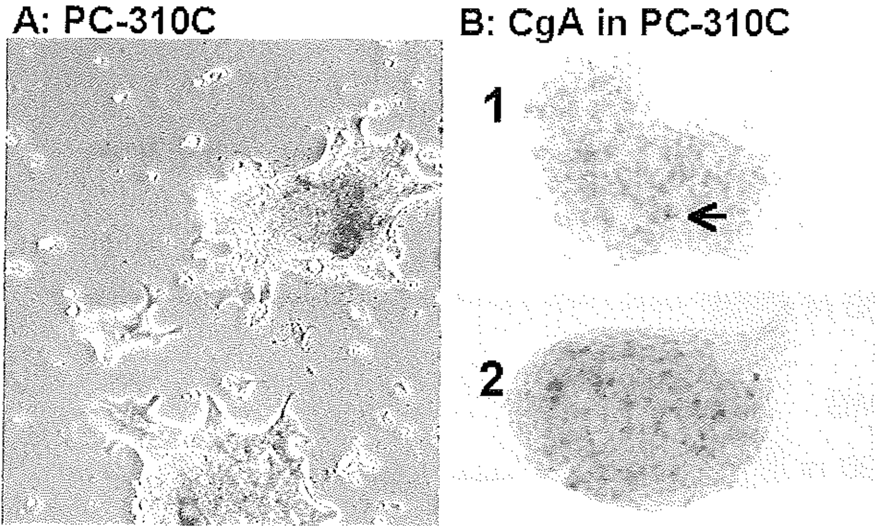

Figure 6: NE differentiation in the in vitro PC-310C culture after androgen withdrawal.
A: Overview of the PC-310C after 5 passages in culture which grows in monolayer colonies.
B: Comparison of CgA expression in PC-310C cultures growing with or without androgens on glass slides. 14 days post-castration a clear increase in NE differentiation is shown. Magnification 310X.

DISCUSSION

This study of the PC-310 prostatic carcinoma xenograft model aims at a comparison of the kinetics of inducible neuroendocrine differentiation on androgen depletion in an androgen dependent model system both in vivo and in vitro. The androgen dependent human PC-310 xenograft model was established from a primary untreated tumor and behaves as an androgen dependent clinical tumor. The growth and regression profiles of the PC-310 xenograft in the presently described castration experiments was comparable to studies earlier performed (van Weerden, 1996). Hormonal suppression of the model induced an initial increase of apoptosis, decreased proliferation and down regulation of the androgen receptor. This was paralleled by a rapid and prominent decrease of prostate specific antigen (PSA) serum levels, demonstrating the androgen dependent character of the PC-310 model, as was previously demonstrated for the PC-295 model (Jongsma, 1998). As a consequence, the PC-310 tumors regressed, and after 21 days of androgen withdrawal only 30-40 % of the initial tumor volume was left. The rapid loss of androgen receptor expression in the PC-310 tumors was comparable to that observed previously in the androgen dependent human PC-82 xenograft after castration (Ruizeveld de Winter, 1992). In contrast to the PC-82 however, after 5 days of androgen depletion the expression level of the androgen receptor returned to above normal. Constitutive high androgen receptor expression has been found in Trans Urethral Resection specimens of clinical hormone refractory tumors (van der Kwast, 1991), but lower expression of the androgen receptor was noted during regression of hormonal treated prostate cancer (Gil-Diez de Medina,

1998). In the same study, increased levels of the androgen receptor were observed in hormone refractory specimens as well as increased expression of the basal epithelial marker, cytokeratin 5 and the epidermal growth factor (EGF) receptor. Androgen receptor positive prostatic cancer cell lines, like the LNCaP also continue to express the androgen receptor after hormone depletion (Esquenet, 1997; Koivisto, 1998).

NE differentiation was clearly induced at 5 days after androgen withdrawal as shown by expression of CgA. At that time point only a few proliferating non-NE cells were left. Analysis for CgA at both RNA and protein level demonstrated the increased expression of the CgA in time from a low basal expression level in the controls to a maximum at 21 days post-castration. Despite inter-tumoral differences in basal CgA expression levels at the moment of castration, increased CgA expression was observed at 5, 7, 14 and 21 days post-castration at the IHC level, which was confirmed by Western blot analysis, RT-PCR and Northern blot analysis as shown by the induction of the 2.3 kb mRNA transcript of CgA after androgen withdrawal. By comparing the Western blots for CgA and androgen receptor post-castration, it was extrapolated that from day 7 post-castration on there were both NE cells present as well as cells expressing the androgen receptor. However, IHC double staining for AR and CgA confirmed that CgA and androgen receptor did not colocalize. Thus post-castration, the NE cells represent the androgen independent, non-proliferating part of the prostatic tumor.

From the stainings for CgA and MIB-1, it was seen that the proliferation at 7 days post-castration was near zero, whereas there were still increased numbers of NE differentiated tumor cells. It can be concluded that the greater part of the NE cells was in the G_0-phase of the cell cycle which is in agreement with our previous study of the PC-295 model (Jongsma, 1998) as well as with other studies on NE differentiation and proliferation in prostate cancer (Bonkhoff, 1994a; Bonkhoff, 1994b). In clinical prostate cancer specimens, Bonkhoff et al (Bonkhoff, 1994b) found that androgen receptor negative NE cells expressed cytokeratins 18 and 5, and that these cells were found in the proximity of proliferating cells. In the PC-310 experimental tumor model, during a period of 21 days post-castration NE cells were found scattered over the tumors and were definitively not associated with proliferative activity of the surrounding non-NE cells.

The kinetics of NE differentiation, after androgen withdrawal was clearly demonstrated by the time related induction of other secretogranins and processing enzymes belonging to the regulated secretory pathway (RSP) (Holthuis, 1995) next to the observed induction of CgA. RT-PCR analysis of the RSP markers clearly showed the time dependent induction of 7B2, SgIII, PC1, PC2, and PAM in the PC-310 NE cells. The kinetics of NE differentiation and the maturation of secretory granules in prostatic NE cells was also shown by IHC in the PC-310 model. Increased expression at protein level of SgIII by IHC and 7B2 by Western blotting was detected from 5 to 7 days post-castration on, whereas induction of PC1, PC2 and PAM was shown later, i.e. at day 14 and 21 post-castration during apparent maturation. These results indicate that the NE cells in the PC-310 model possess an active RSP and also demonstrate the time dependent maturation in the secretory granules as shown by the increased expression of the prohormone convertase, PC1 and the increased processing of CgA coinciding at 14 days post-castration (Figure 2b). Clearly, the 78 and 68 kD CgA protein (Noordzij, 1996), is processed by PC1 to form products and processed proteins ranging from ~ 49 kD to ~ 31 kD and ~ 18 kD as was shown before by others (Barbosa, 1991; Metz-Boutigue, 1993; Eskeland, 1996).

PAM is most likely expressed in NE cells, as the protein activates neuropeptides by amidation mainly in the secretory granules of the RSP, but expression in non-NE

cells has also been described (Saldise, 1996). The increase of PAM in the PC-310 model after castration coincides with the time-related induction of expression of RSP associated proteins. It has yet to be proven whether PAM is expressed in NE cells or in the neighboring non-NE cells, but preliminary IHC data indicate increased PAM expression with a granular 'NE like' staining pattern 14 days post-castration (data not shown).

VEGF, GRP and 5-HT, but not VIP and calcitonin were only expressed post-castration in the PC-310 model. The expression of VEGF co-localized in the NE cells and the expression was induced shortly in time after the expression of CgA and SgIII. Expression of GRP and 5-HT was only found in a small part of the NE cells relatively late, at 14 and 21 days post-castration. This indicates that possibly other neuropeptides and growth factors for endothelial cells can be induced post-castration in the PC-310 model. Apparently, the PC-310 tumors respond to castration by induced VEGF, GRP or 5-HT expression that may induce proliferation of endothelial cells or non-NE cells. Induction of VEGF may be a consequence of NE differentiation, but alternatively VEGF expression can be induced by hypoxia (Semenza, 1998), which is a common condition observed in prostatic tumors (Zhong, 1998). Possibly, increased hypoxic stress after androgen withdrawal induced VEGF expression in the NE differentiated cells.

Previously, we suggested that the condition of NE differentiation and the absence of androgen receptor expression plays a key role in the survival of prostate cancer cells, whereas Bcl-2 protects the remaining androgen independent cells from undergoing apoptosis (Jongsma, 1998). Here we show another unexpected behavior of androgen dependent cells, being the drop in androgen receptor expression shortly after androgen withdrawal followed by a relapse of expression after 5 days post-castration. Part of the initially androgen dependent PC-310 cells differentiated into NE cells following androgen withdrawal, whereas the remaining carcinoma cells are behaving as androgen sensitive tumor cells surviving in an androgen deprived situation. In contradiction to what has been reported about the LuCap 23 model (Liu, 1996) and to our previous observations in the PC-295 model, here we showed an alternative route of overcoming cell death after androgen deprivation by both a strong induction of NE differentiation and a transient down-regulation of the androgen receptor, without inducing Bcl-2 expression.

The establishment of in vitro cultures from PC-310 epithelial cells in a specialized culture medium has enabled us to study the in vitro effect of androgen deprivation on the kinetics of NE differentiation in PC-310C and compare it with the behavior of PC-310 in vivo. To our knowledge PC-310C is the first human cell culture of a primary untreated prostatic adenocarcinoma containing cells with the NE phenotype and which can be induced to differentiate into NE, i.e. CgA and SgIII positive cells after androgen depletion. The in vitro model PC-310C resembles the in vivo model for most parameters like the decrease in proliferation, the induction of apoptosis, the regulation of AR expression and the less stronger but significant induction of CgA mRNA expression. The main difference with the in vivo situation is the observation that after an initial decrease of PSA in vitro, the PSA levels increased again to control level. An explanation for this finding is that PSA can not be degraded in the absence of stromal cells or is not removed by the blood stream in vitro. Otherwise, PSA secretion is induced by androgen deprivation, or PSA leaks into the culture medium from dying cells. Alternatively, androgen independent activation of the androgen receptor by growth factors added to the culture medium, e.g. by insulin, EGF or hydrocortisone might be involved.

The difference in the stainings for CgA, SgIII, PC 1 and PC 2, the non-proliferative status of most of the NE cells as observed in the controls (T_0) and at 7 days

post-castration and the temporal increase in apoptosis in the PC-310 model system, clearly show the proliferation independent process of neuroendocrine differentiation and maturation of NE cells as being induced by androgen withdrawal.

Expression of CgA is not a requisite for regulated secretion of bioactive neuropeptides in the PC-310 model, but co-expression in the PC-310 model system with RSP related proteins, like SgIII, 7B2 and the time related induction of PC1 and PAM expression indicates that the NE cells have the potency to actively secrete growth factors like neuropeptides from secretory granules (Holthuis, 1995; Holthuis, 1996a). This statement is fortified by the relatively late induction of GRP and 5-HT expression during granular maturation. Likewise, the induced expression of VEGF may lead to the growth of new blood vessels, which may play a decisive role in progression of prostate cancer to hormone independence. Furthermore, we consider the possibility of androgen independent activation of the androgen receptor, for instance by neuropeptides like GRP, IGF-1 (Chan, 1998) or cytokines like IL-6 which can not be excluded (Hobisch, 1998) as the androgen receptor is still expressed on 50 % of the PC-310 cells in the androgen depleted situation.

As the role of NE differentiation in progression of prostate cancer is still unclear (Krijnen, 1997; Jiborn, 1998), testing of other markers than CgA such as SgIII, 7B2, PC1 and PAM as putative NE-related prognostic markers for the progression of clinical prostate cancer is still relevant. Possibly, changes in expression of components of the regulated secretory pathway like PC1 or PAM might have effects on, for example, overproduction of bioactive growth stimulating neuropeptides in prostate cancer after androgen suppression and thus lead to hormone refractory disease.

The PC-310 model system is a unique model for further studies on induction of NE differentiation both in vivo and in vitro by androgen withdrawal, the role of the regulated secretory pathway in the progression of prostate cancer in particular. In addition, androgen independent growth or prostate cancer progression is currently under investigation in a long-term androgen suppression experiment of the PC-310 in vivo model. Induction of NE differentiation, maturation of NE cells and temporal down regulation of androgen receptor protein expression in prostate cancer might be the same as the clinical behavior of a major part of hormone refractory prostate cancer patients who relapse after initial 1-2 years of remission of the disease. The only cells in the PC-310 tumor left after 21 days are androgen independent, CgA expressing NE cells and androgen receptor expressing androgen sensitive cells, which do not proliferate nor go into apoptosis. Further studies on the PC-310 model system will yield information as to whether NE cells play a role in the maintenance of tumor residues by initiating growth induction of androgen receptor positive non-NE cells e.g. by different growth factors.

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CHAPTER 5

DIFFERENT PROFILES OF NEUROENDOCRINE CELL DIFFERENTIATION EVOLVE IN THE PC-310 HUMAN PROSTATE CANCER MODEL DURING LONG-TERM ANDROGEN DEPRIVATION.

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Submitted

ABSTRACT

Neuroendocrine (NE) cells are androgen independent cells and secrete growth-modulating peptide hormones via a regulated secretory pathway (RSP). We studied NE differentiation following long-term androgen withdrawal in the androgen dependent human prostate cancer xenograft PC-310. Tumor-bearing nude mice were sacrificed at 0, 2, 5, 7, 14, 21, 47, 84 and 154 days post-castration. Half-life of the PC-310 tumor was 10 days, with a stabledormant residual tumor volume of 30-40% after 21 days and longer periods of androgen deprivation. Proliferative activity and prostate specific antigen (PSA) serum levels decreased to zero post-castration, whereas cell cycle arrest was manifested by increased $p27^{kip1}$ expression. A temporary downregulation of androgen receptor (AR) expression was observed after androgen deprivation. The granine family expression of CgA, SgIII and 7B2 increased in vivo from 5 days post-castration on. Subsequently, prohormone convertase 1 (PC1) and peptidyl α-amidating monooxygenase, as well as the vascular endothelial growth factor were expressed from 7 days post-castration on. Finally, growth factors like gastrin releasing peptide and serotonin were expressed in a small part of the NE cells 21 days post-castration, but strong expression was induced late during androgen deprivation, i.e. 84 and 154 days post-castration, respectively. Androgen deprivation of the NE differentiated PC-310 model induced the formation of NE, AR^- and non-NE, AR^+ tumor residues. The NE residues actively produced growth factors via a RSP, among others VEGF inducing angiogenesis, which may lead to hormone refractory disease. The dormant non-NE, AR^+ tumor residues were shown to remain androgen sensitive even after longterm androgen deprivation. In the PC-310 xenograft, a time dependent NE differentiation and subsequent maturation was induced after androgen depletion. In conclusion, the androgen dependent PC-310 model constitutes an excellent model for studying the role of NE cells in the progression of clinical prostate cancer.

INTRODUCTION

Neuroendocrine (NE) differentiated cells form an androgen independent subpopulation of the prostatic glandular cells (Abrahamsson, 1996) and are considered to be non-proliferating cells which do neither express the androgen receptor (AR) (Krijnen, 1993) nor the anti-apoptotic oncogene Bcl-2 (Xue, 1997). Prostatic NE cells may regulate homeostasis and secretion of prostatic fluid, by interaction with non-NE prostatic glandular cells. Variable numbers of NE cells occur in nearly all clinical prostatic adenocarcinomas (Abrahamsson, 1986; di-Sant'Agnese, 1992b; Aprikian, 1993; Bonkhoff, 1995; di-Sant'Agnese, 1995; Noordzij, 1995a; Noordzij, 1995c). NE cells are often found near Bcl-2 positive prostate cancer cells (Colombel, 1992; Cohen, 1994). Many studies on the prognostic value of chromogranin A (CgA), the general marker for NE differentiation, have been performed. Although suggested otherwise, no clear correlation between CgA expressing NE cells and poor prognosis or progression of prostate cancer has been shown (Abrahamsson, 1986; di-Sant'Agnese, 1992b; Aprikian, 1993; di-Sant'Agnese, 1995; Noordzij, 1995a; Noordzij, 1995c). However, a recent study did show a correlation between NE differentiation with response to endocrine therapy of prostate cancers (Krijnen, 1997). Other studies revealed that androgen deprivation of clinical prostate cancer induced either NE differentiation or tumor residues (Van de Voorde, 1994; Chen, 1997; Guate, 1997; Jiborn, 1998; Pruneri, 1998). Other investigators identified non-NE related changes concerning AR status and tumor staging (de Vere White, 1997; Reuter, 1997; Culig, 1998).

NE cells can be identified by immunohistochemistry with specific antibodies against secreted products including serotonin (5-HT) (Xue, 1997) and gastrin-releasing peptide (GRP) or secretion-associated proteins, like CgA (O'Connor, 1986; Abrahamsson, 1989; Schmid, 1994; Hendy, 1995). Based on its possible role in progressive behavior of different types of cancer, e.g. small cell lung cancer and gastrinoma (Alexander, 1988; Chu, 1996; Kelley, 1997), also in prostate cancer research more attention is payed to GRP in recent years (Aprikian, 1998).

NE cells have an activated regulated secretory pathway (RSP) (Holthuis, 1996b) besides the lysosomal and the constitutive pathway. The RSP consists of a sequence of processes linked from transcription/translation of various factors to final secretion of peptide hormones (Smeekens, 1992; Tucker, 1996) at the plasma membrane from secretory granules (Holthuis, 1995). Different RSP markers can be identified, such as the granular markers Secretogranin III (SgIII) and Secretogranin V (7B2) (Martens, 1989; Sigafoos, 1993; Braks, 1996), carboxy peptidase E and the processing enzymes prohormone convertase 1 and 2 (PC1 and 2). The enzyme peptidylglycine α-amidating monooxygenase (PAM) (Vos, 1995; Vos, 1996) is expressed in or in the near vicinity of NE cells.

Recently, several groups have developed both *in vitro* (Romijn, 1996) and *in vivo* prostatic cancer models (Liu, 1996; Nagabhushan, 1996; van Weerden, 1996). NE differentiation was studied in the panel of *in vivo* human prostate cancer xenograft models developed at our institution (Noordzij, 1996). The androgen dependent PC-295 and PC-310 models constitutively express the NE phenotype. In both tumor models, androgen deprivation induced NE differentiation. The kinetics of short-term NE differentiation have been studied in both the PC-295 (Jongsma, 1998) and the PC-310 model (Jongsma, 2000b). Data from both model systems clearly showed a proliferation independent induction of NE differentiation after androgen deprivation. In the completely androgen dependent PC-295 model, the tumor rapidly regressed and like the non-NE cells, the NE cells eventually disappeared. In contrast, the PC-310 xenograft did not totally regress after androgen withdrawal. Therefore, the latter model was considered

particularly suitable for studying the process of NE differentiation and the consecutive granular maturation of NE cells after long-term androgen deprivation and the role of NE cells in tumor progression. For this purpose we analyzed the time dependent expression patterns of CgA, different markers of the RSP, and different growth factors like 5-HT, GRP, vasoactive intestinal peptide (VIP) and vascular endothelial growth factor (VEGF) after androgen suppression. The effect of long-term androgen deprivation on the non-NE tumor population was investigated with the markers MIB-1, $p27^{kip}$, and AR. Furthermore, we studied tumor histology and effects on tumor vascular density during long-term androgen deprivation. Also, the ability of the dormant tumor residues to respond to testosterone re-supplementation was investigated to assess the androgen sensitivity of the PC-310 tumor residues.

RESULTS

Androgen deprivation of the PC-310 xenograft model showed a 50% decrease in tumor volume within 9 days which confirmed previous studies with this model (Jongsma, 2000b). Between 14 and 21 days post-castration the decrease in tumor volume stagnated at 30-40 % of the initial tumor volumes and remained constant throughout the rest of the experimental period (figure 1). The decline in tumor volume was associated with a rapid decrease of proliferating, MIB-1 expressing, cells from 20 % in the controls to zero at 7 days post-castration (figure 1). $p27^{kip}$ expression increased from day 2 on to maximum positivity of 85 % of the tumor cell population

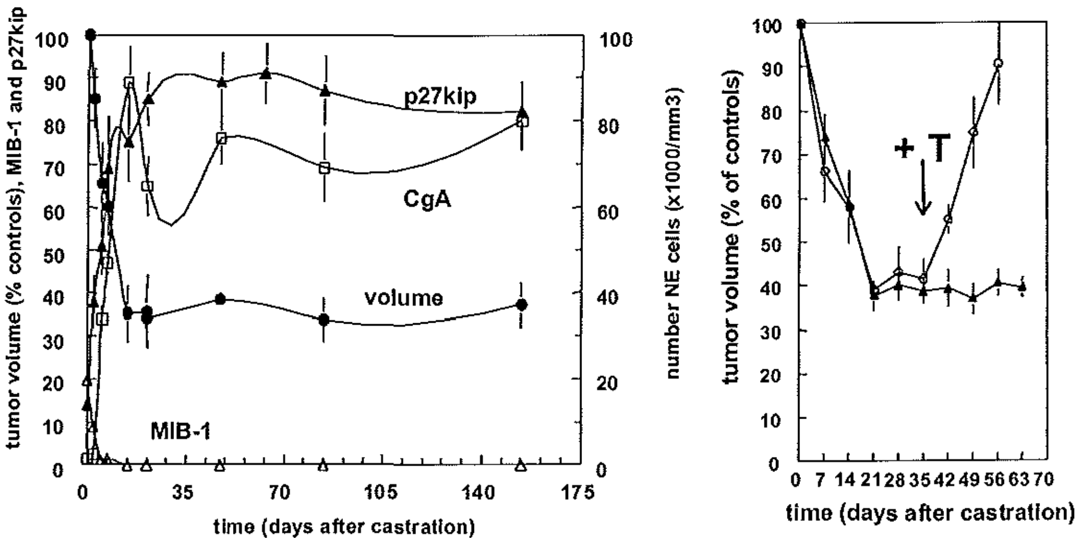

Figure 1: Androgen deprivation of PC-310 xenograft model. Effect on tumor volume, proportion of MIB-1 and $p27^{kip}$ expressing cells and the number NE cells per mm^3.
A) The effects of androgen withdrawal on tumor volume (●), MIB-1 expression ( ), $p27^{kip}$ expression ( ) and the number of NE cells marked by CgA (□) at different time points post-castration (n=3). in the PC-310 model.
B) Tumor volume in androgen deprived (●), androgen re-supplemented (O)PC-310 tumors after castration at different time points post-castration (n=3).

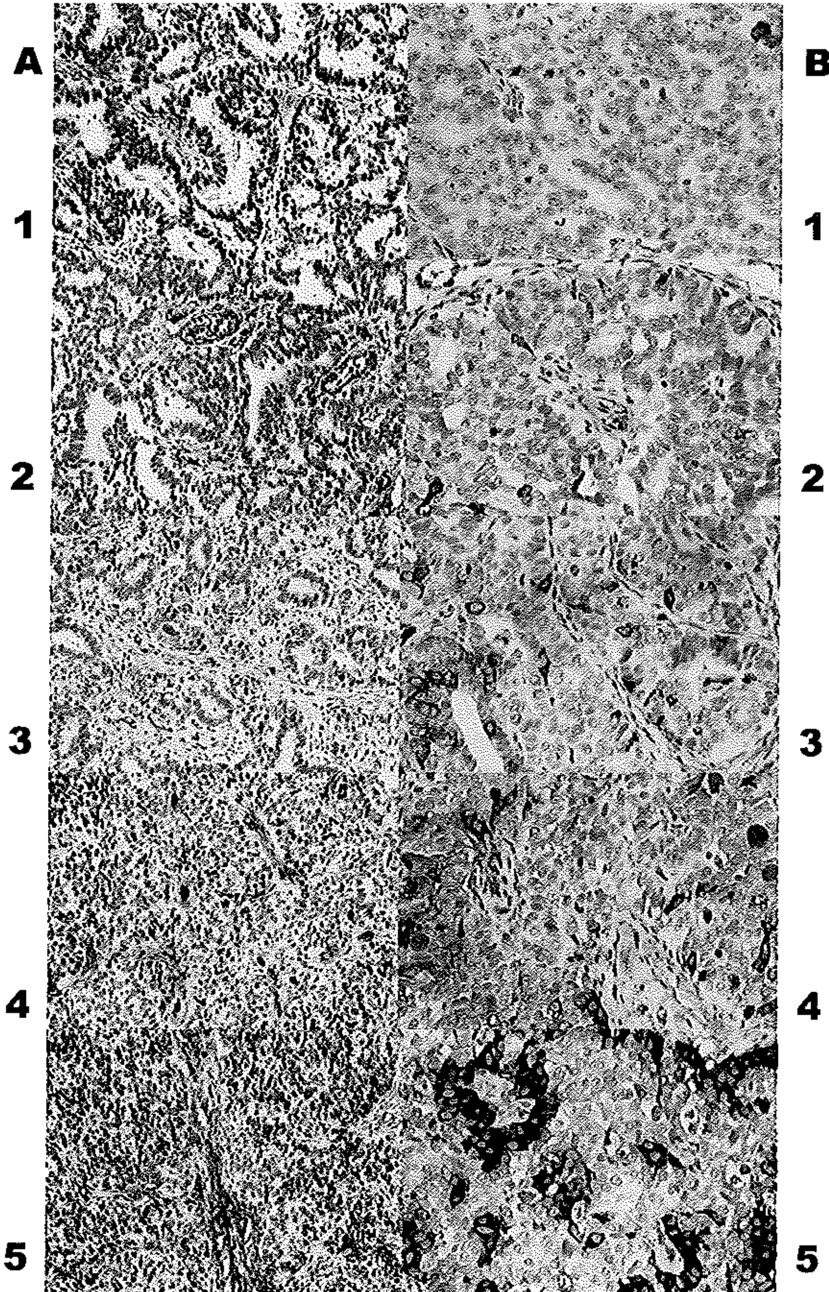

Figure 2: Expression of the androgen receptor (AR) and Chromogranin A (CgA) following castration in the PC-310 xenograft model. The left panel show the time points T_0, T_{-2}, T_{-5}, T_{-7}, T_{-14} after androgen withdrawal for expression of the androgen receptor (**2A**). In the right panel (**2B**) CgA expression is shown at day T_0, T_{-2}, T_{-7}, T_{-21}, and T_{-154}.

(figure1). Apoptotic counts were increased at day 2, 5, and 7 post-castration, but returned to below control levels after 14 days (data not shown). The serum PSA level dropped rapidly post-castration and remained just above zero from 14 days post-castration on, whereas the NE serum marker CgA increased from day 7 during the post-castration period (table I). Expression of the 110-112 kD AR decreased directly after castration but increased from 7 days post-castration onwards as was observed by both Western blot and immunohistochemistry (figure 2a).

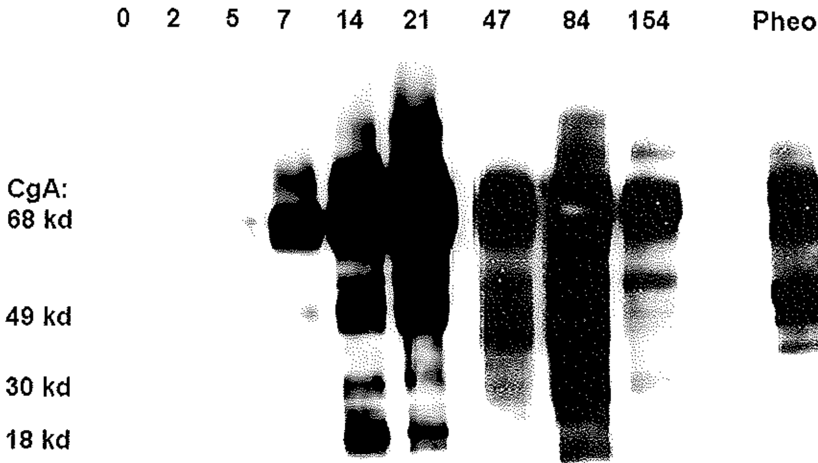

Figure 3: Expression of NE marker CgA on Western blot in the PC-310 model system after androgen withdrawal. The increased CgA signal can be found between 68 and 78 kD in the lower panel, which is the highest band in the control pheochromocytoma (Pheo). After 7 days clear processing of the protein is shown by smaller fragments at 49 kD, 30 kD and 18kD.

The proportion of CgA positive tumor cells increased rapidly up to approximately 40-fold at 14 days after androgen deprivation (figure 1). Immunohistochemistry showed that islands of NE differentiated cells developed after longer time intervals of androgen deprivation (figure 2b). These islands were surrounded by areas of non-NE tumor cells. Double labeling studies showed that about 50 % of the epithelial tumor cells expressed the AR (CgA^-, AR^+), and the other 50 % were NE cells lacking the AR (CgA^+, AR^-). On Western blot, expression of CgA (M_w 78 and 68 kD) was associated with increased processing of the protein into 49, 31 and 18 kD proteins from 14 days post-castration on (figure 3). Increased mRNA levels for CgA were found in Northern blot analysis (data not shown). Reverse transcribed mRNA of the same series of tissues was used in subsequent PCR reactions analyzing the time dependent expression of CgA and other markers of NE differentiation. Expression of markers of the regulated secretory pathway (RSP) was found to be increased after androgen deprivation of PC-310 (figure 4). Expression patterns of SgIII and 7B2 were found to follow a similar pattern as observed for CgA expression. Temporal induction of mRNA expression of PC1 and PC2 (data not shown) was found at day 5 and 7, respectively. Additionally, PC1 expression was again up regulated at day 84. PAM-mRNA expression was temporarily induced at day 7 and again at days 47 and 154 post-castration, with respect to the expression of the largest of the three PAM splice variants (Vos, 1995). The time dependent increase in the expression of the different RSP markers at mRNA level was confirmed by immunohistochemistry (table I). From this table, it is clear that the protein expression of SgIII was already apparent at day 5 and 7 post-castration, whereas the processing

enzymes PC1 and PC2 were expressed at a later stage in the NE cells, i.e. at day 7 with its highest expression at day 14 and 21 post-castration. Moderate levels of PAM expression were observed in non-NE cells during short-term castration, but after 47 and 84 days of androgen deprivation strong PAM expression was observed in part of the NE cells. Double-labeling co-localized strong PAM expression in the secretory granules of CgA positive NE cells, whereas the moderate expression of the protein was observed in the non-NE cells.

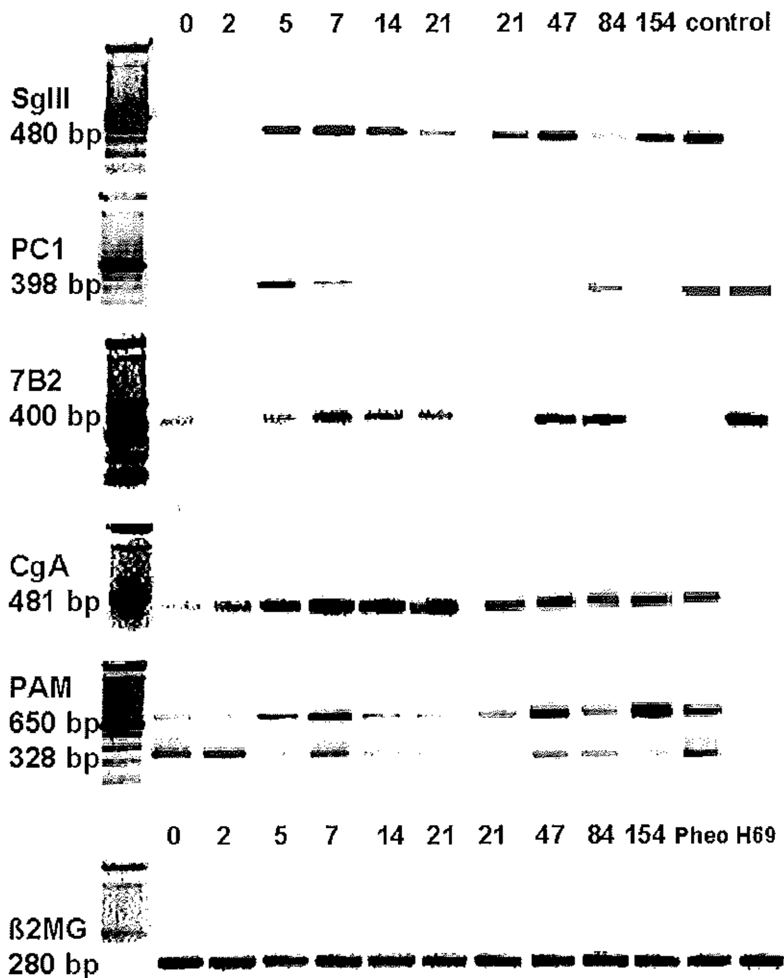

Figure 4: NE differentiation and regulated secretory pathway in PC-310. RT-PCR was performed for expression of SgIII, PC1, 7B2, CgA, PAM and ß2MG in PC-310 xenograft after castration. H69 was used as positive control for PC1, 7B2 and PAM analysis and Pheo for SgIII and CgA. The RT-PCR products are indicated as the length in basepairs (bp).

Table I: Expression of NE markers, neuropeptides and growth factors in the PC-310 xenograft after long-term androgen deprivation as determined by immunohistochemistry and mouse plasma levels.

Time (days)	PSA (pl) (ng/ml)	CgA (pl) (ng/ml)	CgA	SgIII (Sg3)	PC1	PAM	GRP	5-HT	VEGF	CD31
Control	196.9	0	+	±	-	-	-	-	-	±
2	11.3	0	+	±	-	-	-	-	-	±
5	7.7	0	++	+	-	-	-	-	-	±
7	5.4	12	+++	+	±	+	±	-	+	±
14	1	39	++++	+++	++	++	+	±	++	±
21	0.9	82	+++++	++++	+++	++	±	+	++++	+
47	0.3	3960	+++++	+++	+++	+++	++	++	++++	++
84	0.1	6391	+++++	++++	++	+++	++	+++	++++	+++
154	0.1	2838	+++++	++++	++	+++	++	++++	++++	+++

CgA, SgIII, PC1, PAM, GRP, 5-HT, VEGF and CD31 were all determined by IHC. Expression was scored as following: –, no expression, **±**, sporadic expression, **+**, focal expression, **++**, few cells positive per optical field, **+++**, clustered cells positive per optical field, **++++**, more clusters of positive cells per optical field, **+++++**, 50% of tumor positive. pl= plasma; PSA plasma levels are mean values (n=3).

Up to 7 days postcastration, expression of the vascular endothelial growth factor (VEGF), was not found whereas it was clearly increased from 14 days post-castration. VEGF was exclusively detected in the vast majority of NE cells at day 47 and later. Tumor vascularization, defined by CD31 positivity, was visible at day 47 and became marked at day 84 and 154 (table I). Interestingly, expression of the NE growth factors, GRP and 5-HT could clearly be demonstrated as a relatively late event of NE differentiation from 14 days post-castration on, with its highest expression found at day 84 and 154 post-castration (table I). Vasoactive intestinal peptide or calcitonin was not detectable during the whole experimental period of 154 days.

21 Days of androgen re-supplementation to long-term androgen deprived PC-310 tumor bearing mice resulted in rapidly increasing tumor volumes (figure 1B) and plasma PSA levels returned to normal in these mice (table II). Increased proportions of MIB-1 expressing and strong nuclear AR expressing cells were observed after day 56, whereas the proportion of cells expressing $p27^{kip}$ (data not shown), CgA and other NE markers slightly decreased at both protein and mRNA levels (table II and figure 5).

Table II: Effects of testosterone (T) re-supplementation on PC-310 tumor residues. MIB-1, CgA, and AR expression and PSA plasma levels are given as mean values ± S.D. (n=3).

Time (days)	MIB-1 cells (%)	NE cells (cells/m^2)	AR (%)	PSA plasma levels (ng/ml)
Control	12.7 ± 2.1	0.8 ± 0.9	89.0 ± 2.3	234 ± 63
35	0.2 ± 0.2	64.0 ± 14.0	37.2 ± 5.0	0.3 ± 0.2
63	0.9 ± 0.9	57.3 ± 22.0	35.0 ± 7.4	<0.1
35+21 T	10.8 ± 3.2	17.8 ± 6.8	50.7 ± 4.5	247 ± 85

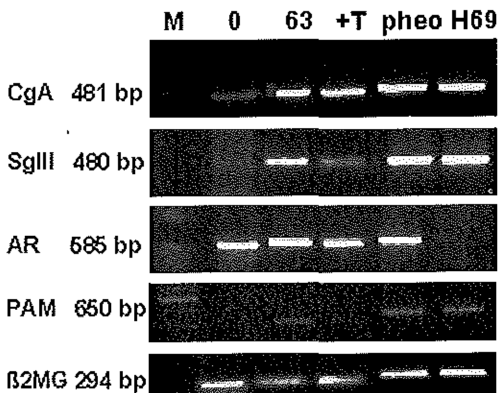

Figure 5: Effect of testosterone re-supplementation on NE differentiation in longterm androgen deprived PC-310 tumor tissue. Expression of CgA, SgIII, PC1, PAM, and AR in RT-PCR at different time points post-castration with or without supplementation of testosterone during three weeks. ß2MG is used as a control for equal amounts of mRNA and the PCR products are given in basepairs (bp) for each marker used. Pheochromacytoma (pheo) and H69 (SCLC cell line) were used as controls. (M=100bp ladder)

DISCUSSION

This study of the PC-310 prostatic carcinoma xenograft model focused on the behavior of the PC-310 model after long-term androgen deprivation with regard to time dependent NE differentiation, granular maturation and the subsequent sequential cellular changes in expression of NE cell products resulting in secretory signals. The growth and regression profiles of the PC-310 xenograft in the presently described androgen deprivation experiments was comparable to those of previous studies (van Weerden, 1996; Jongsma, 2000b). A temporal down regulation of the AR in non-NE cells was observed. This was paralleled by a rapid and prominent decrease of prostate specific antigen (PSA) plasma levels. Androgen deprivation of the PC-310 model induced temporally increased apoptosis, continuously decreased proliferation, and induced expression of the cell cycle inhibitor $p27^{kip}$. From 14 days post-castration on 30-40 % of the initial PC-310 tumor volume was maintained as dormant tumors. The initial loss of AR expression in the PC-310 tumors postcastration was comparable to that observed previously in the androgen dependent human PC-82 xenograft (Ruizeveld de Winter, 1992). By contrast, after 5 days of androgen depletion the expression level of the AR in non-NE PC-310 tumor cells returned to control levels.

The induction of NE differentiation from 5 days post-castration on is shown by a constitutively increased proportion of cells expressing CgA and the time related induction of other secretogranins and processing enzymes associated with the regulated secretory pathway (RSP) namely SgIII, PC1, PC2, PAM and 7B2 (Holthuis, 1995). The sequence of induction of three subsequently evolving maturation waves inside NE cells combined with the AR relapse in the PC-310 prostatic tumor residues after long-term androgen deprivation is unique. First, CgA and the other secretogranins, SgIII and 7B2 are expressed to mark the NE status. Secondly, the processing enzymes PC1 and PC2, associated with peptide hormone production, are expressed. And finally, all kind of growth factors or neurotransmitters, like GRP and 5-HT are expressed which may play a mitogenic role in the PC-310 tumor residues. The processing of CgA protein by PC1 to smaller proteins was shown before in other systems by others (Barbosa, 1991; Metz-Boutigue, 1993; Eskeland, 1996). Additionally, the increased and substantially high CgA plasma levels found in PC-310 bearing mice from 14 days post-castration on (table 1) demonstrated that maturating NE cells in this prostatic cancer tissue actually secrete members of the granin family and possibly neuropeptides as well. Expression of the neuropeptides, GRP and 5-HT, was initially found in a few NE cells after 14 and 21 days post-castration, but became prominently expressed in the long-term androgen deprived tumors at 154 days post-castration. Other neuropeptides and growth factors may be induced during long term androgen deprivation that potentially can stimulate non-NE, endothelial and murine stromal cells in the PC-310 model. It might be hypothesized that the time dependent induction of GRP and 5-HT expression in the maturating NE cells in the PC-310 tumor residues may cause induction of androgen independent growth of the AR positive non-NE cells. The importance of the presence of bombesin/GRP family in androgen independent prostate cancer has already been shown by others (Aprikian, 1998).

PAM, a two-enzyme modality, is predominantly expressed in NE cells, as the protein activates neuropeptides by amidation during maturation of the secretory granules. However, expression in non-NE cells has also been described (Saldise, 1996). The moderate PAM expression in non-NE cells observed in the PC-310 model shortly after androgen deprivation coincided with the induction of secretogranins expressing NE cells. After long-term androgen deprivation we have now demonstrated the presence of numerous PAM positive NE cells at 47, 84 and 154 days post-castration. PAM expression was shown inside the secretory granules of NE cells by co-localization with CgA. This NE cell specific expression is assumed to be associated with amidation of neuropeptides during the sequential maturation of NE cells after androgen deprivation in PC-310 tumor residues. Time dependent changes in expression profiles of the RSP markers, like PC1 or PAM may affect the overproduction of bioactive growth stimulating neuropeptides in prostate cancer during longterm androgen deprivation and might induce the development of hormone refractory disease. The relation between maturation of NE cells with mitogenesis or innervation has already been shown for lung cancer (Cuttitta, 1993; Sorokin, 1997).

VEGF co-localized in the NE cells and its expression was induced shortly after the manifestation of CgA and SgIII positive cells. Induction of VEGF expression may be the consequence of NE differentiation. The expression of VEGF may have contributed to an increased formation of CD31 positive murine blood vessels in PC-310 tumor residues at day 84 and 154. This might play a role in progression of prostate cancer towards hormone independence, e.g. by inducing a second angiogenic switch (Hanahan, 1996a).

Double immunostaining confirmed that CgA and AR did not co-localize in the neither of the longterm androgen deprived PC-310 tumor residues. Thus, the NE cells in the prostatic tumor residues represent androgen independent cells, which are surrounded, by AR positive non-NE tumor cells. Part of the initially AR positive PC-310 cells differentiate into NE cells during androgen deprivation, whereas the remaining cells re-express the AR and persist in an androgen deprived situation. We studied the effect of androgen (testosterone) re-supplementation and observed increased proliferation of AR positive non-NE tumor cells in the presence of testosterone, resulting in rapidly increasing tumor volumes. The expression of $p27^{kip}$ decreased rapidly as testosterone induced progression of the cell cycle and high plasma PSA levels were restored. This clearly showed that the dormant PC-310 tumor residues were still androgen sensitive. A decrease in the percentage CgA expressing NE cells was expected as these cells do not express the AR and cannot be induced to proliferate by androgens. The rapidly proliferating AR positive non-NE cells overgrow the non-dividing NE cells. SgIII-mRNA expression tended to decrease more rapidly then that of CgA suggesting that maturation of the RSP in NE cells is inhibited by the re-supplementation of androgens. This experimental study clearly shows that the AR sensitive pathway is still intact in the PC-310 model after longterm androgen deprivation.

The increasing role of the AR in the progression of prostate cancer as being adapted, hyperactivated, mutated or overexpressed has been shown by other groups (Culig, 1998; Gil-Diez de Medina, 1998). AR positive cells can

be grown in androgen deprived situation through activation of autocrine or paracrine routes (Culig, 1998; Jongsma, 2000a), activation of oncogenes, e.g. Bcl-2 and ras, or inactivation of tumor suppressor genes (Jenster, 1999). High AR expression has also been found in transurethral resection (TUR) specimens of clinical hormone refractory prostatic tumors (van der Kwast, 1991), but low expression of the AR was noted during regression of hormonal treated prostate cancer (Gil-Diez de Medina, 1998). The AR positive prostatic cancer cell line LNCaP also continue to express the AR after hormone depletion (Esquenet, 1997; Koivisto, 1998). Previously, we assumed that induction of NE differentiation and absence of the AR would play a key role in the survival of PC-295 prostate cancer cells, whereas induction of Bcl-2 expression would protect the remaining cells from undergoing apoptosis (Jongsma, 1998). In contrast to our previous observations in the PC-295 model and to what has been reported about the LuCap 23 model (Liu, 1996), the PC-310 model exhibits both a strong induction of NE differentiation and a transient down-regulation of the AR, but not an induction of Bcl-2 expression (data not shown). Both NE and AR upregulation after longterm androgen deprivation might be an alternative route to bypass androgen deprivation induced cell death, independent of an induced Bcl-2 pathway.

Hormonal escape of prostate cancer can be the result of androgen independent activation of an AR sensitive pathway (Jenster, 1999). The possibility of androgen independent activation of the AR in PC-310, for instance by neuropeptides like GRP, or by IGF-1 (Chan, 1998) or cytokines, like IL-6, is an interesting option (Hobisch, 1998), particularly because the AR is well expressed in 50 % of the PC-310 cells in the long-term androgen deprived situation. Data of a pilot study of continuous administration for 21 days of relatively high concentrations of GRP to PC-310 tumor bearing mice which were deprived of androgens for 35 days showed minor tumor volume increases and GRP had no effect on the level of NE differentiation or AR expression. In 2 out of 3 mice small but significantly increased amounts of PSA (±4 ng/ml) were measured in the mouse plasma at day 56 (Data not shown). We have shown *in vitro* that androgen independent growth of androgen deprived prostate cancer cells can be specifically induced by GRP (Jongsma, 2000a). Although, we did not observe androgen independent growth induced by neuropeptides in the PC-310 xenograft model during the 154 days of androgen deprivation, we cannot exclude that progression to androgen independence will occur after a longer period of androgen deprivation. A possible explanation might be that the AR positive non-NE PC-310 tumor cells have not been able to adapt the AR sensitive pathway. Adaptation of the AR sensitive pathway has been shown by Zhu *et al* through MAP kinase dependent activation of hormone receptor dependent gene transcription (Zhu, 1997).

The precise role of NE differentiation in progression of prostate cancer is still difficult to assess. Different clinical studies have shown an induction of NE differentiation after neo-adjuvant hormonal treatment of patients undergoing a radical prostatectomy or transurethral resection of the prostate (Van de Voorde, 1994; Guate, 1997; Jiborn, 1998; Pruneri, 1998). These studies either showed a relation with high Gleason scores or suggested that there is a possible relation of NE cells with androgen independent prostate cancer. However, these studies did not prove that CgA positive NE cells can

be of prognostic significance. One other study supported the view that NE differentiation in prostatic adenocarcinomas is a prognostic factor for progressive disease under subsequent hormone therapy (Krijnen, 1997). Plasma CgA levels in hormone refractory patients were also found to be significantly elevated (Kadmon, 1991; Cussenot, 1996b), as we observed in our PC-310 xenograft model too after long-term androgen deprivation. Their findings suggests that a hormonally pretreated tumor with induced NE differentiation may eventually lead to hormone refractory prostate cancer. As CgA cannot provide enough prognostic value in the progression of prostate cancer, testing of other markers as putative NE-related prognostic markers, such as SgIII, 7B2, PC1 and PAM for the progression of clinical prostate cancer seems relevant.

The observed induction of NE differentiation, the sequential maturation of NE cells and temporary down regulation of AR protein expression in experimental prostate cancer might well represent the initial clinical behavior of hormonally treated prostate cancer patients (Van de Voorde, 1994; Guate, 1997; Pruneri, 1998). Longterm hormone deprivation of PC-310 during the whole experimental period did not lead to hormone refractory cancer. Instead of this, dormant tumor residues were induced in which the maturating NE cells express different markers for NE differentiation evolving after longer periods of androgen deprivation, including growth modulatory neuropeptides like GRP and 5-HT. The truly dormant part of the PC-310 tumor residues consisted of the androgen sensitive AR positive residual tumor cells.

The androgen dependent human PC-310 xenograft model may be very useful as a model for further studies on induction of NE differentiation after androgen deprivation, the possible role of the maturating RSP in progression of prostate cancer in particular. The present results showed that the NE cells in the androgen deprived PC-310 model possess an active peptide hormone producing RSP and demonstrate sequential NE maturation patterns resulting in different secretory signals. Further long-term androgen deprivation studies or growth modulatory studies in the PC-310 model will yield information as to whether the NE cells play a role in the 'dormant' tumor residues by initiating growth induction of AR positive non-NE cells by different growth factors that were released through the RSP. Otherwise, the role of the AR in formation of androgen independent prostate cancer is of particular interest. Further detailed studies on these different aspects of the PC-310 xenograft model may lead to a better understanding of prostate cancer behavior during hormonal treatment and the mechanism underlying prostate cancer progression.

Acknowledgements

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MATERIALS AND METHODS

PC-310 xenograft model

The nude mouse human prostate cancer xenograft model PC-310 was established from a primary prostatic tumor after radical prostatectomy of a previously untreated patient (van Weerden, 1996). The tumors grow with a doubling time of about 16 days and a lag phase of 2-3 months. The model represents a strictly androgen dependent and histologically moderately differentiated relatively slow growing tumor, organized in solid sheets and microacini. Briefly, PC-310 tumors were implanted subcutanously at both shoulders of intact NMRI male nude mice (Harlan, Horst, The Netherlands). Optimal growth conditions were reached by supplementation of PC-310 transplanted mice with testosterone implants, as previously described (van Steenbrugge, 1984). Subcutanously growing tumors developed within 2-3 months and were grown up to a maximum volume of 2000 mm^3. Tumor volume changes were followed weekly by perpendicular diameter measurements (D1 and D2) after which the volume was calculated from the formula: $V=(\Pi/6)(D1 \times D2)^{3/2}$.

Hormonal manipulation of PC-310 tumor bearing mice

Androgen deprivation experiments were performed with testosterone-supplemented PC-310 bearing male mice (Harlan, Horst, The Netherlands) at the Erasmus Center for Animal Research. Androgen deprivation was performed by castrating the mice under hypnorm anesthesia (Janssen Pharmaceuticals, Oxford, UK) and by removing the silastic testosterone implant. Three mice were sacrificed at 0, 2, 5, 7, 14, 21, 47, 84 and 154 days after castration.

After blood samples were taken for determining serum prostate specific antigen levels, mice were sacrificed. Tumor volumes were measured and tumor nodules were removed. The tumors were cut into small pieces which were either fixed in 4% buffered formalin and paraffin embedded for immunohistochemical analysis or snap frozen in liquid nitrogen and stored at -80°C for biochemical analysis. The paraffin embedded material was processed routinely for hematoxylin and eosin staining.

9 Tumor bearing mice were castrated in a separate experiment and 2 control mice were used. Three castrated mice were sacificed after day 35 and 63 each. Three castrated mice were resupplemented after 35 days with a testosterone containing silastic implant and sacrificed at day 56. Tumor volumes were measured, serum samples were taken just before sacrifice and tumor tissue was sampled for either formalin fixation or storage at –80°C for biochemical analysis.

Immunohistochemistry

To identify the fraction of cells expressing the NE phenotype, paraffin embedded tissue sections of the PC-310 xenografts were stained with antibodies against CgA (polyclonal rabbit anti human CgA, DAKO), SgIII (rabbit polyclonal antibody; provided by the Department of Animal physiology, University of Nijmegen) (Holthuis, 1996a) and PC1 and PC2 (Alexis Biochemicals, 10P's, Breda, The Netherlands). For identification of the proliferative capacity, tissue sections were stained with an antibody against

the proliferation associated Ki-67 antigen (MIB-1, Immunotech, Marseille, France) and the $p27^{KIP}$ antigen. In addition, apoptotic cells were identified by counting the apoptotic bodies in hematoxylin and eosin staining of each tissue. Other antibodies used were directed against the AR (clone F39.4, kindly provided by Dr. A.O. Brinkmann, Department of Endocrinology and Reproduction, Erasmus University); the Bcl-2 antigen (clone 124, Dako, Glostrup, DK), VEGF (rabbit polyclonal, DAKO) and against the neuropeptides bombesin (rabbit polyclonal GRP, DAKO; and anti-GRP MoAb 2A11; kindly provided by Dr F. Cuttitta, NCI, Bethesda, USA), serotonin (5-HT; rabbit polyclonal antibody, DAKO), vasoactive intestinal polypeptide (VIP, rabbit polyclonal, DAKO) and Calcitonin (rabbit polyclonal, DAKO).

4 µm sections for single immunostaining and 2 µm for double immunostaining were incubated overnight at 60°C. The slides were deparaffinized and endogenous peroxidase activity was blocked with 3.3% hydrogen peroxide (H_2O_2) in methanol for 10 minutes. Antigen retrieval was then performed in 10 mmol/L citrate buffer (pH 6.0) in a microwave at 700 watt for an initial 10 minutes and a subsequent 5 minutes(Shi, 1991). The slides were allowed to cool down to room temperature and then put into the sequenza immunostaining system (Shandon, Uncorn, UK) and rinsed with phosphate buffered saline (PBS). All slides were pre-incubated with normal goat serum (DAKO, Glostrup, DK) for 15 minutes diluted 1:10 in PBS. The primary antibodies were incubated overnight at 4°C. The secondary antibody was incubated for 30 minutes, being either horseradish-peroxidase conjugated goatαmouse or goatαrabbit (1:50), or biotinylated goatαmouse and goatαrabbit (1:400) for monoclonal and polyclonal antibodies, respectively. In case of biotinylated goatαmouse and goatαrabbit, a horseradish-peroxidase streptavidin1:400 in PBS was incubated for a subsequent 30 minutes. Between the subsequent steps, the slides were rinsed four times with PBS. The bound horseradish-peroxidase was visualized in 10 minutes with diaminobenzidine (DAB, Fluka, Neu-Ulm, Germany) in PBS containing 0.075% H_2O_2 as substrate. Slides were rinsed extensively in tap water and finally counterstained in Mayer's Hematoxylin, dehydrated through a series of alcohol and embedded in malinol.

To assess the properties of the NE cells, double staining of CgA respectively with MIB-1 and AR was performed. The double staining procedure consisted of two consecutive stainings with two primary antibodies. The first being always a horseradish-peroxidase-related stable diaminobenzidine complex, whereas the second staining was performed with an alkaline phosphatase conjugated goatαrabbit secondary antibody which was subsequently stained with a Fast blue staining protocol. In between both stainings, the slides were rinsed with PBS for one hour and again boiled in a microwave in 10 mmol/L citrate buffer (pH 6.0) for 10 minutes. As negative control, PBS replaced the primary antibody in all stainings. Radical prostatectomies, containing normal as well as cancerous prostatic tissue were used as positive control for CgA, SgIII, PC1, PC2, AR, GRP, 5-HT, VEGF, Bcl-2 and MIB-1 expression.

For MIB-1, CgA, SgIII and $p27^{kip}$, the number of positive cells was determined by quantitative counts of all cells in tumor squares at 300x magnification from which the number of positive cells per square mm was calculated. In total, positive cells were scored as percentage of the total cell

number, in ≥ 10 squares. For AR, VEGF, CD31, GRP, 5-HT, PC1, and PC2 the level of immunostaining was assessed semi-quantitatively.

Western blot analysis

We further confirmed the expression patterns of CgA, SgIII, Bcl-2, 7B2 and AR by Western blotting. As positive controls, material of human pheochromocytoma was used for CgA, of rat pituitary for SgIII and of the human in vitro cell line LNCaP for AR expression. The procedure of protein extraction was done as previously described (Noordzij, 1996). Briefly, frozen tissues of the PC-310 xenografts were crushed in a liquid-nitrogen-chilled metal cylinder. The tissue homogenates were transferred into a lysis buffer (10mM TRIS (pH 7.4), 150 mM NaCl (Sigma), 1 % Triton X-100 (Merck, Germany), 1 % deoxycholate (Sigma), 0.1 SDS (Gibco), 5 mM EDTA (Merck) and protease inhibitors (1mM phenylmethylsulfonyl fluoride, 1mM aprotinin, 50 mg/L leupeptin, 1 mM benzamidine and 1 mg/L pepstatin; all from Sigma). After centrifugation of the mixture at 100,000 rpm at 4°C for 10 minutes, the protein content of the supernatants was measured by the Bradford method (Bio-Rad protein assay, München, Germany).

20 µg of each sample was transferred to a SDS polyacrylamide gel and gel electrophoresis was performed with pre-stained markers as size standards (Novex, San Diego, CA). The gels were blotted to a 0.45µm cellulose nitrate membrane (Schleicher & Schuell, Germany). The immunoblot was blocked with PBS (pH 7.7) containing 0.1% Tween-20 (Sigma) and 5% dry milk for one hour. The CgA, 7B2 (Mon 102/144, kindly provided by Prof. Dr WM van der Ven, Leuven, Belgium), Bcl-2, androgen receptor or SgIII antibodies were added in their optimal concentration and incubated overnight on an orbital shaker at 4°C. After rinsing four times 15 minutes with PBS, incubation for one hour was performed with the secondary horseradish-peroxidase-conjugated antibodies and goatαmouse for mouse monoclonal, goatαrabbit for rabbit polyclonal antibodies, respectively. Subsequently, a short incubation with a mixture of 10 ml luminol and 100 µl oxidizing agent (BM chemiluminescense kit, Boehringer Mannheim GmbH, Germany) followed, after washing for four times 15 minutes with PBS. Excess reagent was removed and antibodies were visualized by exposure of the blots to an X-ray film.

Reverse Transcriptase-polymerase chain reaction

RNA was isolated by using the single step RNAzol™ B method (Campro, The Netherlands (Chomczynski, 1987). Frozen tissue (100mg) or cell culture pellets were homogenized in 1 ml of RNAzol™. Chloroform (0.1ml) was added and the mixture was vortexed for 15 seconds followed by 5 minutes incubation on ice. The homogenate was centrifuged at 4 °C at 12,000 g for 15 minutes. The upper water-phase containing the RNA was removed and mixed with an equal volume of iso-propanol. This mixture was then kept at 4 °C for 15 minutes and after that centrifuged at 4°C at 12,000 g for 15 minutes. The supernatant was removed and the RNA pellet was washed twice with 75 % ethanol by vortexing and centrifugation at 4°C at 12000 g. The pellet was then dried and resuspended in sterile H_2O. The concentration was determined at OD 260 and solutions of 1µg/µl were prepared for further use in

Reverse Transcriptase-polymerase chain reaction (RT-PCR) or Northern blot analysis. The quality of the isolated RNA was checked by determining the 260/280 ratio and by formaldehyde gel-electrophoresis to check the ribosomal (28 S and 18S) bands.

RT-PCR was performed for CgA, SgIII, PAM, PC1 and 2 and ß2-microglobulin (ß2MG) with a standard protocol. Reverse transcriptase reaction was performed with a mastermix containing 5 mM $MgCl_2$, PCR buffer, 10 mM dNTP's, RNAse inhibitor (10 units), Reverse transcriptase (25 units), 2.5 mM random hexamer primers and 0.5 µg RNA in a total volume of 10 µl covered with 50 µl of mineral oil. The mastermix was then processed at 42°C for 60 minutes followed by 15 minutes incubation at 99°C and the reaction was stopped at 4°C for five minutes. The cDNA mix that was formed was then used totally with the mastermix of the polymerase chain reaction (PCR) protocol. In this protocol, the mastermix contained reaction buffer, Supertaq polymerase (HT Biotechnology Ltd., Cambridge, UK; 1 unit), 15 µM sense and antisense primer in a total volume of 40 µl. All samples were first denatured at 94°C for 10' and then amplification was performed for 35 cycles of 1' 94°C, 1' 60°C and 1' 72°C and a final extension at 72°C for 10'. The PCR product was checked on a 1% agarose gel and, if necessary, followed by Southern blotting. The internal control of human ß2MG clearly showed the equal amounts of mRNA that were used for each time-point.

Northern blot analysis

Northern blot analysis was performed by running a formaldehyde gel in MOPS buffer containing 20 µg of each sample including the controls. The RNA content of the gel was subsequently blotted overnight onto a hybond-N^+ filter in 10 x Standard saline citrate (10xSSC; 1.5 M sodium chloride and 0.15 M sodium citrate). The filter was checked for RNA and ribosomal bands are marked. After rinsing the filter in 2xSSC, the blot is cross-linked in a GS gene linker™ UV chamber (Bio-RAD, München, Germany) and ready for (pre)hybridization with different probes against CgA, SgIII, PAM and B2-microglobulin as control.

CHAPTER 6

PEPTIDYLGLYCINE ALPHA-AMIDATING MONO-OXYGENASE AND PROADRENO-MEDULLIN N-TERMINAL 20 PEPTIDE NEUROENDOCRINE EXPRESSION IS INDUCED BY ANDROGEN DEPRIVATION IN NEOPLASTIC PROSTATE

Nuria Jiménez, Johan Jongsma, Alfonso Calvo, Theo van der Kwast, Anthony Treston, Frank Cuttitta, Fritz Schröder, Luis Montuenga, Gert J van Steenbrugge

Submitted

Abstract

Most prostatic carcinomas show neuroendocrine differentiation. Several studies have tried to correlate neuroendocrine expression with disease status but the reported findings have been contradictory. Some prostatic neuroendocrine products, such as proadrenomedullin N-terminal 20 peptide (PAMP), are amidated. Peptidylglycine α-amidating monooxygenase (PAM) is the only Carboxy-terminally peptide amidating enzyme known to date. We studied the expression of PAMP and PAM in the normal prostate, and in prostatic tumors (clinical specimens and human xenograft models), with or without prior androgen-deprivation therapy. We found a wide distribution of PAMP and PAM expressing neuroendocrine cells in the normal and malignant situation, although both markers were not always co-expressed by the same cells. There was no significant correlation of either marker to tumor grade, clinical progression or disease prognosis in the clinical specimens. However, higher PAMP or PAM expression was found after androgen-blockade therapy. In the PC-310 and PC-295 androgen-dependent xenograft models PAMP or PAM neuroendocrine differentiation was induced after androgen withdrawal. The higher induction of both markers in the PC-310 might explain the long-term survival of this model after androgen deprivation. Our work shows an induction of the expression of neuroendocrine markers in prostate cancer after endocrine therapy.

Introduction

Prostate cancer is the most commonly diagnosed cancer in elderly men[1] and is becoming the leading malignant cause of death for men in this age group.

Together with the basal and secretory cells, the normal prostatic epithelium has a well developed neuroendocrine (NE) cell population[2]. In prostatic carcinoma (PC), the most common pattern of NE differentiation corresponds to scattered isolated NE cells or clusters of NE cells focally distributed among the neoplastic cells[3]. The reported incidence of NE cells in PC has increased as the detection methods have improved[4,5]. The widespread distribution of NE cells in prostatic tumors has promoted many studies about the relationship of NE differentiation to prognosis in PC (reviewed by di Sant' Agnese PA[6]), but contradictory findings have been reported. Prostate cancer is androgen-dependent in early stages but usually relapses to an androgen-insensitive state after androgen-withdrawal therapy[7] which implies more aggressive tumors and a poor prognosis for the patient. Since prostatic NE cells lack androgen receptor this population has been suggested to be androgen-independent[8,9] and therefore, assumed to be involved in the development of refractory tumors.

The function of the NE population in the normal and malignant prostate is not known. Together with generic NE markers such as chromogranin A (CgA), chromogranin B or neuron-specific enolase[10,11], NE cells produce biogenic amines and neuropeptides (reviewed by di Sant' Agnese PA[3]). The effects of some of these NE products on prostatic cells have been demonstrated *in vitro* in some instances. Thus, serotonin and bombesin/gastrin-releasing peptide (GRP) act as growth stimulating factors[12,13], while somatostatin is a growth inhibitor[14]. The production and probable secretion of these kind of substances suggests that NE cells may play a role in the regulation of growth, differentiation and local homeostasis of the normal and neoplastic prostate.

Proadrenomedullin N-terminal 20 peptide (PAMP) is a 20 amino-acids peptide originated from the post-translational processing of pre-proadrenomedullin[15]. Recently, we have detected PAMP in NE cells of the human normal prostate[16]. NE expression in other tissues, such as the rat gastric mucosa, is also associated with the production of PAMP[17]. However, this peptide has first been described in the vascular wall, and in endocrine organs in normal and pathological situations[18,19]. PAMP is a pluripotent peptide: it was initially identified as vasodilator[15], but it can additionally act as neural transmission inhibitor[20], endocrine secretion inhibitor[21] or as cell growth suppressor[22]. Some of these roles could contribute to the regulation of prostatic homeostasis or functioning.

At least 3 peptides produced by the prostatic NE cells (PAMP, GRP, and calcitonin) are Carboxy-terminally amidated peptides *in vivo*[18,23]. The C-terminal α-amidation is a two-step post-translational modification process necessary for many peptides to be completely bioactive[23]. It is sequentially performed by the peptidylglycine α-hydroxylating monooxygenase (PHM), and the peptidyl-α-hydroxyglycine α-amidating lyase (PAL)[24]. Both enzymes are included in a precursor, the peptidylglycine α-amidating monooxygenase (PAM). PAM has been detected mainly in endocrine tissues[25,26]. At present,

different PAM mRNAs have been detected in rat prostate[27] and in human prostatic xenografts grown into nude mice[28,29].

We presently performed a study on PAMP and PAM expression in prostatic human NE cells by means of their detection in a variety of specimens obtained from a broad spectrum of histopathological subgroups. Our aim was to get more insight into the possible role of NE subpopulations in the normal and the neoplastic prostate by studying the dynamics of the NE cell population in relation to tumor malignancy and after androgen deprivation treatment.

Materials and Methods

Patients

Two control prostates were obtained from autopsies of healthy young individuals (17 and 23 years old) killed in traffic accidents. Neoplastic tissues were obtained through radical prostatectomies (RPs) during 10 years in the University Hospital Rotterdam, or through trans-urethral resections of the prostate (TURPs) mainly performed in the University Hospital Rotterdam, and Reinier de Graaff Hospital, Delft, The Netherlands. RPs were performed on 61 patients with non-metastasized, clinically localized or locally advanced prostate cancer. The patients were followed regularly for a mean period of 82 months (range 13-178). Clinical progression, defined as cytologically or histologically proven local recurrence or the appearance of distant metastasis, occurred in 27 of the 61 patients. Tumor specific death, defined as death due to direct tumor effects or metastasis or death due to tumor therapy, occurred in 11 of the 27 patients showing disease progression. TURPs were obtained from 29 patients with extensive tumors. Fourteen of them were pre-treated with androgen-blockade therapy, i.e. by anti-androgens, flutamide, androcur or with orchiectomy (P-TURPs). The rest (n=15) were untreated before undergoing surgery (U-TURPs). All tissue procurement protocols were approved by the relevant institutional committees.

Prostates were fixed in 10% buffered formalin, dehydrated in alcohols, and embedded in paraffin for *in situ* detection. Slides of each case stained with hematoxilin and eosin (H&E) were used for determining the grade of the tumors according to Gleason's system (Gleason sum score, GSS).

Human prostate tumor xenograft models

PC-295 and PC-310 models[30] were established respectively from a pelvic lymph node metastasis and from a primary prostatic carcinoma at the Department of Experimental Urology, Erasmus University, Rotterdam. These models are androgen-dependent and exhibit NE differentation which was demonstrated to be significantly increased after androgen ablation[28,29,31]. Briefly, small pieces of tumor were heterotransplanted in NMRI athymic nude mice by subcutaneous implantation. The male or female mice (obtained from the Harlan Company) were androgen-supplemented through Silastic implants containing testosterone (Sigma, St. Louis, MO). The tumors were grown up to a maximal volume of 2000 mm^3 . For castration experiments androgen withdrawal was achieved by removing the Silastic implants and by castrating the male mice under hypnorm anesthesia (Janssen Pharmaceuticals, Oxford, UK). The mice were sacrificed at short-term intervals after androgen ablation: days 0, ½, 1, 1 ½, 2, 4, 7, 14, and 21 for PC-295 and days 0, 2, 5, 7, 14 and

21 for PC-310. A long-term castration experiment was also performed for PC-310, with mice sacrificed at 0, 21, 47, 84, 90 and 154 days after androgen withdrawal. Prostatic tumors were then either fixed in 4% buffered formalin and paraffin embedded for immunohistochemical analysis or snap-frozen in liquid nitrogen and stored at -80°C for Western blotting analysis.

In this study were also included other models[30,32], both androgen-dependent or independent, established at the Department of Experimental Urology, Erasmus University, Rotterdam: PC-82, PC-133, PC-135, PC-324, PC-329, PC-339, PC-346, PC-346I, and PC-374. All the tumors were grown in mice as described above. Although these tumors do not exhibit NE differentiation after androgen withdrawal[31], a small-scale castration experiment was designed: some of the mice bearing the mentioned tumors were sacrificed at 14 days after androgen withdrawal. Tissues were processed for immunohistochemical analysis.

Antibodies and peptides

Immunoreactivity for PAMP was demonstrated using a previously characterized polyclonal antiserum[33] raised to the C-terminal peptide $PAMP_{YY13\text{-}20AMIDE}$. It was applied at 1:3000 on the histological sections and at 1:1500 for Western blotting.

To detect PHM/PAM, two monoclonal antisera (clone G8 and clone E10, produced by Dr. Anthony M. Treston, NCI, NIH, USA&) were used. They were raised against bovine PHM region ($bPAM_{288\text{-}310}$; GenBank Accession number M18683) which shows a high homology with the human $PAM_{288\text{-}310}$ sequence. Both antibodies were applied at 1:2000 on sections of clinical material and at 1:1000 on sections of xenograft-derived tissues.

A monoclonal antiserum raised against human CgA (Boehringer Mannheim, Germany) was applied to sections at a concentration of 2 μg/ml to confirm whether or not PAMP and PAM expressing cells had the NE phenotype. Peptides $PAMP_{YY13\text{-}20AMIDE}$ and $bPAM_{288\text{-}310}$, used for obtaining the specific antisera, were used for the absorption controls.

Immunohistochemistry

Sections 4 μm thick were obtained from the paraffin-embedded tissues and placed on StarFrost@ slides. Some reverse-face sections were mounted to assess co-localization of molecules. Sections were deparaffinized through xylene and 100% ethanol, and endogenous peroxidase activity was blocked with 3% H_2O_2 solution in methanol for 30 min. After rinsing with tap water and distilled water, a microwave pre-treatment was applied for antigen retrieval. Slides were placed in a 0.01 M citric acid (Merck, Darmstadt, Germany) buffer (pH 6) and heated at 700 W for 12 min and other subsequent 5 min after adding distilled water. The slides were allowed to cool down to room temperature (RT), rinsed with PBS and placed into the Sequenza Immunostaining System (Shandon, Uncorn, UK). Thereafter, sections were incubated with normal goat serum (NGS; Dako, Denmark) diluted 1:10 in PBS for 20 min and incubated overnight at 4°C with the specific primary antiserum at the optimal concentration. After rinsing with PBS, sections were incubated at RT for 30 min with horseradish-peroxidase-conjugated goat anti-rabbit or

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anti-mouse (according to the primary antiserum applied) immunoglobulins (both from Dako) diluted 1:50 in PBS containing 3% NGS. The slides were rinsed four times with PBS before developing. Peroxidase activity was detected with 3-3'-diaminobenzidine tetrahydrochloride dihydrate (DAB; Fluka, Neu-Ulm, Germany)- H_2O_2 and after rinsing in tap water, slides were counterstained with Mayer's hematoxylin, dehydrated and embedded in malinol.

Absorption controls were performed to test the specificity of the antisera. Solutions containing the specific antiserum pre-incubated overnight at 4°C with 10 nmol/ml of its respective peptide were applied onto the slides instead of the primary antiserum.

Immunocytochemical double-staining

A previously described double-staining protocol was applied[34]. In summary, sections were deparaffinized, rehydrated and endogenous peroxidase was inhibited, afterward they were microwave preheated as described above. Slides were incubated for 20 min with NGS (1:20) at RT, and at 4°C overnight with the specific antisera mixture (containing one monoclonal and one polyclonal antiserum). Then, slides were treated with horseradish-peroxidase-conjugated goat anti-rabbit immunoglobulins and with unlabelled goat anti-mouse immunoglobulins (Dako), diluted 1:50 and 1:100 respectively, for 30 min. Tissues were incubated with monoclonal alkaline phosphatase-anti-alkaline phosphatase (APAAP, mouse, monoclonal; Dako) at 1:50 for 30 min. Goat anti-mouse antiserum was applied again, followed by APAAP, for 10 min each. The alkaline phosphatase was developed using naphthol AS-TR and new fuchsin as substrate. Peroxidase activity was detected with DAB and nickel enhancement. Slides were embedded in PBS-glycerol.

Quantification of immunostaining

For the clinical specimens (RPs and TURPs) tissue sections adjacent to the immunostained slides were stained with H&E and the neoplastic zones were identified and marked. The markings were copied on the slides immunolabeled for PAM or PAMP. The evaluation of NE cells positive for PAMP or PAM was done semi-quantitatively. Ten fields of tumor tissue were randomly selected at 160x and cells showing granular cytoplasmatic immunostaining were counted. The quantitative data were transformed to a semi-quantitative final score considering the number and distribution of the positive cells (Table I). Therefore, every tumor was assigned two semi-quantitative scores, one for PAMP and another for PAM.

In the case of the xenograft models a quantitative evaluation of the PAMP expression was performed. Ten fields of neoplastic epithelium were randomly selected and scored at 400x by counting the number of positive cells. The final value assigned to each tumor was the arithmetic mean of all the values obtained.

Statistical analysis of the data

Statistical computer package SPSS was used to perform the statistical analysis. The PAMP or PAM score of each patient was correlated to the respective GSS. PAMP scores were correlated with PAM scores. Scores from

experimental models were correlated with the post-castration time. The correlation was studied in every case calculating the non-parametric Spearman correlation coefficient (r). Scores from patients who underwent RP were also related to clinical progression and survival time (Kaplan-Meier method). The frequencies of PAMP or PAM induction for the untreated and pre-treated TURPs were compared by the Fisher's exact test.

Protein extraction and Western blotting

PC-310 prostatic tissues stored at –80°C were crushed in a metal cylinder cooled down at -80°C. The tissue homogenates were transferred into a lysis buffer containing 10 mM TRIS (pH 7.4), 150 mM NaCl (Merck), 1% Triton X100 (Merck), 1% deoxycholate (Sigma), 0.1% sodium dodecyl sulphate (SDS; Fluka), 5 mM EDTA (Merck), and protease inhibitors (Complete™, Boehringer Mannheim). After centrifugation of the mixture at 100.000 rpm at 4°C for 10 min, supernatants were recovered and the protein content was measured spectrophotometrically by the Bradford method (Bio-Rad protein assay, München, Germany).

For the Western blotting detection of PAMP, 35 μg of each protein sample was mixed with sample buffer, heated to 95°C for 3 min, and transferred to 10-20% tricine SDS-PAGE gels (Novex, San Diego, CA). Prestained markers were used as size standards (Novex). 5 ng of synthetic PAMP were loaded in separate wells as positive controls. Proteins were electrophoretically separated at 100 V for 2 hr under reducing conditions (5% β-mercaptoethanol). The gels were blotted to polyvinyldifluoride membranes (Immobilon PVDF; Millipore, Bedford, MA) at 30 V for 2 hr. The immunoblot was blocked overnight at 4°C with PBS (pH 7.7) containing 5% dry milk. The anti-PAMP antiserum diluted in PBS/milk was added subsequently, and the membranes were incubated for 1 hr on an orbital shaker at RT. After rinsing the membranes four times for 15 min each with PBS containing 0.1% Tween-20 (Fluka Chemika, Switzerland) an incubation for 1 hr was performed at RT with the horseradish-peroxidase-conjugated anti-rabbit secondary antibody (Amersham, Life Science, England) diluted 1:5000 in PBS/milk. Membranes were rinsed again with PBS/Tween as explained above, and finally incubated for 1 min with Western blotting luminol reagent (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Excess reagent was removed and blots were exposed to high performance chemiluminiscence film (Hyperfilm™ ECL™, Amersham).

Results

Immunodetection and quantification of PAMP in the clinical specimens

PAMP immunostaining was exclusively detected in NE cells, both in the normal and the neoplastic prostates (Figures 1A-D). The intensity of the labeling, granular and cytoplasmatic, was usually strong, although some cells did stain more intensively than others. As previously observed in normal prostate[16], double immunostaining demonstrated that PAMP positive cells were a subpopulation of the CgA positive cells in the neoplastic specimens. PAMP immunoreactive cells in the neoplastic glands were distributed scattered either as isolated or clustered cells (Figures 1B-D), whereas in the

normal glands they were mainly isolated (Figure 1A). The specificity of the staining was confirmed by the absorption controls (Figure 1F).

49 out of 61 (80%) RPs, 12 out of 15 (80%) U-TURPs and 11 out of 14 (79%) P-TURPs exhibited NE PAMP expression. PAMP scores in relation to the respective GSS are shown in Tables II and III. There was no correlation between the PAMP score and the GSS for the RPs (r=-0.012, p=0.924) and for the U-TURPs (r=-0.078, p=0.782). However, the P-TURPs showed a positive correlation between the scores (r=0.489) although it proved to be statistically not significant (p=0.076). There was a very noticeable increase in the percentage of patients expressing high PAMP (score 3) from untreated (1/15=7%) to pre-treated (5/14=36%) TURPs (p=0.08). Concerning the patients who underwent radical prostatectomy, 78% (21/27) of those showing disease progression and 82% (9/11) of patients who died because of the tumor, exhibited PAMP-associated NE differentiation. On the other hand, 82 % (28/34) of the patients without disease progression and 80% (40/50) of patients without tumor specific death showed also PAMP NE expression. Kaplan-Meier curves did not show a prognostic value of PAMP expression in relation to clinical progression and tumor specific survival (data not shown).

Table I. Semi-quantitative values for NE expression

Semi-quantitative score	Expression level	Evaluation criteria
0	Not detected	No positive cells observed
1	Low, irregular	Positive cells in less than 5 fields
2	Intermediate, regular	Positive cells in at least 5 fields
3	High	More than 6 positive cells in at least 5 fields or clusters of 10 or more positive cells in at least 1 field

Table II. Relationship between PAMP score and GSS in the RPs

TUMOR PAMP SCORE	GLEASON SUM SCORE							TOTAL
	4	5	6	7	8	9	10	
0	0	1	1	4	3	3	0	12
1	1	1	7	13	3	7	2	34
2	1	0	0	3	0	0	0	4
3	0	1	0	4	3	3	0	11
TOTAL	2	3	8	24	9	13	2	61

Table III. Relationship between PAMP score and GSS in the TURPs.

TUMOR PAMP SCORE	Pre-treated patients					Untreated patients				
	GSS				TOTAL	GSS				TOTAL
	7	8	9	10		7	8	9	10	
0	1	2	0	0	3	0	0	2	1	3
1	1	2	3	0	6	1	1	4	5	11
2	0	0	0	0	0	0	0	0	0	0
3	0	2	2	1	5	0	0	1	0	1
TOTAL	2	6	5	1	14	1	1	7	6	15

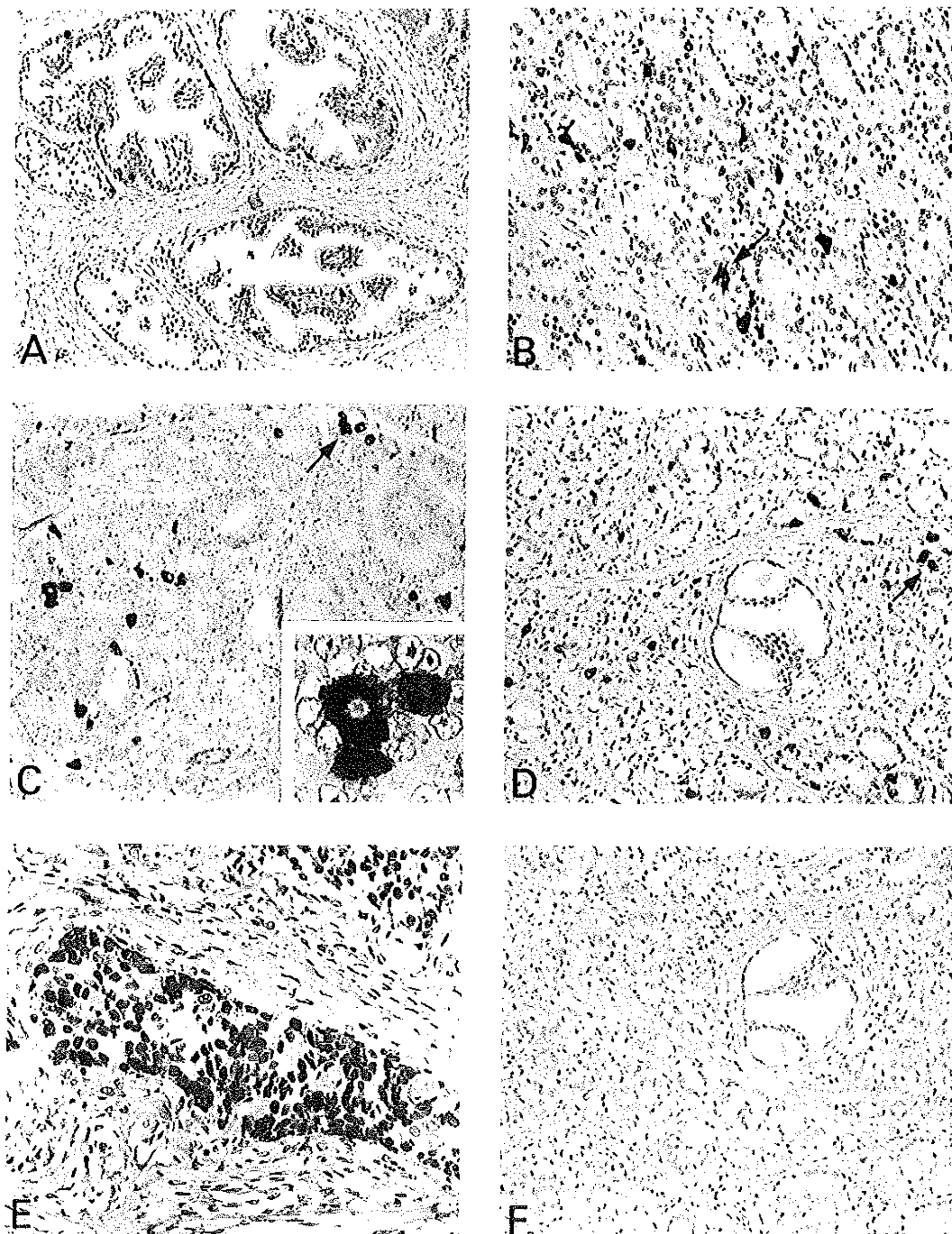

Figure 1: NE PAMP expression (A-E) and PAMP absorption control (F). PAMP cytoplasmatic and granular expression is well appreciated at the insert (C). The arrows point some of the PAMP expressing clustered cells. Note the very high PAMP expression in the neoplastic epithelium of the PC-310 model (E). **A**: normal prostate; **B** and **C**: RPs from patients with GSS 7 and 8, respectively; **D** and **F**: TURP with assigned GSS 8 from a patient pre-treated with androgen blockade; **E**: tumor from the PC-310 model obtained at T_{-90} post-castration. Magnification: A-D and F, 125x; E, 250x; insert, 500x. Counterstaining: Mayer's hematoxilin.

Immunodetection and quantification of PAMP in the xenograft models

PAMP expressing cells showed a remarkable increase in the PC-310 model with the post-castration time (Table IV). The first PAMP positive cells were observed at 48 hours after androgen ablation in randomly selected fields. At 7 days after castration (T_{-7}), the expression started to increase mainly in the peripheral area of the tumor. However, the PAMP positive cells became numerous also in the tumor central zone with the androgen-deprivation time (Figure 1E). The maximal PAMP expression was reached after a period of 90 days of androgen withdrawal. There was a positive and strong significant correlation ($r=0.976$, $p<0.001$) between the post-castration time and the number of PAMP expressing cells.

In the short-term castration experiment done with the PC-295 model, the PAMP-associated NE differentiation was much lower than in the PC-310 model. The first PAMP cells were detected at T_{-7}, and the maximum of PAMP positive cells (mean value 0.9 ± SEM) was observed at T_{-14}. A positive and significant correlation ($r=0.822$, $p=0.007$) was found between the number of PAMP expressing cells and the time during which androgens were withdrawn.

Table IV. Time course of the presence of PAMP positive cells in the androgen-deprivation experiment for the PC-310 prostate xenograft model.

POST-CASTRATION DAYS	PAMP CELLS (mean value)	SEM
0	0.0	0.0
2	0.2	0.1
5	0.6	0.3
7	5.8	1.2
14	12.5	2.9
21	10.3	2.6
47	24.4	3.8
84	19.7	2.7
90	35.0	5.8
154	42.7	5.9

Both PC-310 and PC-295 models, showed a high increase of CgA expressing cells after androgen deprivation. CgA positive cells were found at low levels at T_0 but became numerous in areas from the peripheral zone to the central zone of the tumors after longer periods of androgen deprivation, as shown previously[28,29]. All PAMP positive cells in these xenograft models were CgA expressing cells, but not conversely, which confirmed the PAMP cells as a subpopulation of the total prostatic NE cells. It is worth noting that in both models CgA cells showed a trend to be the main tumor cell type later during the post-castration period. This trend was also observed for the PC-310 model PAMP population, but not for the PC-295.

Most of the other xenograft models did not exhibit PAMP expression at all, neither at T_0 nor at T_{-14}. Some sporadic immunostained cells were observed at T_{-14} in the androgen-dependent PC-82 (PAMP mean value=0.1) and PC-329 (PAMP mean value=0.3) models, and in the androgen-independent PC-374 model (PAMP mean value=0.2).

Immunodetection and quantification of PAM in the clinical specimens

As described in the Material and Methods section, two monoclonal anti-PHM antisera were used for immunolocalizing the PAM/PHM reactivity. In a pilot study, the distribution of the staining derived from the use of both antibodies was found to be similar. As the application of one of them (clone G8) resulted in a more sensitive detection, this antibody was used for the scoring of PAM expressing NE cells in the complete series of neoplastic specimens.

As described for PAMP, PAM-associated NE expression was granular and cytoplasmatic in epithelial scattered cells of the normal and neoplastic prostates (Figures 2A, B, E and F). The scattered PAM immunolabeled cells were assessed as a subpopulation of the CgA expressing NE cells by serial reverse-face staining (Figures 2A and C). Double immunostaining and immunostaining on reverse-face sections performed to co-localize the amidating enzyme (PAM) and the amidated peptide (PAMP) revealed three types of NE cells both in the normal and in the tumor glands: First, positive cells for PAM but not for PAMP; second, positive cells for PAMP but not for PAM; and a third subpopulation of cells which were immunostained for both PAMP and PAM (Figures 2B and D). Most of the positive cells belonged to the third group exhibiting double immunoreactivity. PAM immunolabeling of NE cells was confirmed as specific by the appropriate absorption controls (Figures 2G and H).

In the normal prostate, the PAM-stained NE cells were found distributed as single isolated cells scattered in the epithelium of ducts and acini (Figures 2A and B), and were more abundant in the periurethral than in the peripheral glands. Furthermore, other prostatic structures such as the utriculus and the urethra exhibited many PAM expressing cells. In the tumors, PAM was observed in single or clustered NE cells (Figures 2E and F), as described above for PAMP expressing cells in the neoplastic tissues.

Specific PAM immunoreactivity was also detected in non-NE epithelial, endothelial, and muscle (smooth and striated) cells, as well as in neurons and nerves (Figures 3A-D).

Table V. Relationship between PAM NE score and GSS in the RPs.

TUMOR PAM SCORE	GLEASON SUM SCORE							TOTAL
	4	5	6	7	8	9	10	
0	1	2	5	12	6	5	2	33
1	1	1	3	9	2	6	0	22
2	0	0	0	1	0	1	0	2
3	0	0	0	2	1	1	0	4
TOTAL	2	3	8	24	9	13	2	61

With respect to the scoring of the immunostaining in the neoplastic specimens, 28 of 61 (46%) RPs, 4 of 15 (27%) U-TURPs and 5 of 14 (36%) P-TURPs exhibited NE-associated PAM expression (Tables V and VI). Spearman coefficients indicated no correlation between GSS and PAM score for the RPs ($r=0.070$, $p>0.05$). In the case of the TURPs it was observed a

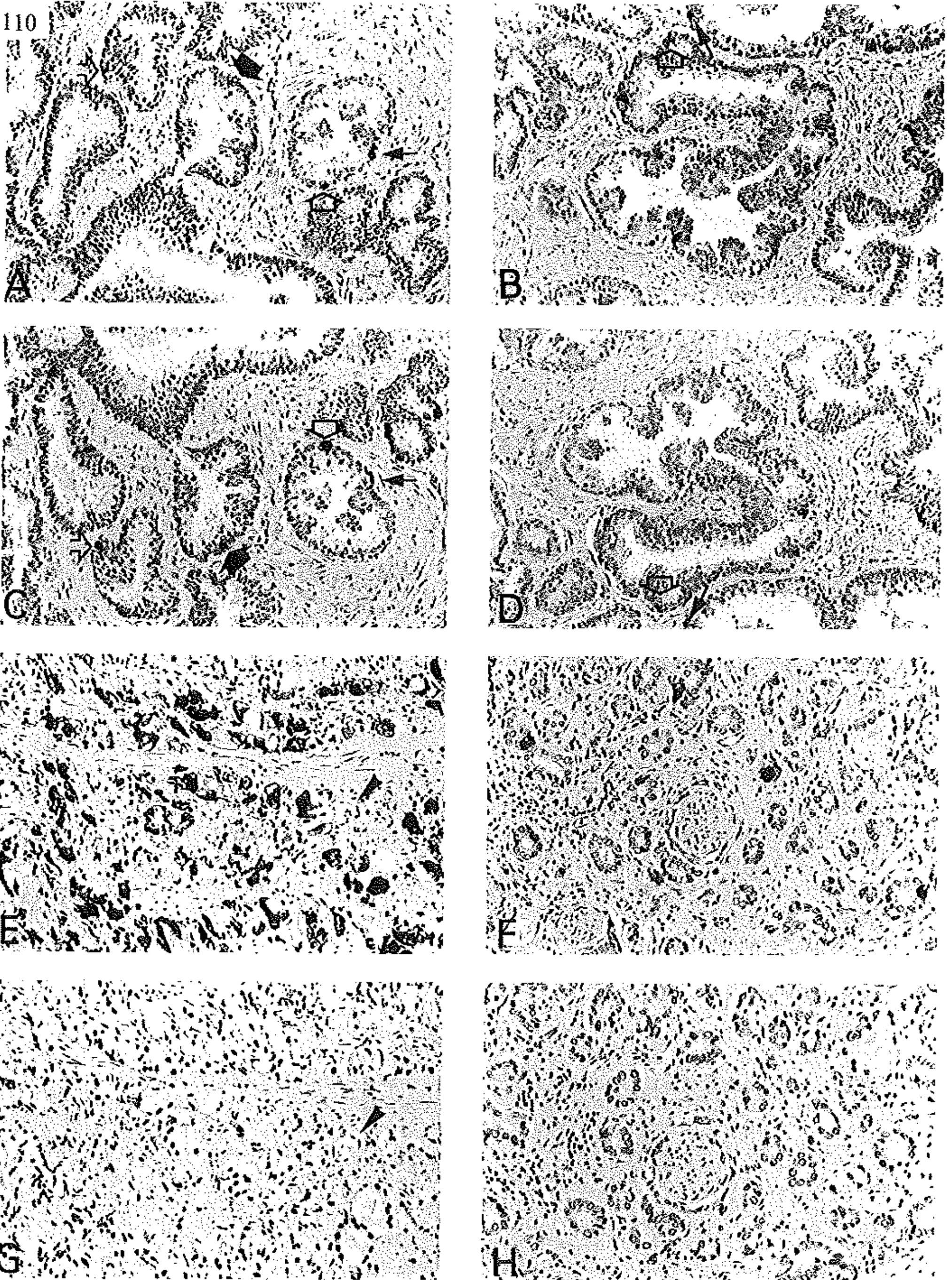

Figure 2: NE PAM expression (A, B, E and F), co-localization with CgA (C) and PAMP (D), and absorption controls (G and H). Same type of arrows point to the same cells immunostained for PAM-CgA (A and C) or PAM-PAMP (B and D). Arrowheads in F and H point to the same vessel.
A-D: normal prostate; E and G: P-TURP, GSS 10 ; F and H: RP, GSS 9.
Magnification: A-E and G, 140x; F and H, 125x. Counterstaining: Mayer's hematoxilin.

Table VI. Relationship between PAM NE score and GSS in the TURPs.

TUMOR PAM SCORE	Pre-treated patients					Untreated patients				
	GSS				TOTAL	GSS				TOTAL
	7	8	9	10		7	8	9	10	
0	2	4	3	0	9	1	1	3	6	11
1	0	0	1	0	1	0	0	3	0	3
2	0	0	0	0	0	0	0	0	0	0
3	0	2	1	1	4	0	0	1	0	1
TOTAL	2	6	5	1	14	1	1	7	6	15

negative correlation between the scores from specimens of untreated patients (r=-0.302) and, conversely, a positive correlation for the scores from specimens of pre-treated patients (r=0.347), but none of them was statistically significant (p>0.05). 27% (4/14) of the P-TURPs and 7% (1/15) of the U-TURPs exhibited high PAM expression (score 3). These percentages were not significantly different (p=0.169, n=29). In the patients who underwent RP, PAM-associated NE differentiation was detected in 44% (12/27) of patients with disease progression and 36% (4/11) with tumor specific death. Furthermore, PAM-associated NE immunolabeling was found in 47% (16/34) of the patients having no disease progression and 48% (24/50) of patients who did not die because of their tumor. Kaplan-Meier curves did not show a prognostic value of PAM expression in relation to the clinical progression or the tumor specific survival (data not shown).

In general, PAM was less detectable than PAMP in every kind of tissue. To assess whether the relationship between PAM and PAMP expressions exists, the correlation between both scores was calculated. For the RPs, there was a positive and significant correlation (r=0.494, p<0.001). For the TURPs, the correlation was smaller and not significant: r=0.208 (U-TURPs) and r=0.307 (P-TURPs).

Immunodetection and quantification of PAM in the xenograft models

The PC-310 xenograft tumors showed two kinds of PAM immunoreactivity (Figure 4A). A faint diffuse staining was found in the non-NE neoplastic epithelium, whereas a usually strong and granular labeling was localized in some epithelial cells later assessed as NE cells. Likewise, expression of PAM was found in the PC-295 model. During long-term androgen deprivation of the PC-310 model, we observed an increase in the number of PAM positive NE cells. The increase in PAM NE differentiation appeared later in time after androgen deprivation than PAMP differentiation. In PC-310 model PAM positive NE cells were observed for the first time at T_{-21} but became numerous from T_{-84} onwards. Although an increase in the PAM NE cells was observed in time after androgen deprivation, we cannot present quantitative data because the parallel increased staining of non-NE PC-310 tumor cells made the scoring of the PAM expressing NE cells unreliable. Strong PAM expression co-localized with PAMP and CgA expressing NE cells

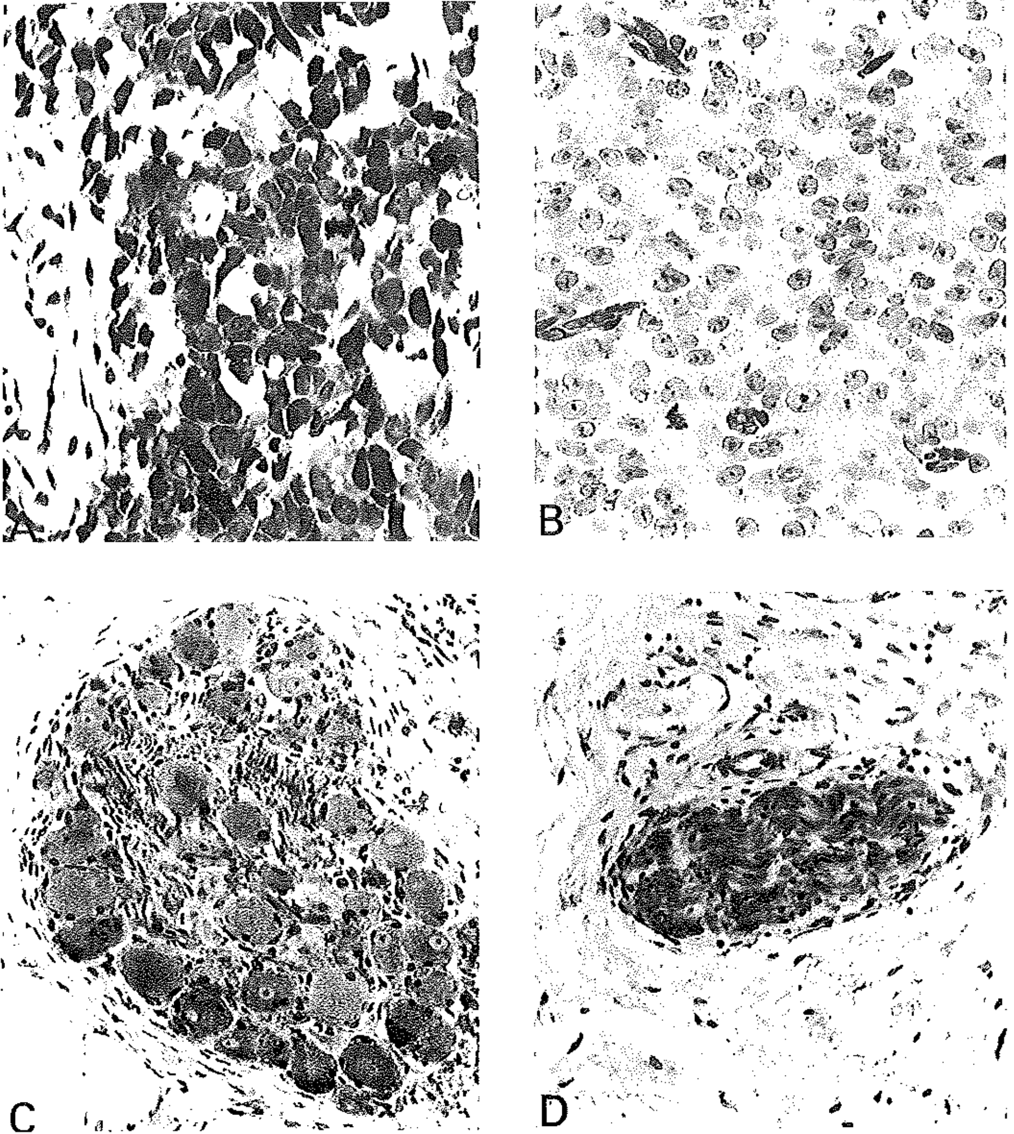

Figure 3: Non-NE PAM expression. (A) Tumor epithelial cells showing a light but general cytoplasmatic labeling. (B) Stained endothelia irrigating a tumor zone. (C) Neurons in a prostatic ganglion. (D) Nerve.
Magnification: A and B, 500x; C and D, 250x. Counterstaining: Mayer's hematoxilin.

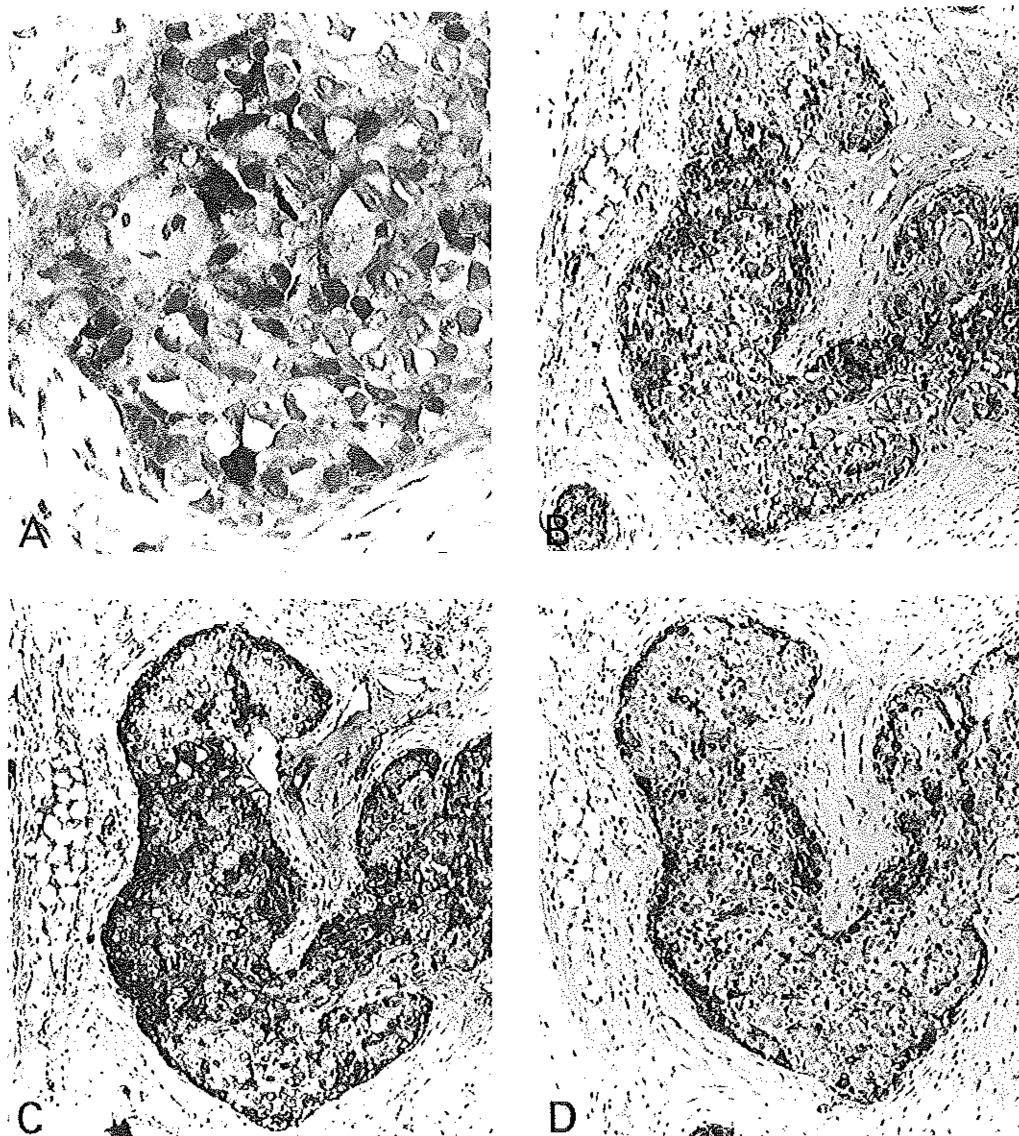

Figure 4: Immunoreactivity in the PC-310 model at T_{-90}. PAM (A and B) is expressed in NE and non-NE epithelial cells. Note that the more stained PAM cells in (B) are situated in coincident zones with the cells immunostained for CgA (C) and PAMP (D). Figures B-D correspond to the same epithelial node. Magnification: A, 500x; B-D, 125x. Counterstaining: Mayer's hematoxilin.

(Figures 4B-D). In a short-term castration experiment the PC-295 model showed a slight increase in PAM expressing NE cells.

The other models showed different epithelial staining patterns. In the androgen-dependent PC-82, PC-329 and PC-346 models a pattern of low and diffuse expression was observed, while some parts of the endothelium were stained strongly. The androgen-independent PC-133, PC-135, PC-324, PC-339, and PC-374 showed a stronger overall staining pattern, whereas the androgen-independent PC-346 subline, PC-346I, exhibited areas with strong epithelial staining surrounded by areas with low PAM expression. In these models the expression levels overall increased during 14 days of androgen deprivation, but these were assessed as non-NE, i.e. CgA negative, tumor cells. Exceptions were a high PAM expression in the peripheral zone of PC-133 tumors, a strong PAM positivity in numerous PC-346I tumor cells and an induction of NE-like PAM expressing cells in the PC-329 model during androgen deprivation.

PAMP Western blotting

The study was performed for the PC-310 model since it exhibited the highest number of PAMP positive cells as determined by immunohistochemistry. Total protein extracts from short-term (0, 2, 5, 7, 14 and 21 post-castration days) and long-term (0, 21, 47, 84, and 154 post-castration days) experiments were studied (Figure 5). The 3 kDa band, corresponding to fully processed PAMP, was not detectable in any of the xenograft extracts. However, other larger bands were observed from T_{-21}. Some of these bands have been previously described as PAMP precursor peptides (14 and 22 kDa). Other bands could not be interpreted so far. The intensity of the signal increased with the androgen deprivation time. Thus, the 14 and 22 kDa bands became stronger from T_{-21} to T_{-84}, although were fainter at T_{-154}. In the absorption controls no signal was detected (data not shown).

PHM/PAM quantification by Western blotting technique was not feasible in the xenograft tumors due to the strong background derived from the mouse immunoglobulins present in the extracts, which were recognized by our detection system (data not shown).

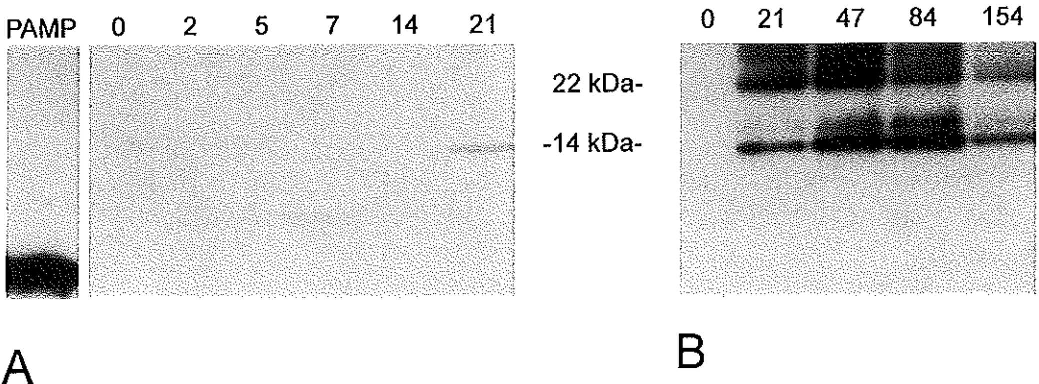

Figure 5: PAMP Western blotting in the short-term (A) and long-term (B) PC-310 post-castration experiments. Synthetic PAMP was loaded in a separate well (A).

Discussion

NE differentiation in prostatic carcinoma has been widely studied during recent years. Focal NE differentiation in most of the prostatic tumors has become evident as the sensivity of the techniques has been improved and the spectrum of tested NE markers has been enlarged[4,5,35]. The generalized presence of NE cells in PC and their characteristic of being androgen-independent[8,9] has suggested that NE population may be involved in the progression of the disease and in the development of refractory tumors after endocrine therapy. Thus, several studies aimed at the possible implication of the prostatic NE population in the prognosis of prostate cancer considering pan-NE markers such as CgA, and other endocrine factors. So far conclusions have been contradictory, from an independent prognostic significance, to a null correlation[6]. The different results are likely due to the heterogeneity of the clinical material used in each study.

We have studied a great variety of prostatic tissues for comparing the expression of two NE markers in different situations including: 1) normal prostate, 2) low and high grade PC obtained through RP from patients showing clinically localized or locally advanced disease, 3) high grade PC obtained through TURP from patients without prior treatment, 4) high grade PC obtained through TURP from patients pre-treated with androgen blockade and, 5) experimental models such as human prostate tumor xenograft models with different degrees of NE differentiation after androgen withdrawal.

With immunohistochemical techniques two new markers were detected which were widely expressed in the NE population of human normal and malignant prostate: proadrenomedullin N-terminal 20 peptide (PAMP), and peptidylglycine α-amidating monooxygenase (PAM) enzyme. PAMP or PAM positive cells were distributed with similar patterns than other NE markers in the normal prostate[16,36]. Thus, they were detected in ducts and acini, were more numerous in the periurethral than in the peripheral zone, and were also present in the prostatic urethra and utriculus. Since PAMP and PAM expressing NE cells were always subpopulations of the CgA expressing cells none of these markers can be considered more sensitive than CgA for localizing NE cells. PAMP is an amidated peptide and PAM is the only amidating enzyme known. It seems logic to think that the PAMP cells should express also PAM, although not conversely, since other prostatic neuropeptides like calcitonin and GRP also require amidation[23], and these could be expressed in other cells than PAMP NE subpopulation. So far, a PAMP/PAM expressing subpopulation, and likely a PAMP-negative and PAM-positive subpopulation could be expected. However, in every group of patients it was also detected third subpopulation, positive only for PAMP but not for PAM. This may account for the weak correlation between PAMP and PAM scores we found in the clinical specimens. The single PAMP subpopulation might reflect the accumulation of peptides in the cells after a period of active synthesis that will require the co-expression of PAM for their amidation. Alternatively, an unidentified amidating enzyme might be involved in the activation of PAMP in these cells, as suggested for other tissues[37].

Previous works have reported variable incidences of focal NE differentiation in PC[4,5,35], what can be partially attributed to the different degree of expression detected by immunohistochemistry for each NE marker[5].

In the clinical material we detected PAMP in approximately 80% of tumors obtained both by RP or TURP. The percentage of samples showing PAM-associated NE differentiation was lower and more variable: 46% (RPs), 27% (U-TURPs) and 36% (P-TURPs). It is conceivable that PAMP, PAM and other regulatory substances are produced by specific NE subpopulations which could exhibit different levels of metabolic or secretory activity and thus, which potentially will have diverse effect on neighboring non-NE tumor cells.

Some investigators have found an increase of NE differentiation with decreasing tumor differentiation[38]. Others, have reported no correlation between NE differentiation and tumor grade[39]. In this work, we also investigated the relationship between the PAMP or PAM NE differentiation and the grade of the tumors. It is known that the histologic grade represented by the Gleason sum score is strongly correlated with the grade of dedifferentiation and the malignancy of the PC[40]. The TURPs specimens received high grade scores (GSS from 7 to 10), while RPs included GSS from 4 to 10. In the RPs no correlation between the PAM or PAMP expression and the GSS was found. In the TURPs from pre-treated patients the correlation coefficients were positive (PAMP: r=0.489; PAM: r=0.347) while in the TURPs from untreated patients were negative (PAMP: r=-0.078; PAM: r=-0.302). Among these coefficients, only the one corresponding to PAMP/GSS in the P-TURPs almost reached statistical significance (p=0.076). Therefore, when considering every clinical subgroup separately, PAMP or PAM NE differentiation does not seem to be directly associated with the tumor grade. However, the increase shown by the correlation coefficients calculated for the P-TURPs with respect to the U-TURPs suggests that, at least in high grade tumors, PAM and PAMP NE cells may acquire functional relevance in tumor dedifferentiation or regulation after endocrine therapy. Furthermore, at least PAMP differentiation is induced in the hormone refractory tumors after anti-androgen therapy. We found high PAMP or PAM NE expression more frequently in the palliative TURPs (PAMP: 36%, PAM: 27%; n=14) than in the untreated TURPs (7% for both markers; n=15) being the PAMP incidences almost significantly different (p=0.08). Possibly, PAMP, PAM, or other substances produced and secreted by NE cells can act as alternative growth factor after androgen depletion. To elucidate whether or not these products are responsible for the emergence of hormone refractory tumors, the expression of their respective receptors by the neighboring neoplastic cells should be investigated whenever possible.

Patients who underwent RP had a long follow-up (13-178 months) for clinical progression and tumor specific death. PAMP or PAM differentiation showed no prognostic value for disease progression or cancer related death for this group of RPs. This lack of prognostic value agrees with a previous work based on CgA expression performed on a similar group of patients[39]. However, some authors have reported that NE differentiation in PC is a prognostic factor for progressive disease, although this was concluded for a group of patients who had received endocrine therapy[41].

Beyond the extensive studies of PC-295 and PC-310 models, all remaining xenografts studied did not show any PAMP or PAM NE differentiation after androgen depletion. This result was expected since induction of NE cells by androgen deprivation has not been previously observed in these models[31]. Androgen deprivation experiments with the

androgen-dependent PC-295 and PC-310 models have been extensively described[28,29,31]. Both models exhibit tumor regression after androgen deprivation but PC-310 tumors survive at least 5 months as dormant tumor residues, while PC-295 tumor epithelium regresses completely in a relatively short period of time[28]. This different behavior of the models explains why the long-term androgen-withdrawal experiment was carried out just in the PC-310. As reported by Jongsma et al.[28,29], both models show a remarkable induction of NE differentiation with CgA positive cells becoming the main epithelial cell type after prolonged androgen withdrawal. In the present study, PAMP-associated NE differentiation was found to be induced in the PC-310 and PC-295 in different ways. The number of PAMP cells raised in PC-295 was very small, but it increased sharply parallel to the CgA positive cell population in the PC-310. High PAM NE differentiation was induced relatively late in the PC-310 model, whereas a limited induction of PAM positive NE cells was observed in the PC-295. It has been previously reported that PC-295 and PC-310 tumors do express PAM mRNAs at every time-point of the androgen withdrawal experiment[28,29]. At sight of our observations, this mRNA could be mainly attributed to the non-NE PAM cells for PC-295, while in the PC-310, PAM mRNA probably corresponds to both NE and non-NE PAM expressing cells. The evidence of a distinct induction of NE subpopulations in PC-310 and PC-295 models may be related to the different evolution of these tumors after hormone deprivation. PAMP or PAM NE subpopulations could be supplying factors alternative to the androgens making survival of the dormant PC-310 tumor residues possible.

Western blotting analysis performed with PC-310 derived material detected some bands (M_W of 14 and 22 kDa) corresponding to PAMP precursors. Other detected bands cannot be explained, but could correspond to binding-proteins associated PAMP, as suggested for its gene-related peptide, adrenomedullin[42]. The signal corresponding to PAMP precursors (specially the 14 kDa) increased as the post-castration time advanced. However, at T_{-154} their intensity apparently diminished, very likely due to the increase of the mouse stromal volume observed in the PC-310 model at this late time-point. The absence of a processed PAMP peptide in the PC-310 extracts could be due to its rapid secretion or degradation.

Assuming the androgen-insensitive nature of NE cells the regulation of PAMP and PAM expression should be considered androgen-independent. Nevertheless, the increase of PAMP and PAM expression in the PC-310 model after androgen ablation, and the increased percentage of high PAMP and PAM scores found in the patients pre-treated by androgen-blockade therapy showed that androgen depletion results in PAMP, PAM and more generally, NE cell differentiation. Some authors[43] have not found the typical hormone responsive elements required for activating gene transcription by steroid hormones in the human pre-proadrenomedullin gene, which is in concordance with our findings concerning PAMP. On the other hand, it might be possible that androgen exerts a transcription repressor effect on the pre-proadrenomedullin gene, which could explain the increase of PAMP after androgen deprivation.

NE cells produce a great variety of regulatory factors both in normal and neoplastic situations. Serotonin and GRP act as growth factors[12,13,44], while somatostatin is a growth inhibitor[14], as tested *in vitro*. The production of

this regulatory substances suggests that the presence and activity of NE cells may affect growth, differentiation and local homeostasis of the prostate. This is supported by the observation of a increased proliferative activity in exocrine cells surrounding NE tumor cells[45]. PAMP exerts a variety of effects on different cell types, acting as vasodilator substance[15], neural transmission inhibitor[20], or growth suppressor[22]. Our observations suggest that PAMP may be responsible for the long-term survival of the androgen-dependent PC-310 model as opposed to the rapid regression of the PC-295 tumors after androgen ablation. Furthermore, it is possible that PAMP induces vasodilatory effect on mouse stromal vessels. The detection of PAMP binding sites in the neoplastic cells, and the supplementation of the PC-295 or PC-310 tumor bearing mice with synthetic PAMP or with inactivating anti-PAMP immunoglobulins may be possible ways to study the precise effect of PAMP on prostatic carcinoma in the future.

We found PAMP expression exclusively associated to the NE cell type. In contrast, PAM was also detected in non-NE cells such as endothelial, epithelial, or neuronal cells and nerves. This spread PAM detection is understandable since peptides requiring C-terminal amidation (like adrenomedullin[46] or endothelin) have also been reported in some of these cell types.

In summary, the study of PAMP and PAM expression in human prostate has demonstrated that they are two markers produced by specific subpopulations of NE cells widely distributed in normal and malignant tissue. Their expression is induced by androgen deprivation in some xenograft models and refractory tumors from patients, showing a similarity between the experimental and the clinical settings. In non-palliative patients receiving no prior hormonal treatment PAMP and PAM presence is not related to the tumor grade, clinical progression or survival time. In the patients previously treated, the trend to a positive correlation between PAMP or PAM NE differentiation with the tumor grade suggests a possible change in the functional relevance of these cells after androgen depletion. The possibility of a significant functional role for PAMP in prostatic tumor biology is supported by the fact that the androgen-dependent PC-310 tumors (showing an increasing PAMP NE differentiation) demonstrate long-term survival after androgen deprivation.

To date, no particular functional role has been demonstrated for prostatic NE cells, neither in normal nor in neoplastic biology. Our study shows that specific NE subpopulations, such as those expressing PAMP or PAM, may be implicated in the progression of the disease after anti-androgen therapy.

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CHAPTER 7

GENERAL DISCUSSION

NE cells and the regulated secretory pathway

Endocrine cells have an important function in organs like the pituitary and pancreas, producing all kinds of peptide hormones and neuropeptides. The endocrine cells form the islets of Langerhans in the pancreas in which the processing and regulated secretion of insulin or glucagon takes place via the regulated secretory pathway. In *Xenopus laevis,* the light to dark background adaptation (Holthuis, 1995) is triggered by production of pro-opiomelanocortin (POMC) by NE cells in the intermediate lobe of the pituitary. POMC is processed to smaller hormones, among others melanocyte stimulating hormone (MSH), which induces melanin production giving the dark skin pigment. The coordinated expression of different proteins from the regulated secretory pathway is necessary for a controlled production of peptide hormones by NE cells. A role for endocrine cells in differentiation of the hypothalamo-pituitary gonadal axis and in the embryonal or pubertal development of different epithelia, like the prostatic epithelium has been postulated (Pearse, 1969; Grube, 1986; Thoss, 1995; Sanchez-Montesinos, 1996; Kosut, 1997; Reinecke, 1997; Xue, 1998a). Sanchez-Montesinos et al. (Sanchez-Montesinos, 1996) showed that the development of the sympatho-adrenal system in the adrenal medulla consists of three different expression patterns. Firstly, expression of the early markers like CgA and CgB was observed, followed secondly by induced expression of NE markers of the regulated secretory pathway like SgIII, 7B2, PC1 and PC2 and thirdly the late appearing markers like SgII, PC3 and neuropeptide Y were expressed. This very much resembles the sequential events of NE cell maturation that we

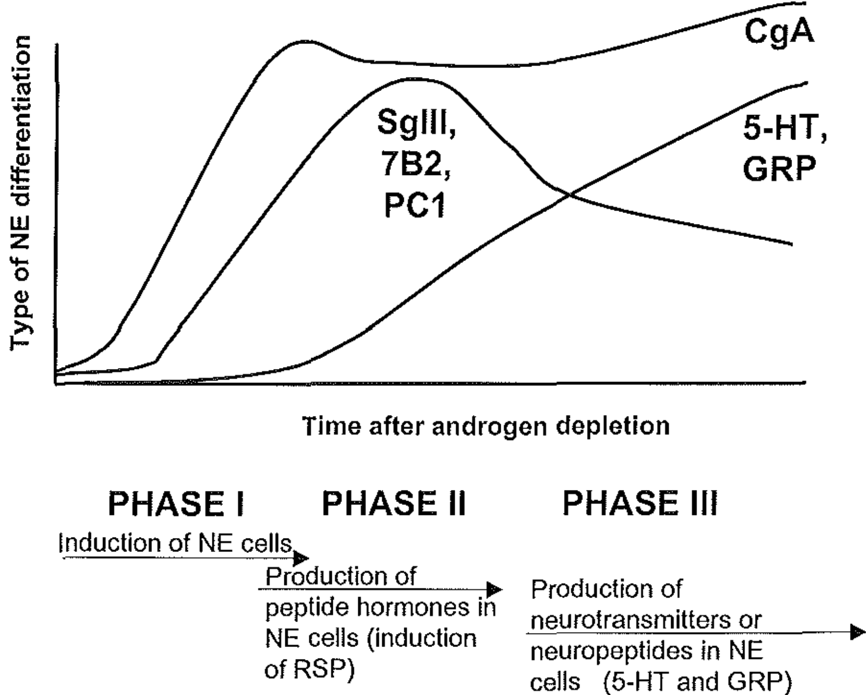

Figure 1: Schematic presentation of the process of NE cell maturation

observed by studying the expression patterns of the different markers of the regulated secretory pathway in the PC-310 xenograft model during long-term androgen deprivation (Chapter 5). Figure 1 schematically shows that androgen deprivation of hormone dependent prostate cancer induces a process of NE differentiation, evolving in subsequent maturation steps. The first step of PC-310 NE cell differentiation is the induced expression of early differentiation markers, like CgA. Prolonged maturation leads to expression of the typical peptide hormone production markers, like SgIII, PC1 and PC2. Subsequently, further NE cell maturation in the PC-310 model leads to the production of neurotransmitters and growth factors, like 5-HT and GRP. This latter time dependent production of growth modulating peptides may be important for either maintaining tumor dormancy or in development of hormone refractory prostate cancer.

NE cells and clinical prostate cancer

Different patient studies of hormonal pretreatment by either chemical castration with an LH-RH analogue or by combination of endocrine monotherapies, like flutamide and a cytostatic agent such as estramustine phosphate (EMP) before radical prostatectomies or trans-urethral resections of the prostate have shown the concomitant induction of NE differentiation (Van de Voorde, 1994; Guate, 1997; Jiborn, 1998; Pruneri, 1998). However, induction of NE differentiation in most of these studies has not yet been proven to be of prognostic significance in relation to prostate cancer progression. Rather, the strong induction of NE differentiation could be a bystander effect of androgen deprivation, without playing a role in tumor progression. This is confirmed by the results of the PAM and PAMP studies performed both in clinical specimens as well as in the PC-310 in vivo model after long-term androgen deprivation (Chapter 6). The only role that one may be certain about is that the induced NE cells are necessary for maintenance of PC-310 tumor residues (Chapter 5). Hormonally pre-treated patients with induced NE differentiated tumor do not necessarily have a shorter disease free survival time compared to patients without NE differentiation (Van de Voorde, 1994; Jiborn, 1998; Pruneri, 1997). On the contrary, prostatic tumors with higher CgA expression showed a significant correlation with bad prognosis (Krijnen, 1997). Still, the exact role of NE differentiation in progression of prostate cancer is difficult to assess.

NE differentiation in human prostatic xenograft models

Previously, NE differentiation in both the xenograft models and the initial patient material was studied on basis of CgA expression (Noordzij, 1996). The differentiation status of the available *in vivo* human prostate cancer xenograft models (described in Chapter 1) and of recently developed sublines of some of these models was re-evaluated. By using an improved and more sensitive staining technique for CgA expression we found more models with NE tumour cell differentiation than previously reported (Noordzij, 1996). The expression patterns of CgA and the marker for the regulated secretory pathway, SgIII (table I) as well as the AR status (data not shown) were studied.

MODEL	Androgen dependent	NE differentiated CgA/SgIII
PC-82	+	±
PC-133	–	–
PC-135	–	–
PC-295	+	+
PC-310	+	+
PC-324	–	+
PC-329	+	+/±
PC-339	–	±
PC-346	+	–
PC-346B	++	±/+
PC-346I	–	–
PC-346BI	+	–
PC-374	±	±

Table I: Status of androgen dependence and expression of the NE markers, CgA and SgIII, in human prostate xenograft models.

Seven of the thirteen presently available tumor models at our institution showed NE differentiation as defined by immunoreactivity against CgA as well as SgIII. These models are the androgen dependent models PC-82, PC-295 (Chapter 2), PC-310 (Chapters 4,5 and 6), PC-329, the BALB/c (B) subline of the PC-346 xenograft (PC-346B) and the androgen independent models, PC-324 and PC-339. The CgA expression pattern in these models closely resembles the pattern of NE differentiation that was found in clinical prostatic tumors (Cohen, 1991; Aprikian, 1993; Krijnen, 1993; Noordzij, 1995a). In the other models, PC-133, PC135, PC-346, and its sublines PC-346I and PC-346BI no expression of CgA was found in the presence of androgens whereas a low but detectable level of NE differentiation was observed in tumors grown on androgen deprived mice.

The androgen dependent models, PC-295, PC310, PC-82, PC-346B and PC-329 can easily be hormonally manipulated and show a homogeneous response to hormone withdrawal. The NE differentiated PC-295 and PC-310 are the two models mainly studied for induction of NE differentiation after androgen deprivation, because the induction is relatively fast and these models had previously been shown to continuously express CgA (Noordzij, 1996). In the PC-295 model, NE differentiation is induced immediately after androgen withdrawal. This model regresses rapidly due to strong induction of apoptosis and a sharp decrease in proliferation. After three weeks of androgen deprivation the tumors only contain small dispersed tumor fields consisting of either NE differentiated or Bcl-2 positive cells. The PC-310 model shows the same pattern of induction of NE differentiation and decreased proliferation after androgen deprivation, but in this model the level of apoptosis was not as high as observed in the PC-295 model.

Morphologically, the PC-324 and PC-339 tumors are very poorly differentiated and the tumor cells stain with CgA and SgIII in a pattern known from small cell prostate cancer (SCPC). Using RT-PCR, high expression levels of CgA, SgIII and PC-1 mRNA were found whether PC-324 tumors were grown with or without androgens (unpublished results). Furthermore, over-expression of p53 and Bcl-2 was found. Apparently, morphology and immunohistochemical features of PC-339 are consistent with a SCPC model.

Other *in vivo* prostate cancer models have been reported, like the

SCPC model UCRU-PR-2 (Haaften-Day, 1987; Jelbart, 1988; Jelbart, 1989), CWR22 and derived sublines (Pretlow, 1993; Wainstein, 1994; Nagabhushan, 1996; Agus, 1999) as well as transgenic mice models, like CR2-T-Ag (Garabedian, 1998) and Gγ/T or ßh1/T (Perez-Stable, 1996; Perez-Stable, 1997). To our knowlegde these models do not show NE differentation as determined by CgA expression.

The PC-346 model (van Weerden, 1996) developed at our institution and the CWR22 (Pretlow, 1993; Wainstein, 1994) xenograft model are unique, as they both relapse after androgen deprivation and androgen independent sublines, PC-346I and CWR22R, respectively, could be generated. PC-346I does not express CgA, and to our knowledge CWR22R does not express the NE phenotype. This strongly argues against a direct role of NE differentiation in progression of prostate cancer as these two progressive prostate cancer models do not show the NE phenotype observed in the PC-310 model.

In present prostate cancer research, the androgen dependent human PC-310 xenograft model still remains a unique model for further studies on induction of NE differentiation following androgen deprivation both *in vivo* as well as *in vitro*. Our studies on the PC-310 model showed a clear induction of the regulated secretory pathway during androgen deprivation. In particular, the expression patterns of different markers like SgIII, 7B2, PC1 and PC-2 implicate that in androgen deprived prostate cancer, the complex process of regulated secretion of different hormone peptides can be induced. Regulated secretion of POMC through this NE pathway might induce systemic effects. ACTH dependent Cushing's syndrome, which is a rare occurring disease in hormonally treated clinical prostate cancer, might then be a consequence (Matzkin, 1987). It is useful to study the long-term effects of androgen depletion on hormone dependent prostate cancer. PC-310, PC-295, PC-346 and PC-82 are relevant androgen dependent models to further study the induction of NE differentiation and the regulated secretory pathway. In conclusion, the hypothesized role of NE cells in the progression of prostate cancer from the androgen dependent state to an androgen independent or hormone refractory state has not been supported by our observations in the androgen dependent prostate cancer xenograft models. Nevertheless, on the basis of our data a role of NE cells in the progression to androgen independent growth cannot be excluded.

Hormone dependence, apoptosis and the role of the androgen receptor

Hormone deprivation in the PC-310 model system induced apoptosis, NE differentiation and decreased proliferation (Chapter 4 and 5). Furthermore, there is a transient down-regulation of AR expression *in vivo*, giving an strong nuclear expression in approximately 50 % of PC-310 tumor residues after long-term androgen deprivation. Long-term androgen deprivation of the PC-310 model leads to the formation of tumor residues consisting of mixed cell types of both NE differentiated and AR positive cells. After androgen deprivation, AR expression is transiently down-regulated in all androgen dependent models. At longer time intervals, AR is partly re-expressed in most of these models except for PC-295 and PC-329, as AR expression in the PC-295 model completely disappeared. In two of the androgen independent

models, PC-374 and PC-346I, AR expression levels increased after androgen deprivation. The androgen independent xenografts PC-133, PC-135, PC-324 and PC-339 never express the AR in either presence or absence of androgens.

In vitro, the PC-310 model showed the temporal decrease of the AR only in the short-term androgen deprived cultures. In PC-310C cultures AR expression could not be detected by Western blot after 56 days of androgen deprivation, although the cells did express the AR up to 28 days after androgen deprivation (data not shown). This loss in expression may be explained either by induced NE differentiation of the AR positive cells or by loss of the cells through detachment of the AR positive cells from the culture flasks. Detachment of cells could be caused by defective cell-cell adhesion or by induction of apoptosis. Induction of apoptosis in the AR positive PC-310C cells means that the balance between cell cyle arresting proteins (p16, p53, p27 and p21) and apoptosis related proteins (Bcl-2, Bax, Caspase 3 and 9, etc) has favoured the cells to undergo apoptosis.

Androgen re-supplementation to the long-term androgen deprived PC-310 tumor residues in the in vivo xenograft model clearly demonstrated that the non-NE AR positive tumor cells still had the capacity to proliferate upon androgen stimulation. This was associated with decreased $p27^{kip1}$ expression and consequently with rapidly increasing tumor volumes and high PSA production (Chapter 5).

Many investigations are performed to study the potential role of the AR in progression of prostate cancer where the AR is being adapted, hyperactive, mutated or overexpressed (Culig, 1998; Gil-Diez de Medina, 1998). Constitutive high AR expression has been found in trans-urethral resection specimens of clinical hormone refractory tumors (van der Kwast, 1991), and decreased expression of the AR was noted during regression of hormonally treated prostate cancer (Gil-Diez de Medina, 1998). These results are in line with the observed high expression levels of the AR after long-term androgen depletion of PC-310 and temporal decrease in AR expression during PC-310 tumor regression following short-term androgen withdrawal.

Development of hormone refractory prostate cancer has been related to several causes like mutations or amplifications of the AR gene or direct stimulatory effects on the AR of the anti-androgens used. Recent studies have shown agonistic effects of the non-steroidal anti-androgen bicalutamide (Casodex), which directly led to withdrawal of the anti-androgen (Scher, 1996; Kelly, 1997; Wirth, 1997; Iversen, 1999). Different mechanisms of adaptation of AR pathway during androgen independent activation are under investigation. Mutations of the AR, AR gene amplifications or induction of anti-apoptotic escape pathways (Bcl-2, p53) may account for androgen independence of prostate cancer (Culig, 1998; Jenster, 1999). In particular, adaptation of the AR sensitive pathway has recently gained a lot of interest, e.g. involvement of the MAPK pathway (Hobisch, 1998; Jenster, 1999). Activation of PSA expression via androgen responsive elements (ARE's) in the PSA promotor region may be activated independent of androgens. One explanation offered in the literature is that secretory products of NE cells, e.g. GRP can activate this pathway. The AR might be either directly activated by GRP or indirectly via activation of the MAPK pathway (Culig, 1998). This kind of androgen independent activation of the AR, for instance by neuropeptides

like GRP, IGF-1 (Chan, 1998) or cytokines like IL-6 cannot be excluded (Hobisch, 1998).

In the PC-346 model, which also demonstrated the temporal down-regulation in AR expression following androgen deprivation, this mechanism of androgen independent activation of the AR may be applicable, as different hormone refractory sublines of this model were recently developed (Van Weerden, personal communication). Allthough one of these independent (I) sublines, PC-346I, has an induced AR mutation that is known as the LNCaP mutation (Veldscholte, 1992), the other spontaneous AID sublines have a wild type AR. In the latter cell lines, NE differentiation induced or alternative androgen independent activation of the AR might well play an important role. Androgen deprived prostate cancer models may initially contain heterogenous clones that will eventually result in the outgrowth of progressive androgen independent strains, like the independent cell lines after androgen depletion of the PC-346 model. We have clearly shown the formation of dormant tumor residues that contain both maturating NE cells and androgen sensitive, AR positive cells in the *in vivo* PC-310 model. Development of tumor dormancy during hormone therapy may well be the first step in developing hormone refractory prostate cancer. The development of hormone refractory prostate cancer might be the consequence of (in)direct mitogenic or innervating effects of NE differentiation on (dormant) tumor cells, as is believed to be the case in lung cancer (Cuttitta, 1985; Sorokin, 1997), but this has yet to be proven in prostate cancer. *In vitro*, we have shown that prostate cancer cell lines can be stimulated to grow in the absence of androgens by neuropeptides, like GRP. However, we have not been able to observe androgen independent growth induced by NE growth factors in the PC-310 *in vivo* model. In conclusion, the PC-310 model did not progress as a consequence of induced NE differentiation or by expression of the AR in long-term androgen depleted PC-310 tumor residues.

Model of possible interactions in hormone deprived prostate cancer tumor residues

Based upon the observations in the PC-310 model system (Chapters 4 & 5), the in vitro studies (Chapter 3) and combined with data in literature, a model is proposed for interactions between the NE prostatic tumor cells with both the non-NE, AR positive tumor component on the one hand and with the surrounding stromal tissue compartment on the other under long-term androgen deprived circumstances (figure 2). Typical products of NE cells, like GRP, 5-HT, VIP, CT, PAMP and other related products like PAM and CgA may be secreted either constitutively or via the regulated secretory pathway (RSP). These secreted products will be partly transported from the tumor area by the blood stream to exert their growth modulating activity systemically. The secreted products can most likely exert local activity, which may be either autocrine or paracrine. For this kind of interactions, it is necessary that the non-NE cells express receptors for the secreted growth factors. Several studies have shown that prostatic tumor cells can express receptors for GRP, CT, VIP, 5-HT and other growth factors (Fischer, 1992; Reile, 1994; Shah, 1994; Aprikian, 1996; Zamora, 1996; Wasilenko, 1997; Aprikian, 1998). So far, we did not consider the presence of such receptors. Receptor expression studies in both clinical prostate cancers and in the available human xenograft

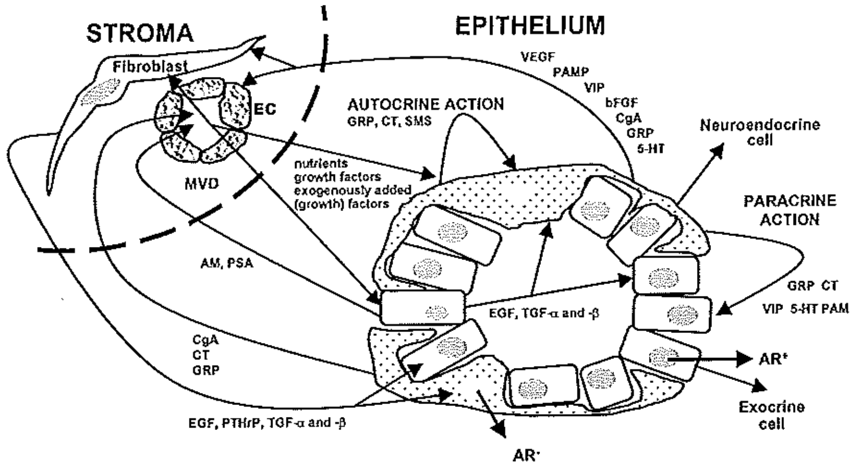

Figure 2: Schematic drawing of the different possible interactions in androgen depleted PC-310 tumor residues. MVD= microvessel density; EC= endothelial cell;AM= adrenomedullin; PAMP= proadrenomedullinN-terminal peptide

models are mandatory, however. Paracrine interaction may result in stimulation of the non-NE, AR positive epithelial cells, e.g by GRP, VIP, and CT, as has been shown in a study of in vitro prostate cancer cell lines (Chapter 3). This stimulatory role of bombesin or other members of the GRP family in prostate cancer growth has already been shown by others (Aprikian, 1998). In lung cancer, the positive relation of NE cell maturation with mitogenesis and innervation has further been shown by Sorokin *et al* (Sorokin, 1997). Other products of NE cells, like bFGF, PAMP and VEGF, may have a paracrine effect on stromal fibroblasts and the endothelial compartment. Vice versa, stromal fibroblasts can produce growth factors, like EGF, TGF-alpha and –beta, and PTHrP, which may play a role in progression of prostate cancer (Cunha, 1994; Desruisseau, 1996; Lee, 1996; Culig, 1997a; Kooistra, 1997; Yang, 1997; Lamm, 1998). The relationship between the three main cell components present in the prostatic tumor residues could be seen as a cellular triangle, if indeed fibroblastic growth can be induced by NE growth factors. In this cellular triangle, fibroblast-derived growth factors, like EGF, TGF-α and -β and PTHrP can act on both the NE and the non-NE epithelial cells (Cramer, 1996; Peehl, 1996a; Peehl, 1997). Continuous activity of this cellular triangle might eventually lead to prostate cancer progression. Alternatively, cytokines supplied via the blood, like IL-1alpha/beta and IL-6, hormonal peptides or growth factors, like IGF-1 may also affect the prostatic tumor residues, e.g. by tumor maintenance or induction of proliferation. In theory, the proposed model may offer an explanation for the growth of non-NE tumor cells in the absence of androgens. Now, we have to test this in xenografts in vivo and in vitro if this is really true.

Functional implication of NE cells in the progression of prostate cancer

This thesis represents the experimental data of both *in vivo*, i.e. in human prostate cancer xenografts, and *in vitro* studies on the kinetics and the possible role of tumor cells with neuroendocrine (NE) differentiation in prostate cancer during androgen deprivation. In addition, the NE related

markers, PAMP and PAM were studied in experimental models as well as evaluated for their prognostic significance in clinical prostate cancer specimens with long-term follow-up. NE cells normally represent a relatively small part of the prostatic epithelium and are well recognized by their frequently displayed dendritic processes which may extend to neighbouring tumor cells (di-Sant'Agnese, 1992b). NE differentiated cells are found in 70% of all clinical prostate cancer specimens. In our laboratory two experimental androgen dependent, NE differentiated *in vivo* models, PC-295 and PC-310, were available.

Since androgen deprivation in clinical prostate cancer (Van de Voorde, 1994; Guate, 1997; Jiborn, 1998) and in human xenografts ((Noordzij, 1996), this thesis) have shown to result in induced NE differentiation, the role of NE tumor cells in the progression of androgen responsive prostate cancer to hormone refractory tumors remains a major topic of discussion. From most clinical studies on the prognostic value of NE differentiation it has been shown that CgA expression does not have a prognostic value. However, patients treated with TURP had a better prognosis when they did not have a high expression of CgA in the TURP specimen. Our results from the expression of new NE markers revealed a tendency towards higher tumor stage and more clinical progression in the higher PAM expressing tumors. In conclusion, our data cannot define a role of NE cells in progression of prostate cancer from the androgen dependent stage to the hormone refractory stage.

Concluding Remarks

Androgen deprivation of the human prostate cancer xenograft models, PC-295 and PC310 induces proliferation independent NE differentiation.

Long-term androgen deprivation of the PC-310 model results in a process of NE cell maturation, leading to the sequential production of both peptide hormones via a regulated secretory pathway as well as the secretion of growth modulating neuropeptides.

The temporal down-regulation of AR expression under androgen deprivation in the PC-310 model as well as in PC-346, PC-82, PC-329 reflects the AR expression pattern found in clinical specimens of regressed prostate cancer, under endocrine therapy. This indicates that these models reflect (in part) the behaviour of clinical prostate cancer

Induction of NE differentiation and subsequent NE cell maturation in the PC-310 model after long-term, i.e. 6 months, androgen deprivation does not lead to the initiation of androgen independent growth. Thus, our data did not provide evidence that NE differentiation plays an important if any role in progression of prostate cancer. However, referring to the clinical situation, the time between initiation of hormonal treatment and final clinical progression in prostate cancer patients ranges from 12 to 36 months. Thus, in periods of androgen deprivation longer than that of 6 months studied for PC-310 until now, there is still a long traject in which NE cells might play a relevant role in progression of prostate cancer. In addition, it cannot be excluded that our xenograft models are not the most appropriate tool to study transition to androgen independence under influence of neurosecretory products.

Future directions for NE related prostate cancer research

The PC-310 model did not show progression as a consequence of induced NE differentiation or by expression of the AR in long-term androgen depleted PC-310 tumor residues. Rather, proliferation was completely inhibited and the volumes of the long-term androgen deprived tumor residues seem to be stable. Thus, one might even argue that induction of NE differentiation in prostatic tumors could be a way to stop androgen dependent tumors from uncontrolled growth. Induction of NE differentiation in androgen dependent tumors like the PC-310 model could then result in growth arrest, tumor regression and less tumor burden for the patient. Following this, it might be of interest to develop alternative strategies to induce NE differentiation in prostate cancer. Besides androgen deprivation, there are numerous other methods to induce NE differentiation in prostate cancer, for example by application of dibutyryl-cyclicAMP, phenylacetate, vitamin A and D. At the department of Urology, we have available both the in vitro models as well as in vivo xenograft models to study treatment of prostate cancer with different concentrations of these agents in time. The detailed kinetic studies that were performed in the PC-310 model provided knowledge of a complete set of NE markers, including CgA and the time related markers of the RSP which can now be used for characterization of the degree of induced NE differentiation by these substances.

As CgA and AR did not colocalize in any PC-310 tumor cell studied, the presence of the AR positive cells in prostatic tumor residues adjacent to induced NE differentiated cells should not be neglected. The fact that the AR is expressed in 50% of the residual tumor cells makes the PC-310 model a useful model to study the upregulation of the AR sensitive pathway after the temporal decrease which might eventually lead to tumor progression. As we did not observe progression in the PC-310 model during six months of androgen deprivation, it may be possible that the AR positive PC-310 tumor cells lack the molecular pathways to stimulate the AR sensitive pathway. Adaptation of the AR sensitive pathway to the androgen deprived situation might also require longer periods of androgen deprivation than six months. The production of specific neuroactive substances, like 5-HT and GRP, occurred rather late during NE cell maturation. Such specific products of fully maturated NE cells might be essential to induce growth of AR positive PC-310 tumor cells. Androgen withdrawal experiments in the PC-310 model system should be performed that exceed six months to study potential long-term effects of NE cell maturation on induction of androgen independent growth.

In view of prostate cancer progression, three androgen dependent xenograft models are at our disposal displaying different properties of hormonally treated clinical prostate cancer, i.e. progression of AR positive tumors (PC-346), induction of NE differentiated tumor residues (PC-310) and formation of androgen sensitive, AR positive, tumor residues (PC-82). The tumor cells in the PC-346 model, which develop into androgen independent cells should be characterized in detail. By using the androgen dependent models in co-transplantation studies, i.e. transplantation of a mixed cell population of two xenograft models, we might be able to initiate progression of the two growth arrested models, PC-310 and PC-82. Detailed kinetic studies with comparable long-term androgen deprivation as has been done for PC-

310 (Chapter 4) should be performed with PC-82 and PC-346 bearing mice to study the behavior of these tumors during long-term androgen deprivation. Androgen withdrawal in all three tumor models induces a temporal decrease in AR expression. In PC-82 and PC-310, $p27^{kip}$, a cell cycle growth arrest marker, is highly expressed and MIB-1 expression decreases rapidly to zero, whereas in the PC-346 model a low level of proliferation continued after androgen deprivation. If the induction of NE differentiation in PC-310 does play a role in inducing progression, we might find androgen independent growth during co-transplantation of PC-82 and/or PC-346 with PC-310 under androgen deprived conditions. In all experimental designs expression of different cell cycle markers, AR, CgA, different NE growth modulating peptides, proliferative markers, and factors involved in the MAPK pathway are relevant to be studied.

The induction of NE differentiation by androgen deprivation and the mitogenic products released after long-term depletion indicate the possibility of MAPK activity induction in neuropeptide receptor positive tumor cells. Long-term mitogenic and growth inhibiting studies in the PC-346, PC-310 and PC-82 xenograft models should be performed under androgen deprived conditions. Therefore, these tumors should carefully be studied for the presence of neuropeptide receptors. Both in vivo and if possible in vitro studies will yield information as to whether NE growth factors can play a role in prostatic tumor residues by initiating growth induction of the AR positive non-NE cells. Large groups of tumor bearing mice should be studied for growth modulating effects by different concentrations of GRP and other neuropeptides under androgen deprived conditions and over different periods of time.

Clinically, a large study could be performed on prostate cancer patients that are undergoing hormonal treatment followed by either a consecutive radical prostatectomy or not. The main objective of this study is to examine whether NE differentiation is induced, whether NE growth factors are produced and whether these growth factors can play a role in clinical prostate cancer progression. Tumor mass and level of NE differentiation can be determined by measuring plasma levels of PSA and CgA. The relation between AR expression, induction of NE differentiation and subsequent expression of late evolving NE markers like 5-HT and GRP should be studied as well as the presence of receptors for NE growth factors on residual tumor cells. Patients should be followed for tumor progression, disease related death with respect to differences in therapy and in relation to the induced levels of NE differentiation.

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Summary

Assessing growth modulatory effects of neuropeptides in prostate cancer models *in vitro* and *in vivo*, and studying the kinetics of induction of NE differentiation upon androgen withdrawal in human prostate cancer xenografts and *in vitro* cell lines has contributed to a better understanding of the role of NE cells in androgen deprived prostate cancer.

Experimental work described in Chapter 3 demonstrated that cell proliferation of the prostatic cancer cell lines, LNCaP, DU-145, PC-3 and PC-346C under the condition of androgen depletion can be modulated by neuropeptides, like serotonin, gastrin releasing peptide (GRP), calcitonin and vasoactive intestinal peptide which are known to be produced by neuroendocrine cells. The way the four neuropeptides affected cell growth differed from one cell line to the other irrespective of their androgen dependent status. Although androgen independent PC-3 cells did not respond to exogenously added GRP, results of incubation with the anti-GRP monoclonal antibody 2A11 (MoAb 2A11) did show that PC-3 proliferation is strongly dependent on GRP as ^{3}H-thymidine incorporation dropped by 50%. Probably, GRP is produced endogenously by this prostate cancer cell line. MoAb 2A11 is a strong and specific inhibitor of GRP-induced proliferation, e.g. in GRP producing cell lines, in prostate cancer, as was shown before for small cell lung cancer cell lines. In conclusion, GRP is a potent NE growth factor for prostate cancer cell lines that acts specifically under androgen deprived conditions via induction of the second messenger cAMP in both androgen independent (DU-145, PC-3) and androgen dependent (PC-346C) cells.

We established FGC-DCC, an androgen independent derivative of LNCaP after long-term androgen deprivation. The parental LNCaP and FGC-DCC subline show a clear difference in their androgen responsiveness status, which showed the charateristic biphasic relation for DHT in LNCaP whereas FGC-DCC cells did not respond to increasing DHT concentrations, indicating that FGC-DCC cells grow androgen independently. The FGC-DCC cells have a neuronal appearance, produce low levels of PSA and show an induction of Bcl-2 expression shortly after androgen withdrawal. Neither the LNCaP nor the FGC-DCC cell line showed any expression of the NE marker CgA or a significantly increased expression of basal SgIII or NSE levels. In spite of their characteristic neuronal morphology, FGC-DCC is not a NE-differentiated cell line. Like for the PC-3 cells, MoAb 2A11 decreased proliferation of FGC-DCC cells. FGC-DCC cells possibly have adjusted to an autocrine growth pattern after androgen suppression, by producing growth factors like GRP endogenously.

Studying NE differentiated in vivo models like PC-295 and PC-310 has contributed to implicating new functions for NE differentiation in clinical prostate cancer. In Chapter 2, we showed the effects of short-term androgen deprivation in the completely androgen dependent xenograft model PC-295. Upon androgen withdrawal, proliferation and PSA serum levels decreased rapidly to zero. AR expression is downregulated and there is a strong induction of apoptosis, which resulted in near complete regression of the tumor volume. Induction of NE differentiation was a proliferation independent process and independent of tumor volume changes. After longer periods of androgen deprivation, the PC-295 model could no longer grow in the presence or absence of androgens.

In Chapters 4 and 5 we have shown that the hormone depletion of the PC-310 model resulted in induction of apoptosis and $p27^{kip1}$ expression, decreased proliferation and PSA serum levels, and temporal down regulation of the androgen receptor (AR). Like in the PC-295 xenograft, proliferation independent induction of NE differentiation was observed in PC-310 from 5 days post-castration on. This was shown by increased expression of CgA and by the time related induction of the secretogranins, 7B2 and SgIII and the processing enzymes, PC1, PC2 and PAM, factors all belonging to the regulated secretory pathway (RSP). The time dependent induction of these markers in the PC-310 NE cells showed that they possess an active RSP and demonstrate the maturation inside the secretory granules. Additionally, the serum CgA levels were clearly increased post-castration, which shows that the PC-310 NE cells can actually secrete neuropeptides and secretogranins.

When we looked at the behavior of the PC-310 model during long-term castration with regard to NE cell differentiation and maturation, the production of growth regulatory peptides or hormones and the expression of the androgen receptor, we found additional markers for the maturation of the NE cells. NE growth factors, like 5-HT and GRP are expressed after a relatively long period of androgen deprivation. Expression of GRP and 5-HT was initially found in a small part of the NE cells at 14 and 21 days post-castration, but became evidently expressed 154 days post-castration. PAM expression is also induced in the NE cells at 84 and 154 days post-castration. This late NE specific expression points at a direct role in amidation of neuropeptides during the maturation of NE cells. The expression of VEGF co-localized in the NE cells and was induced shortly after the expression of CgA and SgIII.

The *in vivo* PC-310 xenograft model regresses after androgen deprivation, but not as strong as the PC-295 model. Between 14 and 21 days, the tumor volume is stabilized at 30-40 % of the initial tumor volume. During longer periods of androgen deprivation the tumor residues remains

constant and the PC-310 model has become dormant. In these dormant tumors there are two main cell types, which are the AR positive cells and the NE cells. Both cell types may play a role in the formation of the PC-310 tumor residues. On the one hand, the AR positive cells remain androgen sensitive as shown by tumor regrowth upon androgen reconstitution. On the other hand, these non-NE AR postive cells might be the tumor cells that escape androgen wtihdrawal by means of different hypothetical pathways, through mutations of the AR, altered specificity of the AR or ligand independent activation of the AR sensitive pathway.

Chapter 6 shows expression patterns of the NE markers, PAM and PAMP in *in vivo* studies of the experimental PC-310 model as well as in clinical prostate cancer patients. Induction of both markers clearly correlated with induction of NE differentiation in the PC-310 model. In a group of immunohistochemically evaluated specimens of prostate cancer patients, no strong correlation of PAM and PAMP expression was observed with either tumor stage nor with clinical progression, although there was a tendency towards higher expression of PAM and PAMP in late stage tumors, i.e. at higher gleason sum scores.

With the in vitro studies androgen independent growth of prostatic cancer cell lines was shown to be induced either by growth modulating neuropeptides or by androgen deprivation of an androgen dependent cell line (LNCaP) which lead to the development of an androgen independently growing cell line (FGC-DCC).

Induction of NE cell differentiation and maturation in the PC-310 model by androgen deprivation does not lead to androgen independent growth. Our data did not provide evidence that NE cell differentiation plays an important if any role in progression of prostate cancer. However, the time between initiation of hormonal treatment and clinical progression in prostate cancer ranges from 12 to 36 months. Thus, androgen deprivation of the PC-310 model for periods longer than 6 months might still yield a role of NE cell differentiation in prostate cancer progression.

Samenvatting

In dit proefschrift staat de rol van neuroendocriene (NE) cel differentiatie in prostaatkanker centraal. NE cellen zijn aanwezig in normale prostaatklierbuizen en in veel prostaatkankers. Producten die door deze cellen worden uitgescheiden, ook wel neuropeptiden genaamd, kunnen mogelijk de groei van nabij gelegen non-NE prostaatkankercellen moduleren. Voor het bestuderen van de groeimodulerende effecten van neuropeptiden en het bestuderen van NE differentiatie werd gebruik gemaakt van veschillende experimentele prostaattumormodellen. Deze modellen omvatten enerzijds prostaatkankercellen die gekweekt worden in weefselkweek flesjes (*in vitro* onderzoek) en anderzijds androgeen-afhankelijke humane prostaattumoren die onderhuids groeien in immuundeficiente muizen (*in vivo* onderzoek). Het zowel *in vitro* en *in vivo* vaststellen van de groeimodulerende effecten van neuropeptiden in prostaatkankermodellen en de studies naar de kinetiek van het induceren van NE differentiatie heeft bijgedragen tot een beter begrip van de rol van NE cellen in prostaatkanker na androgeen deprivatie.

Experimenteel werk beschreven in hoofstuk 3 laat zien dat proliferatie van prostaatkankercellijnen, LNCaP, DU-145, PC-3 en PC-346C gemoduleerd kan worden door neuropeptides, nl. serotonine (5-HT), bombesine (GRP), calcitonine (CT) en vasoactief intestinaal peptide (VIP) onder condities van androgeen deprivatie. De manier waarop de neuropeptides de celgroei beinvloeden veschilt tussen de 4 celllijnen onafhankelijk van de status van androgeengevoeligheid. Monoclonaal antibody 2A11 (MoAb 2A11) is een specifieke remmer van door GRP-geinduceerde proliferatie zoals studies in kleincellige longkankercellijnen hebben aangetoond. Hoewel de androgeen-onafhankelijke cellijn PC-3 niet reageert op exogeen toegevoegd GRP, tonen incubaties met het anti-GRP MoAb 2A11 aan dat proliferatie van PC-3 afhankelijk is van GRP. Hieruit kan afgeleid worden dat GRP wordt geproduceerd door deze prostaatkankercellijn. Concluderend kan gezegd worden, dat GRP in potentie een NE groeifactor is voor prostaatkankercellijnen welke specifiek werkt via intracellulaire inductie van cAMP in zowel androgeen-onafhankelijke (DU-145, PC-3) als androgeen-afhankelijke (PC-346C) prostaatkankercellen.

Een androgeen-onafhankelijke cellijn, FGC-DCC is ontstaan door langdurige androgeen deprivatie van kweken van LNCaP cellen. LNCaP en FGC-DCC cellen vertonen een groot verschil in androgeengevoeligheid. Zoals eerder beschreven toont LNCaP de karateristieke bi-fasische groeicurve voor DHT, terwijl FGC-DCC cellen niet reageren op toenemende

DHT concentraties, hetgeen aangeeft dat FGC-DCC cellen onafhankelijk van androgenen groeien. Morfologisch lijken FGC-DCC cellen op zenuwcellen, ze produceren in tegenstelling tot de oorspronkelijke LNCaP geringe hoeveelheden prostaat specifiek antigeen (PSA) en vertonen Bcl-2 expressie na androgeen onttrekking. Noch LNCaP, noch FGC-DCC cellen laten expressie zien van de NE specifieke marker chromogranine A (CgA) of een significante toename van de basale niveaus van secretogranine III (SgIII) of neuron specifiek enolase (NSE), ondanks hun karakteristiek neuronale uiterlijk. Evenals in PC-3 cellen, is MoAb 2A11 in staat om de proliferatie van FGC-DCC cellen te remmen. Mogelijk hebben FGC-DCC cellen na androgeen deprivatie het vermogen tot autocriene groeistimulatie, door endogene productie van groeifactoren zoals GRP.

Het bestuderen van NE gedifferentieerde *in vivo* modellen als PC-295 en PC-310 heeft bijgedragen tot een verdere opheldering van mogelijke functies van NE differentiatie in klinische prostaatkanker. In hoofdstuk 2 worden de effecten beschreven van kortdurige androgeen onttrekking in het prostaatkanker xenotransplantatie model PC-295, dat voor groei volledig afhankelijk is van androgenen. Proliferatie en PSA serum niveau's dalen snel tot nul na androgeen deprivatie. Androgeen receptor (AR) expressie is sterk verlaagd en er is een aanzienlijke inductie van geprogrammeerde celdood of apoptose. Dit resulteert in een bijna complete regressie van de tumor. De inductie van NE differentiatie bleek een proliferatie onafhankelijk proces. Hergroei van PC-295 model is niet induceerbaar in aan- of afwezigheid van androgenen na langdurige periodes van androgeen deprivatie. Oftewel, in dit model zijn NE cellen niet in staat om de tumor te doen groeien in afwezigheid van androgenen.

Hoofdstukken 4 en 5 omvatten gegevens die laten zien dat hormoon deprivatie in het PC-310 model resulteert in inductie van apoptose en cel cyclus blokkade, aangetoond door verhoogde $p27^{kip1}$ expressie, een afname van proliferatie en PSA serum niveaus, en een tijdelijke verlaagde AR expressie. Evenals in PC-295 wordt in het PC310 model een proliferatie onafhankelijke inductie van NE differentiatie waargenomen vanaf 5 dagen na androgeen deprivatie. Dit werd duidelijk aan de hand van toegenoemen CgA expressie en, in verloop van de tijd, een inductie van achtereenvolgens de secretogranines, 7B2 en SgIII, de prohormoon enzymen, prohomoon convertase 1 en 2 (PC1, PC2) en het peptidyl-glycine alfa-amiderend mono oxygenase (PAM). Deze laatste factoren zijn geassocieerd met de gereguleerde secretie route (RSP). De tijdsafhankelijke inductie van deze specifieke markers in NE cellen van het PC-310 model laat zien dat die cellen over een actieve RSP beschikken en demonstreren het rijpingsproces binnen de secretoire granulas. Overigens werden in de xenotransplantaat

dragende muizen ook sterk toegenomen CgA serum niveaus waargenomen hetgeen erop duidt dat NE cellen in het PC-310 model neuropeptiden en secretogranines uit kunnen scheiden.

Vervolgens werd gekeken naar het gedrag van het PC-310 model gedurende langdurige androgeen deprivatie. Dit werd bestudeerd met betrekking tot NE cel differentiatie en rijping, de productie van groeimodulerende peptiden of hormonen, en de expressie van de AR. Er bleken additionele specifieke markers voor rijpende NE cellen te zijn. Vasculair endothelium groeifactor (VEGF) kwam in NE cellen tot expressie en werd geïnduceerd kort na de expressie van CgA en SgIII. NE groeifactoren, zoals 5-HT en GRP komen pas tot expressie na een relatief lange periode van androgeen deprivatie. De expressie van GRP en 5-HT was aanvankelijk na 14 en 21 dagen beperkt tot enkele NE cellen, maar kwam met name sterk tot expressie na langere perioden van androgeen deprivatie (154 dagen). Ook PAM kwam specifiek en relatief laat (84 en 154 dagen na androgeen deprivatie) tot expressie in NE cellen. Deze relatief late NE specifieke PAM expressie leek te duiden op een directe rol van PAM in de amidatie van neuropeptiden gedurende de rijping van NE cellen.

Hoewel *in vivo* PC-310 xenotransplantatie tumoren in regressie gaan na androgeen deprivatie, geldt dit in veel mindere mate als voor het PC-295 model. Tussen 14 en 21 dagen na androgeen deprivatie stabiliseert het tumor volume zich rond 30-40 % van het begin volume. Gedurende langere perioden van androgeen deprivatie blijft het volume van de tumorresiduen constant. In deze 'slapende' (dormant) tumoren komen twee tumorceltypes voor. Dit zijn de AR positieve cellen en de NE cellen. Aan de ene kant blijven de resterende AR positieve cellen androgeen gevoelig, zoals blijkt uit tumor hergroei na opnieuw toedienen van androgenen. Aan de andere kant kunnen deze niet-NE, AR postieve cellen de tumorcellen zijn welke ontsnappen aan langdurige androgeen deprivatie, Hieraan kunnen verschillende mechanismen ten grondslag liggen: door mutaties van de AR, veranderde specificiteit van de AR of androgeen onafhankelijke activatie van AR afhankelijke groeistimulatie.

Hoofdstuk 6 beschrijft de expressie patronen van twee NE markers, PAM en proadrenomedulline-N-terminaal peptide (PAMP) in zowel *in vivo* studies van het experimentele PC-310 model alswel in tumoren van prostaatkankerpatienten. In het PC-310 model komt het expressiepatroon na inductie van beide markers overeen met die van NE differentiatie gebaseerd op CgA expressie na androgeen deprivatie. In een groep tumoren van prostaatkankerpatienten vinden we geen sterke correlatie van PAM en PAMP expressie met hetzij tumorstadium of klinische progressie. Er lijkt een tendens te zijn voor hogere expressie van PAM en PAMP in slechter gedifferentieerde prostaattumoren.

Uit de *in vitro* studies blijkt dat androgeen onafhankelijke groei van prostaatkankercellijnen geïnduceerd kan worden door enerzijds groeimodulerende neuropeptides en anderzijds door androgeen deprivatie van een androgeen afhankelijke cellijn (LNCaP) hetgeen leidt tot de ontwikkeling van een androgeen onafhankelijk groeiende cellijn (FGC-DCC).

Inductie van NE cel differentiatie en het daarop volgende rijpingsproces in het PC-310 model door androgeen deprivatie leidt niet tot androgeen onafhankelijke groei van dit model. Uit de huidige studie is niet gebleken dat NE cel differentiatie een directe rol speelt in progressie van prostaatkanker. Echter, de tijd die verstrijkt tussen start van hormonale behandeling en klinische progressie bij prostaatkankerpatiënten varieert van 12 tot 36 maanden. Dus androgen onttrekking van het PC-310 model gedurende perioden langer dan de 6 maanden die tot op heden werden bestudeerd zou alsnog kunnen leiden tot progressie van deze tumor als gevolg van androgeen-onafhankelijke groei. In dat geval kan een mogelijke rol van NE cellen in progressie van prostaatkanker in meer detail worden bestudeerd.

List of Publications

Jongsma J, Oomen MH, Noordzij MA, Van Weerden WM, Martens GJ, van der Kwast TH, Schroder FH, van Steenbrugge GJ.
Androgen deprivation of the PC-310 human prostate cancer model system induces neuroendocrine differentiation.
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Johan Jongsma, Monique H. Oomen, Marinus A. Noordzij, Wytske M. Van Weerden, Gerard J. M. Martens, Theodorus H. van der Kwast, Fritz H. Schröder, and Gert J. van Steenbrugge.
Different profiles of neuroendocrine cell differentiation evolve in the PC-310 human prostate cancer model during long-term androgen deprivation
Submitted

Nuria Jiménez, Johan Jongsma, Alfonso Calvo, Theo van der Kwast, Anthony Treston, Frank Cuttitta, Fritz Schröder, Luis Montuenga, Gert J van Steenbrugge
Peptdylglycine alpha-amidating mono-oxygenase and proadreno-medullin N-terminal 20 peptide neuroendocrine expressioin is induced by androgen deprivation in neoplastic prostate
Submitted

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Curriculum vitae

De schrijver van dit proefschrift werd geboren op 18 juni1969 te Marum. In 1987 behaalde hij het Atheneum B aan het "Drachtster Lyceum" te Drachten. Vervolgens studeerde hij Biologie aan de Rijksuniversiteit te Groningen van 1987 tot 1993. In 1993 behaalde hij het doctoraalexamen, met als hoofdvak Biochemie. Stages werden verricht bij de afdelingen Moleculaire Microbiologie (Prof W.N. Konings, Haren) en de afdeling Klinische Immunologie (Prof L. de Ley, AZG, Groningen).

Het in dit proefschrift beschreven onderzoek werd in oktober 1995 aangevangen bij de afdeling Urologie van de Erasmus Universiteit Rotterdam op een door het KWF gefinancierd onderzoeksproject (EUR-95-1029). Sinds 1 maart 2000 is hij werkzaam op het Nederlands Kanker instituut /Antonie van Leeuwenhoek binnen de afdeling Moleculaire Genetica op het door het KWF gefinancierde project NKI 1999-2058.