Hormonal dependence of human prostate tumors transplantable in nude mice:

The importance of low androgen levels in prostate tumor growth.

Hormonale afhankelijkheid van humane prostaattumoren transplanteerbaar in de naakte muis:

Het belang van lage androgeenspiegels bij de groei van prostaattumoren.

Proefschrift

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Ter nagedachtenis van Oom Piet,

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Contents

List of abbreviations

A	androstenedione
ACI rat	August x Copenhagen inbred rat
ACTH	adrenocorticotrophic hormone
AR	androgen receptor
ARTIS	androgen receptor transcription initiation site
BPH	benign prostatic hyperplasia
BRDU	bromodeoxyuridine
C	corticosterone
CNS	central nervous system
CPA	cyproterone acetate
CRBC	chicken red blood cells
DAB	3,3'-diaminobenzidine
DNA	deoxyribonucleic acid
DHEA	dehydroepiandrosterone
DHT	5α-dihydrotestosterone
DMBA	7,12-dimethylbenzo(a)anthracene
E	estrogen
hEF	human elongation factor 1-alpha
LH	luteinizing hormone
LHRH	luteinizing hormone releasing hormone
LN	lymph node
L-W rat	Lobund x Wistar inbred rat
mRNA	messenger ribonucleic acid
MNU	N-nitroso-N-methylurea
17OH-P	17-hydroxyprogesterone
P	progesterone
PA	prostatic adenocarcinoma
PAP	prostatic acid phosphatase
PC	prostatic carcinoma
PSA	prostate specific antigen
RNA	ribonucleic acid
SD	standard deviation
SEM	standard error of the mean
SHBG	sex hormone binding globulin
T	testosterone
TP	testosterone propionate
TRPM-2	testosterone repressed prostate messenger-2
TURP	transurethral resection of the prostate

Scope of the thesis

After lung cancer, cancer of the prostate is the main cause of cancer death in males in most industrialized countries in Western Europe and North-America. The high mortality of prostate cancer is primarily due to the fact that the majority of patients have advanced (metastatic) disease at the time of diagnosis. For this stage of the disease, when the tumor is no longer confined to the prostate, no cure exists. In 1941 Huggins and colleagues [1,2] were the first to show that prostatic cancer, like the normal prostate, is dependent on androgens for its growth. Ever since this discovery, the principal aim of treatment of advanced prostate cancer consists of manipulating the hormonal environment of the tumor by some type of androgen suppressive therapy, i.e. by blockade of testicular androgens by surgical or medical castration. The effectiveness of the therapy, however, is only temporary; after an initial response the tumor escapes from endocrine regulation and becomes insensitive to anti-hormonal treatment. The progression of the tumor to hormone independency inevitably leads to the death of the patient as no effective therapy (chemotherapy) has been developed for the control of hormone-refractory prostate cancer. Although standard hormonal treatment (surgical or medical castration) has no long-term therapeutic result, it is still the basis for treatment of prostate cancer (Chapter 1).

The mechanisms which lead to the progression of prostatic tumors to autonomy are largely unknown. For a better understanding of the biology of prostate cancer and its escape from hormonal regulation, adequate in vivo and in vitro models are essential. Most information on the hormonal regulation of prostate cancer has so far been obtained from (tumor) models of rodent origin. Using these experimental models new theories about the origin of prostate cancer progression from hormone dependency to hormone independency have been developed (Chapter 2). In our laboratory we have the unique opportunity to study two human prostate tumor models, PC-82 and PC-EW, which are serially transplantable in athymic nude mice. These human tumor models are androgen dependent and in this respect they mimic clinical prostate cancer. The aim of the studies presented in this thesis was to investigate several aspects of androgen regulated growth of prostatic carcinoma.

Androgens stimulate prostate tumor growth through induction of tumor cell proliferation. The proliferative activity of tumor tissue is an important parameter of the biological potency of a tumor. Ki-67, a proliferation-associated antigen and bromodeoxyuridine (BrdU), a thymidine analogue which is incorporated into the DNA, were used as markers of the proliferative activity of hormonally manipulated PC-82 and PC-EW prostate tumors (Chapter 4). In addition to the stimulatory effect of androgens on prostate (tumor) cells, androgens also have an inhibitory effect upon the process of cell death. The PC-82 and PC-EW tumor models, although both dependent on androgens for their growth, respond differently to androgen withdrawal. This appears to be partly the result of a different castration-induced cell death pathway (Chapter 3). Androgenic effects are regulated through the androgen

receptor. The effects of castration and androgen resupplementation on the androgen receptor expression have been studied in the PC-82 tumor (Chapter 9).

It was suggested as early as 1945 that adrenal androgens, which are still present in the circulation after castration, might be involved in the progression of prostatic carcinoma. This approach was first advanced by Huggins & Scott [3] in 1941 and tested by surgical adrenalectomy in addition to orchiectomy. The complications experienced with surgical, and later with medical, adrenalectomy as well as the questionable results of this therapeutic approach largely discouraged its further application. In the early 1980s, the concept of total androgen blockade was (re)introduced by Labrie and his group [4]. New developments provided the clinic with potent analogues of LHRH (medical castration) and with anti-androgens. Ablation of androgens from all sources can now be achieved by a combination treatment.

The need for a "total androgen blockade" was disputed by others who believed that already existing androgen independent cells were more likely to be responsible for the relapse in prostate tumor growth observed in most patients. From this point of view, a combination therapy of androgen suppression and chemotherapy would be more effective than a therapy aiming at total ablation of all androgens. Many clinical trials have since been conducted to investigate the efficacy of a "total androgen blockade" treatment over standard hormonal therapy. The possible advantage of this concept is still a matter of debate.

The studies presented in this thesis provide experimental data which could contribute to the discussion on whether adrenal androgens are, directly or indirectly, capable of inducing growth stimulation of human prostate tumor tissue. Hormonal titration experiments were conducted to investigate whether there is a critical androgen level for growth stimulation of human prostate tumors and whether or not this threshold level exceeds the androgen levels found in castrated men (Chapters 5 and 7). Since the adrenals of rodents do not secrete androgens (Chapter 6), castration of the mouse can be regarded as total androgen withdrawal. The effect of adrenal androgens on human prostate tumor growth was studied in PC-82 tumor-bearing mice supplemented with adrenal androgens, androstenedione and dehydroepiandrosterone (DHEA) (Chapters 7 and 8).

In the general discussion (Chapter 10) an attempt is made to integrate experimental data and the derived ideas presented in this thesis with clinical experience on prostate cancer. Hopefully this thesis will contribute to a better understanding of the mechanisms of androgen regulated growth of human prostatic carcinoma which, together with the outcome of the necessary future experiments with human xenograft models, will result in a more effective treatment of patients with advanced prostatic cancer.

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Chapter 1

General introduction

1.1. Prostatic cancer

Benign and malignant disorders of the prostate are probably the most common neoplastic diseases in men. Approximately 40 to 50% of men over 65 years of age have benign prostatic hyperplasia (BPH): an enlargement of the prostate gland, which frequently results in obstruction of the urethra [1]. In contrast to BPH, which is not a life-threatening disease, carcinoma of the prostate is the second main cause of cancer death in males in the Western world. From autopsy studies it is estimated that 15 to 30% of males over 50 years of age have histological evidence of prostatic adenocarcinoma, the incidence increasing dramatically with age [2].

Latent (microscopic) prostate cancer is evenly distributed throughout the world, while the incidence of clinical prostate cancer varies widely. Incidence rates (annual new cases per 100,000) for prostate cancer range from two to five in China and Japan to approximately eighty in the US, with intermediate rates in the European countries [3]. These data show that less than 1% of the latent prostatic lesions will actually develop into a clinical cancer. Migration of (low risk) Japanese or Chinese men to the USA is also associated with a considerable increase in the incidence of prostate cancer [4,5]. These data strongly suggest that environmental factors, such as diet, are involved in promoting latent prostate cancer to clinical cancer. To date, epidemiological studies could not detect factors which are associated with an increase in prostate cancer risk [6].

1.2. The prostate gland

The prostate is the largest accessory sex gland in men and is located at the base of the bladder, surrounding the urethra. The prostate consists of alveoli lined with columnar epithelial secretory cells and is embedded in a fibromuscular stroma [7]. The stroma plays an essential role in the normal development and differentiation of the gland. It is assumed that a multitude of properties of the adult gland is mediated through stromal-epithelial interactions [8]. The epithelial secretory cells of the gland secrete a fluid which maintains the viability of the semen. The three main secretory proteins of the epithelial cells are prostatic acid phosphatase (PAP), prostate specific antigen (PSA) and beta-microseminoprotein (beta-inhibin) [9]. These proteins have clinical significance as markers for diagnosis and as monitors androgen dependent prostate cancer [10].

BPH and prostate cancer are independent entities that originate in different regions of the prostate. BPH develops from the prostatic periurethral (transition) zone, whereas clinical prostate cancer originates almost exclusively in the peripheral

zone of the prostate [11]. The differentiation grade and the growth pattern (glandular, cribriform or anaplastic) of prostatic cancers are important characteristics as progressive loss of differentiation is associated with increased malignant potency [12].

1.3. The endocrinology of prostate (cancer) growth

1.3.1. Androgen dependency of the (malignant) prostate

The development, growth, morphology, and functional activity of the prostate gland depend primarily on androgenic stimuli as atrophy of the prostate is induced after castration. The almost complete castration-induced involution of the prostate demonstrates that remaining androgens of adrenal origin (see below) are insufficient to compensate for the loss of testicular androgens [13,14]. The observation that men with hypogonadotropic hypogonadism or castrated before puberty (no testicular function but normal adrenal function) and men with panhypopituitarism (neither testicular nor adrenal function) have atrophic prostate glands further demonstrates the lack of effect of the adrenal androgens [15].

The androgen dependency of prostatic cancer was discovered in 1941 by Huggins and Hodges, who observed that suppression of circulating testosterone (T) induced a decline in the (secretory) activity of the prostate cancer [16]. At the time of diagnosis 60 to 80% of prostate adenocarcinomas respond to anti-androgen treatment, indicating that these tumors have retained part of their normal androgenic regulation. However, essentially all tumors eventually escape from this control and progress to an autonomous state in which androgens are no longer required for growth stimulation [17].

1.3.2. Mechanisms of androgen action

Testosterone is the most important circulating androgen in the male and more than 95% of this steroid is produced by the testes. The remaining 5% of T comes from peripheral conversion of the androgens androstenedione and dehydroepiandrosterone (DHEA), secreted by the adrenal glands. 98% of plasma T is bound to albumin and sex hormone binding globulin (SHBG) and is not physiologically active [18]. Free T may directly diffuse into target cells. After T has entered a prostatic cell the hormone migrates to the nucleus where it is reduced by the enzyme 5α-reductase, which is an integral component of the nuclear envelope, into the active compound 5α-dihydrotestosterone (DHT) [19]. This conversion may be considered as an amplification of androgen action, because the binding affinity of DHT to the nuclear androgen receptor (AR) is twice that of T, and the rate of dissociation of T from the receptor is five times faster. Therefore, the biological

potency of DHT is greater than that of T [20]. DHT interacts with the AR to form a receptor-steroid complex, which binds to specific high-affinity acceptor sites on the chromatin (DNA). The steroid-receptor interaction leads to gene transcription, followed by translation of the specific messenger RNAs on the polyribosomes, finally resulting in the synthesis of new gene products [21].

The AR, whether or not occupied by steroid, is localized exclusively in the nucleus of intact (prostate) cells [21]. With the elucidation of the primary structure of the human AR [22], antibodies against specific domains of the AR were developed [23]. Immunohistochemical localization of the AR revealed that in the adult human prostate luminal epithelial cells were strongly positive for AR, whereas basal cells and the majority of stromal fibroblasts were AR negative [24,25]. A considerable heterogeneity in AR expression was observed in multiple samples taken from single prostatic carcinoma specimens [26].

1.3.3. Androgens in prostate (tumor) regulation

Evidence that DHT is the key androgen in the prostate with T acting as a pro-hormone, stems from several studies demonstrating that incubation of prostate cells with T resulted mainly in the accumulation of DHT within prostatic nuclei. A study of male pseudohermaphrodites with 5α-reductase deficiency showed that DHT treatment could induce growth of the vestigial prostates of these men. Experiments with 5α-reductase inhibitors finally resulted in the DHT-hypothesis suggested by Petrow [27]. The intraprostatic DHT content has since been suggested as an indicator of androgen dependency of prostate cancer and, consequently, of treatment response. Interestingly, Geller et al. [28] and Belis et al. [29] reported that high prostatic DHT levels seemed to be associated with a good treatment response. Indeed, Chodak et al. [30] and Haapiainen et al. [31] showed that high pretreatment plasma levels of T corresponded with a favorable treatment response. No such associations could, however, be demonstrated by others [32,33].

Androgens have a dual effect on the prostate. Besides having an agonistic ability to stimulate prostatic cell proliferation, androgens have an antagonistic ability to inhibit prostatic cell death [34]. It was postulated by Isaacs [35] that these two androgenic effects are distinct processes in the prostate. The rapid involution of the prostate after castration was suggested to be the result of both a decline in antagonistic and a decreased agonistic effect of androgen on the prostate.

1.4. Treatment modalities for prostate cancer

1.4.1. Clinical course of prostate cancer

The high mortality from prostatic cancer is primarily due to the fact that 40 to

50% of the patients have metastatic disease at the time of diagnosis [36]. At this stage of the disease androgen ablation might delay the cancer process by interfering with differentiation and growth rate of the tumor, but it does not cure the disease. Approximately 70% of patients respond to first line anti-androgen therapy, but ultimately all patients will become refractory to further endocrine treatment [37]. The current selection of hormonal treatment regimens can be divided into those that are designed to suppress testicular androgens and those that attempt to block androgen action at the target site [38,39]. Figure 1 gives a schematic representation of the possible targets for treatment modalities in advanced prostate cancer.

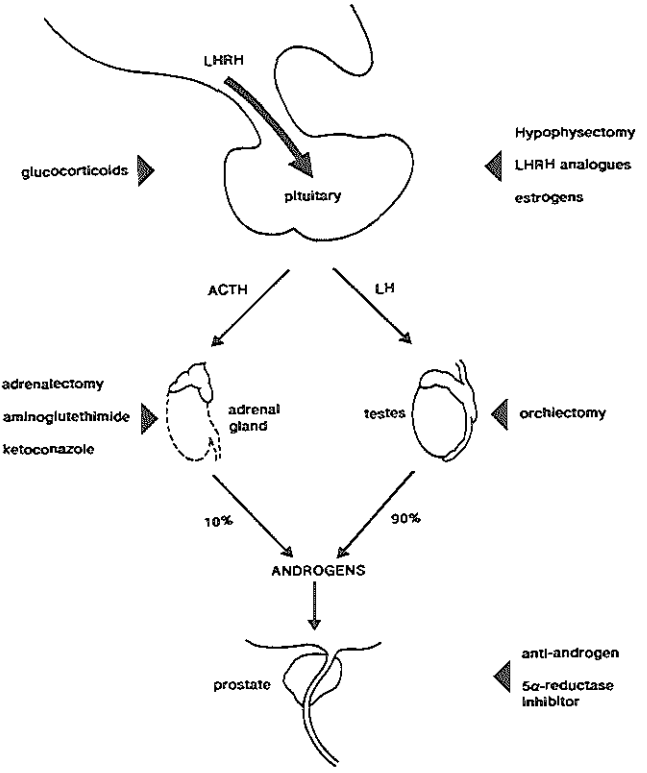

Figure 1. Schematic representation of the treatment modalities in metastatic prostate cancer as indicated by arrow heads.

1.4.2. Androgen deprivation treatment modalities

Androgen deprivation by castration or estrogen treatment was the first known therapy against prostate cancer [16]. To date surgical castration is still considered as the standard hormonal therapy, which results in the optimal suppression of T and a favorable treatment response with minimal side-effects [38]. High dose estrogen therapy (diethyl-stilbestrol) is based upon the indirect suppression of T through inhibition of the hypothalamus-pituitary-gonadal axis, resulting in decreased synthesis of testicular T. Although clinical studies confirmed the efficacy of estrogen treatment, later studies revealed significant cardiovascular side-effects [40,41]. For this reason, estrogens are no longer considered as first line therapy in advanced prostate cancer.

As with estrogens, inhibition of the hypothalamus-pituitary-gonadal axis can also be achieved by the recently developed luteinizing hormone-releasing hormone (LHRH) agonists and antagonists [42]. Treatment with LHRH analogues initially causes a rise in plasma T, but with continuous administration of the agonist castration levels of T are achieved [43]. The transient rise in plasma T can be effectively blocked by anti-androgens [44]. LHRH therapy is nowadays considered as standard hormonal therapy.

1.4.3. Androgen blocking treatment modalities

Antiandrogens block the androgen receptors in the presence of normal or even increased levels of DHT. Cyproterone acetate (CPA) is a steroidal antiandrogen, which not only blocks the androgen receptor but also has some progestational antigonadotropic activity [45]. Monotherapy of prostate cancer patients with CPA has shown to be as effective as castration, although some cardiovascular toxicity has been reported [46]. The non-steroidal or "pure" antiandrogens (flutamide, nilutamide, casodex) displace the androgen from the receptor thereby preventing androgen-induced activation. However, this also leads to a compensatory rise in LH, which in turn results in increased levels of T [47]. This unintended rise of T, in addition to the reported side-effects, probably limits the use of these compounds as monotherapy [48]. Most of these pure antiandrogens are used in combination with castration or LHRH agonists (see below) [49].

1.4.4. Total androgen blockade

After 50 years of endocrine treatment only a limited impact on the overall survival of advanced prostate cancer patients is observed [50]. Although the treatment seems to delay tumor progression, it was ultimately shown to be only palliative. The continued presence of the adrenal androgens has been proposed as the cause of this incomplete response [51,52]. For this reason a "total androgen blockade" is proposed aiming at suppressing the effects of both testicular and adrenal

androgens by using a combination of surgical or medical castration and a pure antiandrogen [53]. Many clinical trials have been conducted to establish the advantage of this treatment over standard hormonal therapy. Unfortunately, comparison of the results obtained by these studies is difficult as patient characteristics, response criteria and endpoints differ considerably [54].

Recently, Crawford et al. [55] reported a longer progression-free survival and an increase in the overall survival of patients treated with an LHRH agonist (leuprolide) plus flutamide over those receiving leuprolide alone. However, other reports suggest that there is no advantage of the combined treatment over standard hormonal therapy when judged by the small differences between results in the various treatment groups in these studies [56].

1.4.5. 5α-reductase inhibitors

Since the establishment of DHT as the active androgen in prostatic cancer, drugs have been developed which inhibit the enzyme responsible for the conversion of T to DHT [57]. In preliminary studies, one of these compounds, finasteride (MK-906), has been shown to specifically block DHT production while maintaining libido and potency [58]. Further experimental and clinical studies will be necessary to establish the role of this promising compound in the treatment of prostate cancer.

1.4.6. Hormono-chemotherapy

Endocrine treatment is a good palliative first line treatment for advanced prostate cancer patients. Chemotherapy is, therefore, mainly used for patients who have relapsed from endocrine treatment. Unfortunately, only a limited number of hormone-refractory patients exhibited a favorable response to a variety of different chemotherapeutic agents. This is probably due to the slow doubling time of the tumor and the large tumor burden, together with the poor condition of the relatively older prostate cancer patient [59]. A combined therapy of anti-androgenic treatment with chemotherapeutics has been proposed as a more effective means of attacking all prostate tumor cells [60].

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Animal models in the study of progression of prostate cancer and breast cancer to endocrine independency

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2.1. Introduction

The majority of patients (60 to 80%) with cancer of the prostate or breast initially respond to hormonal ablation therapy. However, nearly all these patients eventually progress to a state that is unresponsive to further anti-hormone therapy [1,2]. The factors involved in the progression of a hormone-dependent cancer to hormone independency and the underlying mechanisms are poorly understood. Appropriate in vitro and in vivo models are essential to investigate biological aspects of human cancer. Establishing human prostate tumor cell lines in culture and in vivo is difficult and has only a low rate of success [3]. For this reason, the number of available models is limited. This review will focus upon those animal studies which have been important for our understanding of hormonal regulation and the mechanisms responsible for the progression of breast cancer and of prostatic carcinoma in particular.

2.2. Tumor models in prostate cancer research

2.2.1. In vitro prostate tumor models

To date eight in vitro models of human prostatic cancer have been described (Table 1). All the models, except the LNCaP (Lymph Node Carcinoma of the Prostate) cell line, are androgen independent with little or no secretion of the prostate specific enzyme prostatic acid phosphatase (PAP) or the prostate specific antigen (PSA). The LNCaP cell line is the only cell line showing androgen responsive growth. In contrast to all other in vitro cell lines listed in Table 1, the LNCaP was shown by many investigators to be poorly tumorigenic in athymic nude mice. Recently, Gleave et al. [12] showed that growth of LNCaP cells in vivo could be induced by prostate and bone fibroblasts. The derived tumor tissue appeared, however, not to be permanently transplantable. In our laboratory we, recently, succeeded in permanently growing the LNCaP cell line in (high dose) estrogen substituted nude mice [13]. Whether a high dose of androgen will also result in the development of transplantable LNCaP tumors is currently under investigation.

2.2.2. In vivo prostate tumor models of animal origin

The available in vivo prostate tumor models can be classified by their origin: spontaneously developed, hormonally or chemically induced, transplantable rodent carcinomas, and heterotransplantable human carcinomas [14]. Recently, Isaacs and Coffey published two extensive reviews concerning experimental in vivo model systems used in prostatic cancer research [14,15]. Table 2 summarizes the various in

vivo models of non-human origin used in prostate cancer research, some of which are discussed in more detail below.

Table 1. In vitro prostatic cell lines.

Tumor line		Origin	Androgen-dependence	Ref.
EB-33	1973	primary tumor	-	4
Du-145	1975	metast. CNS	-	5
PC-3	1976	metast. bone	-	6
LNCaP	1977	metast. LN	+	7
TSU-PR1	1980	metast. LN	-	8
JCA-1	1987	primary tumor	-	9
PPC-1	1989	primary tumor	-	10
DuPro-1	1991	xenograft LN	-	11

CNS = central nervous system, LN= lymph node.

Spontaneous prostate tumors. Cancer of the prostate is a rare disease in animals. Yet, transplantable tumor models have been established from some incidentally occurring prostatic adenocarcinomas. In 1975 Shain et al. [18] reported a high percentage (30 to 40%) of spontaneously developed carcinomas in the ventral lobe of the prostate in aged ACI/Seg rats. Transplanting these tumors into androgen-treated ACI rats resulted in the serially transplantable tumor lines designated as LSC-AXC [19]. From these tumors continuous in vitro cultures were established. This tumor system now constitutes a unique model to study the earliest events in the development of latent to clinically manifest prostatic cancer.

Similar, although less frequent (10%), spontaneous prostatic carcinomas were observed by Pollard et. al. [20] in aged Lobund Wistar rats. Transplanting four of these carcinomas into Lobund Wistar rats resulted in the serially transplantable tumor lines PA I, II, III, and IV [21]. These sublines were also established as in vitro tumor lines. The Pollard sublines have a high ability to metastasize via the lymphatics and/or blood, which makes them useful for the study of mechanisms of metastasis in prostate cancer.

Inducable prostatic cancers in the rat. In 1977 Noble et al. [22] observed the incidental development of adenocarcinomas of the dorsal lobe of the prostate in aging Noble rats. The incidence could be increased from 0.45% to 18% by

implanting pellets with testosterone or testosterone plus estrone in young Noble rats. The transplantable hormonally induced tumors could be classified as being androgen responsive, androgen independent, or estrogen responsive [23]. The presence of both hormone dependent and independent prostate tumors and the occurrence of metastases makes the Noble prostate tumor system a suitable model for investigating the progression of prostate cancer to autonomy.

Table 2. The most commonly used animal models for the study of prostatic cancer.

Category	Name	Origin	Androgen-dependence	Ref.
PROSTATE				
spontaneous		dog	+	16
		L-W rat	+	20
		ACI rat	+	18
inducable				
E + TP		Noble rat	+	22
MNU + TP		L-W rat	+	24
transplantable	CPA-1	dog	+/-	17
	Dunning	Copenhagen rat	+/-	26
	Noble	Noble rat	+/-	23
	Shain	ACI rat	+/-	18
	Pollard	L-W rat	-	21
BREAST				
transplantable	Shionogi	mouse	+/-	31

+/- = both androgen dependent and independent sublines exist.
ACI = inbred of August x Copenhagen rats
L-W = inbred of Lobund x Wistar rats
MNU = N-nitroso-N-methylurea
E = estrone
TP = testosterone propionate

Prostatic adenocarcinomas could be induced in Lobund Wistar rats by long-term treatment with high levels of exogenous testosterone. The incidence of grossly manifest prostate cancer could be largely increased from 16% to more than 50% by treating rats with testosterone implants plus N-nitroso-N-methylurea (MNU). This model allows the very early steps of prostate carcinogenesis to be studied [24,25].

Transplantable rat prostate tumor models. Much of the knowledge of androgen regulated growth and progression of prostatic cancer comes from investigations with the Dunning rat prostate carcinoma system R3327. This model system was established in 1961 by Dunning from a spontaneously developed tumor of the dorsal lobe of the prostate of an aged Copenhagen rat [26]. Following serial in vivo passage of the original R3327 tumor, eight different sublines were obtained, including a relatively fast growing, poorly differentiated, but still androgen sensitive subline (G-subline). The original R3327 has been propagated at several laboratories resulting in various sublines with different biological characteristics. The R3327 passaged at the John Hopkins Hospital, designated as the H-tumor (H for Hopkins) is one of the best characterized sublines. The H-tumor is a well differentiated, androgen dependent tumor with a relatively slow growth rate [27]. Androgen deprivation of tumor-bearing rats induces tumor regression, but in time the H-tumor progresses to autonomy no longer requiring androgens for its growth [28]. This makes the H-tumor an interesting animal model to study the relapse phenomenon frequently seen in clinical prostate cancer.

Due to the genetic instability of the H-tumor continuous passage occasionally resulted in tumor progression, giving rise to a number of androgen independent sublines: the metastatic MatLu and MatLyLu and the anaplastic AT-1, AT-2 and AT-3 [15]. Establishing these sublines in vitro greatly increased the usefulness of the Dunning system [29]. Recently, Aumüller et al. [30] presented some provocative immunohistochemical data, suggesting that the Dunning R3327-H tumor is not prostate derived. However, these findings, if correct, do not alter the relevance of the model to study growth regulation of androgen-sensitive tumors.

The Shionogi mouse mammary tumor model SC-115 was established by Minesita & Yamaguchi in 1964 [31] from a spontaneous tumor in a female mouse of the DS strain. After passage of this hormone independent tumor in male DS mice, an androgen dependent subline of the tumor arose. Although the Shionogi tumor is of non-prostatic origin, the model system is used in prostate cancer research because of its androgen dependent growth behavior. A number of androgen dependent Shionogi sublines as well as estrogen dependent and estrogen independent sublines, have been developed [32]. Extrapolating the results to human prostate cancer is, however, strongly restricted by the mouse mammary origin of this model.

2.2.3. Heterotransplantable human prostate tumor models

The discovery of an athymic mutant mouse, with a significantly impaired cell-mediated immune response, was a breakthrough in experimental oncology, as athymic nude mice, first introduced by Rygaard and Povlsen, proved to be excellent hosts for human tumor tissue [33]. Although the nude mouse differs from man in a variety of immunological, pharmacological, and endocrine factors, the major

advantage of heterotransplantable models over the animal models reviewed above is the possibility to study human tissue [34]. The acceptance of hetero-transplanted human neoplastic tissue in athymic nude mice was shown to vary considerably for tumors of different origin. Certain types of human cancers (melanoma, colon) appear to grow readily in nude mice, whereas others such as prostate and mammary carcinomas, are more difficult to establish. Hormonal effects, nutritional deficiencies and immunological defence mechanisms might be responsible for the differences in success rate for the various malignancies [35].

In our laboratory, since 1977 over 200 specimens from primary prostatic carcinomas and a limited number of metastatic lesions (mostly lymph node) have been grafted into Balb/c athymic nude mice. Transplantation of tumor tissue was routinely carried out by implanting small tumor pieces into the subcutaneous space of both shoulders of intact males or T or DHT supplemented male mice (Figure 1). To date only three permanently growing cell lines have been established: PC-82, PC-133 and PC-135. A relatively high take rate of prostate carcinoma specimens was achieved when NMRI nude mice supplemented with DHT [36].

Recently, it was shown that growth of inoculated human prostate tumor cells can be significantly improved by mixing the cells with a reconstituted basement membrane gel (Matrigel) [37,38]. Using this technique six out of ten primary human prostatic carcinomas could be transplanted successfully [39]. Conversely, the DuPro-1 in vitro cell line could be established in vitro from a primary prostate xenotransplant using Matrigel-coated culture disks [11].

Table 3. Human prostate tumor models growing in nude mice.

Name		Androgen-dependence	AR	PAP/PSA	Ref.
PC-133	1981	-	-	-	49
PC-135	1982	-	-	-	49
PC-82	1977	+	+	+	40
PC-EW	1981	+	+	+	43
Honda	1977	+	+	+	46
TEN/12	1985	+	+	+	47
PC-EG	1988	+	+	+	48

AR = Androgen receptor.

In 1977 a primary prostatic carcinoma was transplanted subcutaneously in athymic male Balb/c nude mice by Hoehn in our laboratory. The tumor line derived from this transplant was denoted as PC-82 and was the first permanent in vivo human prostatic tumor line to be described [40]. The original tumor was graded as being a moderately differentiated adenocarcinoma of the cribriform type, as were all subsequent transplant generations of the PC-82 tumor line. The androgen dependency of the PC-82 tumor has been studied extensively [41,42]. In 1981 Hoehn and co-workers established another transplantable hormone dependent human prostate tumor line (PC-EW) in nude mice from a lymph node of a patient with prostate cancer [43]. The PC-EW tumor of the 16th transplant generation was made available to the Urological Institute in Rotterdam in 1984. The PC-EW tumor line consists of moderately and poorly differentiated tumor tissue with both cribriform and small glandular arrangements.

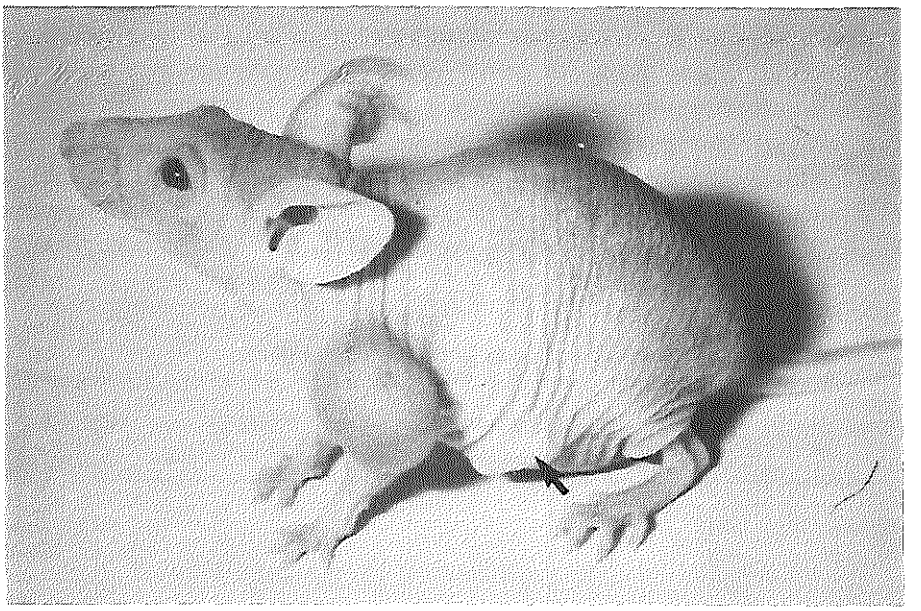

Figure 1. Athymic nude mouse bearing the human prostate tumor PC-82 and supplemented with a T-containing Silastic implant (arrow).

The PC-82 and PC-EW tumors share many of the properties known from clinical prostate cancer, such as the presence of androgen receptors and secretion of PAP and PSA (Table 3) [44,45]. Both models are extremely useful in the study of androgen dependent prostate cancer. However, the apparent inability of either of the

tumor models to progress to an androgen-insensitive state, as observed in the Dunning rat prostatic model, limits the usefulness of the models. A more detailed characterization of both models in respect to their androgen dependency is given in Chapter 3.

Other hormone dependent human prostatic xenografts in nude mice are the Honda tumor derived from a metastatic (testicular) lesion of a prostate cancer, described by Ito et al. [46]; the TEN/12 tumor, established by Harper et al. [47] from a transurethral resection of a primary prostatic carcinoma (TURP), and the PC-EG tumor also established from TURP material and described by Csapo et al. [48]. The presence of androgen receptors and the secretion of PAP and PSA by these three androgen dependent tumors were comparable to those of the PC-82 and PC-EW tumor models (Table 3).

After establishing the PC-82 model, continuous efforts to establish more prostate xenograft models in our institute resulted in two androgen independent cell lines (PC-133 and PC-135) with an undifferentiated (anaplastic) histological pattern [49]. Neither in the PC-133 nor in the PC-135 tumor could any of the prostate-specific markers or androgen receptors be detected. Clearly, these two models represent the hormone-unresponsive, undifferentiated counterpart of prostatic carcinoma. Continuously growing in vitro cell lines could not be established from any of the seven tumor lines that grow permanently in nude mice. As an exception, the TEN/12 tumor was reported to survive when cultured in semi-solid agar, thus also providing an experimental in vitro model of prostate cancer [47].

2.3. Animal models for the study of breast cancer

Mammary tumors in rats and mice, spontaneous or induced, have been widely used as models for human breast cancer [50]. Huggins et al. [51] discovered that carcinogens, such as dimethyl-benzo(a)anthracene (DMBA), could induce breast tumors in female rats. These breast tumor models were found to be estrogen dependent initially, but to progress rapidly to a hormone independent phenotype. The extensive literature on carcinogen-induced mammary carcinomas has been reviewed by Welsch [52].

Hormone dependent mammary tumors could be induced in Noble rats by high doses of estrogen [53]. These tumors remained dependent on estrogen when serially transplanted into estrogen-treated animals. In contrast to DMBA-induced mammary tumors, only very few of the estrogen-induced tumors showed a progressive change to estrogen independency. This estrogen-induced mammary tumor system is a suitable model for studies on tumor progression. In addition to these rodent breast

tumor models, several human breast tumors have been established by heterotransplantation in athymic nude mice [54].

2.4. Progression to hormonal independency

2.4.1. Theories of tumor progression

Hormonal therapy rarely cures breast or prostate cancer patients because in the course of treatment, the tumors progress to autonomy being unresponsive to further hormonal therapies [2,55]. The origin of this relapse phenomenon has been investigated intensively by many research groups. Basically, three hypotheses have been proposed to explain the inefficacy of endocrine therapies to definitely stop prostate cancer growth. The first hypothesis is based upon the theory that the cellular composition of these carcinomas is heterogeneous with both hormone dependent and hormone-independent clones of cells present in various proportions [56]. A second hypothesis assumes the presence of both hormone-sensitive and hormone-insensitive stem cells with the capacity of self-renewal [57]. Finally, a third hypothesis suggests the development of hormone-sensitive tumor cells, which are thought to have adapted themselves to grow at a bare minimum of stimulating hormone [58]. The failure of hormonal ablation treatment to improve therapeutic results is explained by the first two hypotheses as a relapse in tumor growth caused by the selective outgrowth of unaffected hormone independent (stem) cells. The latter hypothesis considers inadequate suppression of stimulating hormones as the cause of the tumor relapse.

The behavior of tumors, with respect to their hormonal environment, can be defined as hormone (un)responsive, hormone (in)dependent, or hormone (in)sensitive. The responsiveness of a tumor to hormones is thought to be the result of its cellular composition. Tumors may be homogeneously or heterogeneously composed of hormone (in) dependent and/or hormone (in) sensitive tumor cells. Hormone dependent cells have an absolute need for the stimulating hormone, whereas hormone sensitive tumor cells grow faster with the hormone but do not require it. Tumors consisting of these types of tumor cells are hormone responsive as they react to the presence of hormones. Independent insensitive tumor cells are unresponsive to hormonal manipulation and tumors composed of these cells can be indicated as resistant or autonomous [59].

2.4.2. Is tumor progression coupled to tumor regression?

The stepwise irreversible change towards autonomy is generally assumed to take place in the absence of a stimulating hormone [60,61]. A putative link between progression and endocrine ablation was first made by Foulds [60] who observed that

changes in hormonal status seemed to trigger progression of the carcinomas. The idea that regression seemed to be an accelerator rather than a prerequisite for autonomous change was further substantiated by Noble. He suggested that hormone levels intermediate to those found in castrated and normal rats could control progression [61,62]. Furthermore, regression itself could well be a selection mechanism in favor of pre-existing but dormant independent cells capable of surviving hormone withdrawal. Therapies based upon suppressing the stimulating hormone would, thus, favor progression. In this respect, the presence of small amounts of the hormone could delay or even prevent tumor progression [62,63].

Although hormone depletion kills the major part of the hormone dependent tumor, a few cells may remain dormant indefinitely. Estrogen-induced, hormone dependent Noble tumors remained dormant, but viable in the absence of estrogen and only start to grow after estrogen restimulation [61]. These cancers only progress very slowly, if at all, to hormone independency. The androgen dependent human prostate cancer PC-82 remains dormant when transplanted into female or castrated male nude mice and can be stimulated to grow by androgens, even after long-term depletion. However, the tumor never progressed to autonomy [64] (Chapter 3). The dormant state of these cancers may resemble the situation of androgen ablated prostate cancer in men. The selective outgrowth of independent cells may be a consequence of the heterogeneous nature of the tumors.

2.4.3. Heterogeneity of normal and malignant breast and prostate tissue

Tumor heterogeneity can be described as the presence of different cell types within one tumor. It should be noted that cellular heterogeneity is not a property unique to tumors, but one they share with normal tissues [65]. Previously, investigators have reported evidence of multiple distinct tumor subpopulations within single cancers [66,67]. Such populations could be distinguished from each other by a variety of parameters such as antigens, DNA ploidy and chromosomal karyotype, steroid receptor content, metastatic ability, and hormonal and drug sensitivity [68].

2.4.4. The stem cell theory

The development of resistance to hormonal regulation in advanced prostate and breast carcinomas contrasts with their normal counterparts. Growth and regression of the mammary gland during and after lactation, are controlled by estrogens and do not lead to autonomy [69]. The normal rat ventral prostate rapidly involutes after androgen withdrawal and can be restimulated to grow by androgens. The restimulated growth is constrained as proliferative activity of the cells returns to normal when the gland has reached its normal size. Repeated cycles of castration and restimulation do not drive the prostate to androgen resistance [70].

The indefatigable regrowth of the prostate and breast to their original size,

without loss of growth control, has been explained by the existence of stem cells [71]. The principal function of these cells lies in their capacity of self-renewal. After androgen withdrawal, the prostate regresses but the stem cell population remains and, after androgen stimulation, will start to proliferate again [57,71]. Bruchovsky et al. [72] studied the stem cell composition of the androgen dependent Shionogi tumor. They observed that the recurrent androgen independent Shionogi tumors contain an increased number of androgen independent stem cells compared with the parental tumor. Since the parental line was shown to have only a very small population of androgen independent stem cells, the authors suggested that these stem cells survived androgen ablation by adapting to the altered hormonal environment.

2.4.5. Environmental adaptation

A hormonally depleted environment may force androgen sensitive cells to adapt to very low hormone levels. Indeed, clones of the Shionogi SC-115 tumor exposed to various concentrations of androgen showed a wide range in growth response [73]. In the presence of very low (castrate) levels of androgen, so-called hypersensitive clones of cells developed from an androgen sensitive Shionogi subline. In vitro incubation of androgen sensitive Shionogi cells in the absence of androgens ultimately resulted in a total loss of androgen responsiveness [74,75].

In vivo it was shown that autonomous tumors developed from Shionogi SC-115 xenografts during one passage in androgen-depleted hosts [76], although these changes were found to be partly reversible [74]. Androgen responsiveness of the Shionogi clones could be maintained by DHT but, suprisingly, also by the antiandrogen flutamide in the absence of androgen [75]. The rapid loss of androgen sensitivity of Shionogi tumors could be delayed or even prevented when cells were treated with estrogens [77,78] or glucocorticoids [79]. The development of hypersensitive cell clones is thought to be the result of the original androgen dependent tumor cells adapting to the hormone depleted environment.

2.4.6. Genetic instability coupled with clonal selection

The development of cancer is assumed to be the result of a series of events accumulating within a cell. With each step, which may involve activation of oncogenes and/or inactivation of suppressor genes, the cell becomes more susceptible to a subsequent event. The stepwise disregulation of the cell ultimately results in a total loss of cellular control [80]. Genetic instability is an intrinsic characteristic inherent to tumor cells and has been demonstrated in animal and human studies. Moreover, multifocal cancer areas of multiple histological types are commonly found within the breast or prostate [68].

The androgen responsive Dunning R3327-H tumor shows spontaneous regrowth after an initial castration-induced regression, resulting in an androgen independent

HI-tumor [31]. Comparing the growth characteristics of parental H-tumor fragments with those of cell suspensions of the H-tumor revealed that in castrated rats growth of tumor fragments differed widely, whereas cell suspensions showed no variation in tumor growth. In normal male rats no such growth difference could be detected [31]. It was concluded from these experiments that the H-tumor is (initially) composed of a mixture of pre-existing clones of androgen dependent (80%) and androgen independent (20%) tumor cells present in distinct foci in the parental tumor. So, clonal selection rather than adaptation seems to be the mechanism which induces the relapse in growth of the Dunning R3327-H tumor.

Progression to endocrine autonomy has been shown to involve genetic instability coupled with clonal selection. Despite the fact that occasionally distinct sublines spontaneously evolved, it has been possible to maintain the original androgen-sensitive Dunning H-tumor. Once progression occurred within the Dunning R3327-H tumor, accompanying chromosomal changes were detected [81]. Genetic instability does not seem to be a constant characteristic of the H-tumor, as the phenomenon has not been demonstrated during all subsequent passages. Genetic instability could, therefore, well be a dormant characteristic which is expressed only under certain, still unknown, conditions.

One consequence of genetic instability is the origin of a heterogeneous tumor with distinct clones of cells. Outgrowth of one clone of cells due to its selective growth advantage, is an inevitable result of the heterogeneous nature of a tumor. The result of clonal selection is a less heterogeneous tumor, which is more adapted to the tumor environment. Such clones of tumor cells may remain stable indefinitely until there is an exogenous selective pressure or further genetic changes occur [82].

2.5. Hormone independent growth

Hormone independent tumor cells are capable of surviving and dividing in the absence of the stimulating hormone. Resistance to cell death as well as stimulation of cell division by growth stimulating factors are required for these cells to grow. Since androgens act both as agonistic stimulators of cell division and as antagonistic inhibitors of cell death, castration induces suppression of cell proliferation as well as an increase in the cell death rate [83]. The process of programmed cell death (apoptosis) is induced in hormone ablated (tumor) tissue [84]. This process is associated with a cascade of molecular events. One of the genes associated with apoptosis is the testosterone repressed prostate message-2 (TRPM-2) [85]. In the regressing rat ventral prostate, and in androgen deprived human prostatic PC-82 tumor tissue, increased expression of TRPM-2 was detectable [86,87].

In the androgen independent Dunning AT-3 subline programmed cell death was

induced by a rise in intracellular calcium, in the absence of TRPM-2 expression [88]. Likewise, Montpetit & Tenniswood [89] reported the absence of TRPM-2 expression in an androgen independent epithelial cell line derived from the rat ventral prostate. They postulated that the observed absence of apoptosis in these cells as indicated by the absence of TRPM-2 expression, renders the cells resistant to castration-induced cell death. However, in recurrent androgen independent Shionogi tumors, TRPM-2 was constitutively expressed at levels exceeding those found in regressing Shionogi tumors [90]. From this observation it must be concluded that the TRPM-2 gene does not seem to be a mediator of cell death, but it rather seems to protect against cell death. Apparently, both androgen dependent and androgen independent cells in the prostate share a common pathway of cellular death. A defect in this pathway in independent cells, however, seems to be responsible for the fact that cell death is no longer activated by androgen ablation in these cells [91].

Hormone dependent growth is suggested to be mediated via (hormone induced) growth factors. Independent tumor cells may overcome the lack of hormone dependent growth stimulation by constitutively producing and responding to autocrine and paracrine growth factors in the absence of hormone [92]. Both androgen dependent and independent human prostate tumor cells have been shown to produce and respond to a variety of growth factors [93,94]. Recently, Smith et al. [95] reported that androgen dependent tumor cells of a hamster leiomyosarcoma were able to use alternative pathways for growth stimulation in an androgen ablated environment. The cellular signal transduction pathway in this system could be induced by many growth factors along with testosterone. This observation suggests that androgen dependent cells are capable of adapting to a hormone deprived environment by changing the sensitivity of cellular processes for other (non-androgenic) factors.

2.6. Concluding remarks

The main problem in establishing effective treatment for breast and prostate cancer is the heterogeneity in hormonal responsiveness of the tumor cells. It may be obvious from this review that malignant breast and prostate tissues are composed of different populations of epithelial cells. The relapse in tumor growth, frequently seen in hormonally treated patients, is assumed to be the result of clonal selection of hormone independent clones of tumor cells. Whether hormone independent cells develop by genetic instability inherent to a cancer or result from adaptation to very low levels of androgens caused by the incomplete anti-hormonal treatment is still a matter of discussion. This paper summarizes the experimental evidence and arguments for both theories.

The consequences for the treatment of metastatic prostate cancer differ considerably; if independent tumor cells exist at diagnosis, then standard hormonal treatment (castration) should be combined with chemotherapy to prevent selective outgrowth of independent cells. If adaptation of cells occurs due to an incomplete hormonal suppression, then a "total androgen blockade" should be considered to prevent the development of independent cell clones. Although many experimental and clinical studies have been conducted to elucidate the mechanisms of tumor progression, the phenomenon is still not understood.

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Castration-induced changes in morphology, androgen levels and proliferative activity of human prostate cancer tissue grown in athymic nude mice

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Submitted

Abstract

The transplantable human prostate tumor lines PC-82 and PC-EW regress after androgen depletion. The castration-induced decline in tumor volume was faster in the PC-EW tumor (half-life 6 days) than in the PC-82 tumor (half-life 18 days), despite similar castrate androgen levels of less than 4 pmol/g tissue. The proliferative activity (BrdU index) of PC-82 and PC-EW tumor tissue declined from 3% to less than 1% after castration. Some proliferative activity remained after androgen depletion, the major part of which was localized in the (murine) stromal compartment of the tumors. Androgen ablation of the PC-82 tumor induced apoptosis, whereas massive necrosis was observed in the PC-EW tumor. In contrast to the PC-EW tumors, regrowth of androgen-ablated PC-82 tumors was rapidly induced by androgen resubstitution. The differences in response of these tumor models to androgen depletion and repletion appear to be related to the putative involvement of different cell death pathways. The role of the stroma in these processes is unclear.

Introduction

The recognition that prostate cancer, like the normal prostate gland, depends of androgens for its growth has been the basis of endocrine therapy of prostate cancer for half a century now [1]. Although most patients with advanced prostate cancer have significant palliative benefit from this treatment, almost all tumors subsequently relapse to an androgen insensitive state leading to death from progressive disease [2]. The process of progression of prostatic carcinomas from an androgen dependent to an autonomous state is still poorly understood [3]. Detailed observation of the changes occurring in the tissue of prostate tumor models after androgen depletion might give more insight into the mechanisms of prostate cancer progression.

The establishment of human prostatic tumor models in vivo is difficult and has resulted in only very few suitable models [4]. Four human prostate tumor lines (PC-82, PC-EW, PC-133 and PC-135) are permanently growing in athymic nude mice in our laboratory. The PC-82 and PC-EW tumors are androgen dependent and in this respect they mimic clinical prostate cancer before relapse [5,6], whereas the PC-133 and PC-135 tumor models are androgen independent and represent the hormone unresponsive counterpart of prostate cancer in men [7]. In addition to these models other in vivo human prostate tumor lines have recently been established [8].

In the present study, the PC-82, PC-EW and PC-135 tumor models were used to study castration-induced changes in human prostatic tumor tissue. After androgen deprivation of tumor-bearing animals changes in tumor volume and in tumor morphology as well as the decline of intratumor levels of testosterone (T) and 5α-dihydrotestosterone (DHT) were monitored. Growth potential of the tumors was assessed by estimation of the fraction of S-phase cells in these tissues using an anti-bromodeoxyuridine (BrdU) antibody [9].

Materials and methods

Tumor characteristics

The PC-82 tumor has been developed in 1977 from a primary prostate adenocarcinoma [10]. The tumor is moderately differentiated with a cribriform growth pattern. The PC-EW tumor originates from a lymph node metastasis of a prostate adenocarcinoma [11] and consists of moderately and poorly differentiated carcinoma tissue. Both the PC-82 and PC-EW tumor models contain androgen receptors, are androgen dependent and secrete the prostate specific proteins, prostate acid phosphatase (PAP) and prostate specific antigen (PSA) [12,13]. The PC-135

tumor was established in 1982 from a primary prostate tumor and has an undifferentiated histological pattern [7]. The tumor is androgen independent and it neither contains the androgen receptor nor secretes PAP and PSA [7,14]. During many years of serial transplantation these tumors have kept their original characteristics.

Hormonal manipulation

Small tumor fragments were subcutaneously transplanted at both shoulders of male or female athymic nude mice of the Balb/c background. Mice were supplemented with Silastic implants (Talas, Zwolle, The Netherlands) of 0.8 cm length filled with crystalline T (Steraloids, Pawling, NY). With this method stable plasma levels of T of approx. 10 nmol/l are obtained, which support tumor take (80-85%) and tumor growth [15]. Mice with established tumors were deprived of androgens by castration and removal of the T-implants. Resubstitution with T was achieved by reimplantation of T-containing tubings. Transplantation of tumor tissue and removal or installation of the implants was carried out under light ether anesthesia. Castration was carried out via the scrotal route under anesthesia with tribromoethanol (Aldrich, Beerse, Belgium). Mice were sacrificed at various points of time after castration and/or androgen repletion. Plasma and tumor tissue were sampled.

Tumor volume

Changes in tumor volume were followed weekly by caliper measurements. Tumor volume was calculated from the formula: $V = \pi/6\ (d_1 \times d_2)^{3/2}$, d_1 and d_2 being two perpendicular tumor diameters.

Androgen levels

Tissue levels of T and DHT were assayed in whole tumor homogenates. Homogenization and extraction of the steroids were carried out as described in more detail previously [16]. T and DHT were separated on Silica columns (Silicagel-60; 70-230 mesh ASTM; Merck, West-Germany) as described by Hämäläinen et al. [17]. After chromatography, T and DHT were estimated in the fractions according to Verjans et al. [18]. Plasma samples were analysed for total T [18].

Proliferative activity

One hour prior to sacrifice mice were injected i.p. with BrdU (1 mg/kg), a thymidine analogue, which is incorporated into DNA in the S-phase of the cell cycle. BrdU incorporation was visualized in paraffin embedded material by incubation with a monoclonal anti-BrdU antibody (Eurodiagnostics BV, Apeldoorn, The Netherlands) followed by an indirect peroxidase staining procedure [19]. The

BrdU index indicates the number of BrdU-positive cells per 1000 cells counted.

Immunohistochemistry

Frozen sections of PC-82 tumor tissue were incubated with the monoclonal antibodies directed against specific keratins and stromal markers: RGE-53 (glandular epithelial cells), RCK-102 (all epithelial cells), RCK-103 (basal cells), vimentin (mesenchymal cells) and desmin (smooth muscle cells) [20].

Hoechst-33258 staining

Sections of PC-82 tumor tissue were deparaffinized and stained at room temperature with a 4 μg/ml solution of bisbenzimide (Hoechst 33258, Sigma, St. Louis, MO). The specimens were examined using a fluorescence microscope (Axiophot, Zeiss, FGR). The differences in fluorescence pattern of Hoechst-containing DNA clearly distinguishes human from murine DNA [21].

Ultrastructural analysis

Fresh samples of PC-82 tumor tissue were minced into 2 mm^3 cubes and immediately fixed and stored as described by Kros et al. [22]. Subsequently, the specimens were aceton-dehydrated and Epon-embedded. After ulthrathin cutting (35 nm), the sections were collected on mesh 100 copper grids and counterstained with uranyl acetate and lead citrate. Transmission micrographies were made on a Zeiss 902 transmission electron microscope at 80 kV.

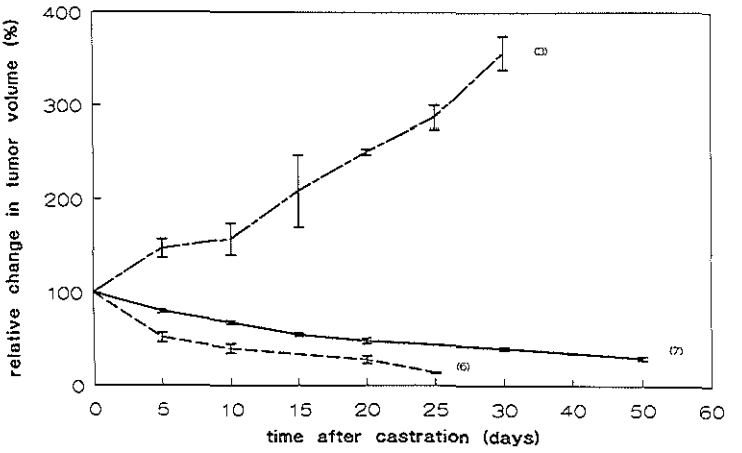


Figure 1. Relative change in tumor volumes of PC-82 (——), PC-EW (- -) and PC-135 (— - —) human prostate tumor models after androgen withdrawal. Data are expressed as means ± SEM.

Results

Effect of castration on tumor volume and morphology

The PC-135 tumor continued to grow after castration of tumor bearing mice, whereas tumor volumes of the PC-82 and PC-EW tumors declined (Figure 1), the regression of the PC-EW tumor being faster than that of the PC-82 tumor (half life of 6 and 18 days, respectively). PC-EW tumors regressed completely, leaving small nodules consisting of fibrotic tissue. In contrast, approximately 20% of the initial volume of the PC-82 tumor remained irrespective of the length of androgen depletion. Additional treatment of castrated PC-82 tumor bearing mice with the pure antiandrogen flutamide for 9 days did not induce a further decline of the residual tumor (result not shown).

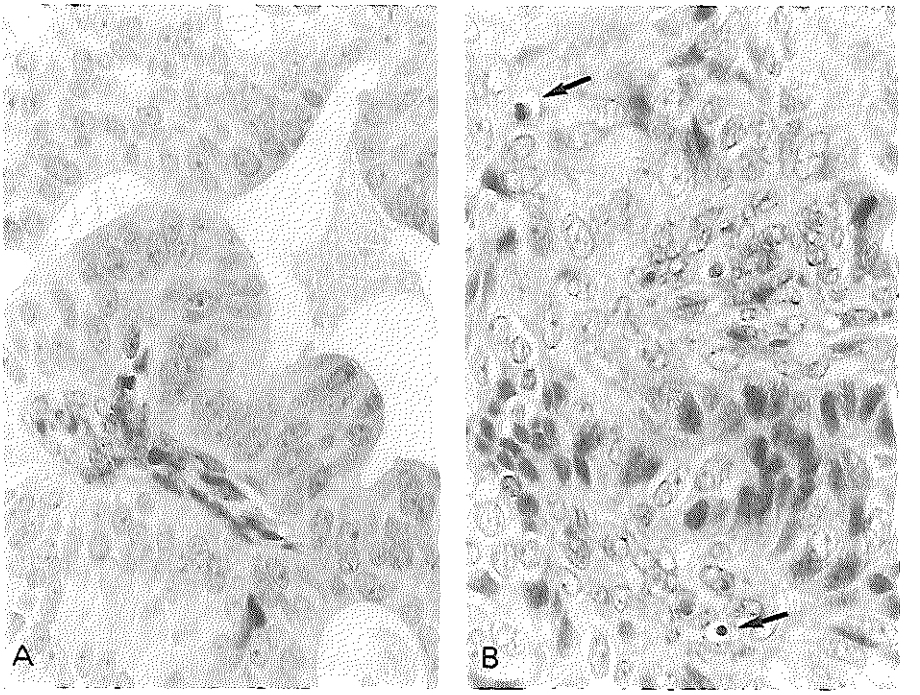


Figure 2 A,B. Morphological appearance of the human prostate tumor PC-82 before (A) and after 30 days of androgen depletion (B) (magnification: 425x). B) Arrows indicate apoptotic bodies.

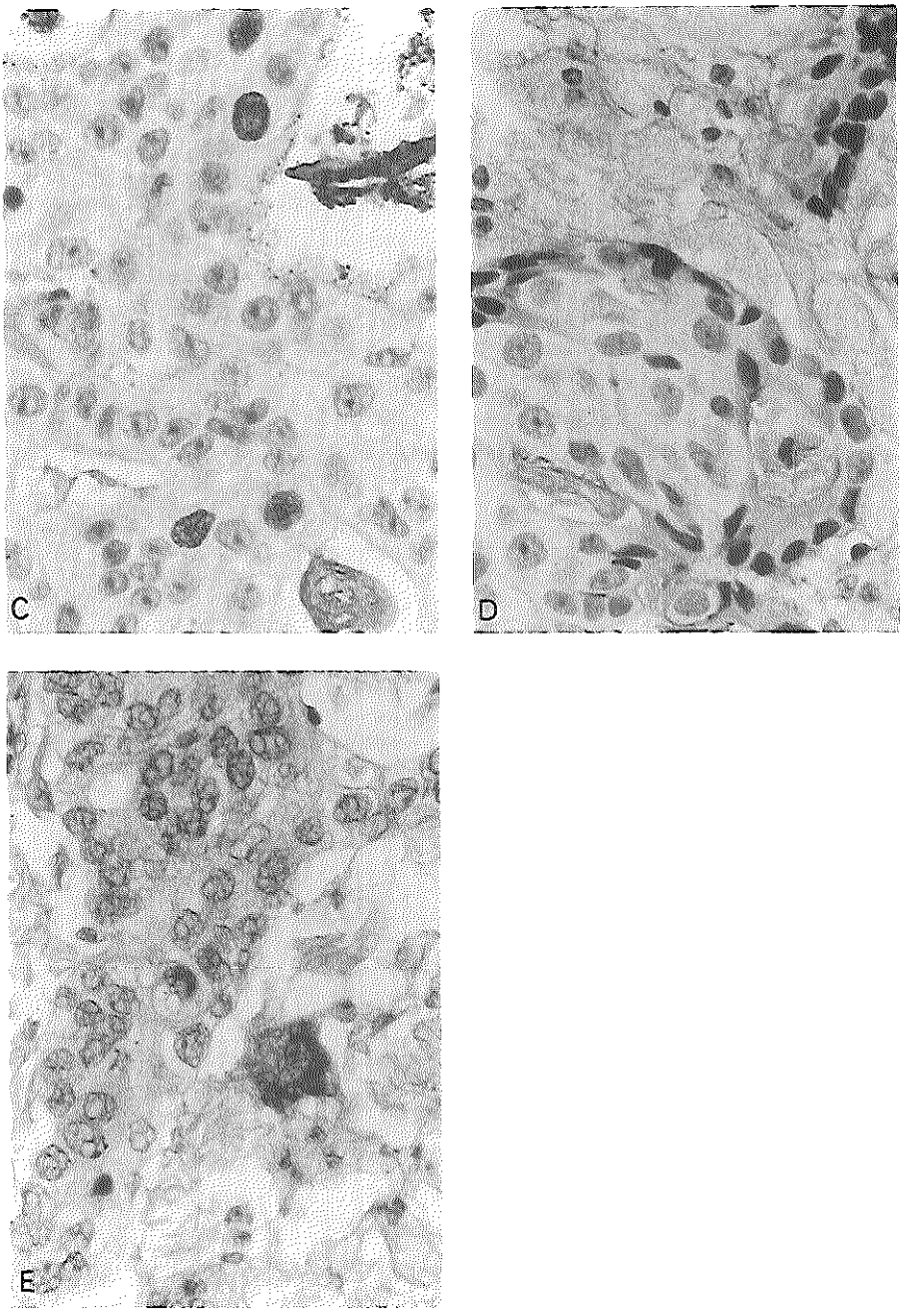


Figure 2 C,D,E. Morphological appearance of the human prostate tumor PC-EW before (C) and after (D, 15 days and E, 35 days) androgen depletion (magnification: 425x).

In spite of a clear reduction in tumor size, the histological appearance of the PC-82 tumor tissue was not dramatically affected by androgen depletion. Although cytological changes were clearly visible and apoptotic bodies could be detected, the glandular structure was largely preserved (Figure 2 A, B).

However, castration of PC-EW tumor-bearing mice induced severe destruction of the epithelial structure of the tumor tissue with a relative increase in stroma and marked necrosis of epithelial tumor tissue (Figure 2 C, D, E). The PC-EW tumor could only be restimulated to grow when tumor regression was limited (< 50%), i.e. during a relatively short period of androgen depletion (< 10 days). When regrowth occurred it took at least a week before tumor volume actually increased (result not shown). In contrast, resubstitution of PC-82 tumor bearing mice with T resulted in an almost immediate regrowth of the tumor (Figure 3). Similar results were obtained with PC-82 after androgen-depletion periods of longer than 90 days (results not shown).

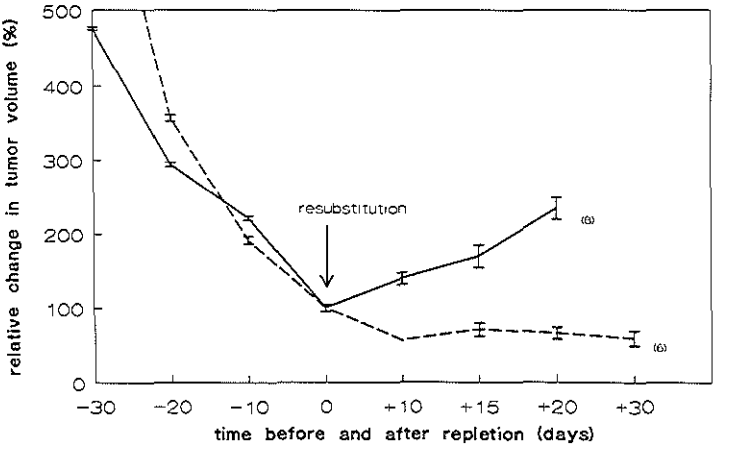


Figure 3. Effect of androgen-depletion and -resubstitution on growth of PC-82 (——) and PC-EW (---) prostate tumors (means ± SEM, number of animals in parentheses).

Castration-induced decline in tumor DHT

Concomitant with the castration-associated fall in plasma T a decline in intratissue T and DHT levels was observed. Intratumor concentrations of T in PC-82 and PC-EW tumors were not significantly different and fell within one day to castrate levels of less than 4 pmol/g tissue (Figures 4 and 5). Before androgen depletion, DHT concentrations in the PC-EW tumor were significantly lower than

those found in PC-82 tumor tissue. The depletion of DHT from PC-82 tissue appeared to be slower than that in PC-EW tumors: after one day of castration DHT declined with 65% and 80%, respectively.

However, similar levels of DHT of less than 2 pmol/g tissue, which means a 80-90% reduction, were detected at 3 days after castration of PC-82 and PC-EW tumor-bearing mice (Figures 4 and 5). DHT levels in PC-135 tumor tissue in T-substituted mice were already at the castrate level of the PC-82 and PC-EW tumors (0.5-2 pmol/g tissue) and in this tumor no effect of castration on androgen levels was observed.

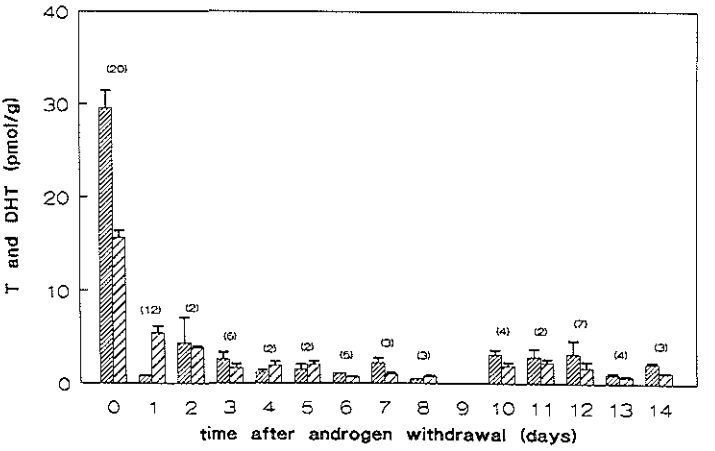


Figure 4. Castration-induced decline of tissue T (fine hatched bars) and DHT (coarse hatched bars) in the PC-82 prostate tumor (means ± SEM; numbers of samples have been indicated).

Castration-induced decline in proliferative activity

Figure 6 shows the decline in proliferative activity of PC-82 and PC-EW tumor tissues after castration as assessed by BrdU incorporation. The fraction of cells in S-phase at day 0 varied between individual tumors (range 0.6-6.1) with a mean of approximately 3% for both the PC-82 and PC-EW tumor. Within 1 day after castration BrdU incorporation declined to one-third of the initial percentages and after 2 days the proliferative activity of both tumors had declined to levels below 1%. However, in some cases a relatively high BrdU index (>1%) was observed, which could, in part, be attributed to BrdU incorporation in the stromal cells surrounding the epithelial tumor cells. The number of BrdU positive cells in regressing PC-EW tumors is probably overestimated since the undifferentiated

character of the PC-EW tumor did not allow corrections to be made for BrdU positive stromal cells, such as used in PC-82 tumors. Figures 7 A and B show the presence of BrdU positive cells within the stromal compartment of regressing PC-82 tumors.

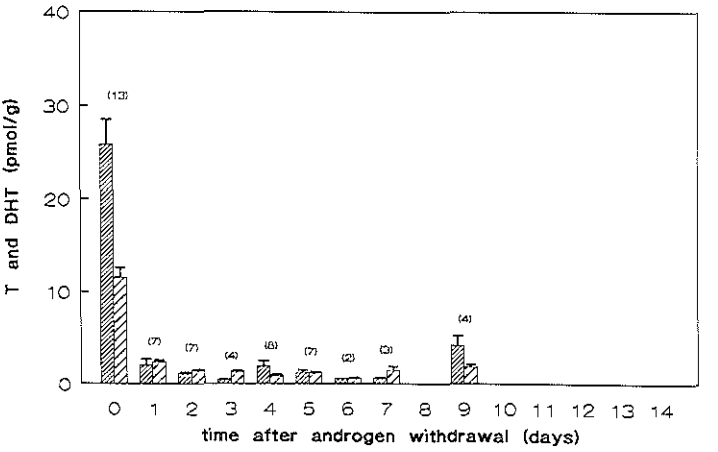


Figure 5. Castration-induced decline of tissue T (fine hatched bars) and DHT (coarse hatched bars) in the PC-EW prostate tumor (means ± SEM; numbers of samples have been indicated).

Staining of PC-82 and PC-EW tumor tissue with a panel of antibodies directed against specific keratins and stromal markers demonstrated that these tumors consist of glandular epithelium. Basal cells could not be detected. Small islands of keratin-negative, vimentin-positive cells represented the stromal part of these tumors (Figure 8). BrdU-positive cells localized in the stromal fraction of the PC-82 tumor were identified by Hoechst staining as from murine origin (Figure 9). Ultrastructural analysis showed considerable amounts of rough endoplasmic reticulum, Golgi apparatus and collagen deposits, without the characteristics of epithelial-like cells, such as the presence of desmosomes or tonofilaments. The cells were thus identified as fibroblast-like cells (Figure 10).

Discussion

The PC-135 tumor is clearly androgen independent as its growth is not influenced by androgen depletion. The androgen dependency of the PC-82 and PC-

EW tumor models is demonstrated by their castration-induced regression. The decline in tumor volume of the PC-EW tumor is significantly faster than the regression of the PC-82 tumor, and is comparable to the castration-induced regression of the normal rat and mouse ventral prostate [15,23]. In contrast, the androgen dependent rat Dunning R3327-H prostate tumor model shows cessation of tumor growth with only a slight reduction in tumor volume after androgen deprivation [24].

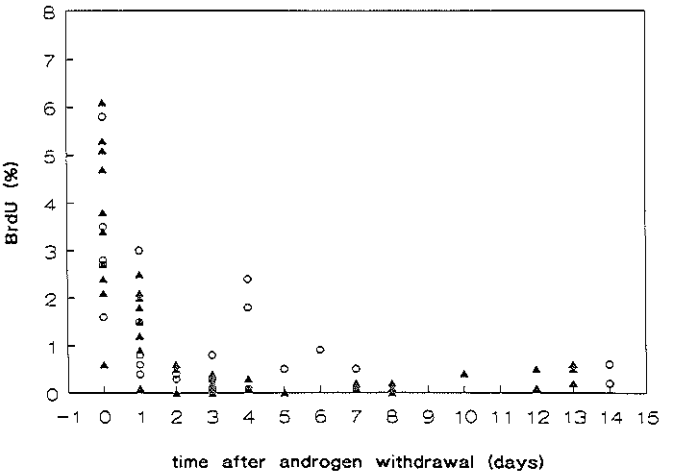


Figure 6. Reduction of BrdU incorporation in PC-82 (▲) and PC-EW (○) prostate tumor tissue after androgen depletion.

The intratumor concentration of T and DHT found in the transplantable androgen dependent tumors presently studied are within the (wide) range reported for androgen-dependent clinical prostate cancer tissues [25,26]. The lower DHT content in the PC-EW tumor corresponds with DHT levels reported for lymph node metastases of prostate cancer patients; such levels were indeed half the levels found in primary prostate tumors [27]. In spite of differences in DHT content, the PC-82 and PC-EW tumors do not differ in their sensitivity for androgenic stimulation of growth, since earlier studies revealed that both tumor models have a similar androgen threshold (3 pmol/g tissue) for growth stimulation [6].

The castration-induced decline in tumor volume of PC-82 and PC-EW tumors is preceded by the reduction in intratumor androgen content. The androgens in both tumors reached similar castrate levels after 3 days of androgen ablation (Figures 4 and 5). These levels of T and DHT were comparable to those found in androgen depleted tissue from the rat ventral prostate, the rat prostate Dunning R3327-H

tumor and the Shionogi tumor model, all characterized as androgen dependent target tissues [24,28,29].

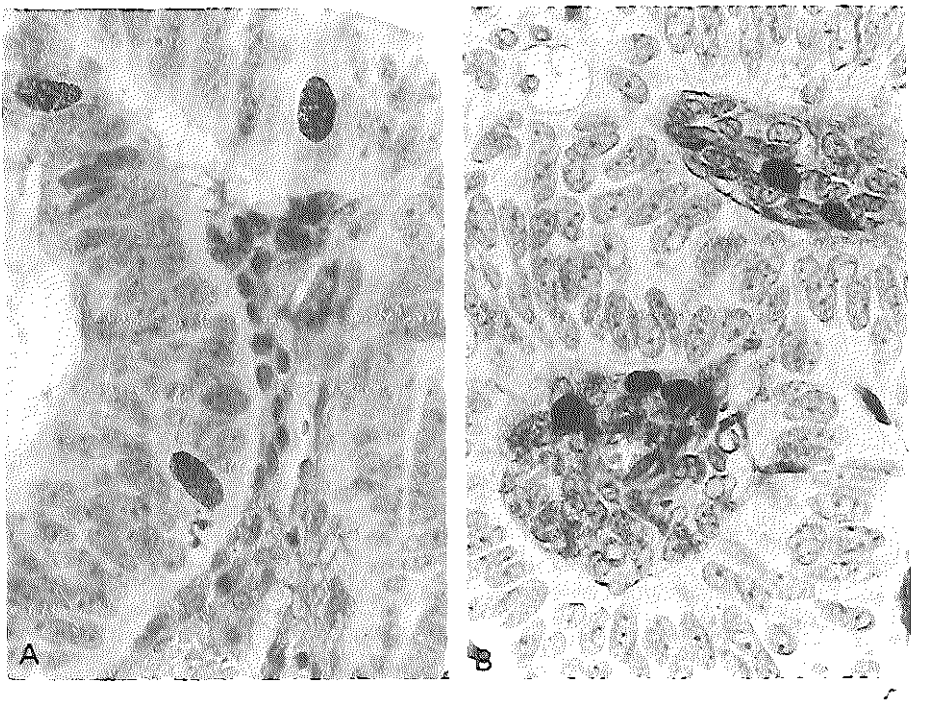
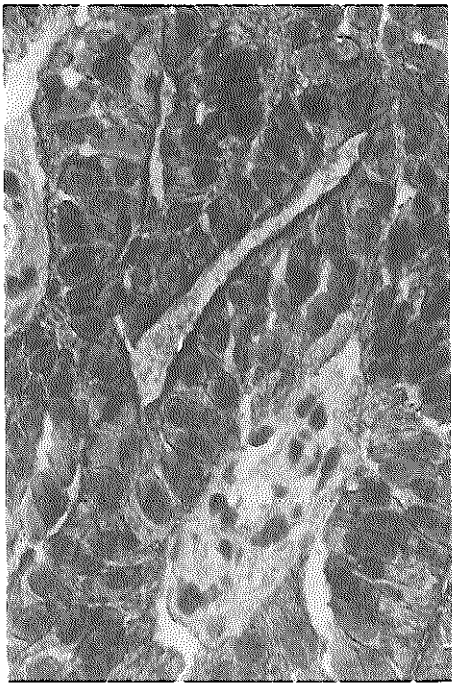


Figure 7. BrdU staining in PC-82 tumor tissue. BrdU positive cells in the epithelium of normal growing tumors (A) and BrdU staining of fibroblast-like stromal cells of androgen depleted tumors (B) (magnification: 425x).

The faster decline of intratissue DHT in the PC-EW tumor within the first two days after androgen depletion as compared to that in the PC-82 tumor may be associated with the more pronounced regression of this tumor. Although accumulation of DHT is almost exclusively due to its binding to and its retention by the androgen receptor [30], the lower DHT content of the PC-EW tumor and the faster depletion of tissue DHT are most likely not due to differences in androgen receptor characteristics. The affinity of the receptor, as analyzed for R1881, was

similar (0.1 nM) with a receptor capacity of 80 versus 120 fmol/mg protein for the PC-EW and PC-82 tumor, respectively [31,32]. A difference in the activity of 5α-reductase, the enzyme which converts T into DHT, cannot explain the difference in disappearance rate of tissue DHT, since neither of the tumor models contain detectable activity of 5α-reductase. It is inferred that DHT in the tumor tissue is entirely derived from conversion of T in the host. This makes it likely that a faster metabolism of DHT in the PC-EW tumor is responsible for the differences observed.



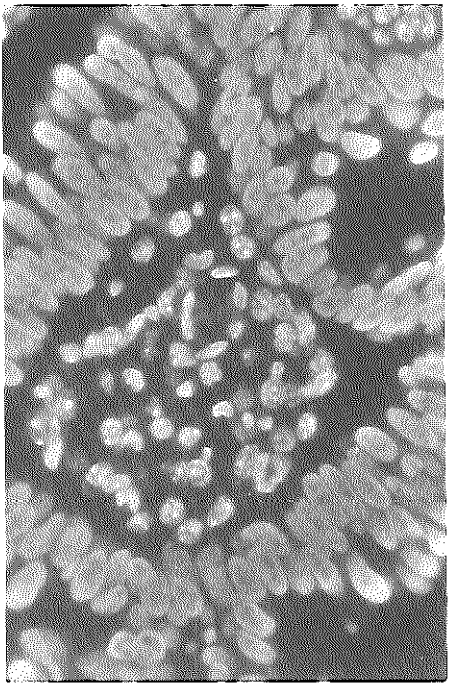


Figure 8. **Figure 9.**

Figure 8. Keratin staining of PC-82 tumor tissue (magnification: 425x).

Figure 9. Visualization of murine cells in the PC-82 tumor detected by Hoechst 33258 fluorescence (magnification: 425x).

The castration-induced involution of the normal prostate is associated with an active process referred to as programmed cell death or apoptosis. In contrast to apoptosis, necrosis is an uncontrolled degenerative process [33]. The regressing PC-EW tumors showed disruption of the epithelial structure and widespread necrosis (Figure 2 D, E), whereas this was not observed in regressing PC-82 tumors. Moreover, apoptopic bodies were frequently observed in androgen ablated PC-82 tumor tissue (Figure 2 B). Since this was not the case in regressing PC-EW tumors, it seems that in these tumors programmed cell death is not involved. In contrast, the PC-82 tumor, like the normal rat ventral prostate, has retained this castration-induced cell death pathway [34,35].

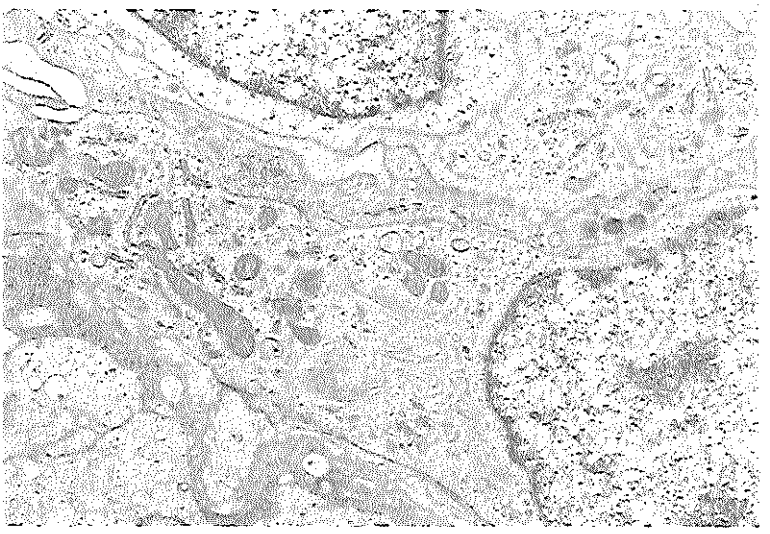


Figure 10. Ultrastructural morphology of BrdU positive stromal (murine) cells in regressing PC-82 tumors (magnification: 7000x).

The normal prostate has the capacity to regress and regenerate repeatedly after androgen depletion and repletion without escaping from androgenic control mechanism [36]. This control mechanism is based on the dual effects of androgens on prostate cells: stimulation of cell proliferation and inhibition of (programmed)

cell death [37]. Apparently, these phenomena also control the PC-82 tumor, which can remain quiescent for long periods of time after androgen deprivation (>90 days), while the PC-EW tumor completely regresses without having the capacity to be restimulated after a relatively short androgen ablation period (> 10 days).

The small residual PC-82 tumors (20% of the initial volume) do not become androgen independent, as in depletion experiments lasting for over 100 days spontaneous regrowth has never been observed. Nevertheless, flutamide treatment for 9 days did not lead to a further regression of the residual PC-82 tumors.

Previous studies have shown that BrdU incorporation in S-phase cells significantly correlates with changes in growth of transplantable human prostate tumors [38]. In the present study, involution of the PC-82 and PC-EW tumors coincided with a significant decline in the BrdU index. A small population of cells, however, still incorporated BrdU, suggesting that DNA synthesis in these cells was not completely ablated by androgen withdrawal. Part of this remaining proliferative fraction could be localized in the stromal compartment of PC-82 tumors. These cells are host-derived (mouse) stromal fibroblast-like cells as judged from the pattern of Hoechst- 33258 staining (Figure 9) and ultrastructural analysis of these cells (Figure 10). The presence of "activated" stromal cells in the regressing PC-82 prostate tumor might be the result of a "normal" reaction to clear away all dead cells. Interestingly, however, no such reaction was observed in the Dunning rat prostate tumor model. English et al. [39] found labelling of stromal cells in the adult rat ventral prostate (2%), which declined to a percentage below 0.5% after castration. In studies of the Dunning rat prostate tumor no thymidine incorporation was observed in stromal cells in the regressing rat R3327-H prostate tumor [40].

The difference between the effects of androgen withdrawal and resubstitution on growth of the PC-82 and PC-EW tumors seems to be related to the different ways of castration-induced cell death in both tumor models. Although the faster metabolism of DHT in PC-EW tumors may account for the delay in regrowth observed in these tumors, castration-induced necrosis strongly restricts the capacity of androgen-induced regrowth of the PC-EW tumor. Regrowth of the PC-82 tumor can be induced at all times after androgen depletion. This is probably due to the controlled proces of cell death in this tumor, although involvement of the stroma in this process remains also possible. Interestingly, in a study of Murphy et al. [41] histological examination of tumor specimens from androgen deprived prostate cancer patients revealed no clear signs of degeneration and necrosis. This made the authors to suggest that suppression rather than ablation of prostatic tumor cells is induced by androgen deprivation. If apoptosis is the major pathway of cell death in androgen ablated prostatic tumors, then the stringent control of this process of cell death may be a factor which limits the effectiveness of the present treatment modalities of prostate cancer.

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Ki-67 expression and BrdU incorporation as markers of proliferative activity in human prostate tumor models

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Submitted

Abstract

The validity of the use of the monoclonal antibodies Ki-67 and anti-BrdU to evaluate proliferative activity of human prostate tumor models was studied. Growth of the transplantable PC-82 and PC-EW prostate tumors, as assessed by tumor volume measurements, was significantly correlated with the proliferative activity as reflected by BrdU incorporation into DNA ($r=0.64$ and $r=0.78$, respectively). The proliferative activity of PC-82 tumors detected by Ki-67 antigen expression parallelled the pattern observed with BrdU ($r=0.51$) and a significant correlation ($r=0.60$) between the results obtained with both markers was found. In growing PC-82 and PC-EW tumors only small variations in Ki-67 and BrdU indices were observed. In contrast, Ki-67 expression in regressing PC-82 tumors varied considerably ($2.7 \pm 2.2\%$). The BrdU index in regressing tumors showed less variation ($1.3 \pm 0.2\%$), but part of the BrdU-positive cells was observed in the stromal (murine) part of the regressing tissue. It is concluded that the Ki-67 and BrdU proliferation markers are reliable parameters to monitor changes in growth of prostate tumor lines, but that in slowly growing or regressing tumors Ki-67 and BrdU data need to be interpreted with caution.

Introduction

The growth potential of a tumor is one of its most important characteristics. In subcutaneously growing tumors, growth can be estimated by measuring tumor size using calipers [1]. Growth activity can also be estimated using a number of biological techniques, such as flowcytometry, counting of mitotic figures and incorporation of radiolabeled thymidine [2,3]. Finally, growth may be assessed by immunohistochemical estimation of the proliferative activity of the tumor cells using the monoclonal antibody Ki-67 and the thymidine analogue bromodeoxyuridine (BrdU).

The Ki-67 antibody detects a proliferation-associated nuclear antigen located on chromosome 10 [4], which is expressed with increasing intensity in the G_1, S and G_2/M phase of the cell cycle but not in the G_0-phase cells [5]. The cell cycle specificity of the Ki-67 antigen was confirmed using phase-specific blocking agents [6,7]. The development of an antibody to the thymidine analogue BrdU made it possible to identify DNA synthesizing cells (S-phase) in situ by histochemical procedures [8]. As DNA synthesis is an integral part of each cell division cycle, BrdU incorporation gives an indication of the proliferative activity in a tumor [9].

BrdU incorporation into DNA and Ki-67 antigen expression have been shown to be useful markers for estimating growth potential of a diversity of tumors, including prostatic carcinoma [10,11,12]. In this study the applicability of the monoclonal antibodies Ki-67 and anti-BrdU to detect changes in proliferative activity of tumors was investigated in the androgen dependent human prostate tumor models PC-82 and PC-EW, and a comparance was made with changes in tumor growth as estimated using calipers.

Materials and methods

Tumor characteristics

The PC-82 and PC-EW human prostate tumor lines are serially transplantable in athymic nude mice [13,14]. They originate from a primary prostatic carcinoma and a lymph node metastasis, respectively. The tumor models are androgen dependent and have preserved many of the characteristics of prostate cancer in man [15]. Both tumors regress after androgen withdrawal, with the PC-EW tumor showing a considerably faster decline in tumor volume than the PC-82 tumor [16].

Experimental design

Small fragments of PC-82 or PC-EW tumor tissue were transplanted

subcutaneously in athymic nude mice of the Balb/c background. Female mice were supplemented with Silastic implants filled with crystalline testosterone (T) [17]. After take of tumor transplants and growth of tumors to approximately 800 mm^3, PC-82 and PC-EW tumor-bearing mice were depleted of T for 12 and 5 days, respectively, to arrest growth of the tumors as well as to reduce intratumor androgen levels. Subsequently, mice were reimplanted with silastic tubes filled with cholesterol mixed with various proportions of T (0-100 %T) to obtain levels of circulating T ranging between 0 and 15 nmol/l [18]. Tumor volume was followed weekly by caliper measurements. After 28 days mice were sacrificed and plasma and tumor tissue were sampled. BrdU (10 mg/kg) was injected intraperitoneally one hour prior to sacrifice of the host animal.

Estimation of hormone levels

T and 5α-dihydrotestosterone (DHT) were estimated in extracts of tumor homogenates after separation on silica columns [19]. The fractions containing T and DHT and plasma T were estimated by radioimmunoassay [20].

Proliferative activity

Proliferative activity of the tumor tissue was assessed immunohistochemically using the monoclonal antibodies Ki-67 (Dakopatts, Denmark) and anti-BrdU (Eurodiagnostics, The Netherlands). Ki-67 expression and BrdU incorporation were visualized using an indirect immunoperoxidase staining procedure applied to frozen and formalin-fixed paraffin-embedded tumor sections, respectively. Aspecific staining of the murine part of the tumor did not interfere with the visualization of Ki-67 and BrdU positive cells, because the stromal (murine) part of these human tumors can easily be discriminated from the epithelial (human) cell fraction.

Antibody stained nuclei were counted in randomly chosen fields at a magnification of x400; their numbers were expressed as percentage of a total of 1000 cells counted.

Flow cytometry

Tissue samples for flow cytometric DNA-analysis were enzymatically dispersed using a solution of 200-400 U collagenase/ml (Worthington, Freehold, NJ) at 37°C and filtered through a 40 μm nylon filter. Single cell suspensions were stored in liquid nitrogen as described by Vindeløv et al. [21]. Thawed cell suspensions were stained with propidium iodide (Sigma) according to the protocol of Vindeløv et al. [22]. Chicken red blood cells (CRBC) were added to the tumor samples as an internal standard and human fibroblasts were used as an external standard. The samples were processed using a FACS-2 cell sorter (Becton Dickinson, Sunnyvale, USA) at an excitation wavelength of 514 nm between 0.5 and 3 h after staining.

Data processing

Data are expressed as mean ± sd with number of samples in parentheses.

Results

Relationship between tumor growth and androgen levels

The differences plasma levels of T introduced in tumor-bearing mice resulted in variable intratumor concentrations of androgens, and consequently, in variations in tumor growth. Figure 1 shows the significant correlation between tumor growth, i.e. changes in PC-82 and PC-EW tumor volumes, and tumor DHT concentrations ($r=0.86$, $n=39$, $P<0.005$ and 0.89, $n=18$, $P<0.005$, respectively, [23]).

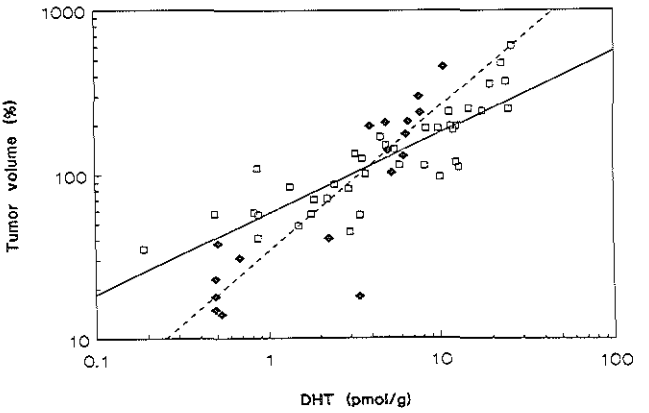

Figure 1. Correlation between tissue DHT levels and PC-82 (□, extended line) and PC-EW (◆, broken line) prostate tumor growth.

Castration-induced effect on the proliferative activity

Figures 2 A and 2 B show the Ki-67 and BrdU antibody staining of growing PC-82 tumors; the percentages of BrdU and Ki-67 positive cells are 5.4 ± 2.4 ($n=6$) and 10.4 ± 1.9 ($n=6$), respectively. The percentage of BrdU positive cells in tissue taken from the central part of the tumor (5.2 ± 1.6%, $n=13$) was not significantly different from that in tissue from the peripheral zone of the tumor (6.7 ± 2.8%, $n=13$). Castration of PC-82 tumor-bearing mice resulted in a decline of the BrdU and Ki-67 indices to 1.3 ± 0.2% ($n=4$) and 2.7 ± 2.2% ($n=14$), respectively.

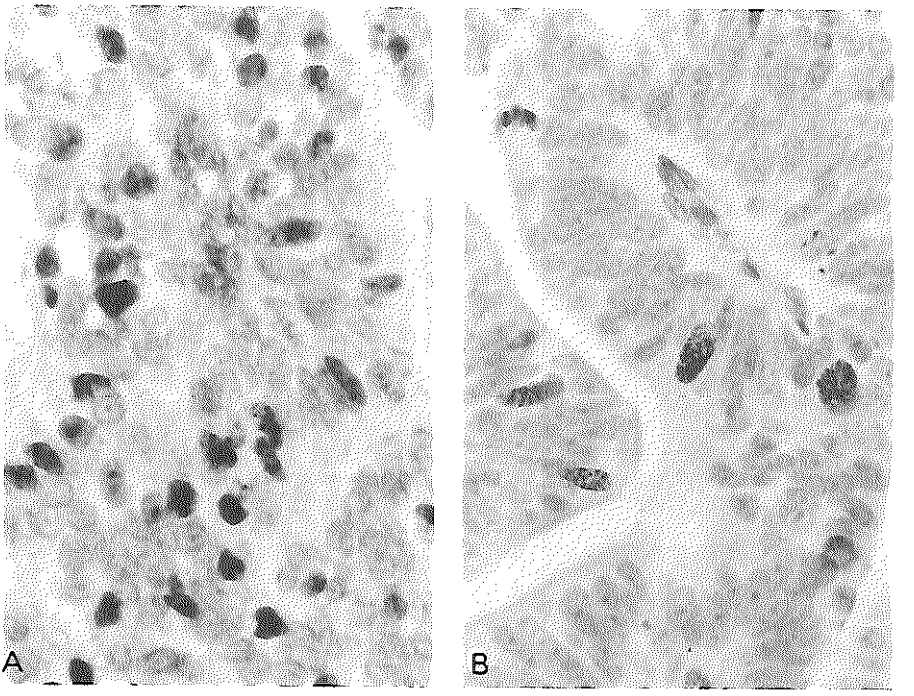

Figure 2 A,B. Ki-67 expression (A) and BrdU incorporation (B) of PC-82 tumor tissue (magnification 425x).

In Figure 3 the flow cytometric distribution of DNA in PC-82 tumor tissue before and after androgen withdrawal is shown. The fraction of cells in G_0/G_1-phase was calculated to be 85-90%; approximately 8% of the cells was in G_2/M-phase. The fraction of cells in S-phase was estimated not to exceed 5%. After castration no cells in the S or G_2/M phases could be detected.

Growing PC-EW tumors had BrdU and Ki-67 indices of 3.8 $\pm$ 1.7% (n=3) and 5.2 $\pm$ 1.2% (n=3), respectively. After androgen withdrawal both indices decreased to percentages below 0.2 (n=3).

Correlation between proliferative activity and tumor growth

Changes in PC-82 and PC-EW tumor volumes were significantly correlated

with the fraction of BrdU-positive cells ($r=0.64$, $n=26$, $P<0.05$ for PC-82 and $r=0.78$, $n=12$, $P<0.05$ for PC-EW) (Figure 4). PC-82 tumor growth also correlated significantly with Ki-67 expression ($r=0.55$, $n=40$, $P<0.05$, result not shown). The Ki-67 proliferation index parallelled the fraction of BrdU-incorporating cells in the PC-82 tumor ($r=0.60$, $n=20$, $P<0.05$, Figure 5).

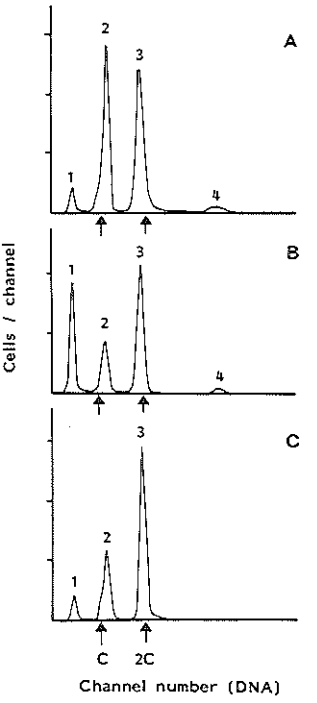

Figure 3. Flow cytometric analysis of the PC-82 prostate tumor before and after androgen depletion.
PC-82 tumor tissue before (A) and 2 (B) and 10 days (C) after androgen deprivation. 1. CRBC-peak; 2. mouse diploid cells; 3. G_0/G_1 PC-82 tumor cells; 4. G_2/M PC-82 tumor cells. The arrows indicate the C and 2C peaks of external diploid reference cells (normal human fibroblasts).

Discussion

The aim of the present study was to investigate if proliferative activity, determined immunohistochemically using the markers Ki-67 and BrdU, reflects changes in tumor volume following hormonal manipulation of the androgen dependent PC-82 and PC-EW prostate tumors. Growing PC-82 tumors had a Ki-67 proliferation index (8-13%, mean 10%) comparable to that observed in a previous study [24]. BrdU indices (2-10%, mean 4-5%) were slightly higher than those found in biopsy specimens of prostate cancer patients (1-5%) [25,26].

The flow cytometric analysis of PC-82 tumor cells supports the immunohistochemical data obtained with the proliferation markers Ki-67 and BrdU. From the DNA histogram it was calculated that approximately 15% of the tumor cells were in the S and G_2/M phase of the cell cycle. The BrdU index of approximately 5% is in agreement with the flow cytometric estimation of the S-phase. The percentages of Ki-67 positive cells indicate that nearly all of the cells in the G_0/G_1 peak of the DNA histogram must be in a quiescent state. The small proportion of cycling PC-82 tumor cells corresponds with the relatively slow growth rate known of these tumors.

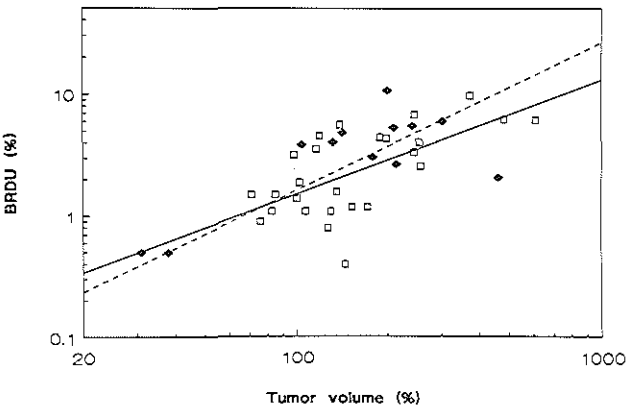

Figure 4. Relationship between changes in PC-82 (□, extended line) and PC-EW (◆, broken line) tumor volume as measured by calipers and the fraction of cells in S-phase estimated by BrdU.

Androgen depletion of the androgen dependent tumors by castration of the host animal, induced a regression in tumor volume accompanied by a decline in the S-

phase fraction to approximately 1%. This was confirmed by flow cytrometric analysis of androgen depleted PC-82 tumor tissue. In androgen ablated PC-82 tumors part of the remaining BrdU positive cells (up to 2%) were found in the stromal compartment of the tumor. The precise origin of these BrdU-positive cells, which were definitely shown to be host-, i.e. mouse-derived, has yet to be determined. A castration-induced decline of Ki-67 expression in the PC-82 tumor resulted in a considerable variation (0-6%, mean 3%). These Ki-67 positive cells may indicate the presence of a small subpopulation of androgen independent cells, although spontaneous regrowth of androgen ablated PC-82 tumors has never occurred as yet [27]. Alternatively, the observed variation could be the result of the slow decline in Ki-67 expression in cells that have passed into the G_0-phase as suggested by Van Dierendonck et al. [28].

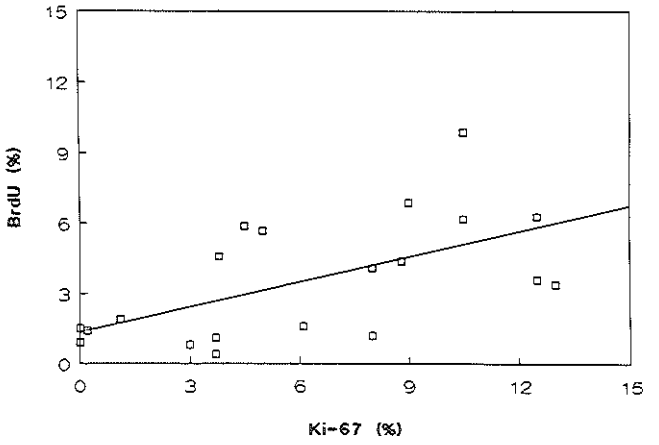

Figure 5. Relationship between the BrdU and Ki-67 positive fractions of cells of PC-82 tumor tissue.

Growth of the PC-82 and PC-EW tumors correlated significantly with BrdU incorporation into DNA (r=0.64 and r=0.78, respectively) and with Ki-67 expression (r=0.55). The Ki-67 and BrdU labelling indices of PC-82 tumor tissue correlated significantly (r=0.60), as was also found in a variety of other human tumors [29,30,31]. Due to the variation in Ki-67 index at low levels of BrdU labelling, such a correlation was not observed in regressing tumors.

A similar pattern was found by Veneroni et al. [32] and Sahin et al. [32], whereas others reported constant Ki-67 to BrdU ratios even at low expression levels [29,30,34]. It was suggested that the Ki-67 antibody did not accurately detect cycling

cells in slowly proliferating tumors due to a variability in Ki-67 expression during the various stages of the G_1-phase [35,36]. The low expression levels of Ki-67 in this phase of the cell cycle could obscure the discrimination between the cells in the G_0 and the G_1 phase, resulting in inaccurate estimates of the growth fraction of the tumor [32,37].

In general, the advantage of BrdU as a proliferation marker is its applicability to paraffin-embedded samples, in which the morphology of the tissue is better preserved. This is an important aspect of this method, as in human xenograft models, such as used in the present study, it is essential to discriminate between the different cell types of the tumors, i.e. human epithelial cells versus host-derived stromal cells. A general disadvantage of this method is that BrdU must be administered in vivo, which is accompanied with potential harmfull properties of this DNA analogue [38,39]. In spite of that, BrdU has been reported to be applied in clinical studies [40]. In contrast, the Ki-67 antibody can be applied directly to tissue specimens. However, this antibody can only be applied to frozen sections, which strongly restricts morphological examination.

It is concluded that Ki-67 and BrdU can be used to monitor the proliferative activity of (hormonally) manipulated prostate tumors. Both markers gave accurate and detailed information about the proliferative activity and growth of tumors in addition to caliper measurements of tumor nodules. However, caution is needed when BrdU and Ki-67 are applied to slow growing or regressing tumors as indicators of tumor growth.

Nevertheless, Ki-67 has succesfully been applied in a follow-up study of hormonally treated prostate cancer patients [41,12], which warrants further investigations into this proliferation marker.

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Chapter 5

Assessment of the critical level of androgen for growth response of transplantable human prostatic carcinoma (PC-82) in nude mice

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Abstract

The androgen dependent prostatic carcinoma of human origin, PC-82 was used as a model system to investigate the effect of various levels of androgen on the growth of prostatic tumor tissue. Plasma testosterone levels in mice were correlated to tumor growth and intratumor concentrations of testosterone and 5α-dihydrotestosterone. PC-82 tumor burden remained stable at plasma testosterone levels of 0.8 nmol/l, whereas tumor growth occurred at higher levels and tumor regression was observed at lower plasma levels. This critical level of testosterone corresponded with intratumor testosterone and 5α-dihydrotestosterone concentrations of 6 to 10 and 3 to 4 pmol/g tissue, respectively, which are significantly above the levels found in castrated non-supplemented animals (3.1 and 1.4 pmol/g, respectively). This indicates that, remaining concentrations of dihydrotestosterone, which amount two to three times the castrate level, are not stimulatory for tumor growth in the model of the androgen dependent PC-82 tumor.

Introduction

After an initial response to hormonal therapy all prostatic cancer patients eventually relapse to a state in which the tumor is unresponsive to further androgen ablation therapy [1]. At present there is a fundamental difference of opinion concerning the question why androgen sensitive prostate cancer progresses to an androgen insensitive state. Some investigators assume that all prostate cancer cells are more or less sensitive to androgens and that the relapse phenomenon can be seen as a consequence of inadequate suppression of androgens [2]. These authors indicate that in men, androgens of adrenal origin which are still secreted after chemical or surgical castration, may cause continued growth of so-called hypersensitive androgen dependent prostate cancer cells after this treatment. Other investigators hold the opinion that prostate cancer is composed of different clones of cells, with different dependency on androgens. Androgen withdrawal therapy would only affect androgen dependent clones of cancer cells leaving the pre-existing androgen independent cancer cells to survive and grow [3,4]. To ascertain the validity of either of these two concepts it is important to investigate if there is a minimal amount of androgen needed to support tumor growth.

In order to elucidate the effects of varying low levels of circulating testosterone (T) on the growth of human prostatic cancer tissue, the transplantable androgen dependent PC-82 tumor line has been used as a model. Differences in take and growth of the tumor, due to various levels of plasma T, were studied and emphasis was put on the relationship between the levels of circulating T, the corresponding intraprostatic androgen concentrations T and 5α-dihydrotestosterone (DHT) and, ultimately, the growth response of the tumor tissue.

Materials and methods

Tumor model

The PC-82 tumor is a moderately differentiated adenocarcinoma initiated in 1977 from a primary human prostate adenocarcinoma. Its origin and features have been described extensively [5,6,7]. Tumors were serially transplanted into athymic nude mice of the Balb/c background. In this study both female and castrated male mice were used. One week prior to start of the experiments, mice were castrated via the scrotal route under total anesthesia with tribromoethanol (Aldrich, Beerse, Belgium). Tumor transplantation was carried out under light ether anesthesia.

Hormonal manipulation of the nude mice

Androgen substitution of mice was performed by subcutaneous implantation of

Silastic tubing (Talas, Zwolle, The Netherlands) packed with crystalline steroid (Steraloids, Pawling, New York). This technique has been shown to be suitable to maintain a constant level of the hormone for at least 40 days [8]. An optimal take rate (80-85%) and growth of the tumor tissue was obtained using T filled implants with a lenght of 0.4 cm, resulting in stable plasma T levels of 10-15 nmol/l. Because, the release rate of T from these implants appeared to be too high to obtain near castrate levels of circulating androgen, Silastic capsules of 0.6 cm lenght were packed with cholesterol mixed with different proportions of T (0, 1, 5, 10, 25 and 100 %T).

Female nude mice, substituted with these implants for seven days, showed various plasma T levels ranging from 0.2 to 19.4 nmol/l (Table 1). The use of cholesterol as a carrier did not result in changes of plasma levels of cholesterol in the implanted mice (result not shown). It was assumed, therefore, that these capsules did not influence steroid metabolism. Light ether anesthesia was used for implantation or removal of the Silastic tubes.

Experimental protocols

Protocol A. To investigate the effect of low levels of plasma T on tumor take and tumor growth, castrated male mice were transplanted with tumor and simultaneously substituted with different doses of T (0, 5, 10, and 100 %T). Tumor volume was measured by caliper measurements and tumor volume was determined according to the formula: $V = \pi /6 \ (d_1 \times d_2)^{3/2}$, in which d_1 and d_2 are two perpendicular tumor diameters. The take rate, the percentage of tumors reaching the volume of 100 mm^3, was calculated. After 90 days mice were sacrificed and blood and tumor tissue were sampled.

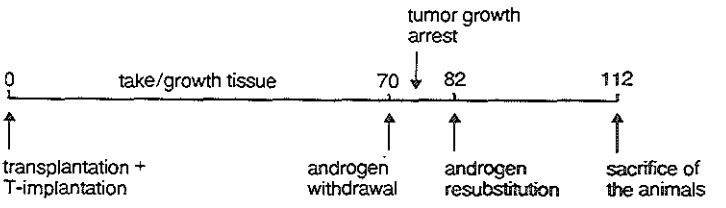

Figure 1. Scheme of experimental protocol (B) for investigation of the effect of various levels of T on the growth of the PC-82 tumor grown on nude mice.

Protocol B. To eliminate the possible effect of low concentrations of plasma T on the development of the tumors, another experimental protocol was applied (Figure

1). Using this approach, differences in androgen requirement during the process of tumor take can be distinguished from the effect of low levels of androgen on tumor growth. These experiments were performed in female or castrated male mice receiving an optimal dose of T (0.4 cm implants; 10 nmol/l) concomitant with tumor transplantation. During the exponential phase of tumor growth, mice were depleted of androgen for 12 days to omit interference of remaining levels of T and the doses of T (0, 5, 10 and 100 %T) that were subsequently resubstituted. Growth of the tumor was followed weekly by caliper measurements. Tumor volume was calculated as indicated above (protocol A). After a period of 28 days mice were sacrificed and blood and tumor tissue were sampled.

Table 1. Plasma T concentration in female nude mice supplemented with different proportions of T.

Proportions of T in the implant (%T)	T plasma (nmol/l)
0	0.2 ± 0.03 (3) *
1	0.3 ± 0.04 (5)
5	1.2 ± 0.13 (5)
10	1.5 ± 0.34 (7)
25	2.2 ± 0.20 (9)
100	19.4 ± 2.22 (7)

Plasma T was analysed 7 days after implantation.
* number of animals are indicated in parentheses.

Hormone estimations in plasma and tissue extracts

To determine concentrations of T and DHT in tumor tissue samples were homogenized in 0.01 M phosphate buffer, pH 7.4, containing 0.9% (w/v) NaCl and 0.1% (v/v) Tween-80. Procedural losses were monitored by adding known amounts of ^{3}H-T and ^{3}H-DHT to each sample. Steroids were extracted from the tissue homogenates by adding a threefold volume of acetone. After centrifugation at 2,000 g for 10 minutes, the supernatant was removed and the pellet was extracted again with the same volume of acetone. Acetone in the combined supernatants was evaporated under nitrogen at 45°C. The aqueous residues were extracted with n-hexane/ether (4:1; v/v), the organic phase was separated from the aqueous phase by freezing and then evaporated. The residue was dissolved in 3% (v/v) acetone in dichloromethane. T and DHT were separated on Silicagel columns (Silicagel-60; 70-230 mesh ASTM; Merck,

West Germany) and eluted with 3% and 50% (v/v) acetone in dichloromethane, respectively, as described by Hämäläinen et al. [9].

After chromatography, part of each fraction was used for estimation of the recovery of the steroids and fractions were analysed for T and DHT by radioimmunoassay using the antiserum described by Verjans et al. [10]. For estimation of plasma levels of T samples were extracted with n-hexane/ ether (4:1; v/v), followed by radioimmunoassay.

Statistical procedures

All data are expressed as the mean ± standard error of the mean (SEM) with number of animals in parentheses. When comparing multiple groups, statistical significance of all treatment differences was determined by Anova analysis followed by the Student-Newman-Keuls procedure. Differences were considered statistically significant when $P < 0.05$.

Results

Plasma T and PC-82 tumor growth

Protocol A. Results of these experiments are summarized in Table 2. The 0 and 10 %T implanted mice had final circulating T levels comparable to those indicated in Table 1. Plasma T levels of mice with 25 and 100 %T implants were still far above the levels found in the 0 and 10 %T groups of mice. The take rate of tumors supported with 25 %T was found to be similar (79%) to that of tumors in control mice receiving 100 %T, whereas, tumors transplanted in 10 %T substituted mice had a significantly lower take rate (40%). Finally, in mice receiving implants with 100% cholesterol no tumor growth was observed. The mean tumor volume reached at 90 days after transplantation was significantly smaller in the 10 and 25 %T implanted mice as compared to tumors in 100 %T implanted animals. The difference between tumor volume in 10 and 25 %T supported mice was not statistically significant. Individual plasma T levels of mice supplemented with 5 or 10 %T implants showed that PC-82 tumor take could only be achieved in those mice having a plasma T level above 1 nmol/l.

Protocol B. Circulating T levels and growth response of the tumor in female or castrated male mice were not significantly different between both sexes for a given percentage of T in the implant (data not shown). Therefore, the results of the experiments with female and castrated male mice were combined. At the end of the experiment (i.e. 28 days) plasma concentrations of T in the various groups of mice were in the range of those shown in Table 1. Plasma T levels showed a lineair increase

with increasing T substitution, except when comparing the 5 and 10 %T substituted groups of mice. During the period of 12 days, in which the animals were deprived of T, the volume of the PC-82 tumor regressed by 30 ± 15 % (n=49). Compared to the 100 %T supported mice, growth of the tumors in all the other groups was significantly retarded (Figure 2). Tumors in 10 %T implanted mice showed a growth curve which was also significantly different from the 0 %T implanted group. Administration of 5 %T implants resulted in stabilization of tumor volume. Plasma concentrations of T were significantly correlated with tumor growth ($r = 0.85$, $n = 35$, $P < 0.001$).

Table 2. Growth properties of PC-82 tumor tissue in castrated male mice supplemented with different doses of T (protocol A).

%T	T plasma (nmol/l)	Tumor take (%)	Tumor volume (mm^3)
0	0.7 ± 0.2 (4)	0 (0/8)	-
10	1.8 ± 0.2 (3)	40 (4/10)[a]	127 ± 27 (4)[b]
25	4.0 ± 0.4 (4)	70 (10/14)	239 ± 42 (9)[c]
100	7.0 (2)	79 (11/14)	742 ± 132 (11)

Mice were sacrificed 90 days after transplantation.
Values are expressed as mean ± SEM with the number of observations in parentheses.
Significantly different from 100 %T-implanted group; a) Chi-squered test; b) and c) Student's t-test; $P < 0.05$.

T and DHT tissue levels and PC-82 tumor growth

In Figure 3 data on tissue levels of T and DHT are summarized. No significant differences were found between the concentrations of T and DHT in tumor tissue of 100 % cholesterol implanted mice and tumors at day 0, i.e. at 12 days after androgen withdrawal. With increasing doses of T substitution an increase was seen for serum T and prostatic T and DHT levels. The difference between each group was significantly except for the 5 and 10 %T supplemented mice. Plasma T levels of 0.8 ± 0.8 nmol/l, causing tumor stabilization in 5 %T substituted mice, resulted in mean intratumor T and DHT concentrations of 10.4 ± 6.7 and 5.1 ± 4.2 pmol/g tissue, respectively. These levels are significantly higher than those in castrated or 0 %T implanted animals. Lower tissue concentrations of androgens were associated with reduction of the tumor volume.

Correlating individual intratumor androgen levels with tumor burden, showed a significant correlation between the intratissue T and DHT concentration, and the change

in tumor volume after 28 days of repletion ($r=0.76$, $n=36$, $P<0.001$ and $r=0.88$, $n=39$, $P<0.001$, respectively). Maintenance of the tumor was achieved when intratissue androgens were in the order of 6-10 and 3-4 pmol/g tissue, for T and DHT respectively.

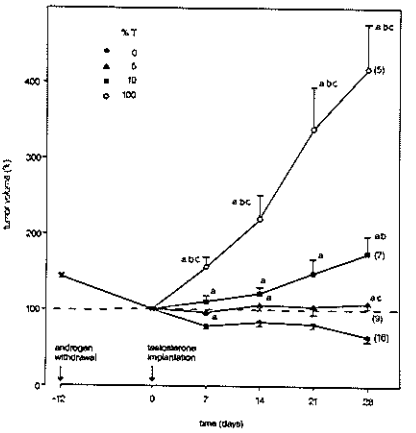

Figure 2. Growth curves of the PC-82 tumor in mice supplemented with various plasma levels of T (protocol B).
Values are expressed as mean ± SEM with number of animals in parentheses. a, b, c: significantly different from 0 %T- 5 %T- and 10 %T-implanted group respectively; $P<0.05$.

Discussion

Surgical or chemical castration of prostate cancer patients suppresses circulating T by over 90 %, but reduces intraprostatic DHT to only 50 % of its normal level [11,12]. It is not clear whether these intraprostatic androgen levels may still have a stimulatory effect on prostate tumor cells. The human androgen dependent prostate tumor cell line PC-82 was used to investigate the effect of similar low levels of androgens on the growth of prostatic carcinoma tissue. This is a very suitable model, because the adrenals of mice, in contrast to those of men, do not synthesize androgens (unpublished results). Therefore, interference of adrenal androgens with hormonal manipulation of female or castrated male mice is not expected.

Hormonal manipulation of mice concomitant with tumor transplantation (protocol

A) demonstrated that below a plasma T level of 1 nmol/l no PC-82 tumor developed. This result was confirmed by the application of low dose T resubstitution in mice bearing already established tumors (protocol B). Growth of prostatic tumor tissue correlated significantly with both peripheral T levels and tissue concentrations of T and DHT. Because in men more then 95 % of plasma T is bound to sex hormone binding globulin (SHBG) [13], whereas mouse plasma does not contain detectable amounts of SHBG [14], in men intratumor levels of androgens may be better parameters to indicate the relationship between androgens and proliferative activity of the prostate cancer tissue than peripheral hormone levels.

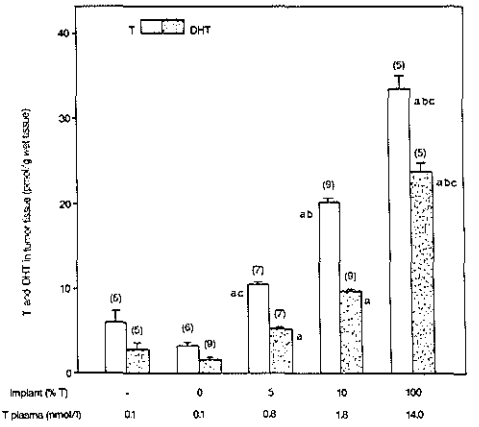

Figure 3. Concentrations of T and DHT in PC-82 tumor tissue grown under different conditions of T-substitution according to protocol B.
Values are expressed as mean ± SEM with number of mice in parentheses. Plasma levels of T are indicated at the bottom of the graph. a, b, c: significantly different from 0 % T- 5 % T- and 10 % T-implanted group; $P < 0.05$.

The threshold level for androgen stimulated growth of the PC-82 tumor was found to be at plasma T concentrations of approximately 0.8 nmol/l (5 %T implant). This threshold level corresponded with tissue T and DHT concentrations of 6-10 and 3-4 pmol/g tissue, respectively, which amount 2 to 3 times the androgen levels in castrated animals.

The present results with the human PC-82 prostate tumor model agree with the

observations made with the Dunning R3327 H-subline, that plasma T levels should be kept below a certain threshold level to prevent growth of the androgen responsive tumor, but do not need to be lowered to castrate levels in order to obtain a suppressive effect on tumor growth [15,16]. Additionally, it was demonstrated in the rat ventral prostate that androgen-induced increase in prostatic cell number could only occur when the concentration of prostatic DHT exceeded a critical threshold value [17]. These data on tumor models in the rat, as well as our results support the finding that relatively low levels of androgens, which are well above the castrate levels of T and DHT found in these animals, have no significant stimulatory effect on prostate tumor growth, although lower levels of androgens will induce a maximal reduction of the PC-82 tumor.

It is well recognized that androgens have an agonistic, stimulatory effect on cell proliferation and an antagonistic effect on cell death in the prostate [18]. It may be argued that the critical level for tumor growth found in the PC-82 tumor model is the level at which the rate of cell proliferation is balanced by an equal rate of cell death. Lower levels of T maximally activate cell death resulting in involution of the tumor. Due to this dose difference of T it seems possible to prevent a total reduction of androgen dependent prostate tumor cells without selectively activate androgen independent tumor cell proliferation.

Very few data are available on androgen concentrations in tissue of human prostatic cancer patients. Recently, Bélanger and associates [19], found a mean prostatic DHT content of 9.29 $\pm$ 5.16 pmol/g in 5 patients who underwent an orchiectomy. Lower levels of intraprostatic DHT were reported by Geller and coworkers [20]: after surgical castration of a group of 38 patients the mean DHT level in prostatic tissue was 4 pmol/g. However, the majority of the patients had DHT levels lower than 4 pmol/g, whereas DHT levels exceeded 6.5 pmol/g in only 7 patients [21]. These clinical data suggest that the intraprostatic concentration of DHT found in some prostatic cancer patients after orchiectomy may still be higher than the threshold level for growth stimulation of the PC-82 tumor model. However, the data obtained with this experimental tumor also show that intraprostatic DHT levels of 3 to 4 pmol/g, which are in the range of those found in the prostate of a majority of prostate cancer patients after surgical or chemical castration, may well be low enough to stop growth of prostatic cancer.

The concept of total androgen ablation therapy as a more effective therapy against prostate cancer than partial androgen withdrawal is based upon the fact that the secretion of androgens of adrenal origin is not affected by castration. It has been argued that these levels of adrenal androgens have a potential stimulatory effect on the prostate cancer cells [22,23]. To ascertain whether or not adrenal androgens have an effect on the growth of human prostate cancer cells, a series of experiments with substitution of adrenal androgens in the PC-82 tumor model is being currently carried out.

In conclusion, the present data indicate that low levels of androgen, which are

well above castration levels found in nude mice, do not stimulate PC-82 prostate tumor growth, suggesting that submaximal suppression of androgens can stop tumor growth. It remains to be seen whether lower levels of androgens, which cause maximal regression of the PC-82 tumor, may be relevant for a subgroup of prostatic cancer patients.

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Chapter 6

Adrenal glands of mouse and rat do not synthesize androgens

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Abstract

Human adrenal glands produce considerable amounts of the C-19 steroids dehydroepiandrosterone (DHEA) and androstenedione (A). To investigate the capability of rodent adrenals to produce these steroids, cell suspensions of mouse and rat adrenal glands were incubated in the absence and presence of adrenocorticotropic hormone (ACTH). Corticosterone levels in the incubation medium increased dramatically in the presence of ACTH, but no significant amounts of 17-hydroxyprogesterone or A could be detected. This indicates that the adrenals of rat and mouse lack the enzyme 17α-hydroxylase. Absence of plasma cortisol in the presence of high levels of corticosterone confirmed these data. Plasma levels of A were significantly decreased in castrated male rats as compared to levels observed in intact males, showing the contribution of the testes to the plasma content of A. Very low levels of A were observed in female, male and castrated male mice. Plasma concentrations of DHEA were not detectable in intact and castrated male mice and rats. It is concluded that rat and mouse lack the enzyme necessary to synthesize adrenal C-19 steroids and that the adrenals in these animals, therefore, do not contribute to plasma levels of A and DHEA.

Introduction

Androgens in men are secreted by the testes and the adrenal glands. Quantitatively, the most important androgens secreted by the human adrenal cortex are dehydroepiandrosterone (DHEA), its sulfate DHEA-S, and androstenedione (A) [1]. Although A and DHEA have limited androgenic potential they can be metabolized into more potent androgens in peripheral target tissues, such as the prostate [2,3]. In prostate cancer these androgens of extratesticular source remain in the circulation after standard hormonal therapy, i.e. (chemical) castration. It has been suggested that these androgens may account for the relapse in tumor growth seen in almost all of these patients [4], but this is still a matter of debate [5].

Rats and mice are commonly used as model systems in studies on the effect of androgens on the (malignant) prostate [6]. The role of the adrenal androgens in the growth of prostatic carcinoma has been investigated extensively using rat prostate (tumors) as well as human prostate tumor xenografts in athymic nude mice as models [7,8,9]. It is necessary, therefore, to know whether these animals produce quantities of adrenal androgens comparable to those secreted by the human adrenal gland. The information on the subject is scarce, however, and to our knowledge the issue is poorly documented and controversial [10,11].

In order to gain more insight into synthesis and secretion of androgens by rodent adrenal glands, incubation studies with adrenals from female rats and mice were performed and 17-hydroxyprogesterone and A concentrations in the incubation media were measured. Adrenals of female nude mice were used since the androgen-supplemented female nude mouse is an important model in our studies on growth regulation of prostate tumors using heterotransplantable human tumor models [12]. To our knowledge no reports exist in which differences in steroid biosynthesis between adrenal glands of male and female mammals are described.

In addition to the incubation studies, plasma levels of corticosterone, cortisol, A and DHEA were determined in female, male and castrated male nude mice and normal rats.

Materials and methods

Adrenal incubations

Adrenal glands of female Balb/c nude mice and female normal Wistar rats were collected under stress free conditions. The tissues were minced and incubated with collagenase in Krebs-Ringer-bicarbonate-glucose (KRBG) at 37 °C under an oxygen-5% carbondioxide gas mixture. After 50 minutes of collagenase treatment, the cell

suspension was centrifugated. The cell pellet was resuspended in 0.5% bovine serum albumin (BSA) in KRBG, filtered and centrifuged for 10 minutes at 100g. The pellet was resuspended again in 0.5% BSA in KRBG, whereafter the suspension was added to a 2% BSA solution and centrifuged for 5 minutes at 10g. The cell pellet was resuspended in 0.5% BSA in KRBG. The adrenal cells were incubated for 150 minutes without ACTH or in the presence of 150 or 300 pg/ml of ACTH. Levels of corticosterone [13], A [14], 17-hydroxyprogesterone [15] and progesterone [16] in the incubation medium were assayed by radioimmunoassay.

Plasma levels

Plasma levels of the adrenal androgens A and DHEA as well as of cortisol and corticosterone were determined in heterozygous nude Balb/c mice and normal Wistar rats. Intact female, male and castrated (14 days) male animals of approximately 20 weeks of age were used. The animals were anaesthesized with ether and blood was taken from the orbital sinus. Plasma samples from individual mice and rats were analysed for A, DHEA and cortisol by coated tube radioimmunoassay (Diagnostic Products Corporation, Los Angeles, California). A chromatographic step using Sephadex LH-20 micro columns was introduced for analysis of DHEA. Detection limits of these assays were 0.4, 1.7 and 25 nmol/l, respectively. Corticosterone was assayed as described above [13].

Results

Levels of adrenal steroid hormones which were determined in the incubation medium with or without ACTH stimulation of the adrenal cell suspensions are summarized in Table 1. The significant increase in corticosterone levels after addition of ACTH indicates that the amounts of ACTH used were effective in stimulating steroid hormone production by the adrenal cells. The maximum response of the adrenal cells was reached with an ACTH concentration of 150 pg/ml. The increase of the levels of 17-hydroxyprogesterone and A in the incubation media can be explained by the crossreactivity with progesterone of the antibodies used (crossreactivity of 4 and 5.5%, respectively).

Plasma levels of cortisol, corticosterone, A and DHEA in female, male and castrated male mice and rats are shown in Table 2. The apparent plasma levels of cortisol amounted to 2-3% of those of corticosterone. Intact male rats had significantly higher A levels compared to castrated males (1.6 ± 0.6 and 0.4 ± 0.2 nmol/l, respectively). A levels in mice did not significantly differ between female, male and castrated male mice (0.8 ± 0.6, 0.5 ± 0.3 and 0.5 ± 0.3 nmol/l, respectively). Levels of DHEA were below the detection limit of the assay in both

intact and castrated male rats and mice.

Table 1. Secretion of steroid hormones by adrenal glands from female rodents after incubation in the absence or presence of ACTH.

Incubation	C (nmol/l)	A (nmol/l)	17OH-P (nmol/l)	P (nmol/l)
Mice:				
control	13.5 ± 1.9	0.04 ± 0.09	0.00 ± 0.04	0.6 ± 0.1
150 pg/ml ACTH	213.0 ± 28.4	0.14 ± 0.11	0.26 ± 0.09	6.4 ± 0.3
300 pg/ml ACTH	203.2 ± 30.7	0.16 ± 0.09	0.34 ± 0.05	5.6 ± 0.6
Rats:				
control	57.7 ± 11.5	0.18 ± 0.10	0.15 ± 0.06	5.0 ± 0.5
300 pg/ml ACTH	318.0 ± 5.8	0.20 ± 0.08	0.55 ± 0.25	15.4 ± 2.2

Data represent the mean ± S.D. of 5 incubations carried out within one experiment.
Abbreviations: C=corticosterone, A=androstenedione, 17OH-P=17-hydroxyprogesterone, P=progesterone.

Discussion

The ability of adrenal glands from female rodents to produce and secrete androgens in vitro has been investigated in the present study. Incubation studies of rat and mouse adrenal cell suspensions in the presence of ACTH revealed that the main precursors of androgen biosynthesis, 17-hydroxyprogesterone and A, were not produced. The rat and mouse adrenals apparently lack the key enzyme for the pathway by which pregnenolone and progesterone are converted into 17-hydroxypregnenolone and 17-hydroxyprogesterone, the precursors for the adrenal androgens DHEA and A.

Contrasting results reported by Edery et al. [17,18] showed that in one out of four mouse strains studied, i.e. only in the F1 (C3H x RIII) hybride, adrenal homogenates incubated with radiolabelled pregnenolone were capable of synthesizing DHEA. Suprisingly enough, only very small amounts of A could be detected. Tait and coworkers [19] observed that human and rat adrenal tissue in vitro were capable of producing A and DHEA when incubated with an intermediate in the sesterpene pathway. They hypothesized that steroid biosynthesis in adrenal glands occurs not only via the cholesterol pathway but may be also, and in the rat preferably, via a

sesterpene pathway. The present observations do not support this hypothesis.

Plasma concentrations of A in rats and mice showed that the ovaries and testes secrete small amounts of A. Castration of male rats resulted in a fall in A levels showing that the testis is the main source of A in the rat and that the adrenal glands do not contribute to the plasma levels of this steroid: the remaining high levels of A in castrated male mice and rats of the present study (Table 2) can be explained by the high levels of corticosterone observed in these animals, which crossreact with the antibody used in the A assay. The levels of A found in this study in rat and mouse plasma are comparable to those reported in the literature [20,21].

Table 2. Plasma levels of adrenal androgens and cortisol and corticosterone in heterozygous Balb/c mice and normal Wistar rats.

	A (nmol/l)	DHEA (nmol/l)	C (nmol/l)	Cortisol (nmol/l)
Mice:				
female	0.8 ± 0.6 (15)	2.0 ± 1.8 (7)	310 ± 127 (6)	6 ± 3.7 (6)
male	0.5 ± 0.3 (6)	<1.7 (8)	312 ± 97 (5)	11 ± 3.6 (5)
castrate	0.5 ± 0.3 (6)	<1.7 (4)	795 ± 261 (3)	11 ± 3.8 (3)
Rats:				
male	1.6 ± 0.6 (7)	<1.7 (7)	372 ± 128 (4)	9 ± 0.5 (3)
castrate	0.4 ± 0.2 (7)[a]	<1.7 (5)	193 ± 31 (3)	4 ± 1.7 (5)

Plasma levels are expressed as mean ± sd with number of animals in parentheses.

[a] significant lower than in intact males ($P<0.05$).

Abbreviations: A=androstenedione, DHEA=dehydroepiandrosterone, C=corticosterone.

Low levels of DHEA were observed in female mice, but not in intact or castrated male mice, indicating some secretion of DHEA by the mouse ovaries. No DHEA could be detected in the plasma of either intact or castrated male rats. In female rats plasma levels of A and DHEA were not different from those observed in males (data not shown). These data clearly show that no significant amounts of DHEA are secreted by the testes or the adrenal glands of these animals.

Ando and coworkers [21], however, reported on the plasma levels of testosterone (T), 5α-dihydrotestosterone (DHT), A and DHEA after castration of male rats. There results are in contrast with the present findings: detectable levels of

these steroids were observed after various times of castration. The changes in the ratios of these steroids suggested that the conversion of DHEA to A could explain the post-castration levels of T and DHT.

Recently, Bélanger et al. [22,23] reported on the concentrations of C19-steroids in guinea pig and rat adrenal tissue and plasma. In castrated rats small amounts of androgens remained present in the adrenal glands. However, plasma levels of these androgens could not be detected after castration, and gas chromatographic-mass spectrometric measurements did not confirm the presence of androgens in the rat adrenal glands. The data obtained in the present study confirm the results of Bélanger et al. for the rat and extend them to the situation in the mouse.

Finally, plasma concentrations of cortisol were near the detection limit of the assay, even after correction for crossreactivity of corticosterone (1.4%) in the cortisol assay. These low levels of cortisol amounted to less then 4% of the levels found for corticosterone. This is also indicative for the lack of the enzyme 17α-hydroxylase in mice and rats.

The present results also explain the absence of any effect of adrenalectomy on basal T secretion in male rats as reported by Feek et al. [24] and by McNeilly et al. [25]. In studies in which the role of adrenal androgens in prostate (tumor) growth was investigated using the the normal rat ventral prostate and the Dunning rat prostate tumor model, adrenalectomy in addition to castration, did not affect prostate (tumor) volume and cell number [26,27]. Plasma levels of T and DHT remaining in the plasma after castration of male rats were reported to become undetectable after subsequent adrenalectomy [28]. Since these very low levels of T and DHT are most probably due to crossreactivity in the radioimmunoassays used, adrenalectomy may not have influenced plasma androgen levels. In conclusion, in research involving endogenous adrenal androgens, rat and mouse are not suitable model systems for extrapolation of results obtained to the human situation.

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Chapter 7

Effects of low testosterone levels and of adrenal androgens on growth of prostate tumor models in nude mice

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Abstract

Two transplantable, androgen dependent prostate tumor models of human origin, PC-82 and PC-EW, were used to study the effect of low androgen levels and adrenal androgens on prostate tumor cell proliferation. Tumor load of the PC-82 and PC-EW tumors could be maintained constant when plasma testosterone levels were 0.8 and 0.9 nmol/l, respectively, corresponding with an intratissue 5α-dihydrotestosterone level of 3 to 4 pmol/g tissue. This critical androgen level for prostate tumor growth stimulation amounted to 2 to 3 times the castration level and proved to be similar for both tumor models. Relatively high levels of androstenedione resulted in physiological levels of plasma testosterone causing androgen concentrations in PC-82 tumor tissue exceeding the critical level for tumor growth. These results indicate that submaximal suppression of androgens can stop tumor growth in these prostate tumor models.

Introduction

Endocrine treatment of prostate cancer patients alone, whatever therapy regimen used, cannot anticipate the relapse phenomenon which always follows the initial response of the tumor to hormonal therapy [1]. It has been questioned whether this transient response to hormonal treatment results from a selective outgrowth of (pre-existing) androgen independent clones of cells [2,3] or is due to an insufficient ablation of androgens [4,5]. In men, androgens of adrenal origin, remaining in circulation after castration, may compensate the castration-induced decline in intraprostatic androgen concentration in part [6]. Many studies, both experimental and clinical, have been conducted in an attempt to reveal whether a more complete androgen ablation than obtained by castration alone, might yield a more favorable result in the treatment of prostatic cancer. Until today there is no definite answer to this question.

In order to study this point in more detail, two androgen dependent prostate tumor models of human origin, PC-82 and PC-EW, were used to investigate the relationship between growth activity of prostatic carcinoma tissue and peripheral and tissue testosterone (T) and 5α-dihydrotestosterone (DHT) concentrations. In addition, the role of the adrenal androgens androstenedione (A) and dehydroepiandrosterone (DHEA) in prostate tumor growth stimulation was studied.

Materials and methods

Tumor model characteristics

Both tumors used in this study, PC-82 and PC-EW, are of human origin and serially transplantable in nude mice. The PC-82 tumor model is derived from a primary, moderately differentiated adenocarcinoma of the prostate [7,8]. The PC-EW tumor originates from a lymph node metastasis and is composed of poorly and moderately differentiated carcinoma [9]. These tumor models share many of the properties seen in clinical prostate cancer. Although tumor take (85%) and tumor doubling time (18 days) are similar for both tumors, the PC-EW tumor regresses much faster after androgen withdrawal than the PC-82 tumor; tumor halftime being 8 and 17 days, respectively.

Manipulation of plasma testosterone levels in mice

Female or castrated male nude mice of the Balb/c background were routinely substituted with T by subcutaneous implantation of Silastic capsules (0.4 cm) filled with the crystalline steroid [10]. Because the release of T from these implants was

too high to obtain relatively low levels of T, in the low dose resubstitution studies Silastic implants (0.6 cm) were filled with either cholesterol (0 %T) or with cholesterol mixed with different amounts of T (5, 10, 25 and 100 %T implants), resulting in various plasma T levels.

Experimental protocol

To study the effect of various levels of androgen on prostate tumor growth without influencing tumor take, mice were transplanted with small fragments of tumor tissue and simultaneously substituted with a standard dose of T (0.4 cm length; resulting peripheral T level: 10 nmol/l) to assure an optimal tumor take and tumor growth. When tumors were in the exponential phase of growth, implants were removed for a short period of time to deplete the tumor tissue of androgens. Because of the difference in regression rate, depletion periods of 12 and 5 days were chosen for PC-82 and PC-EW tumors, respectively. Subsequently, mice were resubstituted with implants containing various doses of T (Figure 1). Tumor volume changes were followed weekly by caliper measurements. Tumor volume was calculated according to the formula $V = \pi / 6 (d_1 \times d_2)^{3/2}$, in which d_1 and d_2 are two perpendicular tumor diameters. After 28 days mice were sacrificed and plasma and tumor tissue were collected.

Substitution with adrenal androgens

The ability of adrenal androgens to support prostate tumor growth was studied. Therefore, by implanting mice with A and DHEA packed in Silastic tubings. As the adrenal glands of mice do not produce androgens (results not shown), endogenous synthesis of adrenal androgens cannot interfere with adrenal androgen substitution in this model. Mice were treated according to the same protocol followed for the T supplementation experiments (Figure 1).

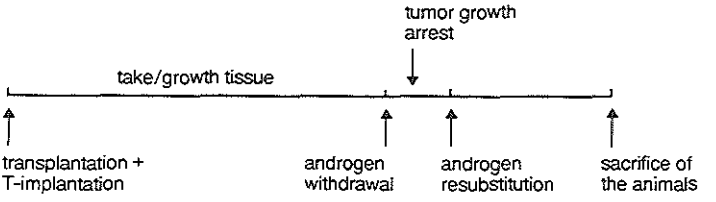

Figure 1. Protocol for the experiments with PC-82 and PC-EW grown under various androgen levels.

Hormone estimations in plasma and tumor tissue

T and DHT levels in tumor tissue were estimated in whole tissue homogenates after separation of the androgens on silica columns as described by Hämäläinen et al. [11]. Plasma androgen content and separated androgen fractions from the tissue homogenates were analysed by a radioimmunoassay [12]. This procedure has been described in more detail elsewhere [13].

Statistical procedure

All data are expressed as the mean ± standard error of the mean (SEM) with number of animals in parenthesis. All group differences were statistically analysed using the Students t-test and were considered significant when $P < 0.05$.

Results

Plasma and intratissue levels of androgen

Substitution of female or castrated male mice with various doses of T resulted in plasma levels ranging from 0 to 20 nmol/l (Table 1). These levels as well as the growth response of the tumors did not differ significantly between implanted females and castrated males. A significant correlation was observed between plasma T and intratumor DHT concentration for both tumor models (PC-82: $r = 0.87$, $n = 34$, $P < 0.001$ and PC-EW: $r = 0.69$, $n = 23$, $P < 0.05$, Figure 2). In all substituted groups PC-EW tumor tissue had a significantly lower DHT content than PC-82 tumor tissue, whereas a similar castrate level of DHT (0 %T) was found in both tumors.

Table 1. Plasma T levels in mice bearing the PC-82 or PC-EW tumor at the end of the experiment (28 days).

Proportion of T in implant (%T)	Plasma level of T (nmol/l)
0	0.1 ± 0.1 (18)
5	0.6 ± 0.2 (12)
10	1.3 ± 0.3 (17)
25	4.5 ± 1.0 (4)
100	15.6 ± 1.3 (8)

Plasma T levels were significantly different between all groups ($P < 0.05$).

Threshold level for tumor growth stimulation

A significant dose-response relationships between intratumor DHT concentration and tumor growth was observed in both the PC-82 and PC-EW tumor model ($r=0.88$, $n=39$, $P<0.001$ and $r=0.91$, $n=17$, $P<0.001$, respectively). Tumor growth in 10 and 25 %T substituted mice was significantly retarded compared to the tumor growth observed in the 100 %T substituted mice.

Maintenance of the PC-82 tumor was achieved at a plasma T level of 0.8 ± 0.8 nmol/l, corresponding with intratissue T and DHT levels of 6-10 and 3-4 pmol/g tissue (Figure 2). Stabilization of the PC-EW tumor volume was observed at similar plasma T levels (0.9 ± 0.9 nmol/l) resulting in intratumor T and DHT levels of 5-9 and 2-3 pmol/g tissue (Figure 3). Both tumors showed a decline in tumor burden in 0 %T substituted mice (0.1-0.2 nmol/l), which was comparable to the regression normally seen in castrated animals. DHT threshold levels for growth stimulation of both PC-82 and PC-EW tumors were approximately 3 pmol/g tissue.

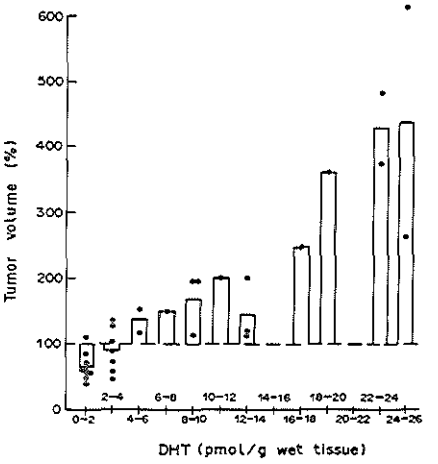

Figure 2. Relationship between intratissue DHT content and PC-82 tumor growth.

PC-82 tumor growth with adrenal androgen substitution

Supplementing mice with DHEA (peripheral levels: 9.2 ± 1.7 nmol/l) did not result in any PC-82 tumor growth stimulation. The tumor regression observed in these mice was equal to that observed in the 0 %T implanted mice (Table 2). Although plasma levels of T in DHEA substituted mice were above castration level

(1.2 ± 0.2 nmol/l), intratissue androgen levels were not significantly different from those of castrated animals (data not shown). Androstenedione substitution caused plasma T levels of 10.7 ± 1.6 nmol/l and accordingly induced a increase in PC-82 tumor volume which was significantly less than that observed in the 100 %T implanted mice (Table 2).

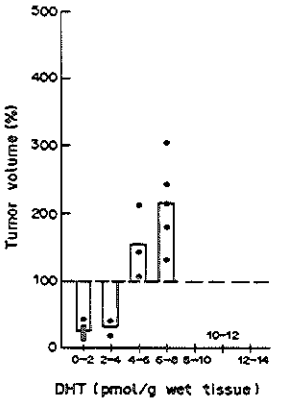

Figure 3. Relationship between intratissue DHT content and PC-EW tumor growth.

Discussion

After castration of prostate cancer patients plasma levels of androgens decrease markedly, whereas relatively high levels of T and DHT remain present in the prostate [14]. Obviously, these levels originate from androgens secreted by the adrenal glands and it is well known that prostatic tissue is capable of metabolizing adrenal androgens into T and DHT [15]. In this study the relevance of these remaining levels of androgens for growth stimulation of human prostate tumor tissue was investigated.

From the experiments presented here, it became evident that growth of PC-82 and PC-EW prostate tumor tissue could be stimulated when intratissue DHT exceeded approximately 3 pmol/g tissue. This intratumor level of DHT, which is 2-3 times the level found in 0 %T implanted or castrated mice, can be regarded as a

threshold level for tumor growth stimulation [13], although, lower levels of androgen did result in a maximal reduction of tumor burden.

The response of the PC-EW tumor to androgen depletion differs from that of the PC-82 tumor, as PC-EW regresses much faster. In addition, PC-EW has considerably lower intratissue levels of DHT as compared to the PC-82 tumor model (Figures 2 and 3). Remarkably, the critical level of intratissue DHT for tumor growth is similar for both tumor models is. The question remains, however, whether all prostatic carcinomas do have a similar cut-off point for minimal stimulating androgen levels.

The adrenal androgen DHEA could not prevent regression nor could it activate growth in both tumor models. In contrast, androstenedione induced a marked increase in plasma T levels, resulting in high levels of intratissue T and DHT levels in the PC-82 tumor. These levels, which exceeded the threshold level for growth stimulation, caused the tumors to grow accordingly (Table 2). It should be mentioned, however, that these plasma levels of A were above the levels normally found in castrated men as in a group of patients treated with an LHRH agonist A levels of 4-5 nmol/l were observed (own clinical data). Experiments with levels of A which are more close to the clinical situation are currently being carried out.

Table 2. PC-82 tumor growth and plasma levels of DHEA A and T in female nude mice supplemented with DHEA A 0 and 100 %T.

	Plasma level (nmol/l)			Tumor volume (mm^3)
Androgen	DHEA	A	T	
0 %T	0.7 ± 0.2 (5)	0.9 ± 0.3 (5)	0.3 ± 0.1 (9)	60 ± 4 (11)
DHEA	9.2 ± 1.7 (5)[a]	ND	1.2 ± 0.2 (10)[a]	71 ± 4 (11)
A	ND	13.5 ± 1.3 (5)[a]	11.9 ± 1.4 (7)[b]	217 ± 15 (8)[b]
100 %T	ND	ND	17.6 ± 2.1 (3)	362 ± 44 (4)

Mice were sacrificed at the end of the experiment (28 days).
ND not detected. a) significantly different from 0 %T ($P<0.05$), b) significantly different from 100 %T ($P<0.05$).

After surgical or chemical castration of prostate cancer patients DHT levels in the prostate remain relatively high: in prostatic tissue of castrated prostate cancer patients average DHT levels of 4 pmol/g were found [16,17]. However, 7 out of the 38 patients had DHT levels exceeding 6.5 pmol/g tissue whereas the majority of the

patients had DHT levels lower than 4 pmol/g [18]. Intraprostatic concentrations of DHT found in a majority of prostate cancer patients after orchiectomy may therefore be considered low enough to prevent prostate tumor growth, whereas in only a few patients DHT levels exceed the level found to be stimulatory for the prostate tumor models PC-82 and PC-EW.

In conclusion, the data presented in this paper indicate that partial androgen withdrawal can stop tumor growth in the prostate tumor models PC-82 and PC-EW. This indicates that total androgen ablation is not needed to prevent growth of these tumors. Relatively high plasma levels of A were found to be stimulatory for PC-82 tumor growth, which implies that adrenal androgens may be involved in clinical prostate cancer.

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Effects of adrenal androgens on the transplantable human prostate tumor PC-82

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Abstract

The potential of the adrenal androgens androstenedione (A) and dehydroepiandrosterone (DHEA) to stimulate prostate tumor growth was investigated in the hormone dependent human prostate tumor model PC-82, propagated in nude mice. Substitution of castrated mice bearing growth-arrested tumors with DHEA for 28 days resulting in peripheral levels of 9.2 $\pm$ 1.7 nmol/l (mean $\pm$ SEM), leading to a decline of tumor burden comparable to that observed in castrated controls. Intratumor testosterone (T) and 5α-dihydrotestosterone (DHT) levels were similar to those detected in the castrated group. In contrast, high-dose A supplementation (peripheral level of 13.5 $\pm$ 1.3 nmol/l) in androgen ablated tumor-bearing mice resulted in tumor growth, although less pronounced than in T resubstituted mice (T level of 18.8 $\pm$ 1.5 nmol/l). Intraprostatic levels of androgens were not different between both groups. Substitution of castrated PC-82 tumor-bearing mice with low-dose A (2.5 $\pm$ 0.4 nmol/l) neither stimulated growth of tumors nor did it lead to regression of PC-82 tumors. Proliferative activity as estimated by BrdU incorporation (S-phase cells) was not induced in these tumors. In conclusion, DHEA does not have a stimulatory effect on growth of PC-82 tumor tissue, but A is capable of inducing PC-82 tumor growth, most likely through peripheral conversion of A into T and DHT.

Introduction

Since the establishment of the androgen dependency of prostate cancer, the treatment of advanced disease has been based on the suppression of testicular androgens [1,2]. Unfortunately, this treatment does not cure the patient as a relapse of prostate tumor growth occurs after an initial period of remission [3]. Androgens of adrenal origin, which are unaffected by standard endocrine therapy (surgical or medical castration), might be the cause of tumor recurrence [4]. The role of adrenal androgens in the regulation of growth of prostate cancer growth has received limited attention, mainly because of complications encountered with surgical or medical adrenalectomy and because of disappointing clinical results [5,6].

New theories on progression of prostatic cancer suggested the pre-existence of androgen independent tumor cells, which are likely to cause the relapse phenomenon since endocrine treatment does not affect these cells [7]. In contrast, Labrie and coworkers postulated that androgen hypersensitive rather then androgen insensitive tumor cells cause prostate tumor progression [8]. Growth of these hypersensitive cells is thought to be induced by low levels of androgens originating from the adrenal gland. These authors advocate a complete inhibition of the action of androgens from all sources by using non-steroidal pure antiandrogens in addition to surgical or medical (LHRH analogues) castration [9]. The beneficial effect of this combination therapy over that of standard hormonal therapy is, however, limited and contradictory [10,11].

The main androgens secreted by the human adrenal glands are androstenedione (A) and dehydroepiandrosterone (DHEA). They account for 5-10% of circulating androgenic activity in adult males [12]. Both steroids have only weak androgenic activities but can be converted into the active androgens testosterone (T) and 5α-dihydrotestosterone (DHT). Although orchiectomy induces a rapid fall in plasma T, residual levels of DHT have been reported in prostate tumor tissue of hormonally treated patients [13,14]. A and DHEA remaining in the circulation after orchiectomy are likely to be responsible for these observed intraprostatic DHT levels. It is questioned, however, whether these residual low levels of DHT are actually stimulatory to the prostate cancer [15].

We, recently, demonstrated that the transplantable human prostate tumor models, PC-82 and PC-EW, have an androgen threshold level for growth stimulation which significantly exceeds the level observed in castrated animals [16]. These data showed that near-castrate levels of DHT, comparable to those observed in clinical prostate tumors after androgen deprivation therapy [17], did not stimulate growth of the experimental prostate tumors. Since the adrenals of mice were shown not to synthesize androgens [18], the mouse is a very useful model to study the effects of substitution with the adrenal androgens DHEA and A. Moreover, castration of these

animals can thus be regarded as a total androgen ablation. The present study was conducted to investigate whether "physiological" levels of A and DHEA in PC-82 tumor-bearing nude mice induce plasma and intratumor T and DHT levels which are capable of stimulating prostate tumor growth.

Materials and methods

Tumor model

The PC-82 tumor model was established in 1977 from the primary tumor of a prostate cancer patient and has since been serially transplanted in athymic nude mice [19]. The androgen dependency of the model is demonstrated by the absence of tumor growth in female mice and by the regression of the tumor after castration of tumor-bearing male mice [20]. The PC-82 tumor contains androgen receptors and secretes considerable amounts of the prostate specific proteins, prostate specific antigen (PSA) and prostatic acid phosphatase (PAP) [21,22].

Experimental protocol

Female and male athymic nude mice were transplanted with small fragments of the PC-82 tumor. Supplementation of mice with T was carried out by implantation of T-containing Silastic devices [23]. Mice with exponentially growing tumors were androgen ablated by removal of the T-implants or by castration via the scrotal route under anesthesia with tribromoethanol. All other procedures were carried out under light ether anesthesia. A short period of androgen deprivation was used to minimize interference of the androgen levels present in the tumor tissue with the levels resulting from resubstitution with the adrenal androgens [24]. After 12 days of androgen withdrawal mice received implants of 1.0 cm length filled with either cholesterol (0 %T, i.e. castrated controls), DHEA, A (100 %A) or T (100 %T). One group of mice received implants containing a mixture of 25% A and 75% cholesterol (25 %A). Tumor growth was assessed using calipers. Mice were sacrificed after 28 days of androgen supplementation and plasma and tumor tissue were sampled.

Hormone estimations in plasma and tumor tissue

Tissue levels of T and DHT were assayed in whole tumor homogenates. Homogenization and extraction of the steroids were carried out as described in more detail previously [16]. T and DHT were separated on Silica columns (Silicagel-60; 70-230 mesh; Merck, Germany) as described by Hämäläinen et al. [25]. After chromatography, the fractions were estimated for T and DHT as described by Verjans et al. [26]. Plasma samples were analysed for T [26]. Plasma levels of A

and DHEA were analysed by coated tube radioimmunoassay (Diagnostic Products Corporation, Los Angeles, USA). A levels were estimated in whole tissue homogenates by radioimmunoassay using the antiserum described by Fröhlich et al. [27].

Estimation of BrdU incorporation into DNA

One hour prior to sacrifice, mice were injected i.p. with bromodeoxyuridine (BrdU, 10 mg/kg), a thymidine analogue, which is incorporated into DNA in the S-phase of the cell cycle. BrdU incorporation was visualized in paraffin embedded material using a monoclonal anti-BrdU antibody (Eurodiagnostics BV, Apeldoorn, The Netherlands) followed by an indirect peroxidase staining procedure as described by Schutte et al. [28].

Statistical procedures

All data are expressed as mean $\pm$ standard error of the mean (SEM). Differences in tumor growth between the various treatment groups were statistically tested using an ANOVA analysis followed by the Student-Newman-Keuls procedure. Differences between hormone levels in the treatment groups were tested for statistical significance by Student's t-test. Differences were considered significant when $P < 0.05$.

Results

Administration of DHEA and two doses of A (25 %A and 100 %A) for 28 days to androgen depleted PC-82 tumor-bearing mice resulted in plasma levels as indicated in Table 1. Levels of DHEA and A in control mice were below or close to the detection limit of the assays. Figure 1 shows the change in PC-82 tumor volume expressed as percentage of the tumor volume at day 0 of supplementation. DHEA did not support PC-82 tumor growth and tumors regressed with a rate similar to that observed in castrated controls. Plasma levels of T in DHEA-treated mice were above castrate level (Table 1). This may be due to cross reactivity of the antibody used in the assay with other steroids present in the samples, since intratumor levels of T and DHT in this group were similar to those observed in the castrated mice (Figure 2).

High levels of A (100 %A) clearly stimulated growth of the PC-82 tumor, although less pronounced than in 100 %T-supplemented animals (Figure 1). Although, plasma levels of T were significantly lower in the A-treated mice as compared to the T-substituted mice (Table 1), intratumor androgen levels between both groups were comparable (Figure 2). The group of mice receiving low-dose A (25 %A) showed no change in tumor volume (Figure 1). Although tumor regression

was not observed in these animals, plasma levels of T were not different from those observed in DHEA treated animals (Table 1). When individual plasma levels of A were related to the corresponding growth data a significant correlation ($r=0.82$, $n=15$, $P<0.001$) was found (Figure 3). A highly significant correlation was also found between T and A in the plasma ($r=0.90$, $n=16$, $P<0.001$, Figure 4).

Table 1. Plasma levels of T, DHEA and A in experimental PC-82 tumor-bearing nude mice.

	A plasma (nmol/l)	DHEA plasma (nmol/l)	T plasma (nmol/l)
0 %T	0.9 ± 0.1 (5)	0.7 ± 0.1 (5)	0.2 ± 0.1 (14)
DHEA	-	9.2 ± 1.7 (5)	1.2 ± 0.2 (10)
25% A	2.3 ± 0.2 (8)	-	1.0 ± 0.2 (12)
100% A	10.9 ± 1.1 (7)	-	11.9 ± 1.5 (7)
100 %T	-	-	18.8 ± 1.5 (7)

Mice were sacrificed after 28 days of implantation.
Data are expressed as mean ± SEM with number of animals in parentheses.

Proliferative activity of PC-82 tumor cells, as detected by BrdU incorporation, in the different androgen substitution groups has been summarized in Figure 5. The percentage of BrdU incorporating cells did not differ between castrated controls, DHEA treated animals or low-dose A substituted mice. The S-phase fraction in tumors from mice supplemented with 100 %T corresponded with the observed tumor growth and an intermediate BrdU index was observed in tumors from mice supplemented with high levels of A. The BrdU index of the individual mice correlated significantly with tumor growth ($r=0.84$, $n=39$, $P<0.001$), as also has been reported previously study [29].

Discussion

Plasma levels of DHEA comparable to those observed in prostate cancer patients (7 nmol/l, [30]), did not result in intraprostatic levels of T and DHT which stimulated PC-82 prostate tumor growth. Low levels of A comparable to those observed in androgen ablated prostate cancer patients (3 nmol/l [30]), did not induce regrowth of the tumor, although a further decline in tumor volume as observed in

castrated and DHEA substituted mice was prevented. This level of A resulted in plasma T levels of 1 nmol/l, which has previously been identified as the threshold level for growth stimulation of the PC-82 tumor [16]. In contrast, A levels which were significantly higher induced intratumor T and DHT concentrations similar to those found in PC-82 tumor tissue of T-substituted animals; regrowth and proliferative activity of the tumor tissue were stimulated accordingly.

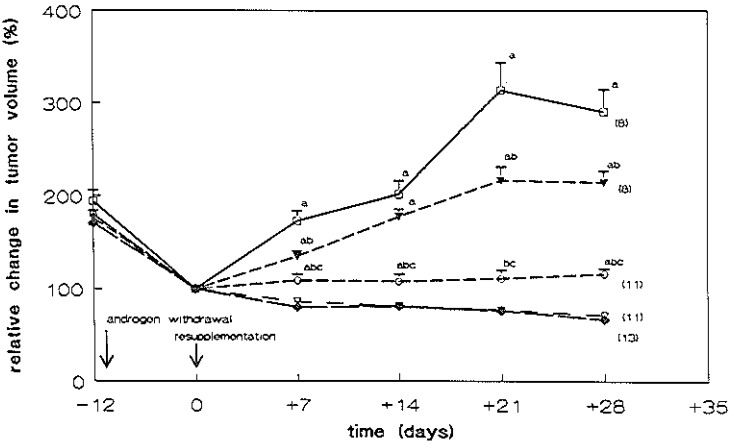

Figure 1. Regrowth of the PC-82 tumor after supplementation of androgen depleted mice with DHEA, A or T for 28 days.

◆= 0%T; ▽= DHEA; ○= 25%A; ▼= 100%A; □= 100%T.

a) significantly different from 0%T ($P<0.05$), b) significantly different from 100%T ($P<0.05$), c) significantly different from 100%A ($P<0.05$).

In contrast, Labrie and coworkers reported that in the rat model levels of DHEA similar to those used in the present study, i.e. 10 nmol/l, induced a 2 fold increase in weight of the ventral prostate in castrated rats, together with a significant increase in intraprostatic DHT content (up to 9 pmol/g tissue) [31]. Lower levels of DHEA were stimulatory for the rat ventral prostate as well [32]. Levels of A similar to those used in the present study, i.e. 2.5 nmol/l, were shown to increase both rat ventral prostate weight (4 fold) and prostate DHT content (up to 13.5 pmol/g tissue)

[31]. Even levels of A of 1.5 nmol/l stimulated growth of the rat ventral prostate [32]. On the other hand, Schiller et al. [33] reported that a mixture of adrenal androgens (A, DHEA, 11β-hydroxyandrostenedione plus DHEA-sulphate) supplemented to castrated rats did not result in regrowth of the prostate and the seminal vesicles. These authors showed, however, that growth of the androgen responsive Dunning rat prostate tumor R3327 was significantly increased by the combination of these androgens as compared to non-supplemented male rats [33].

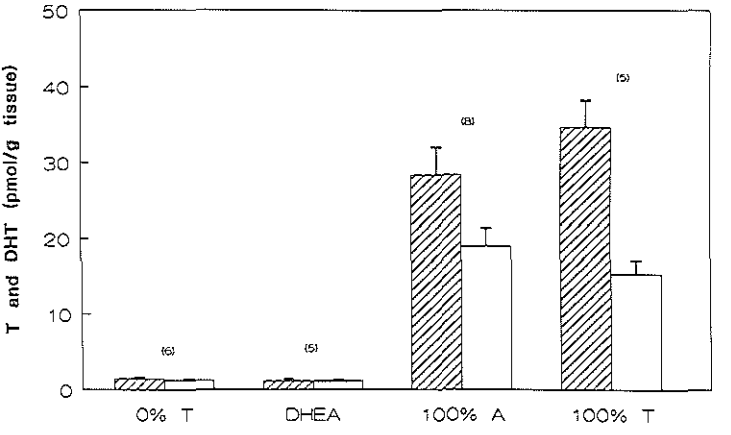

Figure 2. Intratumor levels of T (hatched bars) and DHT (blank bars) after 30 days of androgen supplementation of PC-82 tumor-bearing nude mice.

The present study showed that DHEA supplementation did not induce intratumor DHT concentrations exceeding the levels observed in castrated controls. These results are confirmed by studies with infusion of BPH patients showing that only small amounts of radiolabeled DHEA were recovered as DHT within the prostate tissue (<7%) [34]. In contrast, in vivo infusion of rats with radiolabeled DHEA resulted in a 61% recovery of radiolabel in DHT present in prostatic nuclei [35]. In vitro incubation of human BPH tissue with DHEA revealed a limited conversion of DHEA into DHT of less than 7%, whereas no DHEA metabolism could be observed in incubated rat prostate cells [34,35]. These observations were supported by the metabolism studies of Voigt and Bartsch [36], who did not detect any interconversion of DHEA into one of the active androgens T or DHT in human BPH tissue.

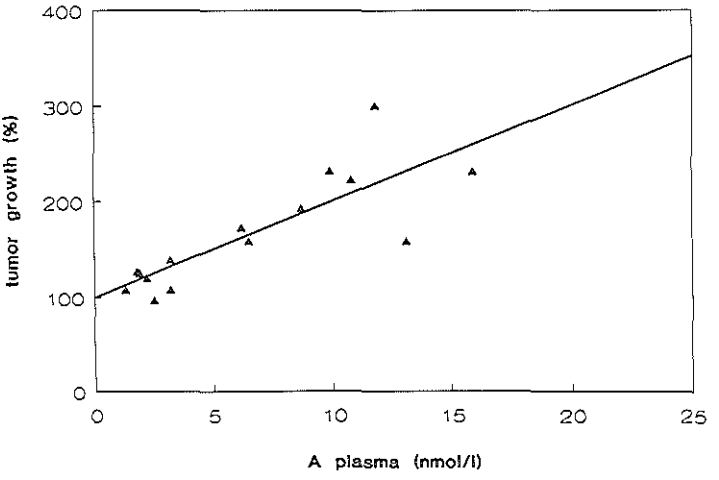

Figure 3. Relationship between plasma levels of A and relative PC-82 tumor growth in PC-82 tumor-bearing nude mice after 28 days of A supplementation (correlation coefficient $r=0.82$, $n=15$, $P<0.001$).

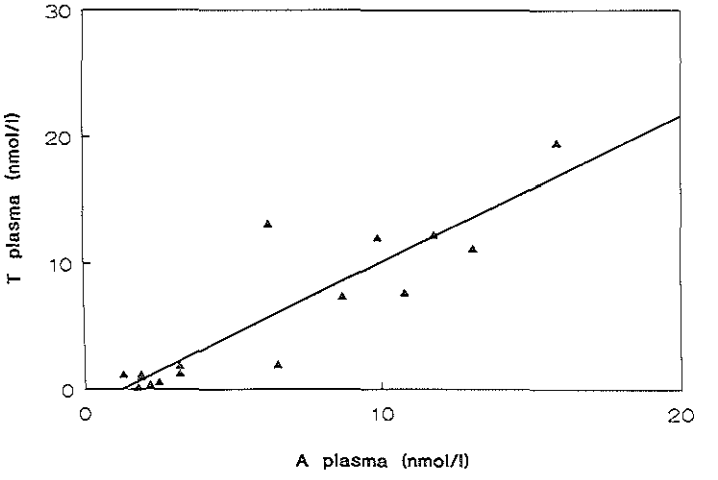

Figure 4. Relationship between plasma levels of A and T in PC-82 tumor-bearing mice after 28 days of A supplementation (correlation coefficient $r=0.90$, $n=16$, $P<0.001$).

A high correlation between plasma levels of A and PC-82 tumor growth (r=0.82, Figure 3) was found in the present study. The question was raised whether A was converted peripherally or locally into T and DHT. Infusion studies with radiolabeled A in the rat showed a recovery of 70% of radiolabel into DHT present in nuclei of the rat prostate [35], whereas only 2-16% of infused radiolabeled A was recovered as DHT in human BPH tissue [34]. In vitro incubation of human prostate tissue and of rat prostate homogenates showed that in both tissues only 3-5% of A was converted into T and DHT [34,35], although 17β-hydroxysteroid dehydrogenase, the enzyme responsible for the conversion of A into T, was demonstrated to be present in the epithelial cells of human BPH tissue [36].

These data indicate that the conversion rates of A into T in vivo are mainly due to peripheral conversion rather than that they reflect the capacity of human and rat prostate tissue to convert A into more active androgens. This is further supported by our observation that plasma levels of A in the nude mouse significantly correlated with plasma levels of T (r=0.90, Figure 4). A similar correlation for plasma A and T (r=0.89) was found in patients treated with aminoglutethimide for up to 3 months [37].

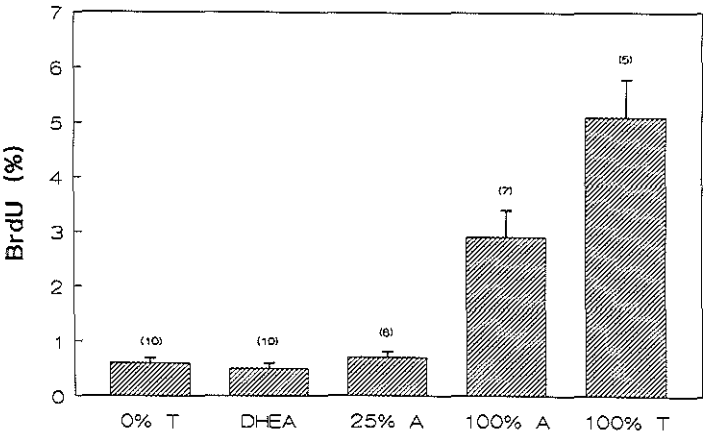

Figure 5. Percentage of BrdU positive cells (S-phase) of PC-82 tumor tissue after 30 days of androgen supplementation.

The inability of the adrenal androgens to induce growth of the regressing rat prostate, observed in the study of Schiller et al. as discussed above [33], is in contrast with the results obtained by Labrie et al. [31,32]. In his experimental

protocol male rats were supplemented with adrenal androgens starting at the time of castration. In this way remaining high concentrations of androgens were likely to be present in the prostate during the first days of treatment, which may in part account for the stimulatory effects reported [24].

Therefore, in the present study a short androgen depletion period was introduced in the experimental protocol to avoid interference of the present concentrations of androgens with the resubstituted levels of adrenal androgens. Moreover, differences in the effects of the adrenal androgens on prostate (tumor) growth may be explained by a species specific difference in the metabolism of androgens. The conversion rate of DHEA to A and T in the plasma of rats was shown to be much higher than that observed in men [34,35] and mice (Table 1). This appears also to be the case for A [34,35]. Although peripheral conversion of A was demonstrated in the mouse (Table 1), the stimulatory effect of A on the rat ventral prostate appeared to be higher. The differences in responsiveness of prostate tissue to adrenal androgens might be explained by a higher sensitivity of the rat prostate for androgen stimulation than of human prostate tumor tissue. In this respect the rat prostate may not be an accurate model to mimic the human situation.

It has been suggested that orchiectomy, although inducing a rapid fall in plasma T in most subjects, does not suppress plasma T to castrate levels in all patients. Remaining high levels of T in some patients originated from the increase in plasma levels of A observed after castration of these men. The adrenal origin of the high levels of T was confirmed by the reduction of both A and T after adrenal suppression with dexamethasone [38]. The suggestion of these authors of a compensatory increase in adrenal androgen secretion after testicular androgen ablation [38] is not supported by other studies [39,40,41]. Moreover, Erikkson & Carlström [42] reported that response to surgical or medical castration could not be correlated to levels of adrenal androgen before or after treatment: A and T levels in non-responders were not significantly higher than the levels observed in responders. Extrapolation of the results of the present study indicates that "normal" postcastration levels of DHEA and A do not stimulate growth of the PC-82 human prostate tumor model. However, higher levels of A may result in plasma levels of T which are stimulatory to the tumor.

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Regulation of androgen receptor expression in the human heterotransplantable prostate carcinoma PC-82

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Submitted

Abstract

In vivo effects of androgen withdrawal and substitution on human androgen receptor (AR) expression were evaluated in the androgen dependent human prostatic carcinoma tumor line PC-82. By application of several antibodies reactive with different epitopes of the AR molecule, AR protein expression was studied in tumor transplants by immunohistochemistry and immunoblotting. AR mRNA levels were quantitated in PC-82 tumor tissue with a S_1-nuclease protection assay. Most PC-82 tumor cells (>97%) from testosterone supplemented mice displayed nuclear AR protein expression immunohistochemically. The almost complete reduction of nuclear AR immunoreactivity after androgen withdrawal (<10%) was restored after androgen substitution within one day. The immunohistochemical data were confirmed by Western blot analysis. In contrast, AR mRNA content of PC-82 cells was not affected by androgen withdrawal. Correlating AR expression with proliferative activity of PC-82 tumor tissue during endocrine manipulation, a rapid castration-induced decline of the percentage of BRdU labeled cells accompanied the loss of AR. Androgen substitution in castrated male mice restored the proliferative activity. However, this increase of proliferative activity lagged at least 24 hrs behind the normalization of the AR protein level. Our data support the concept of AR up-regulation by androgen in contrast to the in other models observed, down-regulation by homologous ligands. Since the AR mRNA levels remained unchanged, expression of the AR in PC-82 is thought to be modulated by translational and/or post-translational mechanisms.

Introduction

Androgens mediate their growth promoting effects on human prostatic cancer cells through their interaction with the human androgen receptor (AR) [1,2]. In both primary and metastatic carcinomas of the prostate the presence of ARs was demonstrated by ligand binding assays, autoradiography and immunohistochemistry [3-7]. The AR, like the other steroid hormone receptors, is a ligand-responsive transcription factor [8]. The genomic organization, primary stucture, mechanistic and regulatory aspects of the steroid hormone receptors, and of the AR in particular, have been reviewed recently [9,10,11]. The AR protein is organized in domains involved in transcription regulation, DNA binding and steroid binding. Ligand binding to the receptor transforms the hormone-receptor complex to a DNA binding state. This activated receptor modulates gene expression resulting in the synthesis of proteins involved in the regulation of cell proliferation and differentiation [11,12].

Reports have been published on the autoregulation of the AR mRNA and AR protein in rodent prostate, other genital organs of the rat, the human prostatic carcinoma cell line LNCaP and human hepatoma cells [13-17]. In some studies androgens were reported to exert a negative influence on AR expression, whereas other studies showed the contrary.

To study autoregulation of AR expression in vivo we used the well- characterized human heterotransplantable prostatic carcinoma PC-82, derived from a primary human prostatic adenocarcinoma [18]. PC-82 shares its histological features, its androgen dependent growth and the secretion of prostate specific glycoproteins with clinical prostatic carcinoma [19,20]. The PC-82 tumor, maintained by serial transplantation in athymic nude mice, slowly regresses in the absence of androgen. Until now, androgen independent growth did never occur in any of the passages of this transplantable prostate carcinoma. Previous studies from our laboratory have shown that PC-82 tissue contains AR mRNA and protein [17,21]. This model allows the simple manipulation of serum and tissue androgen levels by subcutaneous implantation and removal of androgen containing Silastic implants. PC-82 tumor specimens derived from androgen deprived and resubstituted mice were analysed for AR protein content and AR mRNA. Androgen dependent proliferation was evaluated simultaneously.

Materials and Methods

Hormonal manipulation of PC-82

Small fragments of PC-82 tumor tissue were subcutaneously transplanted into athymic nude mice. Male mice were supplemented with testosterone (T) to obtain an

optimal tumor take (80-85%) and tumor growth. Hormonal substitution was achieved by implanting silastic tubings (Talas, Zwolle, The Netherlands) filled with crystalline steroid (Steraloids, Pawling, NY, U.S.A.) [22]. Mild ether anesthesia was used for tumor and steroid implantation. Mice with exponentially growing tumors were deprived of androgens by castration and removal of the T-implants approximately 80 days after tumor inoculation. Castration was carried out via the scrotal route under total anesthesia with tribromoethanol (Aldrich, Beerse, Belgium). Mice which were deprived of androgens for 5 days were subsequently resubstituted with T by reimplantation of T-containing devices for 1 and 3 days (Figure 1). One hour prior to sacrifice the mice were injected i.p. with bromodeoxyuridine (BrdU) (10 mg/kg). After sacrifice, plasma and tumor tissue were collected. Plasma samples were analyzed for T according to Verjans et al. [23].

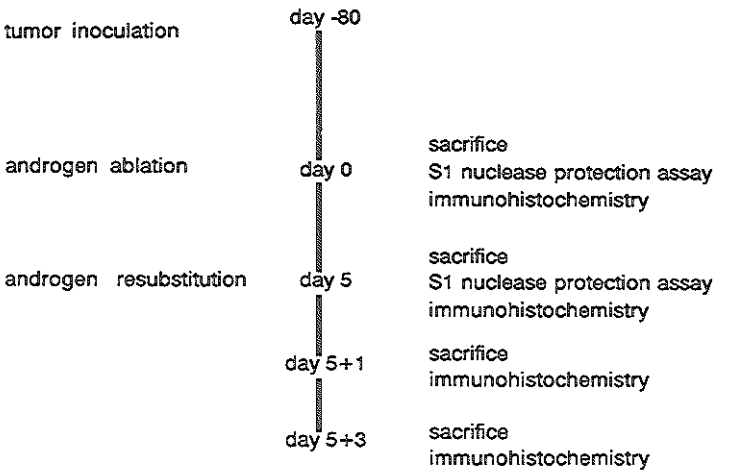

Figure 1. Experimental protocol of endocrine manipulation of PC-82 tumor grown in nude mice.

Antibodies

Monoclonal and polyclonal anti-AR antibodies were prepared and characterized as described previously [24,25]. Conjugates of synthetic peptides corresponding to the amino acid residues 301-322 (Sp061), amino acid residues 593-612 (Sp063) of the DNA binding domain and amino acid residues 892-910 (Sp066) of the steroid binding domain were used for immunization of BALB/c mice (Sp061 and Sp063) or rabbits (Sp061 and Sp066). The specificity of the monoclonal antibody raised against an epitope in the N-

terminal domain, designated F39.4 (Sp061), was demonstrated in sucrose gradient density sedimentation analysis, in immunoprecipitation assays and in Western blots. Moreover, F39.4 is an appropriate antibody for immunohistochemical applications [25,26]. The monoclonal antibody F52.24.4 (Sp063) recognizes the AR, the glucocorticoid receptor and to a much lesser extent the estrogen- and progesterone receptor (Veldscholte: unpublished results). Despite observed crossreactivity, this monoclonal antibody appeared to be a suitable first step reagent for immunoprecipitation of the AR.

The developed polyclonal antibodies against the C-terminal domain (Sp066) recognized the 110-112 kDa AR on Western immunoblots, but they were unable to interact with the native AR in solution complexed with radioactive ligand.

Monoclonal antibody Ki-67 (Dakopatts, Glostrup, Denmark) defines a cell cycle associated nuclear antigen, which is present during most of the G_1-, S-, G_2- and M-phase of the cell cycle [27]. Additionally, the S-phase fraction of PC-82 cells was visualized by the application of an anti-BrdU monoclonal antibody (Becton & Dickinson, Mountain View, California, U.S.A.) [28].

Immunohistochemical techniques

Immunohistochemistry for detection of the AR was performed on cryostat sections as described previously [26]. Immunoreactivity was visualized with the peroxidase anti-peroxidase complex procedure, followed by incubation with 3,3'-diaminobenzidine (DAB) (Sigma, St Louis, MO, U.S.A.).

The monoclonal anti-BrdU antibody was applied on frozen sections after a preincubation in 2N HCl for 30 min at 37 °C and two wash steps in 0.1 M borax buffer (pH 8.5) and PBS, respectively. Immunostaining for Ki-67 and BrdU incorporation was visualized in cryostat sections with a peroxidase conjugated rabbit anti-mouse immunoglobulin as a secondary reagent and DAB [28,29].

AR immunoreactivity, as revealed by monoclonal antibody F39.4, was objectivated by counting three random clusters of hundred cells using a Zeiss bright field microscope, magnification 400, provided with ocular grid. The percentage of BrdU and Ki-67 positive PC-82 tumor cells was determined by counting 500 cells.

Preparation of cytosol and nuclear extract

100 mg of tumor tissue was homogenized in 300 μl of buffer A (40 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10% (v/v) glycerol, 10 mM dithiotreitol, 0.6 mM phenyl-methyl-sulfonyl fluoride (PMSF), 0.5 mM bacitracin and 0.5 mM leupeptin) at 4 °C. The homogenate was centrifugated for 10 min at 12000xg. The supernatant (cytosol) was stored at -80 °C until use. The pellet was washed with buffer A containing 0.2% (v/v) Triton X-100 and then with buffer A without additions. Subsequently, the pellet was extracted with buffer A (pH 8.5) containing 0.5 M NaCl for 1 h at 4 °C. The

extract was centrifugated at 105,000xg for 30 min. The supernatant (nuclear extract) was stored at -80 °C until use.

Immunoprecipitation, SDS-polyacrylamide gel electrophoresis, Western blotting and protein detection with chemiluminescence

ARs from cytosol and nuclear extracts (250 μl samples) of PC-82 tumor tissue and LNCaP control cells (75 μl samples) were immunoprecipitated with the monoclonal antibody F52.24.4 complexed to goat-anti-mouse IgG-agarose as described previously for F39.4.1 [30]. The agarose-antibody-receptor pellets were mixed with SDS-sample buffer, boiled for 2 min and subjected to SDS-polyacrylamide gel electrophoresis on 7% SDS-PAGE-gels. Electrophoresis conditions and the subsequent blotting procedure have been described in detail previously [30]. After blotting, the nitrocellulose paper was incubated with the polyclonal AR specific antibody Sp066, diluted 1:1000 in PBS/0.1% (v/v) Tween 20 (PBS/Tween) for 1 h at room temperature, washed 4 times for 10 min and incubated subsequently for another 60 min with alkaline phosphatase-conjugated goat-anti-rabbit IgG (Sigma, St Louis, MO, U.S.A.) diluted 1:2000 and washed 4 times. The paper was pre-washed with assay buffer (0.05 M Na_2CO_3, 1 mM $MgCl_2.6H_2O$, pH 9.5) and incubated for 5 min with blocking buffer (0.2% I-Block Reagent in PBS/ Tween; according to the manifacturer: Tropix, Inc. Bedford, Mass. U.S.A.), washed 2 times and incubated for 1-2 h with 0.24 mM disodium 3-(4-methoxyspirol(1,2-dioxetane-3,2'-tricyclo-[3.3.1.1^{3,7}]decan-4-yl)phenyl phosphate (AMPPD) (Tropix, Inc.) in assay buffer containing Sapphire enhancer (Tropix, Inc.). Excess substrate solution was removed from the paper and the nitrocellulose filter was exposed to Hyperfilm MP (Amersham, UK).

RNA isolation and S_1-nuclease protection

Total cellular RNA was isolated from PC-82 tumors by the guanidinium thiocyanate method [31]. For S_1-nuclease protection experiments, 50 μg of PC-82 RNA was used in combination with the AR probe; 10 μg of PC-82 RNA was used with the human elongation factor 1 alpha (hEF) control probe. Previous S_1-nuclease assays on LNCaP cells cultured in the presence and absence of androgens demonstrated that hEF mRNA levels did not change after androgen manipulation. The AR and hEF probes are schematically depicted in Figure 2. The AR probe consists of a fragment containing two transcription initiation sites of the AR gene. It was generated by amplification using the primer combination G-I [32]. To obtain the hEF probe, a SphI-SphI fragment (469-1161) from clone HEF 1 was subcloned in a pTZ 19 vector (Pharmacia, Uppsala, Sweden) [33]. An anti-sense primer corresponding to positions 650-669 (5'-CACTTGGCTCCAGCATGTTG-3') was then used in combination with the sequencing primer (15 mer) (NEB, Beverly MA, U.S.A.) to generate the hEF probe. Probes were end-labeled using gamma ^{32}P-dATP and T4 polynucleotide kinase (Gibco BRL, Grand

Island NY, U.S.A.) and separated from unincorporated nucleotides using a Sephadex G-50 column. Approximately $1x10^5$ cpm probe was annealed to the RNA in 80% formamide, 40 mM Pipes (pH 6.5), 0.4 M NaCl and 1 mM EDTA overnight at 50 °C (AR) or 42 °C (hEF) in a total volume of 30 μl. S_1-nuclease (Boehringer, Mannheim, F.R.G.) digestions (400 U) were carried out for 1 h at 37 °C and the resulting protected fragments were analysed on a 6% polyacrylamide/ureum gel [34]. Bands were visualized by exposure of the dried gel to Kodak X-AR films for 48 hrs (AR) or 16 hrs (hEF).

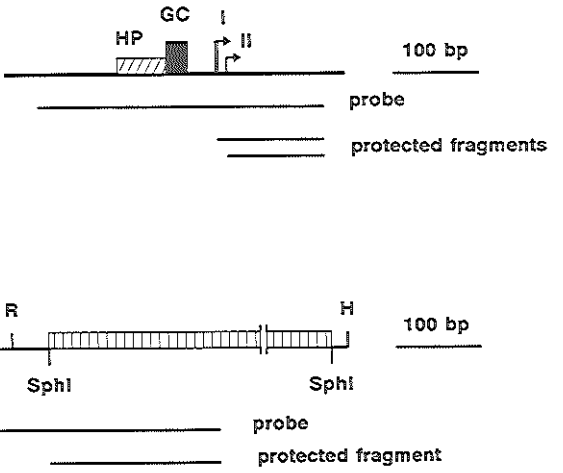

Figure 2. Probes used in S_1-nuclease protection assays:

Top -Shown is the AR gene promoter region. Indicated are the two transcription initiation sites (ARTIS I and II), the position of the homopurine stretch (HP) and the GC-box (GC) with underneath the S_1-probe used and the two expected protected fragments.
Bottom - Shown is the HEF-1 SphI-SphI fragment (striped bar) cloned in pTZ 19 (flanking line) with underneath the S_1-probe used and the expected protected fragment. H: Hind III; R: Eco R1.

Results

Effects of hormonal manipulation on AR immunostaining

To study the effect of androgen manipulation on AR expression in the PC-82 human prostate tumor, this tumor was propagated on nude mice under various different hormonal conditions. The PC-82 tumor, grown in male mice with a T-implant, showed

an uniform nuclear immunoreactivity of moderate intensity with monoclonal antibody F39.4 (Table 1; Figure 3A). No cytoplasmic staining was observed. A small percentage of the PC-82 cells, particularly mitotic cells, did not express the AR. Non-specific immunoreactivity was observed in the stroma aligned epithelial cells. The AR expression in PC-82 tissues of intact male recipients was comparable with immunostaining in T-implanted recipients (Table 1).

Androgen withdrawal from PC-82 bearing mice induced a decline in AR staining of the tumor tissue: percentages of F39.4 reactive cells dropped from approximately 97% in T-implanted male animals to 6% in androgen depleted mice (Table 1; Figure 3A, B).

Repletion of castrated male mice with androgen via a T-implant resulted in a restoration of nuclear AR immunostaining to precastration levels within one day after androgen resubstitution (Table 1; Figure 3C). The staining intensity of tissues at one or three days after androgen resubstitution did not differ (Figure 3C, D). Similar experiments were performed on PC-82 tumor tissue grown in female mice with a T-implant. In female hosts, like in male mice, PC-82 tissue displayed a rapid decrease of AR immunostaining after androgen withdrawal (results not shown).

AR levels in cytosol and nuclear extract

In order to confirm that the observed differences in AR expression under various hormonal conditions, as observed by immunostaining, resulted from variation in AR protein levels, AR expression in PC-82 cells was also studied by a second experimental approach using different antibodies. To this end the AR was immunoprecipitated with the monoclonal antibody F52.24.4.

Legend of Figure 3. on page 127.

Immunohistochemistry of PC-82 tumor tissue for AR (first column: A, B, C, D), Ki-67 (middle column: E, F, G, H) and incorporated BrdU (last column: I, J, K, L). (X 375; nuclear counterstain with haematoxylin). The horizontal rows represent photographs of immunostained PC-82 tumor tissue of intact T-implanted male animals (upper row), of mice depleted of androgens for 5 days (second row), of androgen resubstituted mice for 1 day (third row) and for 5 days (bottom row).

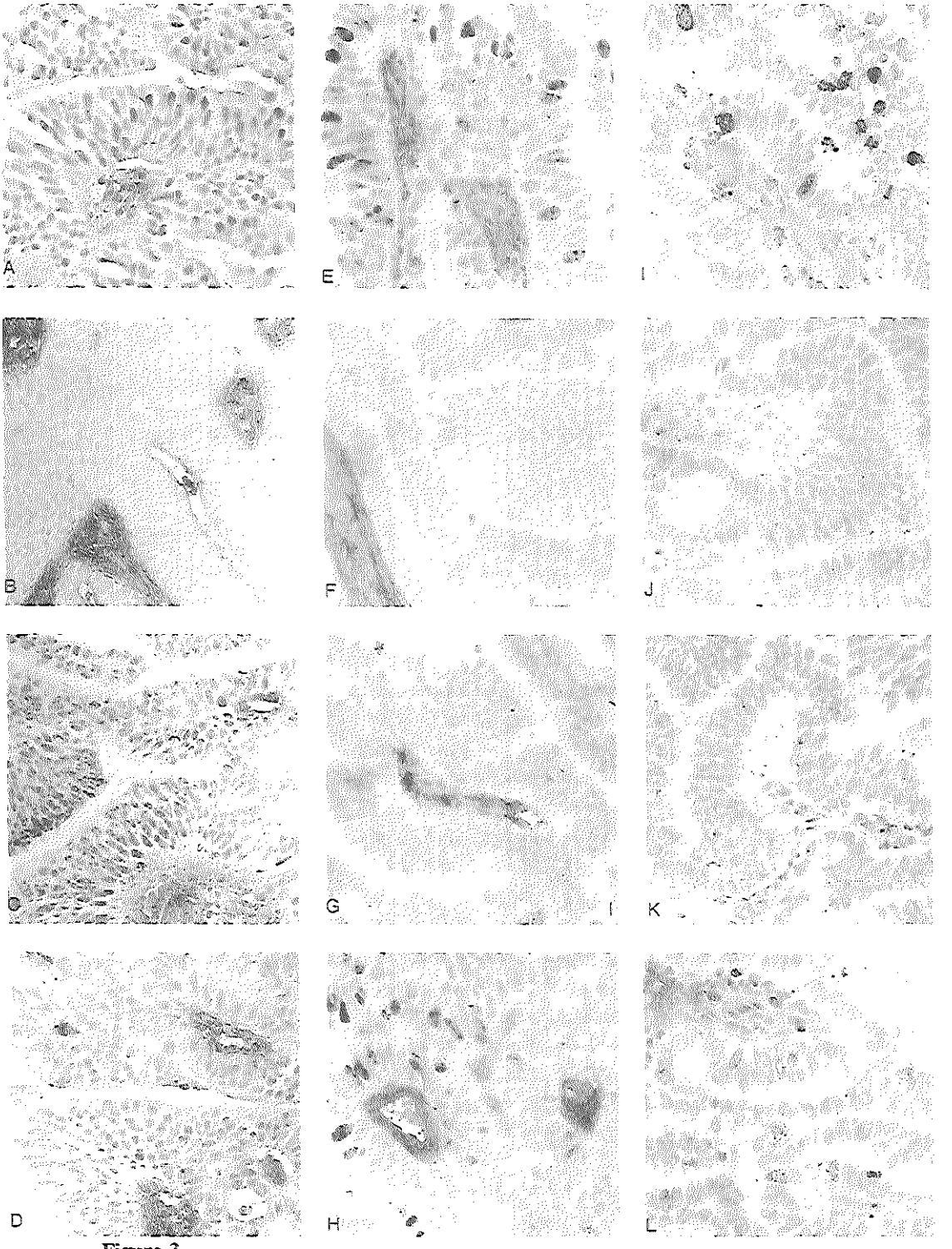

Figure 3.

Table 1. Modulation of androgen receptor expression and proliferation of PC-82 tumor tissue by androgen withdrawal and resubstitution.

Hormonal status of male mice	n	pT (nM)	AR (%)	Ki-67(%)	BrdU (%)
intact	4	ND	97.2 ± 2.1	11.5 ± 1.7	5.5 ± 1.2
castrated + T	6	5.8 ± 1.5	97.5 ± 1.4	14.2 ± 6.1	4.2 ± 1.4
castrated	8	0.4 ± 0.1	6.1 ± 4.0	0.4 ± 0.3	0.5 ± 0.4
castrated + T[2]	5	18.4 ± 4.6	98.4 ± 1.2	0.5 ± 0.2	0.7 ± 0.4
castrated + T[3]	5	14.9 ± 1.5	97.4 ± 1.6	18.0 ± 10.0	5.4 ± 3.1

ND = not done, pT = plasma testosterone
[1] SD, [2] implant for one day, [3] implant for three days.

The isolated receptor complexes were subjected to SDS-PAGE and subsequently to Western immunoblotting with a polyclonal antibody preparation specific for the C-terminal end of the AR (Sp066). The results of these experiments are shown in Figure 4. Lane 5 shows the AR from LNCaP control cells. In the cytosol samples (lanes 1,3) a 110 kDa receptor protein was evident although a considerable degradation to a 50 kDa receptor fragment was observed, despite the presence of several protease inhibitors in the homogenization buffer.

The relative amount of the 110 kDa protein and the 50 kDa degradation product in the cytosol of castrated male mice (lane 1) was low as compared to that in PC-82 tumor tissue from animals with a T-implant (lane 3). The 110 kDa protein (and the 50 kDa band) was absent in the nuclear extracts of castrated animals (lane 2). However, the nuclear fraction of PC-82 carcinoma tissue, grown in T-implanted mice, revealed a considerable amount of the AR protein (lane 4). The protein band at 45 kDa is non-specific staining, caused by the chemiluminescence technique used. Both immunohistochemistry with F39.4 and immunoblotting with the antibodies F52.24.4 and Sp066 indicate a T-induced upregulation of the AR protein, as shown by the restoration of the nuclear AR content after androgen resubstitution.

AR mRNA detection by S_1-protection assay

A S_1-nuclease protection assay was used to study the effect of hormone withdrawal on the level of AR mRNA expression. The results of the S_1-nuclease assay

are displayed in Figure 5. Lane 5 depicts the two protected fragments found when control LNCaP mRNA was analysed. The AR mRNA of PC-82 tumor of castrated nude mice (lanes 3,4) remained at the level found in the tissue of control animals (lanes 1,2)(Figure 5). The presence of two different sized radiolabeled fragments in each of the PC-82 samples analysed for AR mRNA, is due to the probe used. The probe G-BSSH II is partially complementary to sequences of the first exon of the AR gene (Figure 2). Because this AR fragment contains the AR transcription initiation sites, ARTIS 1 and ARTIS 2, two different length AR mRNA fragments were detected by S_1-nuclease protection assay (Figure 2; Figure 5).

The androgen independent hEF mRNA levels did not differ in various samples of PC-82, indicating that equal amounts of PC-82 tumor tissue RNA were analysed (Figure 5).

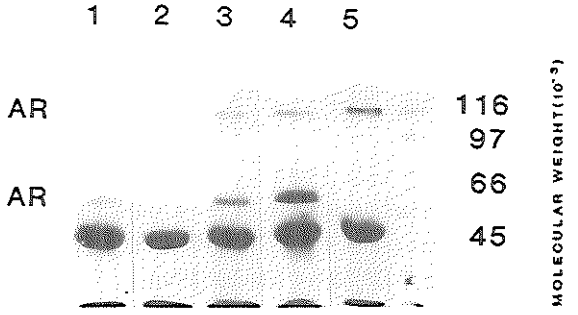

Figure 4. Western blot analysis of cytosol (lanes 1,3) and nuclear fractions (lanes 2, 4) of PC-82 tumor tissue of castrated male mice (lanes 1, 2) and T-implanted males (lanes 3, 4). Lane 5 represents the nuclear extract of LNCaP cells which served as a positive control.

Androgen dependency of proliferation of PC-82

To confirm the presence of a functional AR in the examined PC-82 tissues, the effect of androgen manipulation on the proliferative activity of the tumor tissue was analysed by determination of the percentages of Ki-67 staining and the S-phase fraction by BrdU incorporation. The Ki-67 score, reflecting the number of cycling cells, decreased dramatically after androgen withdrawal from approximately 14% in males with a T-implant to 0.4% in T deprived male mice (Table 1; Figure 3E,F). Interestingly, at the moment AR expression was completely restored, the percentage of cycling cells was still at castration level (Table 1; Figure 3G). Three days after

implantation of T, the mean percentage of Ki-67 positive nuclei increased to the precastration level (18%)(Table 1; Figure 3H).

The BrdU determined proliferation fraction displayed the same time course as the % Ki-67 staining. Almost equal S-phase fractions were observed in precastration and T-resupplemented animals (Table 1; Figure 3I-L).

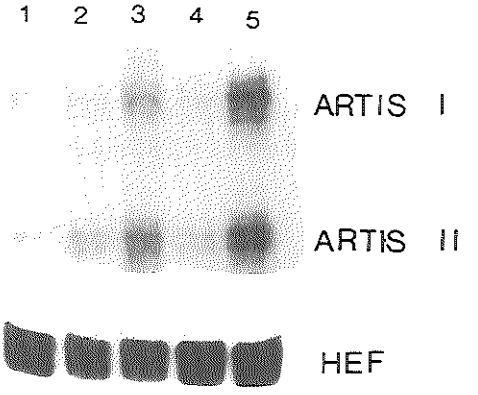

Figure 5. S_1-nuclease protection analysis of PC-82 tumor tissue RNA derived from intact male mice (lanes 1 and 2) and from androgen depleted mice (lanes 3 and 4). Lane 5 is control LNCaP RNA.

Discussion

The availability of AR specific DNA probes and antibodies against the AR [24,26,32, and Veldscholte, unpublished results] has permitted comparative analysis of AR mRNA and receptor protein expression in a well-characterized, strictly androgen dependent human prostatic carcinoma.

In our study, the immunohistochemical staining results of PC-82 tumor tissue with monoclonal antibody F39.4 were in line with immunoprecipitation and Western blot data obtained by application of monoclonal antibody F52.24.4 and polyclonal antibody Sp066. With both methods and different antibodies a decrease in nuclear AR expression after androgen withdrawal and a rapid restoration of AR immunostaining after androgen resubstitution were observed. Comparing intact (non T-implanted) male mice and T-implanted male mice, no significant differences in nuclear immunoreactivity between both groups were revealed by immunohistochemical staining with F39.4.

Our immunohistochemical results are in agreement with a report on the estrogen receptor and the progesterone receptor, stating that steroid receptors reside mainly in

the nuclear compartment independent of the presence of the specific ligand [35]. The AR protein in immunoblots of the PC-82 cytosol fraction, therefore, seems to be due to an extraction artefact and most likely represents a less tightly nuclear bound receptor population.

The observed effects of androgen resubstitution on AR immunostaining in PC-82 tumor samples are in line with AR immunoblotting results of R1881 treated LNCaP cells [13]. The decrease in AR expression of PC-82 at the protein level after androgen depletion is in agreement with some studies of AR expression in rodent prostates as quantitated by saturation and Scatchard analysis of specific ^{3}H-R1881 binding [15,36] or visualized by AR immunohistochemistry [37]. Since both immunohistochemistry and immunoblotting show a diminished AR expression in the PC-82 tumor after androgen withdrawal, we conclude that the decreased receptor expression in this tumor model is due to autoregulation of AR expression and not to a receptor conformation unable to bind the antibody in the absence of ligand nor to technical artefacts, as suggested by Sar et al. [37].

In contrast to our findings, Shan et al. [14] reported a modest increase in the immunohistochemical AR content in rat prostate after castration. These authors attributed the castration induced increase of AR content to a prolonged receptor half life and to an increased rate of receptor synthesis, similarly as has also been described for a hamster ductus deferens smooth muscle tumor cell line [38]. Unfortunately, in their reports no experimental data were provided to substantiate either suggestion.

AR mRNA content of PC-82 was determined in our study by a S_1-nuclease protection assay in order to quantitate AR mRNA of PC-82 tumor cells. The results indicated that in the PC-82 tumor AR mRNA levels were not substantially influenced by androgen withdrawal. This constant AR mRNA expression in PC-82 is in contrast to previously reported results obtained in rodent prostate and in the LNCaP cell line. Northern blot analysis of prostatic and seminal vesicle tissue of castrated rats revealed an increase in AR mRNA content [14]. Furthermore, Quarmby et al. [16] reported in another study on the rat ventral prostate a rapid 4-fold decrease of AR mRNA within 8 h after a single injection of testosterone proprionate. Autologous downregulation of AR mRNA was also observed in LNCaP [16,17]. Using in situ hybridization analysis Takeda et al. reported a 25% reduction of AR mRNA levels in mouse ventral prostate after castration [15]. Like the AR mRNA expression of the PC-82 tumor line the AR mRNA levels of the genital skin fibroblast strain 704 and the osteosarcoma cell line SA-OS did not change in response to androgens [13].

Altogether, our studies suggest that the hormone dependent AR expression in PC-82 tumor tissue is regulated by increased translation rate or by stabilization of the receptor protein. Recent experiments performed by Kemppainen et al. [39] in COS cells transfected with an AR expression plasmid, demonstrated the androgen dependence of AR turnover. In their study, AR degradation half life values were 6 h and 1 h in

presence and absence of androgen, respectively. However, sofar no evidence is available on AR stabilization by hormone under physiological conditions. It is unlikely that phosphorylation plays a role in androgen induced receptor stabilization because glucocorticoid- and progesterone receptor down-regulation have been demonstrated to be independent of phosphorylation [40,41]. Further studies on the kinetics of AR expression after androgen withdrawal and resubstitution are needed to unravel the precise mechanism of AR autoregulation in physiological systems.

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Chapter 10

General discussion

Growth regulation of prostate cancer: experimental results and their clinical implications

Numerous studies have shown that androgen suppressive treatment modalities are similarly effective in achieving a clinical improvement in 60 to 80% of patients with advanced prostate cancer [1]. However, none of the present therapies can cure the patient and, in addition to the 20 to 40% of patients who do not respond to treatment, all patients will ultimately become refractory to androgen ablation [2]. It has been hypothesized that regrowth of prostate tumors is the result of selective outgrowth of pre-existing androgen independent tumor cells [3]. Labrie and associates, however, suggest that the transition of prostate cancer to an androgen independent state involves the selective outgrowth of hypersensitive clones of cells which have adapted to the very low remaining levels of androgens in endocrine treated patients [4]. From this point of view adrenal androgens might be involved in prostate cancer growth.

Several clinical trials have addressed this question by comparing total androgen ablation versus standard hormonal therapy (testicular androgen suppression) [5,6,7]. Still, no clear conclusion can be drawn from these studies as to which treatment is the most beneficial for which patient.

10.1. The importance of human transplantable prostate tumor models

The role of adrenal androgens on prostate cancer growth can be studied effectively using experimental prostate tumor models. The rat (ventral) prostate and the Dunning and Noble transplantable prostate tumors are the models most often used in prostate cancer research (Chapter 2). These model systems have been important for our understanding of the biology of the prostate. However, the rat prostate cannot be directly compared to the human gland, because it does not parallel the human prostate in morphology, biochemistry and response to hormonal manipulation. A recent study has shown that hormonal dependency of the rat prostate appeared to be influenced by the strain and age of the rats, which makes comparison even more difficult [8].

The heterotransplantable human prostate tumors described in this thesis have been shown to be suitable models for studying the effects of endocrine manipulation on human prostate tumor tissue in a physiological situation. Although many aspects of the androgen dependent human prostate tumor models PC-82 and PC-EW indicate a close similarity with the human situation, there are a few limitations of the model. The PC-82 and PC-EW tumors do not show the relapse phenomenon and they do not metastasize, both features which are commonly observed in clinical prostate cancer.

On the one hand, the absence of hormone independent growth, resulting in

tumor relapse after hormone withdrawal, limits the use of these models in the study of mechanisms involved in tumor progression. On the other hand, the homogeneous population of hormone-responsive cells of these two tumors potentiates their applicability to study androgen-regulated processes in prostate cancer tissue. The lack of metastases in the mouse is most probably due to the fact that these tumors grow subcutaneously in athymic nude mice and that there is a substantial activity of natural killer cells in these animals [9].

In addition, the endocrine system of the mouse and rat differ in some important aspects from that of man. The adrenal glands of mice and rats do not synthesize androgens (Chapter 6), whereas relatively large amounts of androstenedione (A) and dehydroepiandrosterone (DHEA) are secreted by the human adrenal gland [10]. Castration of mice and rats, therefore, can be regarded as a total ablation of androgens. For this reason, studies in which the Dunning rat prostate tumor model was used to compare testicular androgen suppression with total androgen withdrawal are of no relevance [11,12]. The role of adrenal androgens in these experimental models can, however, be studied effectively by supplementing these androgens in the animals by implanting androgen-filled Silastic devices (Chapter 8).

The mouse does not have detectable levels of sex hormone binding globulin and consequently the amount of free steroids circulating in mice is much higher than in man [13]. Thus, plasma androgen levels in mice cannot be directly related to the human situation and, therefore, androgen levels in (human) tumor tissue grown in nude mice resemble the clinical situation more closely than the levels of androgens circulating in the mouse. In spite of the limitations of the PC-82 and PC-EW tumor models and the endocrine differences between mouse and man, the heterotransplantable tumor models allow the study of human prostate tumor tissue in this experimental system.

Primary prostate tumors and lymph node lesions of untreated human prostate cancer patients were found to maintain normal levels of testosterone (T) and 5α-dihydrotestosterone (DHT) as compared to the normal prostate, despite reduced activities of the enzymes 17β-hydroxy-steroid dehydrogenase (conversion of A into T) and 5α-reductase [14]. Although the PC-82 (derived from a primary tumor) and PC-EW (derived from a lymph node metastasis) tumor models do not contain detectable levels of 5α-reductase [15], intratumor concentrations of T and DHT were within the range of those observed in untreated prostate cancer patients (Chapter 3). The capacity of human prostate tumor tissue to accumulate DHT indicates the presence of a functionally intact androgen receptor system. This also applies to the PC-82 and PC-EW tumor models (Chapters 3 and 9). As in the clinical situation, androgen depletion of PC-82 and PC-EW tumor-bearing mice resulted in a reduction of intratumor concentrations of androgens, and consequently in regression of tumor

tissue (Chapter 3). The changes in tumor androgen levels are comparable to those observed in endocrine treated patients, which further substantiates the applicability of these tumor models.

10.2. Experimental results and clinical implications

Studies by Geller et al. [16] and Bélanger et al. [17] have shown that surgical or medical castration of prostate cancer patients did not always result in a decline of tissue DHT to castrate levels. Geller et al. [18] observed in seven out of 38 endocrine-treated patients intraprostatic DHT levels exceeding those determined in non-androgenic target tissues. Whether such levels of DHT are of significance for prostate tumor growth has been investigated in the human PC-82 and PC-EW prostate tumor models. Hormonal titration studies revealed that there is a critical level of androgen for growth stimulation of PC-82 and PC-EW tumors (Chapters 5 and 7). PC-82 and PC-EW tumors only grew when plasma levels of T exceeded 1 nmol/l. In these tumors levels of DHT reached 3 to 4 pmol/g tissue. It was shown that DHT levels below 3 pmol/g tissue did not support tumor growth and tumor regression was observed.

Despite the considerable differences between the PC-82 and PC-EW tumor models with respect to intratumor DHT concentrations and, more remarkably, to the rate of the castration-induced tumor regression (Chapter 3), both tumors showed a similar critical androgen level for growth stimulation. More important, this threshold level of DHT exceeded the concentrations of DHT found in most prostate cancer patients after endocrine treatment. However, in some patients unusually high post-castration concentrations of tissue DHT were observed, implying that such levels might be capable of activating prostate tumor growth.

After testicular ablation, adrenal androgens remain in circulation in men. Studies have been made in the PC-82 tumor to determine whether the human physiological levels of A and DHEA are high enough to achieve a level of DHT that exceeds the threshold level for tumor growth stimulation. Levels of A and DHEA comparable to those normally observed in prostate cancer patients were increased in PC-82 tumor-bearing mice (Chapters 7 and 8). DHEA substitution did not result in intratumor levels of androgens which exceeded the levels of castrated controls. In contrast, substituting relatively high levels of A was clearly shown to stimulate PC-82 tumor growth. Levels of A which more closely resembled those observed in endocrine-treated prostate cancer patients [19] clearly arrested tumor regression but did not stimulate tumor growth. The correlation between levels of A and T in the plasma indicated that the conversion of A into T is most likely to occur peripherally and not within the prostatic tissue of the PC-82 tumor (Chapter 8). In conclusion,

high levels of DHT in tumor tissue after castrating patients with prostate cancer may be caused by high plasma levels of A.

The finding of a threshold level of androgen for the stimulation of prostate (tumor) growth is supported by studies of the rat ventral prostate [20,21]. The strong stimulation of the rat ventral prostate by both A and DHEA as reported by Labrie and his associates [22,23] disagrees with the results obtained in our studies. Their observations also do not explain why men castrated at puberty, but with normally functioning adrenal glands, have vestigial prostates, indicating that the adrenal androgens are not capable of maintaining normal functioning of the prostate gland [24]. Stimulation of the ventral prostate by A and DHEA in castrated rats described by Labrie et al. also fails to explain the initial decline in prostate tumor volume observed in 50 to 60% of prostate cancer patients [25].

A large prospective epidemiological study recently showed that A was the only factor associated with an increased risk of developing prostate cancer [26]. It might be suggested that a genetic polymorphism of adrenal androgen secretion exists between normal men which results in the apparent differences in responsiveness to testicular androgen ablation. If in some men secretion and/or conversion of A into the active androgens is increased by constitutional factors then this could result in levels of T high enough to sustain androgen activation after testicular androgen deprivation. It would be of interest to know if subgroups of patients having relatively high levels of intratumor DHT, or those who have progression of tumor growth after gonadal ablation, have correspondingly high levels of A and T. Such data are, however, scarce. Carlström et al. [27] attempted to correlate treatment responses of orchiectomized or estrogen-treated patients with pretreatment plasma levels of T and A. No differences in plasma androgen levels could be found between responders and non-responders, however.

The fact that initial response rates as well as the overall survival rate between groups of patients receiving standard hormonal therapy and a total androgen blockade are no different in most randomized studies, suggests that the subgroup of patients having relatively high remaining androgen levels, and which will thus benefit from the addition of an antiandrogen to the treatment, is small [28]. Indeed, only a limited number of patients who have relapsed from initial endocrine therapy respond to second line treatment by an adrenal androgen blockade through flutamide, aminoglutethimide or ketoconazole [29,30]. These treatment modalities generally resulted in an objective response in 10 to 30% of hormone refractory patients [31,32]. However, the significant toxicity reported for these drugs needs to be weighted against the (objective or subjective) improvement of the individual patient. Nevertheless, the relief of (subjective) symptoms should also be considered an important aspect of treatment in an attempt to improve the quality of life of these patients [29].

This group of patients who might have increased adrenal androgen secretion may not be large enough to show a (statistically significant) difference in survival between the treatment arms of these clinical trials. A meta-analysis of a large number of randomized clinical trials comparing total androgen blockade with standard hormonal therapy is in progress and is especially relevant in determinating the subgroup(s) of patients who will benefit most from combination therapy [33]. Plasma levels of A and T and their ratio should be considered as important indicators for selecting patients with increased secretion of A and who, consequently, are most likely to benefit from the combination treatment.

It can be concluded that adrenal androgen suppression might be necessary in a subgroup of patients, but it has been definitely proven to be of no benefit to most patients. Total androgen blockade has never been shown to prevent tumor progression, so the need to develop other treatment modalities for the control of hormone-refractory prostate cancer remains.

10.3. Future perspectives for the treatment of prostate cancer

Growth of the prostate and of prostate cancer is thought to be predominantly regulated by DHT and not by T [34]. Inhibitors of the enzyme 5α-reductase may become important since they block DHT formation from both testicular and adrenal androgens [35]. Finasteride (MK-906), has been shown to reduce DHT concentrations in human BPH tissue to below levels found after castration and thus the compound may also be effective in controlling malignant growth of the prostate [36]. However, a simultaneous rise in tissue T of approximately ten times the baseline level was observed in the study of BPH patients. The significance of these high levels of T for inducing a residual stimulatory effect on prostate tumor cells has yet to be determined. Studies with finasteride revealed that the difference in dissociation rate of the hormone-receptor complex favors the binding of DHT above that of T, even when DHT formation is minimal [37]. It has been reported that high concentrations of T can compensate for the weaker interaction of T with the androgen receptor when compared to DHT [37]. 5α-Reductase activity itself was found to be controlled by DHT: the activity of the enzyme appeared to be modulated by DHT resulting in an increased formation of DHT [38]. In endocrine-treated prostate cancer patients with a high secretion of adrenal androgens, DHT levels above the normal castrate level might induce this suggested "feed forward" mechanism.

As monotherapy for prostate cancer, the 5α-reductase inhibitors do not seem to be indicated for control of malignant prostate growth. A combination therapy with non-steroidal antiandrogens might be a better approach for an optimal treatment

result [39].

Androgens are thought to have both a stimulatory and an inhibitory effect on prostate (tumor) cells [40]. Androgen deprivation of prostate (tumor) tissue results in a decline in cell proliferation and in activation of programmed cell death (apoptosis) [41]. The limited capacity of the PC-EW tumor to be restimulated by androgens after prolonged androgen depletion might be related to the massive and rapid necrosis of regressing PC-EW tumors. In contrast, regression of PC-82 tumor tissue, like the normal prostate gland, was shown to be associated with apoptosis (Chapter 3, [42]). It is suggested that the process of apoptosis and the absence of necrosis might result in the continued presence of remaining viable cells. This may be a factor which contributes to the limited therapeutic efficacy of the current treatment modalities to definitely cure prostate cancer [43].

The androgen receptor of PC-82 tumor cells seems to be modulated by androgens via (post)translational mechanisms, without the transcriptional process being affected by androgen manipulation (Chapter 9). The androgen receptor remains transcriptionally active after androgen depletion without the protein being expressed. In contrast, the presence of a functional androgen receptor was observed in 13 out of 17 specimens of treatment-resistent androgen deprived prostate cancer patients which showed androgen receptor expression in 80% of the cells [44]. This would suggest that in these patients the androgen receptor is stabilized by either remaining levels of androgens, or by other factors. The absence of the androgen receptor in hormone independent human tumor lines was found to be related to diminished levels of androgen receptor mRNA [45], although genetic alterations of the gene, however, were not demonstrated in these tumor lines [46]. Loss of androgen receptor expression may thus be regarded as a consequence rather than a cause of androgen insensitivity, which appears to be a late event in the process of prostate tumor progression. This is in contrast to suggestions concerning the progressive changes occurring in the Dunning rat prostate tumor system [47].

The androgen receptor, although present in most but not all human prostate tumors, may not be activated but may still be present while it is bypassed by an androgen receptor independent pathway [48]. In such a concept, both androgen receptor mediated and androgen receptor independent mechanisms are thought to be present in the same cell. Androgen mediated growth factors, such as epidermal growth factor and transforming growth factor α, have been demonstrated in the androgen sensitive human prostate tumor cell line LNCaP [49,50]. These and related growth factors have also been found to be secreted by the androgen insensitive DU-145 cell line [51]. Polypeptide growth factors have been shown to modulate cell growth through paracrine and autocrine pathways. Tumor cells may become androgen unresponsive by constitutively activating paracrine and autocrine growth (factor) pathways which are normally under hormonal control [52]. The combination

of a therapy with antipeptidergic substances, such as suramin, and androgen withdrawal may represent a new and promising strategy for the treatment of prostate cancer [53,54].

In conclusion, our studies of the androgen dependent PC-82 and PC-EW tumor models yielded valuable information on the role of (low levels of) androgens in growth regulation and progression of prostate tumors. The problem of prostate tumor progression and of androgen independent growth cannot be solved by the study of androgen dependent prostate tumor models alone. The search for alternative treatment options for hormone refractory prostate cancer, such as interference with the above-mentioned process of apoptosis or targetting the growth factor pathways with anti-peptidergic compounds, can only be successfull when experimental studies comprise both androgen dependent and androgen independent human tumor models. The availability of the described PC-82 and PC-EW models together with two androgen independent xenograft prostate tumor models, PC-133 and PC-135, in our laboratory is a good starting point for such studies. These investigations may ultimately lead to a better understanding of the development of androgen independent tumor growth, which may lead to a better therapeutical approach to the clinical problem of hormone refractory prostate cancer.

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Summary

This thesis describes experimental studies which were conducted to elucidate the role of low testosterone levels and of adrenal androgens in the growth of human prostate cancer. Two human prostate tumor models, propagated in athymic nude mice, were used: the PC-82 tumor which is derived from a primary prostate adenocarcinoma and the PC-EW tumor originating from a lymph node metastasis of a prostate cancer. The PC-82 and PC-EW tumors are both androgen dependent and contain intact and functional androgen receptors. Intratumor androgen concentrations measured in growing tumors were within the range of the levels reported for untreated human prostate cancer tissue.

Chapter 3 describes the effects of androgen deprivation of tumor-bearing mice on PC-82 and PC-EW tumor tissue. Androgen depletion resulted in a reduction of tissue androgen concentrations of both tumor models comparable to those reported for testosterone ablated patients. The tumor volume was affected accordingly. The limited capacity of the PC-EW tumor to be restimulated by androgens seemed to be associated with massive castration-induced necrosis of these tumors. In contrast, the relatively slow regression of PC-82 tumors appeared to be associated with the process of programmed cell death (apoptosis). Due to remaining viable cells the PC-82 tumor could be restimulated by androgen even after prolonged androgen depletion. It is hypothesized that the limited effectiveness of the endocrine treatment modalities for prostate cancer underly the activation of apoptosis rather than the induction of total elimination of prostate tumor cells as occurs with necrosis.

In Chapter 4 it is demonstrated that proliferative activity of tumor tissue is a good indicator of PC-82 and PC-EW tumor growth besides standard diameter measurements. The proliferative fraction as detected by bromodeoxyuridine (BrdU) incorporation (S-phase cells) or Ki-67 expression (all cycling cells) correlated significantly with growth of the tumor models. In regressing or slowly growing PC-82 and PC-EW tumors, however, both markers were less reliable for different reasons, especially in Ki-67 expression. Nevertheless, restimulation of androgen depleted PC-82 tumor tissue resulted in an immediate and clearly demonstrable induction of Ki-67 expression and BrdU incorporation, as described in Chapter 9, which supports the usefulness of these markers in monitoring the response of prostate tumors to endocrine manipulation.

The hormonal titration experiments as described in Chapters 5 and 7 revealed that the PC-82 and PC-EW tumor models have a threshold level of androgen for growth stimulation. Both models only showed a growth response when intratumor levels of dihydrotestosterone (DHT) exceeded the critical level of 3 to 4 pmol/g tissue. Lower levels of DHT resulted in cessation of tumor growth and tumor regression. This androgen threshold level for tumor growth stimulation amounts to two to three times the levels found in tumors of castrated animals. This level is also significantly higher than levels of DHT found in most endocrine treated patients.

Only in a small subgroup of patients, described in some clinical studies, were relatively high levels of DHT detected, which could induce tumor growth according to the outcome of our studies with the xenograft models.

In Chapter 6 incubation studies of the adrenals of mice and rats demonstrate that the adrenal glands of these animals do not synthesize androgens. This observation was further supported by the absence of significant levels of dehydroepiandrosterone (DHEA) and androstenedione (A) in the plasma of rats and mice. Therefore, A and DHEA were supplemented to PC-82 tumor-bearing mice via Silastic implants, resulting in plasma levels comparable to those observed in endocrine treated prostate cancer patients. As described in Chapters 7 and 8, DHEA substitution resulted in intratumor concentrations of testosterone (T) and DHT similar to those of the castrated controls and, consequently, in tumor regression. High levels of A significantly supported tumor growth, although lower (more physiological) levels of A did not. The high correlation between plasma A and plasma T indicated that peripheral conversion of A into T is the major cause of the stimulatory effect of A. It is concluded that relatively high levels of A may result in plasma concentrations of T which induce intratumor levels of DHT exceeding the critical level for the stimulation of prostate tumor growth. Whether these high levels of A are present in those patients having a relatively high intratumor DHT content is unknown as yet; data on plasma levels of A (and T) of prostate cancer patients before and during hormonal treatment are scarce.

The question of whether androgen manipulation of human prostatic tissue affects the androgen receptor is discussed in Chapter 9. Androgen depletion of PC-82 tumors resulted in a decline in androgen receptor expression, while levels of the messenger for the androgen receptor remained unchanged. Apparently, the absence of androgen receptor expression is due more to destabilization of the protein than to a change in the post-transcriptional process. It appears thus that after androgen deprivation the androgen receptor remains present and in a transcriptionally active state.

The conclusion can be reached that an adrenal androgen blockade may be necessary in a subgroup of prostate cancer patients having relatively high levels of A. The presence of an intact and functional androgen receptor in prostate tumor cells after androgen deprivation strengthens the putative importance of remaining levels of androgens. Nonetheless, ablation of androgens from all sources has not resulted in a significant survival advantage for the overall group of prostate cancer patients. Therefore, new therapies, which must involve targeting at both androgen dependent and androgen independent pathways, need to be explored to increase the effectiveness of the treatment of advanced prostate cancer.

Nederlandse samenvatting

De prostaat behoort tot de mannelijke geslachtsorganen en is gelegen rond de urinebuis aan de basis van de urineblaas. Afwijkende groei van de prostaat doet zich bij de man vooral voor op oudere leeftijd. Naast de goedaardige prostaatvergroting die kan leiden tot vernauwing van de urinebuis, kan kwaadaardige groei (kanker) van de prostaat ontstaan. Prostaatkanker is, na longkanker, de meest voorkomende doodsoorzaak door kanker bij mannen in de Westerse wereld. Gezien de toenemende gemiddelde leeftijd van de bevolking in deze landen, alsmede het feit dat de incidentie van prostaatkanker sterk toeneemt met de leeftijd, is het aantal patienten met prostaatkanker de laatste jaren aanzienlijk gestegen.

Prostaatcarcinomen zijn, evenals de normale prostaat, voor hun ontwikkeling en groei afhankelijk van de aanwezigheid van mannelijke geslachtshormonen (androgenen). Indien de tumor niet meer beperkt is tot de prostaat omdat tumorcellen zijn uitgezaaid naar andere plaatsen in het lichaam (metastasen), kan prostaatkanker behandeld worden door de productie van androgenen T door de testikels (zaadballen) te blokkeren. Dit kan gebeuren door verwijdering van de testikels (castratie) of met behulp van medicijnen die de testosteron (T) productie van de testikels remmen (medische castratie). Deze hormoon-suppressieve behandeling is echter slechts tijdelijk succesvol, want na verloop van tijd ontwikkelt de tumor zich tot een tumor die onafhankelijk van androgenen kan groeien. De behandeling is dan niet meer effectief en andere middelen, zoals chemotherapeutica, moeten aangewend worden om de groei van de tumor te stoppen. Chemotherapie is echter tot op heden weinig doeltreffend gebleken in de behandeling van hormoon-ongevoelige prostaatkanker, en de meeste patienten overlijden korte tijd na progressie van de tumor.

Over het proces van progressie van prostaattumoren naar een hormoon-onafhankelijke groeifase bestaan verschillende hypotheses. Enerzijds wordt de progressie verklaard als zijnde het gevolg van de selectieve uitgroei van reeds aanwezige hormoon-onafhankelijke cellen in de tumor na androgeen-suppressieve behandeling. Anderzijds wordt de androgeen-onafhankelijke groei van prostaattumoren gezien als een gevolg van de ontwikkeling van androgeen-hypersensitieve tumorcellen, die door minimale hoeveelheden androgeen stimuleerbaar zijn. Androgenen afkomstig uit de bijnier, die na (medische) castratie in circulatie blijven, zouden een rol kunnen spelen in de hergroei van prostaattumoren. In dit laatste geval zou een complete suppressie van androgenen, zowel afkomstig van de testikels als ook van de bijnieren, noodzakelijk zijn om tumorprogressie te voorkomen.

Een totale androgeensuppressie kan worden gerealiseerd door het werkingsmechanisme van androgenen te blokkeren met behulp van anti-androgenen die de interactie van androgenen met de cel voorkomen. Vele klinische studies zijn opgezet om het effect van een complete androgeenblokkade (castratie plus een

antiandrogeen) te toetsen aan dat van de standaard behandeling (castratie alleen). Tot op heden zijn de resultaten tegenstrijdig en is "totale androgeenblokkade" als therapie voor de behandeling van prostaatkanker nog steeds een onderwerp van discussie.

Dit proefschrift beschrijft de regulatie van het humaan prostaatcarcinoom door androgenen, met name door lage concentraties T en bijnierandrogenen. Omdat studies naar de effecten van hormonale manipulatie op prostaattumorweefsel niet kunnen worden uitgevoerd in de mens, zijn experimentele modellen nodig die de menselijke situatie zo goed mogelijk weerspiegelen. In het onderzoek zoals hier wordt beschreven, zijn twee heterotransplanteerbare humane tumormodellen gebruikt. Dit zijn modellen waarbij stukjes humaan prostaattumorweefsel onderhuids in de naakte, immuundeficiënte (thymusloze) muis worden geïmplanteerd die vervolgens uitgroeien tot tumoren. Door transplantatie van deze tumoren op andere muizen ontstaat een permanente prostaattumorlijn. Hiermee kan de regulatie van tumorgroei door androgenen worden bestudeerd door de hormonale status van de muis te beïnvloeden. Dit laatste gebeurt door een siliconenslangetje gevuld met hormoon onderhuids bij de muis te implanteren, hetgeen resulteert in constante plasmaspiegels van het gesupplementeerde hormoon.

De twee heterotransplanteerbare humane modellen, die zijn gebruikt in het thans beschreven onderzoek, zijn de PC-82 tumor welke is ontwikkeld uit een primaire prostaattumor, en de PC-EW tumor voortkomende uit een lymfkliermetastase van een prostaatkankerpatient. Beide tumormodellen zijn afhankelijk van androgenen voor hun groei en bevatten androgeenreceptoren. Tevens secreteren zij de prostaatspecifieke eiwitten zure fosfatase en het prostaatspecifieke antigen. Deze eigenschappen zijn vergelijkbaar met die bekend voor het klinisch prostaatcarcinoom. T wordt in de prostaat omgezet in dihydrotestosteron (DHT), dat uiteindelijk verantwoordelijk is voor de activatie van androgeen-afhankelijke processen. Het feit dat in humane tumormodellen groeiende op de muis, androgeenconcentraties worden aangetoond overeenkomstig die in tumorweefsel in patienten, bevestigt de bruikbaarheid van deze prostaattumoren als modellen voor het humaan prostaatcarcinoom.

Groei van onderhuids geïmplanteerde tumoren kan eenvoudig gevolgd worden door de diameter van de groeiende tumor te meten en hieruit het volume te berekenen. De groeicapaciteit van een tumor kan echter ook bepaald worden door een schatting te maken van de celdelingsactiviteit van het tumorweefsel. Tumorcellen die zich in de delingscyclus bevinden, kunnen worden aangetoond met behulp van een antilichaam (Ki-67) gericht tegen een celdelingsgeassocieerd antigeen. De fractie tumorcellen welke DNA synthetiseren, kan worden gedetecteerd met behulp van bromodeoxyuridine (BrdU), een stof die tijdens de DNA synthese in het DNA wordt ingebouwd.

Hoofdstuk 4 laat zien dat beide methoden inderdaad een betrouwbare indicatie geven van prostaattumorgroei, maar bij langzaam groeiende of in regressie zijnde prostaattumoren bleek met name de Ki-67 index niet zo nauwkeurig. Het feit echter dat herstimulatie van PC-82 tumoren met T een directe inductie van zowel de BrdU- als ook de Ki-67-fractie tot gevolg had (hoofdstuk 9), toont aan dat de effecten van endocriene manipulatie op het tumorweefsel snel waarneembaar zijn via deze indicatoren.

De effecten van androgeenonttrekking (castratie) op PC-82 and PC-EW tumoren is beschreven in hoofdstuk 3. Castratie van tumor-dragende muizen resulteerde in een afname van de weefselspiegels van T en DHT in PC-82 en PC-EW tumoren overeenkomstig die waargenomen in prostaattumoren van gecastreerde patienten. Het tumorvolume van de PC-82 en PC-EW tumoren nam af, in overeenstemming met de afname van de androgeenspiegels. PC-82 and PC-EW tumoren vertonen geen androgeen-onafhankelijke groei, maar androgeen-gedepleteerde PC-82 tumoren kunnen wel worden gerestimuleerd door T, zelfs na langdurige perioden van androgeenonttrekking. Daarentegen is hergroei van PC-EW tumoren alleen mogelijk na een beperkte periode van androgeendepletie. Dit zou verband kunnen houden met het feit, dat in PC-EW tumoren na androgeenontrekking necrose optreedt, terwijl in gedepleteerde PC-82 tumoren celdood geassocieerd is met apoptosis (geprogrammeerde celdood). Het feit dat bij deze laatste vorm van celdood de weefselstructuur in belangrijke mate gehandhaafd blijft is mogelijk een factor die een rol speelt in de beperkte effectiviteit van de huidige behandelingswijzen van prostaatkanker.

De relatie tussen plasma- and weefselandrogeenconcentraties enerzijds en tumorgroei anderzijds is beschreven in de hoofdstukken 5 en 7. Uit de daar beschreven experimenten bleek, dat bij een plasma T concentratie van 1 nmol/l, waarbij intratumor DHT spiegels van 3-4 pmol/g weefsel gevonden werden, de grenswaarde bereikt is waarbij geen tumorgroei meer plaatsvindt. Lagere concentraties DHT hadden tumorregressie tot gevolg. Deze drempelwaarde voor stimulatie van tumorgroei was 2 tot 3 maal hoger dan de DHT spiegels in androgeen-gedepleteerde tumoren. Bovendien was de kritische DHT concentratie hoger dan de spiegels welke gevonden worden in gecastreerde patienten. In de literatuur zijn in een subgroep van behandelde patienten relatief zulke hoge DHT spiegels gevonden dat deze, uitgaande van de voor de modellen vastgestelde drempelwaarde, groei van de prostaattumor zouden kunnen stimuleren.

In hoofdstuk 6 zijn experimenten beschreven welke aantonen dat bijnieren van muizen en ratten, in tegenstelling tot de mens, geen androgenen produceren. Aanvullende gegevens betreffende plasmaspiegels van dehydroepiandrosterone (DHEA) en androsteendion (A), ondersteunen dit resultaat. PC-82 tumor-dragende dieren werden daarom gesupplementeerd met A en DHEA via met hormoon gevulde

siliconenslangetjes, welke toediening resulteert in plasma A- en DHEA-spiegels overeenkomstig die waargenomen in endocrien-behandelde prostaatkankerpatienten. In de hoofdstukken 7 en 8 is het effect van deze bijnierandrogenen op de PC-82 tumorgroei beschreven. Substitutie van DHEA had geen effect op de intratumor T en DHT concentraties van PC-82 tumoren. De daarmee gepaard gaande tumorregressie was dan ook gelijk aan die welke werd waargenomen na castratie van PC-82 tumor-dragende muizen. Hoge concentraties A resulteerden in groei van de PC-82 tumor. Fysiologische concentraties A konden echter tumorgroei niet stimuleren, hoewel de tumorregressie minder was dan die van androgeen-gedepleteerde tumoren. De sterke correlatie tussen plasma spiegels van A en T doet vermoeden, dat perifere omzetting van A in T de oorzaak is van het stimulerende effect van A op de PC-82 tumor.

De conclusie is dan ook, dat relatief hoge plasmaconcentraties van A, resulterende in relatief hoge spiegels van T, DHT concentraties in het tumorweefsel kunnen veroorzaken, die de groei van prostaattumoren kunnen stimuleren. Onbekend is vooralsnog of deze hoge concentraties A aanwezig zijn in die patienten waar in het prostaattumorweefsel hoge concentraties DHT werden gedetecteerd.

Androgenen oefenen hun effect uit op de prostaat door binding van DHT aan een specifieke receptor; het zo gevormde complex bindt vervolgens aan het DNA, dat de cel aanzet tot het maken van eiwitten. De androgene regulatie van de androgeenreceptor is bestudeerd in de PC-82 tumor en staat beschreven in hoofdstuk 9. Androgeenonttrekking resulteerde in een afname van de androgeenreceptor expressie in PC-82 tumorweefsel, terwijl de transcriptie-activiteit van de androgeenreceptor onveranderd bleef. Het lijkt waarschijnlijk dat het ontbreken van het eiwit (de androgeenreceptor) het gevolg is van een destabilisatie van het eiwit in een androgeen-gedepleteerde omgeving; dit wordt niet veroorzaakt door een verminderde transcriptie. In androgeen-gedepleteerd prostaattumorweefsel is de androgeenreceptor dus aanwezig en functioneel intact.

De conclusie van het in dit proefschrift beschreven onderzoek is dat "normale" spiegels van bijnierandrogenen geen rol spelen in de groei van prostaattumoren na androgeenonttrekking. Echter, alleen in die groep van patienten welke relatief hoge A concentraties in het bloed hebben, zou een totale blokkade van androgenen noodzakelijk kunnen zijn. Een al dan niet complete onttrekking van androgenen heeft echter nooit geresulteerd in een aanzienlijke verlenging van de overleving van prostaatkankerpatienten. Daarom is voortgezet onderzoek op het gebied van zowel androgeen-afhankelijke als ook androgeen-onafhankelijke processen in prostaattumoren van groot belang. Dit onderzoek kan aangrijpingspunten vormen welke noodzakelijk zijn om de effectiviteit van de behandeling van uitgezaaide prostaatkanker te vergroten.

List of additional papers

Pharmacia Award 1990. The biological significance of low testosterone levels and of adrenal androgens in transplantable prostate cancer lines.

Van Weerden WM, Van Kreuningen A, Moerings EPCM, De Jong FH, Van Steenbrugge GJ, Schröder FH.

Urol Res 19 (1991): 1-5.

Castration-induced changes in androgen levels and proliferative activity of human prostate cancer tissue grown in athymic nude mice.

Van Weerden WM, Van Kreuningen A, Elissen NMJ, De Jong FH, Van Steenbrugge GJ, Schröder FH.

In Fiebig HH and Berger D (eds), "Immunodeficient mice in oncology", Contributions to Oncology, Vol 42. In press.

Curriculum Vitae

Schrijfster dezes werd geboren op 26 november 1961 te Wageningen. Na behalen van het VWO-B diploma in 1980 aan "Het Wagenings Lyceum" begon zij in datzelfde jaar met de studie Humane Voeding aan de Landbouw Hogeschool te Wageningen. Het kandidaatsexamen werd behaald in 1985. Tijdens de doctoraalfase van de studie werd onderzoek verricht naar de metabole activatie van carcinogene stoffen in een cocultivatiesysteem (hoofdvak Toxicologie). Voor het tweede hoofdvak Dierfysiologie werd de neuroendocrine regulatie van prolactine afgifte bestudeerd bij ratten. Een bijvak Humane Voeding resulteerde in een literatuurstudie naar de relatie tussen vitamine A opname en het risico op prostaatkanker. Tevens werd een bevoegdheid tot het geven van onderwijs in de biologie verkregen. De praktijktijd werd vervuld aan de afdeling Humane Voeding van de Universiteit van Helsinki waar gedurende een half jaar onderzoek werd gedaan naar de invloed van zware metalen op de seleniumopname in de darm. Sinds haar afstuderen in november 1987 is zij werkzaam bij de afdeling Urologie van de Erasmus Universiteit Rotterdam. Gedurende deze periode werd het onderzoek verricht van welke de resultaten zijn weergelegd in deze dissertatie.