TRANSPLANTABLE HUMAN PROSTATE CANCER (PC-82) IN ATHYMIC NUDE MICE:

A model for the study of androgen-regulated tumor growth

TRANSPLANTEERBAAR MENSELIJK PROSTAATCARCINOOM (PC-82) IN DE THYMUSLOZE NAAKTE MUIS:

Een model voor de bestudering van androgeen-gereguleerde tumor groei

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Cancer is far more than a mere research object, therefore,
this thesis is dedicated to cancer patients and their families

Ter nagedachtenis van mijn moeder

Aan mijn vader

Voor Marti en Wouter

CONTENTS

LIST OF ABBREVIATIONS

AR	Androgen Receptor
ARn	Nuclear Androgen Receptor
ARnT	"Translocated" Nuclear Androgen Receptor
BPH	Benign Prostatic Hyperplasia
CPA	Cyproterone Acetate
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic Acid
E_2	Estradiol
EORTC	European Organization for Research on the Treatment of Cancer
ER	Estrogen Receptor
ERICA	Estrogen Receptor Immunocytochemical Assay
FSH	Follicle Stimulating Hormone
LH	Luteinizing Hormone
LHRH	Luteinizing Hormone Releasing Hormone
McAb	Monoclonal Antibody
n	Number of observations
NK	Natural Killer
Nu	Nude
PA	Prostatic Antigen
PAP	Prostatic Acid Phosphatase
PC	Prostatic Carcinoma
SD	Standard Deviation
SEM	Standard Error of the Mean
SHBG	Sex Hormone Binding Globulin
T	Testosterone
Td	Tumor Doubling Time
Tv(50)	Latency period of a tumor until a volume of 50 mm^3
v/v	Volume/Volume
U	Units

"The etiology of prostatic cancer is not known at the present time so that discussion of treatment must be based on the pragmatism of experiment. In addition, the paucity of information on the human disease enforces reliance upon animal data with all its attendant pitfalls. There must consequently be considerable speculation and oversimplification in a contribution of this type. It is nevertheless hoped that the concepts presented herein will receive sympathetic consideration and will help stimulate interest in a hitherto untried approach to palliative therapy".

"Start New Opinions of an Old Disease", George Crabbe
Letter VII Professions - Physic, The Borough, 1810

RATIONALE AND SCOPE OF THE THESIS

In most countries of the Western world prostatic carcinoma is the second most frequent cause of cancer death among men. The incidence rate and the age-standardized mortality figure in the Netherlands have been estimated as 25.1 and 15.5 per 100,000 male inhabitants respectively [242]. Patients with prostatic carcinoma seem to be in an exceptional position because, as opposed to other human malignancies, this type of cancer can be treated effectively in the disseminated stage [13]: nearly all men with metastatic prostatic cancer actually respond to androgen ablation therapy. However, the annual death rate of prostatic carcinoma has not decreased during the last 30 years [129,220].

Much clinically oriented research has been carried out in the field of prostatic cancer. These investigations focussed primarily on pathological aspects of this disease, on the development of proper prognostic factors and response criteria, on the subject of endocrine dependence, on the mechanism which underlies the relapse phenomenon i.e. the changes that occur with hormone independent growth of prostatic cancer and, finally, on the development of experimental models for this type of tumor. The outcome of these studies has contributed to a better understanding of the tumor biology of prostate caneer and to a better classification of this disease. However, all the research efforts did not result in a prolongation of the lives of prostate cancer patients. It seems that an important step forward in the management of this disease can only be made if the transition from the hormone-dependent to the hormone-independent state is controlled effectively.

In clinical practice many investigations in the field of prostatic carcinoma are hampered by ethical problems, as is the case for studies of all kind of human malignancies. Therefore, there has always been a great need for suitable model systems reflecting the properties of clinical prostatic cancer [28]. Although much has been learned from the studies of a number of excellent prostatic model systems in the rat, the advantage of studying human tumor tissue *in vivo*, i.e. in an animal model, is recognized.

This thesis describes the development and characterization of an animal model system: the human prostatic adenocarcinoma PC-82 which is transplantable in athymic nude mice. This tumor has been employed as a model to study a number of aspects related to clinical prostatic cancer. The various investigations into the PC-82 tumor, discussed in this thesis, have been focussed mainly on its hormone dependence.

The introductory chapter 1 deals with a number of aspects of the prostate and of prostatic cancer in general and it contains a summary of the current knowledge of androgen action. It also includes a survey of the available models of prostatic cancer with special reference to the cell lines and models developed from human prostatic cancer. Finally, it provides a general introduction to the PC-82 tumor model.

The suitability of steroid-containing Silastic implants for the administration of hormones to nude mice was investigated. The experience with this technique, which had been gained prior to its application to the PC-82 tumor model, is described in chapter 2. Chapter 3 contains data on the endocrine manipulation (e.g. castration) experiments with the PC-82 tumor including the results of preliminary experiments with steroids substituted by Silastic implants in this model. For many years prostatic acid phosphatase (PAP) has been used as a valuable serum marker for the presence of prostatic carcinoma, particularly in the metastatic stage. Using a radioimmuno-assay for this enzyme, the impact of endocrine manipulation of PC-82 tumor-bearing mice on the serum and tissue concentration of PAP was studied. The results of this part of the study are described in chapter 4.

A more comprehensive study of the hormonal dependence of the PC-82 tumor and the effect of hormonal manipulation on the tumor tissue is described in the backbone of this thesis, a series of three chapters (5, 6 and 7). These chapters describe the results of studies on the effects of hormonal manipulation on androgen levels in plasma and tumor tissue (chapter 5) and on the content of nuclear androgen receptors in the tumor tissue (chapter 6) and of studies of the effect of estrogens on the growth of the PC-82 tumor tissue (chapter 7). In chapter 8, results of a series of experiments with an analogue of luteinizing hormone releasing hormone (LHRH) are presented. This analogue, the LHRH-antagonist Org-30276, was applied to the PC-82 model to evaluate the potential "castration-like" activity of this compound. Chapter 9 reports some preliminary results of the current research program into the PC-82 model. This includes efforts to determine the minimal amount of androgen needed to support the growth of the PC-82 tumor as well as investigations of the application of proliferation markers to this tumor. Such markers were used in an attempt to monitor short-term effects of hormonal manipulation on the tumor tissue.

Finally, in chapter 10 conclusions are drawn with respect to the use of nude mice and in particular of the PC-82 tumor model for research into human prostate cancer.

GENERAL INTRODUCTION

CHAPTER 1

Part of this chapter is submitted for publication in the Monograph "Scientific Foundations of Urology" (D.G. Chisholm and W.F. Fair, eds.); W. Heinemann, London, 1988.

CHAPTER 1

GENERAL INTRODUCTION

1.1 THE PROSTATE

In 1912 Lowsley [122] published the first detailed description of the anatomy of the human prostate, which is the largest accessory sex gland. In young and middle-aged adults the gland is 3 - 4 cm in diameter and approximately 20 g in weight. The base of the gland surrounds the neck of the bladder and the symphysis [211]. McNeal discussed earlier views on prostate anatomy and described an anatomical model based on detailed dissections and embryological considerations [128,129]. According to this model, the gland can be subdivided into four morphologically distinct parts: the non-glandular stroma, the peripheral zone, the central zone, and the preprostatic segment.

In the human embryo the appearance of the circulating androgen, testosterone (T), signals the initiation of the development of the prostate, the periurethral glands and the seminal vesicles, whereas a metabolite of T, dihydrotestosterone (DHT), is required for differentiation of the endodermal prostate [206]. In the adult, the prostate continuously secretes a thin, milky fluid. This prostatic fluid contains many constituents, the most characteristic of which are: acid phosphatase, citric acid, zinc and spermine. Thus, the prostate must be considered as an exocrine gland. Although the prostate does not synthesize active hormones from cholesterol, as do the gonads and the adrenals, its active metabolism of T to DHT and the return of DHT to the circulation qualifies the prostate as an endocrine organ [5,49]. Most of the processes which occur in the prostate, such as the general maintainance of the gland, nuclear proliferation, mitosis and accompanying protein synthesis and the production and export of secretions, are hormone-dependent [184].

For many years it has been known from histological analyses that the prostate is composed of both epithelial and stromal elements. Morphological [99], functional [24], and embryological [32] studies showed that prostatic epithelium and stroma cannot be regarded as separate functional entities. The importance of the biochemical differences between the two compartments has been recognized since the initial work of Cowan *et al.* [31]. These investigations demonstrated that, the larger part of the enzyme, 5α-reductase, which converts T to its more active metabolite DHT (see section 1.2.1), resides in the stromal fraction. Subsequent studies have shown that the levels of androgen receptor [112], estrogen receptor [104], steroid metabolizing enzymes [77] and steroids [8,113] differ in the two cell types of the prostate. The prostatic stroma plays an important structural and biochemical role in maintaining the normal function of the organ. For example, epithelial

cells from human hyperplastic prostate do not grow well in culture in the absence of the stroma [57]. Furthermore, elegant studies of Lasnitzki and Mizuno [115], and of Cunha and coworkers [32,33] have established the importance of the stroma in the development of the normal morphology of the prostate and in the normal function of the epithelial cells within the gland.

Adenocarcinoma of the prostate originates from the prostatic epithelium, usually at the peripheral zone of the prostate [100] as distinguished from the central or periurethral part of the gland which gives rise to benign prostatic hyperplasia (BPH) [219]. BPH and carcinoma of the prostate frequently occur in the same patient. However, a transition from BPH to prostatic carcinoma is unlikely. The observed coincidence of both lesions seems to be based on their frequency and similar age distribution rather than on a similar etiology (see below 1.3).

1.2 ANDROGEN ACTION IN THE PROSTATE

Growth and function of the prostate are primarily dependent on androgenic stimuli [184,30]. In the normal male the major circulating androgen is testosterone (T), which is almost exclusively (approximately 90 per cent) of testicular origin [119]. The remainder of circulating T is secreted from the adrenal or is peripherally formed from precursor steroids, which are also mainly secreted by the adrenal glands. Although T is the primary plasma androgen causing growth of the prostate gland, it nevertheless appears to function as a prohormone since the active androgen in the prostate is a metabolite of T, 5α-dihydrotestosterone (DHT) [21]. This conclusion follows from the observation that after administration of radiolabeled T to rats, DHT, which is a highly active androgenic steroid, was the major metabolite isolated from the nuclei of the ventral prostate [3,22]. Further support for this hypothesis was obtained from observations of Lasnitzki and Mizonu [114], who showed that induction of the prostate gland in urogenital sinuses of Wistar rats, which were explanted in organ culture, was mediated by DHT and not by T. Additional studies of androgen action in human prostate, e.g. of pseudohermaphrodites with 5α-reductase deficiency but with normal levels of androgen receptors [225] as well as studies with 5α-reductase inhibitors [148], led to the definite conclusion that both in rats and in men, DHT is the main androgen modulating prostate growth with T acting as prohormone [149,150].

1.2.1 Mechanism of Androgen Action

The biologically active part of T in the blood is the part which is not bound to plasma proteins, such as sex hormone-binding globulin (SHBG) which is also known as testosterone-binding globulin (TeBG) [236]. The same protein appears to bind estradiol as well. The steroid-binding proteins decrease the amount of steroid which can affect intracellular processes after diffusion into the cells of the target organ. Hence, they play a role in the control of androgen action. SHBG concentrations in the plasma are increased after administration of estradiol [98,34]. As a consequence, in addition to the estrogen-induced suppression of total androgen levels in the plasma, the concentration of free androgen at the level of the tissue will be further decreased after estrogen treatment.After entry into the cells of the target organ, e.g. the prostate, T is converted to DHT by the enzyme 5α-reductase, which has been shown to be localized in the nuclear membrane [76]. In addition, DHT may also directly enter the prostate from the plasma, in which the level of DHT (approximately 2 nmol/l) is about 10% of the concentration of T [96]. Inside the cell DHT binds to high affinity, low capacity androgen receptors (AR). According to the classical "two-step" model the steroid-receptor complex in the cytoplasm is "activated", whereafter it migrates into the nucleus ("translocation") [124]. In this model cytosolic receptors were supposed to represent the free receptor in the cytoplasm of the cells, whereas receptors in the nuclear extracts were believed to be the occupied receptors.

Recently the understanding of the way in which steroid receptors mediate the response of steroid target cells to steroids has been modified. This is mainly due to the development of monoclonal antibodies against purified receptors, i.e. the estrogen [101] and progestin [147] receptor. The results of immunocytochemical studies have led to the concept that steroid receptors are permanently localized in the nuclei of their target cells [234]. At present, there is general consensus on the nuclear localization of estrogen and progestin receptors. In contrast, glucocorticoid receptors have been shown to be localized in both the cytoplasmatic and in the nuclear compartment [4,59]. Monoclonal antibodies against the androgen receptor, which would allow immunocytochemical detection, are not available yet. Thus, no definite conclusions with respect to a permanent nuclear localization of the androgen receptor can be drawn. A schematic representation of the current view on the possible machanism of action of androgens is depicted in figure 1. All in all, regardless of the localization of unoccupied androgen receptors, there is agreement on the nuclear localization of the occupied, i.e. steroid-bound, receptor.

After binding of the steroid hormone to the receptor, there is an increase in the affinity of the receptor-steroid complex for nuclear components, such as the nuclear matrix [6], DNA-protein complexes and specific DNA sequences. The binding of the androgen-(DHT)-receptor complex to the genome is followed by increased gene transcription and

metabolic changes associated with hormonal activity, i.e. cell growth and function [7]. Testosterone binds to the receptor with a lower affinity than DHT. It is still unknown whether functional testosterone-receptor complexes can bind to the DNA and thus can support prostate growth. Nevertheless, T appears to play a role in some aspects of prostate homeostasis involving maintainance of structure and of secretory activity [5] as well as in prostatic cell death [23,87].

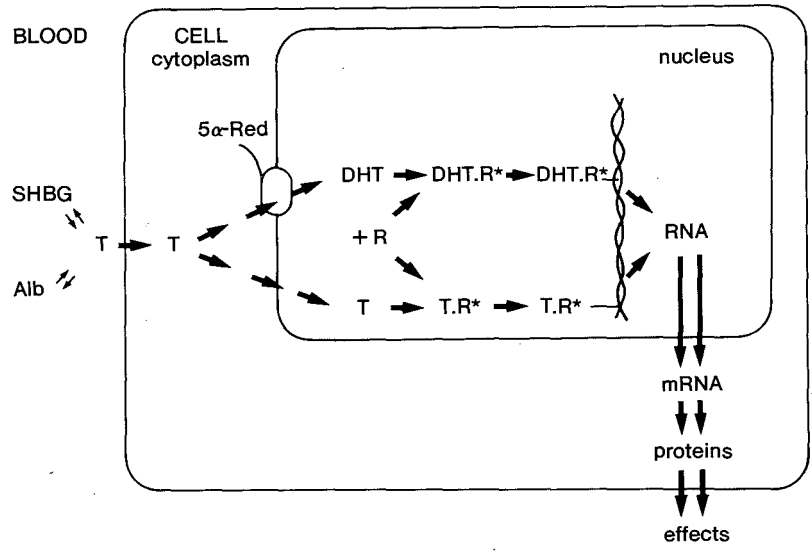

Figure 1 *Schematic presentation of the current view on the mechanism of action of androgens, testosterone (T) and dihydrotestosterone (DHT) and the intracellular localization of androgen receptors (R) and 5α-reductase (5α-Red).*

1.2.2 Growth Factors

Recently it was proposed that autocrine or self stimulatory polypeptide growth factors could exert an additional regulatory role in the hormonal control of cancer cell proliferation [65]. This suggestion that growth factors are involved as "second messengers" was substantiated for the estrogenic regulation of human breast carcinoma cell lines [210,118]. Cells of the MCF-7 breast cancer cell line secrete a variety of polypeptides with growth-promoting potential: a 52 kd protein [176], TGF-α and IGF-1-related growth factors [183,37] and TGF-β, which can act as a growth inhibiting factor [102]. In this way, the growth of estrogen-dependent cells is supposed to be controlled in an autocrine way by

estrogen-induced growth factors, apart from the direct effects of steroid hormones on cell proliferation. In addition, Manni *et al.* [125] reported on the involvement of the polyamine pathway in the growth of breast cancer cells. This pathway is induced by estrogens and is possibly mediated by growth factors. It is very likely that androgen dependent growth of prostate cancer cells is similarly regulated, with the involvement of either known or unknown (transforming) growth factors as was recently reviewed by Wilson and Smith [238]. Prostatic growth factors, i.e. heparin binding mitogens, have been isolated from the Dunning prostatic tumor (section 1.4.2) in the rat [126] as well as from extracts of human BPH tissue [137]. It has been demonstrated that EGF can stimulate the growth of normal and malignant epithelial cells obtained from the prostate of the rat [127]. Likewise, it was recently shown by Schuurmans *et al.* [195] that the growth of human LNCaP prostatic cell line (see section 1.4.3) can be stimulated by EGF and that androgens can stimulate the growth of these cells by increasing the number of EGF receptors.

1.3 PROSTATE CANCER

1.3.1 Epidemiology and Natural History.

In contrast to the nature of the normal prostate and of benign prostatic hyperplasia, carcinoma of the prostate is characterized by its malignant nature, uncontrolled growth and metastatic ability. In most Western countries the incidence of prostatic carcinoma amounts to 10 per cent of all malignancies in men. This makes prostatic carcinoma the second most frequently occurring male malignancy in most cancer statistics; it is only surpassed by carcinoma of the lung [239,51]. Although the incidence of latent prostatic cancer is the same world-wide, there are large differences in clinical incidence and mortality [18]. The lowest incidence rates are found in Asian countries like China, Japan and Singapore, where in 1975 four to five men per 100,000 male inhabitants were found to have clinical carcinoma [51]. However, Chinese and Japanese men living in Hawai at the same time had incidence rates of 26 and 36 per 100,000, respectively [68,2]. Although a satisfactory explanation of this phenomenon is not available yet, this observation indicates that the differences in the incidence of clinically manifest prostatic cancer found in various parts of the world are not only due to racial characteristics but also to environmental (promoting) factors.

Much like other tumors, especially carcinoma of the breast, prostatic carcinoma is frequently encountered at autopsy without its ever having led to clinical symptoms. Such tumors are commonly called latent if they are confined to the prostate and occult if metastases are present [58]. Prostatic carcinoma is called focal carcinoma if one or more small foci are found at routine examination of the organ at autopsy or after surgical removal

of benign prostatic hyperplasia (BPH) due to obstruction. Prostatic cancer is termed clinical whenever the tumor can be detected by routine diagnostic measures, such as rectal examination, determination of serum prostatic acid phosphatase, bone scan, etc. Although the focal tumor must be considered to be the precursor of clinical cancer, the incidence of focal tumors is much higher than that of clinical cancer. Therefore, a different etiology for both lesions is very probable [237]. For the pathogenesis of hormone independent prostate carcinoma, which ultimately leads to the death of the patient, at least three mutational steps seem to be required, according to the following simplified scheme: normal cell ⇉ focal carcinoma ⇉ hormone dependent carcinoma ⇉ hormone independent carcinoma [180]. It is assumed that one or more (promoting) factors are involved in the development of each of the three different entities i.e. focal carcinoma, hormone dependent carcinoma and hormone independent carcinoma of the prostate.

It is obvious from the above cited geographical differences in incidence of prostatic carcinoma and from the migrant studies, that external risk factors play an important role in the promotion of prostatic cancer from the focal to the clinical state. Epidemiological studies actually led to a number of factors related to a higher or lower incidence of prostatic carcinoma [180]. Examples of putative risk factors are: diet, fat consumption, vitamin A, exposure to heavy metals, sexual habits and smoking [209]. Furthermore, the initial endocrine dependence of most clinical prostatic cancers, (see section 1.3.2) strongly suggests that the endocrine environment may play a role in the pathogenesis of clinical prostatic carcinoma. Moreover, it is known that prostatic hyperplasia and prostatic carcinoma very rarely develop in males whose plasma testosterone concentration is at castration level. However, a number of studies have failed to show an increase of free or bound plasma testosterone and dihydrotestosterone for patients with carcinoma of the prostate and several age matched control groups [243,79]. Most likely, androgens will play a role as cocarcinogenic factors [10] in the promotion of the different steps of pathogenesis of prostatic carcinoma [16,158,159] and thus in the early development of this usually fatal form of cancer.

1.3.2 Hormone Dependence and Endocrine Management

For a limited number of types of cancer it has been shown that hormones are involved in the growth of the tumors and in the progression of the disease. Since the early report by Beatson in the year 1896 [9] on the positive effects of ovariectomy in a premenopausal patient with advanced breast cancer, the possibility of hormonal intervention in the development of hormone dependent cancers has gained interest. In the early 1940's, the pioneering studies of Huggins and Hodges [78] delineated the role of testicular androgens

in the regulation of the growth of prostatic carcinoma. Their work, which was later honored with the Nobel Prize, was followed by a large number of retrospective studies based on endocrine management of prostatic carcinoma [48,136,220]. Since these studies, which demonstrated that prostatic cancer can be made to regress after ablation of testicular androgen production by either orchiectomy or by the administration of exogenous estrogens, the treatment of advanced prostatic cancer has been dependent upon suppression of the circulating levels of androgen [226].

This type of endocrine treatment produces substantial palliation in 70 - 80% of all cases and is associated with an objective response in 20 - 40% of all cases. It also causes a significant prolongation of the symptom-free interval. However, androgen ablation hardly ever leads to the cure of a patient; the majority of patients with advanced prostate cancer relapse and die after an initial period of remission [14,130,220]. This relapse phenomenon of an initially androgen-responsive prostatic cancer is characterized by a progression of the tumor to an androgen-resistant stage (see 1.3.3). Moreover, due to the high incidence of competing causes of death, in the age group in which prostatic cancer occurs, many patients die with, rather than of, their prostatic carcinoma.

Recently, with the availability of potent analogues of luteinizing hormone-releasing hormone (LHRH), a new principle of treatment has become available in the management of hormone dependent cancers, particularly breast and prostatic carcinoma [186,241]. This treatment modality is based on the observation that chronic administration of these "superactive" analogues of LHRH results in a paradoxical inhibition of the pituitary-gonadal axis characterized by a decrease of the levels of sex steroids and the atrophy of accessory sex organs [106,175]. In first line endocrine management of human prostate cancer the use of LHRH-analogues has become a well recognized alternative to castration and other means of suppressing plasma testosterone to castration levels. A large number of phase 1 and 2 studies have been carried out proving the value of this treatment modality [217,224,185,165]. The major advantage of this new treatment modality seems to be the absence of side-effects such as the cardiovascular complications associated with high-dose estrogen treatment [64,222].

1.3.3 Tumor Progression

Hormone dependent tumors become independent or autonomous through a stepwise cumulative loss of hormonal sensitivity termed progression [55,139,141]. Recurrent growth of prostatic carcinoma following successful initial therapy by orchiectomy or estrogen administration is almost always caused by the tumor becoming hormone-unresponsive. For the development of androgen-independent prostatic cancer cells from androgen-responsive

cells a number of possible mechanisms, e.g. environmental adaptation, multifocal origin, or genetic instability, have been proposed [91].

Studies of the experimental Dunning prostate tumor system (cf. 1.4.2.) demonstrated that prostatic cancer can be heterogeneously composed of a variety of phenotypically distinct cell clones even before hormonal therapy is started [84]. With regard to androgen responsiveness, there are three distinct phenotypes possible for prostatic cancer cells: androgen-dependent, androgen-sensitive and androgen-independent. Androgen-dependent cells chronically require a critical level of androgenic stimulation for their continued maintainance and growth and in this respect these cells are very similar to the androgen-dependent non-neoplastic cells of the normal prostate. In contrast to the androgen-dependent cells, which die without adequate androgenic stimulation, androgen-sensitive cancer cells grow faster in the presence of sufficient androgen levels but can still grow continuously even without the presence of androgen. Androgen-independent cells neither die nor slow down their continuous growth following androgen ablation, i.e. such cells are completely autonomous to androgenic effects on growth. These androgen-independent cells, once formed in the tumor, proliferate without the requirement for androgenic stimulation, and thus repopulate the tumor producing a relapse after castration. Such an explanation assumes that the changing host environmental conditions following castration are critically involved in inducing the adaptive transformation of an initially androgen-dependent to an androgen-independent tumor cell. Therefore, this process is called the environmental adaptation model. Alternatively, according to the concept of genetic instability [142], changes in the genome of an occasional cancer cell may occur. This can lead to a genetically altered clone of cells [29]. Such a clone, which is no longer androgen-responsive, but is then androgen-independent, can be added to the original homogeneous tumor. The addition of phenotypically new clones of cells, originating from the process of genetic instability, to the tumor population, coupled with the process of clonal selection of these newly developed cells is at least one possible mechanism for the progression of hormone-independent prostatic cancer [86,19].

The optimal treatment of prostatic cancer patients should be directed towards the heterogeneous character of the prostate tumor, i.e. the pre-existing androgen-dependent and -independent cell populations in the tumor. Hence, it is suggested that hormonal therapy must be combined with chemotherapy early in the course of the disease, so that all cancer cell populations will be affected [89].

1.3.4 The Concept of Total Androgen Withdrawal.

Since low levels of non-gonadal (e.g. adrenal) androgens are left following castration, this treatment only induces a partial androgen withdrawal [62]. Some investigators suggest that

a more complete form of androgen withdrawal, in which the very low levels of non-testicular androgens are counteracted (e.g. by simultaneous treatment with an anti-androgen), might be a more effective treatment of prostatic cancer than castration alone [107,166]. In recent years this approach of total androgen blockade, achieved by combining treatment with an LHRH-agonist or castration with the administration of a "pure", i.e. non-steroidal, anti-androgen [133], has been strongly propagated by Labrie and coworkers [108]. This group claims that this combined treatment modality results in considerably higher initial response rates, lower progression rates and in addition, in a smaller chance of transition of the tumor to the hormone-independent state [109,111]. The validity of this approach of complete androgen withdrawal strongly depends on the answer to the question what minimal amount of androgen is necessary to support tumor growth. This appears to be a fundamental question, since Labrie postulated that in a prostatic tumor most, if not all, androgen-insensitive cells develop when tumor cells are exposed to the low androgen milieu provided by the adrenal androgens. This suggestion is based mainly on observations which were made using the Shionogi mouse mammary tumor cell line [132]. A series of different clones of this cell line were shown to be extremely sensitive to androgen stimulation *in vitro* [110]. This behavior is not found in any of the other androgen-dependent tumor models and there is little evidence that human prostatic carcinoma *in vivo* behaves in the same way. It has also been described that the transition of the Shionogi tumor cells to the autonomous state *in vivo* can be prevented by total androgen blockade [123]. This observation has not been reproduced in any of the other model systems. It is even possible that total withdrawal of the growth stimulating hormone from the hormone-dependent tumors favors progression from the responsive to the unresponsive state [139,141]. All in all, in spite of an increasing knowledge about the biological significance of low levels of androgens on the growth of the normal and malignant prostate [61,120,218,105], neither experimental [47,52] nor clinical [143,192,194] evidence has been obtained which substantiates the advantages of complete androgen withdrawal therapy.

1.4 MODEL SYSTEMS FOR STUDIES ON PROSTATE CANCER

Many concepts concerning human cancers have been derived from studies utilizing animal models. Human prostate cancer is a complex disease and the study of several aspects of this type of cancer can only be investigated by using appropriate animal and in vitro systems. Contrary to the frequent occurrence of prostatic adenocarcinoma in men (see section 1.3.1), other mammalian species, including laboratory animals, have a very low incidence of manifest carcinoma of the prostate [174]. As a consequence, the number of permanent transplantable tumor lines, derived from spontaneously occurring prostate cancer, in

syngeneic hosts is very limited [45,154,198]. Likewise, only a few models have been described which were either hormonally [140] or chemically [163] induced in the rat. More detailed information about the available models in the rat is given in the next section (1.4.1).

Although the major model systems stem from cancers which occurred in the prostate of the rat, some other animals, such as the dog [117], the hamster [53,54] and the mastomy [208] are known to develop prostate cancer. However, none of these possible models has been well explored and was of any practical importance up till now. Remarkably, no record exists of any spontaneous prostatic cancer in the mouse, which among rodents is the most widely used laboratory animal and which suffers from many tumors.

The limitations of the above-mentioned animal model systems mainly stem from the restrictions on the direct extrapolation of data derived from animal models to the human disease. Therefore, attempts were made to develop *in vivo* and *in vitro* model systems originating from human prostate cancers [171]. Since the early 1970's a number of investigators have developed a series of models based upon human prostate cancers transplanted as xenografts into immunologically deficient, athymic nude mice. The difficulties found in trying to establish human prostatic cells *in vitro* are typical of the problems in establishing any differentiated cell in culture. Monolayer cell cultures of human prostatic epithelial cells have rarely been continued beyond the state of primary (short-term) cell culture [190,17, 233]. Thus, the number of permanent prostatic cancer cell lines of human origin is limited; a more detailed description of these lines is given below (section 1.4.3).

The usefulness of a model system depends on the extent to which its characteristics reflect the properties of clinical prostate cancer. Therefore, careful characterization of models, once established, is a prerequisite for further experimental studies with such models. In practice this means that a series of known (histological, biological and biochemical) properties of the tissue of origin need to be checked in the model system. The main properties for an ideal animal model of prostatic carcinoma, as extensively described by Coffey *et al.* [28], can be summarized as follows:

* proven origin of prostatic tissue
* adenocarcinoma with histological similarity to human prostatic cancer
* slow growth rate
* hormone-dependence
* biochemical profile similar to prostate (cancer); e.g. secretion of prostatic acid phosphatase, presence of androgen receptors
* malignant and metastatic patterns to bone and lymph-nodes
* relapse to hormone insensitive state.

Very recently, Isaacs [92] published a comprehensive review comprising a catalogue of the available animal model systems of prostate cancer. It contains detailed information on the origin and the major characteristics of the different model systems and presents a list of references which illustate the way these models have been used to study various issues of the tumor biology of human prostate cancer.

1.4.1 Transplantable Tumors in the Rat

The rat prostatic tumor models represent a spectrum of hormone dependence and responsiveness, of metastatic potential and histological differentiation. Four systems of experimental prostatic adenocarcinomas in rats have been described; they are identified as Dunning, Noble, ACI and Pollard prostate tumors.

In 1973 Pollard reported the spontaneous development of prostatic adenocarcinoma in germ-free Lobund Wistar rats [154]. This tumor system comprises four serially transplantable sublines which have been termed PA I, II, III and IV [157]. Each of these *in vivo* tumor sublines was established as an *in vitro* cell line which, when inoculated back into Wistar rats, grows rapidly with histological appearance to the original subline [25]. A special feature of these Pollard tumors is their extensive ability to metastasize. The PA I, III and IV sublines metastazise via the lymphatics, whereas the PA II subline spreads via both the lymphatics and the blood [156]. A great number of studies performed with the Pollard tumors dealt with their metastatic properties and therapeutic approaches to control the growth and metastatic spread of these tumors [155,216,160].

In 1977 Shain and coworkers detected spontaneous adenocarcinomas in the ventral lobe of the prostate of AxC rats [198]. It appeared that aging male ACI rats have a high spontaneous incidence of prostatic carcinogenesis. When ACI rats reach the age of 33 months they invariably have detectable prostate tumors [228], including both microscopic lesions and grossly manifest forms of prostate cancer [199]. Therefore, special attention was paid to the process of tumor progression in this system, i.e. the events required for progression of microscopic to manifest prostatic cancer (see section 1.3.3) [88]. In 1981 Shain *et al.* [200] established a serially transplantable prostatic adenocarcinoma from the ventral prostate of an ACI rat, treated for 4 months with a testosterone-containing Silastic implant. Cells of this tumor could be permanently propagated *in vitro* [200], and clonally derived cell lines were subsequently developed [201]. As the AxC prostate tumors contain androgen and prolactin receptors, the system has been proposed as an appropriate model for studies of hormonal regulation of prostate cancer.

The prostate of the Noble (Nb) rat is markedly susceptible to the development of carcinomas induced by chronic (i.e. > 6 months) exposure to high levels of both testosterone

and estrone [42,139]. In this way, the Noble series of prostatic cancers was established. These transplantable tumors were found to be androgen responsive, androgen-independent or estrogen responsive [140,38]. The Noble rat tumor system has been studied extensively by Drago and coworkers, who used the various hormone-sensitive and autonomous sublines for evaluation of their sensitivity to chemotherapeutic drugs [39,40] and to test the effectiveness of antimetastatic agents [41,43,44]. Data obtained by Noble [139] after hormonal manipulation of sex-steroid dependent tumors in the Noble rat led to the conclusion that the change from hormone dependent to autonomous growth can be promoted by radical withdrawal of the growth stimulating hormone and that a low maintainance dosis of the hormone preserves hormone dependence but slows down growth of the tumor. This interesting finding is at variance with the hypothesis based on observations made of the Dunning tumor system, described below (section 1.4.2). The Dunning tumor model is the most widely applied prostatic tumor system in the rat. Therefore, a more detailed summary of its characterization and further application is given below.

1.4.2 The Dunning Prostate Tumor System

The most important and best characterized model system for prostatic carcinoma is the one which developed from a spontaneously occurring prostatic carcinoma isolated by Dunning in a Copenhagen rat in 1961 [45]. This tumor system, denoted as R3327, primarily arose from the dorsal lobe of the prostate. This model has many properties in common with human prostate cancer and of all available tumor systems it meets the greatest number of the requirements mentioned under 1.4. Voigt and Dunning [221] first reported on the hormone sensitivity of the R3327 tumor and the metabolism of testosterone in the tissue of this tumor. Initially, Dunning isolated eight different tumor sublines, among which the H-tumor is the one studied most often [207]. This tumor was shown to be well-differentiated and hormone dependent and to have a relatively low growth rate. A detailed characterization of this parent tumor at the morphological, biochemical and therapeutic level was accomplished by Isaacs and coworkers [82,83].

The hormone-dependent tumor (H-tumor) was estimated to consist of 20% hormone insensitive cells. This hormone independent component gives rise to hormone insensitive tumors by selective overgrowth if the growth of the hormone dependent cell population is inhibited by castration or estrogen treatment [84]. As discussed before (see section 1.3.3) it has been shown that in the Dunning tumor the process of a tumor becoming hormone independent is a selection phenomenon [84]. The process of genetic instability coupled to clonal selection is the proposed mechanism for the change in tumor phenotype which is associated with tumor progression in the Dunning prostate cancer model [86,223].

Two distinct sublines resulted from the H-tumor. The slow growing hormone insensitive (HI) tumor originated from an H-tumor which was transplanted into a castrated rat [82]. Due to the process of genetic instability of the tumor in an occasional rat, a hormone-insensitive anaplastic tumor (AT) spontaneously developed [82]. An enzymatic index was developed by comparing the biochemical profile of the normal dorso-lateral prostate of the rat with that of the fore-mentioned three sublines, i.e. the H, HI and AT tumors. This relative differentiation index was based upon measurements of the activities of steroid metabolizing enzymes, lactate dehydrogenase and acid- and alkaline phosphatase [83]. After continuous serial passage the first established AT tumor (AT-1), which rarely produced distant metastases [82], gave origin to two distinct and highly metastatic anaplastic sublines [86]. Since one of these sublines produced metastases to both lymphe-nodes and the lung it was termed MAT-LyLu [85], whereas the other which produced lung metastases almost exclusively was termed MAT-Lu [116]. A biochemical metastatic index, which was based on the determination of a variety of hydrolytic enzymes, and which reflected the respective metastatic abilities of the parent and deriving sublines mentioned above could be constructed [121].

As a derivative of the original R3327 tumor after serial transplantation the G-subline developed. This variant grew faster, was histologically less differentiated, but was still androgen sensitive compared to the original parental line [15,27]. The tumor was further characterized with respect to its hormone sensitivity, DNA content and hormone receptors by Pollack *et al.* [151,152]. Shessel *et al.* [204] used the G-tumor to evaluate a series of possible endocrine treatment modalities. It appeared that hypophysectomy, alone or in combination with other forms of endocrine treatment, and orchiectomy plus stilbestrol were the most effective means of suppressing tumor growth. Later studies of the G-subline, carried out by Humphries and Isaacs [80], demonstrated that this tumor is composed of androgen-independent but still androgen-sensitive prostate cancer cells. The androgen sensitivity of the G-tumor appeared to be unusual in that it is not due to androgenic stimulation of cell division but to androgen-induced inhibition of tumor cell loss.

More recently, the androgen-responsive differentiated H-tumor and the poorly differentiated G-subline of the R3327 system were used to ascertain the effectiveness of complete versus partial androgen withdrawal therapy for the treatment of prostatic cancer [47] and to determine the optimal concentration of circulating testosterone for treatment of prostate cancer [218] (see also section 1.3.4). In addition, the Dunning tumor was the experimental model of choice for the initial evaluation of newly developed treatment modalities of prostatic cancer, such as LHRH-agonists or antagonists alone [168,187,153,188] and in combination with anti-androgens [169] or cytostatics [188]. More recently, the first data were reported on the application of analogues of somatostatin to this experimental model system [189]. Finally, an important step was recently taken by Isaacs and coworkers [90],

who established *in vitro* cell lines from seven biologically distinct Dunning R3327 sublines. Four of these *in vitro* cell lines (i.e. G, HI-F, AT-1 and AT-2) retained a low metastatic ability when inoculated back into syngeneic Copenhagen male rats, whereas the other three cell lines (i.e. AT-3, MAT-LyLu, MAT-Lu) retained a very high metastatic ability. A series of genetic (i.e. DNA content per cell, modal chromosome number) as well as phenotypic parameters (i.e. morphology, androgen receptor, 5α-reductase etc) were used to validate that the *in vitro* cell lines retained the major characteristics of the *in vivo* tumor sublines from which these lines originated [90].

1.4.3 Cell Lines of Human Origin

In spite of the efforts of several investigators to establish long-term *in vitro* cultures of prostatic epithelium, only a limited number of continuously growing cell lines have been developed. The six permanent prostatic tumor cell lines described up to now (table IA) were established from primary cultures of prostate cancer tissue which was dispersed by the use of proteolytic enzymes or by mechanical disruption. The main characteristics of these cell lines are presented in table IB.

The first prostatic cell line MA-160, developed by Fraley *et al.* [56], was derived from a culture of tissue of BPH and therefore this line has not been included in table I. Moreover, the nature of MA-160 as well as of the EB-33 cell line (table I) was questioned by a number of investigators [134,234]. These authors suggested that Ma-160 and EB-33 consist of HeLa cells, a reported contaminant of other human cell lines [135]. This assumption was based mainly on the fact that both the MA-160 and the EB-33 cell lines have lost the Y chromosome and that enzymatic and chromosomal characteristics of HeLa cells were found in both these cell lines.

However, Pontes *et al.* [161,162], using specific immunoreagents, demonstrated that EB-33 cells produced prostatic acid phosphatase. Additional evidence that the EB-33 cell line is not contaminated with HeLa, was obtained by Ackermann *et al.* [1], who showed that antibodies directed against antigens obtained from the EB-33 cells can be used to kill EB-33 but not HeLa cells. Based upon the total data, including the hormone-responsiveness of cloned EB-33 cells, Schröder *et al.* [191] indicated that EB-33 cells are not HeLa contaminants.

The history of the PC-93 cell line is comparable to that of the EB-33 cell line. It was established in 1978 at our Urological Department. In contrast with EB-33 cells, PC-93 cells contain an androgen receptor (table IB) [11]. Both the EB-33 and the PC-93 cells were shown to be androgen independent but to a certain extent androgen sensitive i.e., the cells grow continuously without the presence of androgen, but they grow faster in the presence of adequate androgen levels [145, van Steenbrugge *et al.*: unpublished data].

Table IA Prostatic tumor lines of human origin established *IN VITRO*

Tumor line	Established	Origin*	Reference	
EB-33	1973	Primary tumor	Okada and Schroeder	[144]
DU-145	1975	Metast.(CNS)	Stone *et al.*	[215]
PC-3	1976	Metast.(bone)	Kaighn *et al.*	[97]
LNCaP	1977	Metast.(L.N.)	Horoszewicz *et al.*	[74]
PC-93	1978	Primary tumor	Claas and van Steenbrugge	[26]
TSU-PR1	1980	Metast.(L.N.)	Iizumi *et al.*	[81]

* CNS: central nervous system
LN : lymph node

Table IB Main characteristics of human prostatic tumor lines *IN VITRO*

Tumor line	Androgen-dependence	Prostatic acid phosphatase	Androgen receptor
EB-33	±	±	-
DU-145	-	±	-
PC-3	-	-	-
LNCaP	+	+	+
PC-93	±	-	+
TSU-PR1	-	-	-

The characteristics of the TSU-Pr1 cell line, which were recently reported by Iizumi *et al.* [81], appeared to be similar to those of the DU-145 and PC-3 cell lines (table IB). Cells of the early passages of these three cell lines were shown to have an aneuploid karyotype with a variable number of chromosomes including the Y chromosome. In fact, all cell lines, except the LNCaP cell line, consist of undifferentiated hormone-independent cells, which do not secrete prostatic acid phosphatase or which produce this enzyme in very small amounts (table IB).

It has been demonstrated that the cells of all six prostatic cell lines are tumorigenic in

athymic nude mice (compare the references indicated in table IA) [131]. Inoculation of cultured cells of the various cell lines in nude mice (next section) resulted in the development of poorly differentiated, hormone independent tumors which consisted of tissue with no, or very little, secretory activity, and which did not contain androgen receptors. Tumor tissue of the PC-3 xenograft in nude mice did not contain acid phosphatase and 5α-reductase activity, but it did show the prostate specific adenylate-kinase isoenzyme pattern [171].

Ware *et al.* [229,230] described a subline (1-LN-PC-3-1A) of the PC-3 cell line which, when injected into nude mice, consistently metastasized from a subcutaneous site to both regional and distant lymphe-nodes. This subline originated from a spontaneous lymphe-node metastasis which occurred in a nude mouse, which was inoculated with cells of the parental PC-3 line [229]. Further studies of this metastatic subline involved a comparison between the cellular phenotype of this variant and the parental PC-3 cell line [231] and, more recently, chemical modification of the metastatic capacity of the cells [232].

The LNCaP cell line, developed by Horoszewicz [74], is the far most promising *in vitro* model established so far (table I). The LNCaP cells have an aneuploid karyotype including the Y chromosome and a limited number of marker chromosomes [75]. Studies of a number of clones and of several passages of the parental LNCaP line, by Gibas *et al.* [63] and by König *et al.* [103] in our laboratory, showed that the chromosome pattern of this cell line has been preserved fairly well with minor changes of the karyotype. The LNCaP cells were shown to be androgen dependent [75], a property which makes the LNCaP line the only hormone-responsive *in vitro* cell line of human prostatic origin. When inoculated into athymic nude mice LNCaP cells produce rapidly growing tumors, which are not hormone dependent. However, the frequency of tumor development and the mean time of tumor appearance were reported to be different for male and female mice [75]. Cultured cells as well as nude mice supported tumors contain androgen receptors and are positive for prostate specific antigen and prostatic acid phosphatase (PAP). The production of PAP in the LNCaP cells was shown to be regulated by androgens [75,193]. Schulz *et al.* [193] showed that the expression of PAP in the LNCaP cells is modulated by estradiol as well, although the concentration of this hormone appears to be far above the physiological level. The growth of the LNCaP-FGC (i.e. a fast growing colony of the parental line) *in vitro* was shown to be stimulated by androgens in a dose-dependent way [12]. Under optimal conditions the synthetic androgen R1881 (0.1 nM) stimulated the cell growth by a factor of approximately 2.3. In addition, these investigators demonstrated that in the LNCaP cells androgens, i.e. DHT and R1881, stimulate the release of a 40 kD protein. A possible autocrine growth regulating function of this protein was suggested.

In contrast with the fore-mentioned studies, Hasenson and coworkers [70] could not observe a clear stimulatory effect of androgens on the growth of LNCaP cells. They also

found a relatively low content (approximately 16 fmol/mg protein) of androgen receptors in these cells. Consequently, these authors concluded the cells of their LNCaP line were androgen-resistant and they designated this subline LNCaP-r.

During the last few years, the LNCaP tumor model has gained much interest. Various aspects, such as the androgen-dependent pattern of growth, the production and application of monoclonal antibodies and the involvement of growth factors in the growth of LNCaP cells, are currently studied by a considerable number of investigators [35,36,179,195].

1.5 THE XENOGRAFT MODEL

1.5.1 Introduction to the Nude Mice Model

Experimental study of human malignancies *in vivo* relies on the technique of hetero-transplantation of human tumor tissue into immuno-suppressed animals, such as newborn or irradiated rats and mice, or into immunopriviliged sites, such as the anterior chamber of the eye or the cheek pouch of the hamster [212]. Since Rygaard and Povlsen [181] introduced the athymic nude mouse as a possible host for the maintainance of human tumor xenografts, the majority of investigators have used this animal as a model [202]. The mouse mutant nude, first reported by Isaacson in 1962 [93], appears to be homozygous for an autosomal recessive gene bearing the symbol *nu*. This gene is a mutant allele of the nude locus of the VII linkage group on chromosome 11. The phenotype of this mouse was described in detail by Flanagan in 1966 [50]. The nude gene from Balb/c nu/nu mice, which is the most widely used strain of nude mice, has been introduced into a variety of mice of defined genetical background.

Besides the absence of hair, the animals show reduced growth and fertility [167] as well as a relatively short life span [46]. Pandelouris [146] discovered that nude mice lack a normal thymus and consequently these animals are partly deficient in their cell-mediated immune response. However, humoral antibody formation in nude mice is only slightly impaired, whereas the activity of natural killer (NK) cells is even increased in comparison with that in normal immuno-competent mice [71]. Due to the immuno-deficient character of nude mice a "specific pathogen free" environment is required for the maintainance of these animals. Therefore, nude mice should be housed in isolation, by using either filter-supported cages or laminar-air-flow units, which are located in a temperature- and humidity controlled room. All materials, i.e. cages, bedding, food, must be sterilized before use.

Rygaard demonstrated after the successful transplantation of normal rat skin in nude mice [182], that human malignant tissue was readily accepted in these animals [182,164]. Initiation of tumor cell lines into nude mice can be achieved either by grafting of tissue

derived directly from surgical specimens or by inoculation with cells obtained from cultures of permanent *in vitro* cell lines. Substantial differences exist between the "take rate", i.e. the growth and subsequent transplantability, of fresh tumor specimens derived from various tumor types [205]. The overall take of the different tumor types varies from good (50% of samples established) for malignant melanoma and colonic adenocarcinomas, fair (20-50%) for e.g. ovarian, bladder and renal carcinomas, to poor (occasionally) for gastric, breast and prostatic carcinomas [203,196]. The above-mentioned residual immuno-reactivity of nude mice and probably also the hormonal environment in these animals, may account for the very small number of tumor lines from breast and prostatic carcinomas established in nude mice [170,172,197].

1.5.2 Prostatic Tumor Models in Nude Mice

Like the number of human cell lines of prostatic carcinoma *in vitro* (section 1.4.3), the number of permanent prostatic cancer lines in nude mice is very limited. Table IIA shows a list of the seven model systems developed from human prostatic carcinomas which were hetero-transplanted into nude mice. The PC-82 tumor was initiated in the laboratory of the Department of Urology in Rotterdam by Hoehn in 1977. This tumor model, which is the subject of this thesis, was the first transplantable human prostatic carcinoma in nude mice reported in the literature (cf. next section 1.6). Continuing efforts in our institute to increase the number of tumor lines resulted in the development of two other models named PC-133 and PC-135 [van Steenbrugge: unpublished data]. In our laboratory a total number of about 150 prostatic carcinoma specimens have been transplanted during the last 10 years, resulting in three permanently growing cell lines. Thus, we achieved a low primary tumor take of approximately 5 per cent, a figure that has also been recognized by other investigators.

Table IIA Prostatic tumor lines of human origin established *IN VIVO* in nude mice

Tumor line	Established	Origin	Reference	
PC-82	1977	Primary tumor	Hoehn *et al.*	[72]
Honda	1977	Metast.(testis)	Ito *et al.*	[94]
9479	1980	Metast.(bone)	Graham *et al.*	[66]
PC-EW	1981	Metast.(L.N.)	Hoehn *et al.*	[73]
PC-133	1981	Metast.(bone)	van Steenbrugge	
PC-135	1982	Primary tumor	van Steenbrugge	
TEN/12	1985	Primary tumor	Harper *et al.*	[69]

Table IIB Main characteristics of human prostatic tumor lines *IN VIVO* in nude mice

Tumor line	Hormone dependence	Prostatic acid phosphatase	Androgen receptor	Growth rate
PC-82	+	+	+	slow
Honda	+	+	+	moder.
9479	-	-	N.D.	fast
PC-EW	+	+	+	moder.
PC-133	-	-	-	moder.
PC-135	-	-	-	slow
TEN/12	+	+	+	fast

Three of the tumors developed so far (i.e. PC-82, PC-135 and TEN/12) originated from a primary tumor, whereas the remaining four systems (i.e. Honda, tumor-9479, PC-EW and PC-133) were derived from metastatic lesions. From table-IIB, which comprises the main characteristics of the fore-mentioned tumor lines, it can be read that, based on their grade of differentiation, two groups of tumors can be distinguished. The PC-82 [214,215,20], Honda [95], PC-EW [van Steenbrugge: unpublished data] and TEN/12 [69] tumors were shown to be androgen dependent, to secrete prostate-specific acid phosphatase and to contain androgen receptors, whereas the other tumors, are devoid of these properties. The hormone dependence of the former group of models was demonstrated by the observation that none of these tumors grew in androgen-deprived (i.e. castrated male or intact female) mice and that androgen withdrawal from tumor-bearing animals caused growth arrest and subsequent regression of the tumor tissue. The hormone independent tumors were shown to have a similar take rate and to grow equally well in male and female mice.

In addition to the above-mentioned adenocarcinomas of the prostate, a permanent cell line (UM-SCP-1) has been established from a primary squamous cell carcinoma of the prostate, which is transplantable in nude mice as well [67]. Reid and coworkers [173] initiated a tumor line (R-198) derived from a papillary, transitional cell carcinoma of the urinary bladder. In nude mice, the tumor line exhibited properties attributable to both prostatic and transitional epithelium. This tumor was shown to be hormone-responsive, to contain low (insignificant) concentrations of androgen receptors and to produce tartrate-inhibitable acid phosphatase. It was suggested that this tumor might serve as a useful model for studying sex steroid-responsive cells of the urogenital epithelium.

In contrast to observations in the PC-82 tumor model [213] (cf. chapter 4), it appears that the secretion of prostatic acid phosphatase in the Honda tumor is under androgenic control. Furthermore, the properties of the PC-82 and the Honda tumor do not differ

substantially. From none of the seven tumor lines that grow permanently in nude mice, continuously growing *in vitro* cell lines could be established. As an exception, the TEN/12 tumor was reported to be able to survive when cultured in semi-solid agar, thus providing an *in vitro* experimental model of prostatic cancer.

1.6 INTRODUCTION TO THE PC-82 MODEL

1.6.1 Clinical History

The patient from whom the PC-82 tumor line originates was a fifty-eight-year-old Caucasian male at the time of diagnosis. His father had also suffered, and eventually died, from prostatic carcinoma. The patient presented with perineal pain and a burning sensation. Rectal examination revealed an enlarged prostate, estimated to weigh about 60 g, which was indurated on both sides. The induration clearly extented into the left seminal vesicle. Prostatic biopsy confirmed the diagnosis of adenocarcinoma of the prostate on both sides. A complete metastatic work-up, which included a pelvic lymphe node dissection, showed no evidence of metastases in July 1977 and one week after staging lymphadenectomy, a total perineal prostatectomy was carried out. Histological examination showed a very extensive, moderately differentiated prostatic adenocarcinoma, which consisted of small- and medium sized glands as well as of cribriform formations. In several areas the tumor had penetrated the fibrous capsule of the prostate. Sections through the base of the seminal vesicles showed tumor invasion on both sides. The patient did well until November 1978 when he developed a sciatic syndrome. A bone scan revealed tumor metastases in the lumbar spine. Acid phosphatase levels, determined by an enzymatic assay, were not elevated. Orchiectomy was carried out in November 1978, whereafter the pain disappeared. The patient became symptomatic with sciatic pain on the left side in October 1980 again. Various treatment modalities among which radiotherapy, adrenal suppression and treatment with an anti-androgen, were only palliative in nature. The patient died of his disease in 1983.

1.6.2 Development

Small tissue specimens of the original tumor of the patient (see previous section) were transplanted into four male and four female nude mice of the Balb/c background [72]. The transplant was designated with the serial number PC-82. The tissue fragments were grafted subcutaneously in the dorso-lateral wall at each side of the shoulder through an incision in the neck of the animals. At present, this technique of inoculation with tumor tissue is still

applied. Figure 2 shows a nude mouse bearing bilateral PC-82 tumors. The growth of tumors, once palpable, was followed by using calipers. From these tumor measurements growth curves were constructed, as described in more detail in chapter 3 [cf. 177,214].

In the first transplant series tumors with a very slow growth rate developed in the male mice eight weeks after grafting. No tumors developed in the simultaneously transplanted female mice. Due to weakness of the tumor-bearing mice, which occurred between 4 and 6 months after tumor grafting, the tissue had to be transferred to other mice. Since then, the PC-82 tumor has been maintained by serial transplantation. At present, i.e. a good 10 years after initiation, the tumor is in its 38th transplant generation. The original properties of the PC-82 tumor (listed below) appeared to be preserved fairly well during this decade. From the original patient material as well as from many later passages of the tumor in nude mice, it has been attempted to establish the PC-82 *in vitro*. This has not resulted in a continuously growing cell line. Only short-term (2 to 3 weeks) epithelial cell cultures have been obtained [178]. Yet, a permanent fibroblast line descended from the primary culture initiated from the original patient specimen.

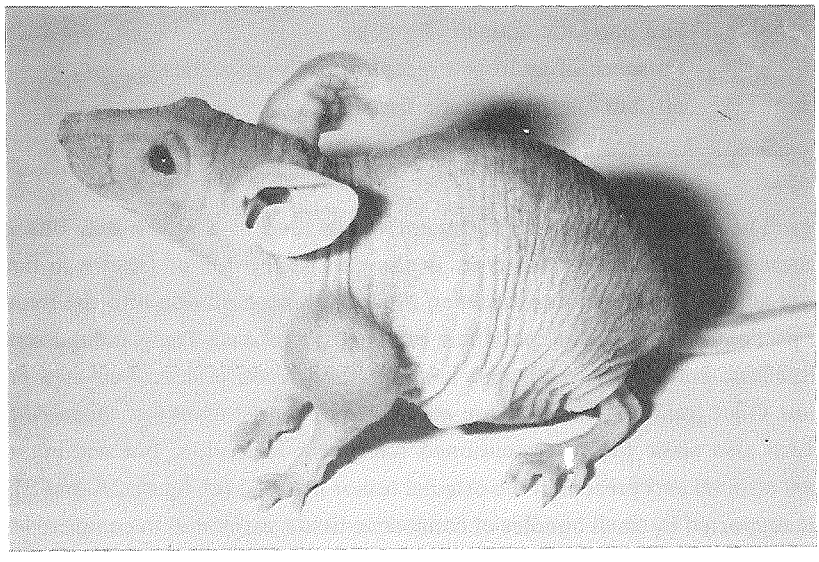

Figure 2 Male nude mouse bearing subcutaneously growing PC-82 tumors

1.6.3 Major Characteristics

The main properties of the PC-82 prostatic tumor model, which are listed in table III, appear to have been preserved fairly well for more than 10 years during the maintainance of

the tumor in nude mice. In particular, the histological feature and hormone dependent growth pattern are the two properties which have frequently been checked. A number of relevant data, dealing with the main characteristics and the further application of the PC-82 tumor model, are given below.

Table III The Main characteristics of the PC-82 prostatic tumor model

* Histology: moderately differentiated adenocarcinoma with cribriform aspects
* Human karyotype including the Y chromosome and with 17 marker chromosomes
* Relatively slow growth rate: tumor doubling time: > 10 days
* Hormone dependence
* Secretion of prostate-specific acid phosphatase and prostatic antigen
* Presence of androgen receptors with properties attributable to prostatic tissue

Histology. The original tumor of the patient was graded as a moderately differentiated adenocarcinoma of the cribriform type. In the part of this tumor (shown in figure 3A), which was adjacent to the tumor specimen used for transplantation into the nude mice, a stromal part and the malignant epithelium can be distinguished. The glandular structure of the epithelium, containing small- and medium sized acini, is described as a cribriform formation. Once transplanted the tissue was shown to consist mainly of sheets of epithelial cells which also show the fore-mentioned cribriform formation, but without the non-malignant stromal part present in the original tumor material (cf. figure 3A and B). Still the tumor is supported by small bundles of connective tissue, estimated to comprise less than 5 per cent of the total tumor. This stromal part of the PC-82 tumor was shown to have descended from the host animal (see next paragraph) and thus to have a non-prostatic origin [103]. By comparing the histological pattern of PC-82 tumors in the 10th, 20th and 30th mouse passage (figure 3B, C and D respectively), it can be seen that the histological grade and glandular formations are identical throughout the subsequent transplant generations. The majority of the glands in the PC-82 tumors are filled with secretory fluid which was shown to contain large amounts of prostate-specific acid phosphatase [177].

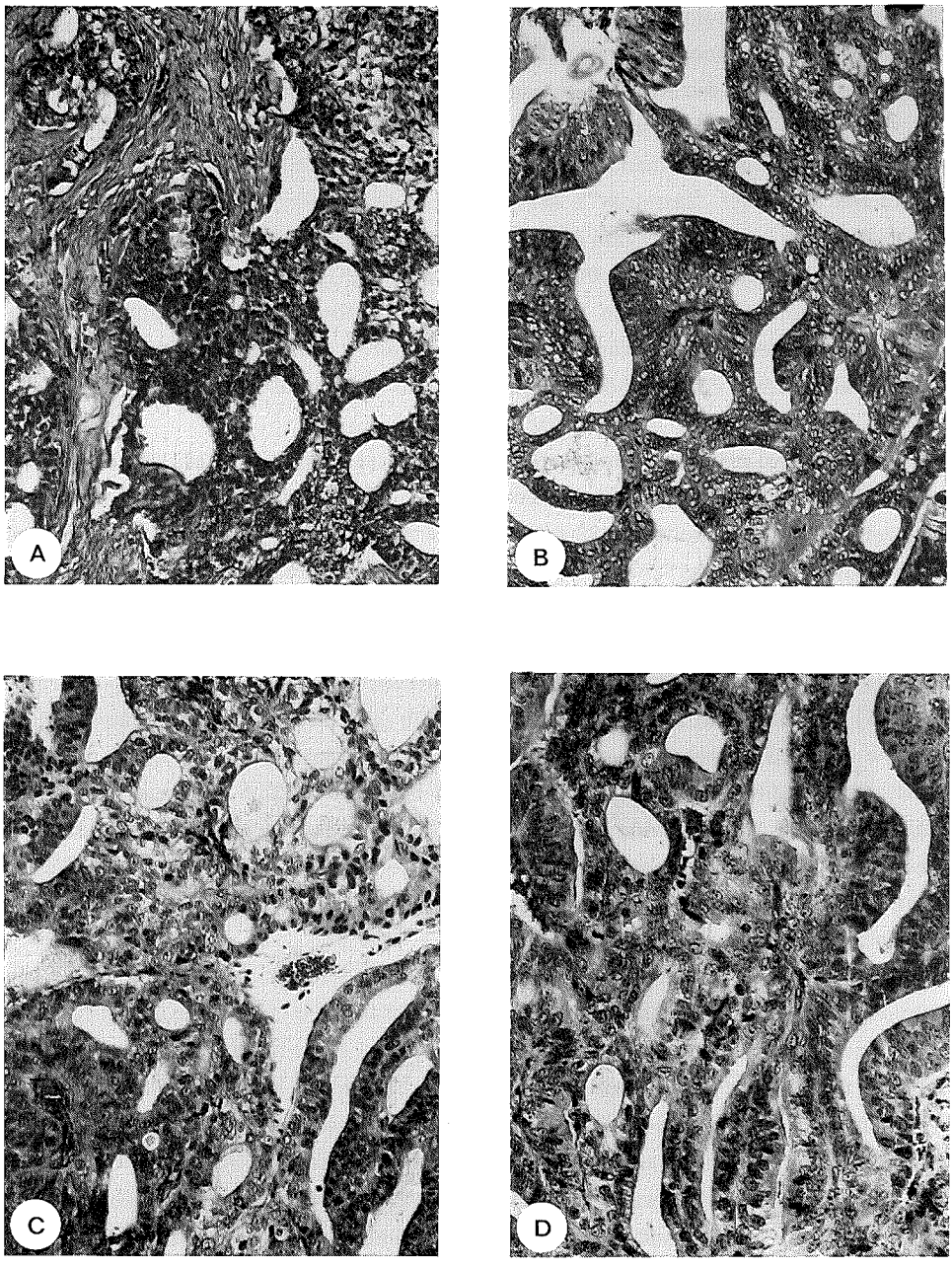

Figure 3 *Histological section of the original patient tumor material of which the PC-82 tumor originates (A), and sections of PC-82 tumors of the 10th (B), 20th (C) and 30th (D) mouse passage.*

DNA Distribution. A representative DNA histogram obtained by flow cytometric (FCM) analysis of a PC-82 tumor is shown in figure 4. A detailed description of the methodology applied is described in the methods section of chapter 9. Analysis of the histogram indicated that this tumor consists of aneuploid cells with a DNA index of approximately 2.0 in comparison with normal human diploid cells. Normal mouse cells, derived from the stromal part of the tumor (cf. "histology"), are present in peak number 2. The PC-82 cells are detected in peak 3 (G_0/G_1), region 4 (S-phase) and peak 5 (G_2/M). It was calculated that 85 to 90 per cent of the cells in the PC-82 tumor are in G_0/G_1 phase, the fraction of cells in G_2/M was found to be less than 10 per cent, whereas the fraction of S-phase cells was estimated not to exceed 5 per cent of the total number of cells. The position of the PC-82 G_0/G_1 peak at 1.9 C corresponds to a chromosome number of 87.

Chromosomes. An extensive cytogenetical characterization of the PC-82 tumor line was recently been completed by König and co-workers [103]. For the cytogenetical analysis of cells derived from the solid PC-82 tumors these autors developed a direct preparation method. In comparison with other methods, this technique is faster and has been shown to yield metaphases of better quality. The number of chromosomes in metaphase cells of tumor tissue of a recent (34th) mouse passage were counted. It can be read from the resulting frequency diagram that the majority of cells were in the hypotetraploid range (figure 5). The modal chromosome number of 85 is in agreement with the estimates made of the flow cytometric DNA histogram mentioned above (figure 4).

Detailed banding analysis was performed on metaphases from different (28 - 35th) transplant generations, showing the presence of 18 consistent marker chromosomes [103]. Among the various rearrangements that of chromosome #1 seems to be a general feature of solid tumors, whereas the involvement of chromosomes #7 and #10 may be more specific for (primary) prostatic carcinomas.

Hormone dependence. The absence of PC-82 tumor growth in intact female and in castrated male mice, which was shown in the first and many of the subsequent mouse passages, indicates the absolute requirement of androgens for the growth of this tumor. The behavior of PC-82 tissue in androgen-depleted animals must be checked at regular time intervals in order to identify hormone independent clones which may arise from the PC-82 tumor following androgen-withdrawal. Moreover, in this way the occurrence of tumors of murine origin, which are rarely found to be induced by the inoculation of human tumor material, can be excluded.

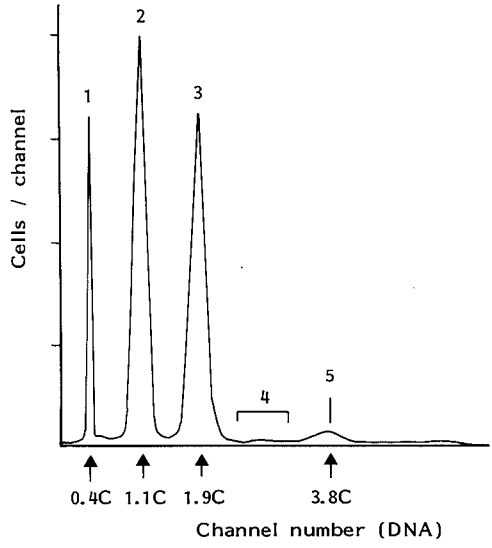

Figure 4 *Flow cytometric DNA distribution of PC-82 cells derived from tissue grown in a T-supplemented nude mouse. 1: chicken erythrocytes (internal DNA reference); 2: normal mouse (diploid) cells; 3: G_0/G_1 cells of the PC-82 tumor; 4: S-phase cells and 5: G_2/M fraction of the cells indicated under peak 3. 1C = the amount of DNA in external diploid reference cells (normal human fibroblasts).*

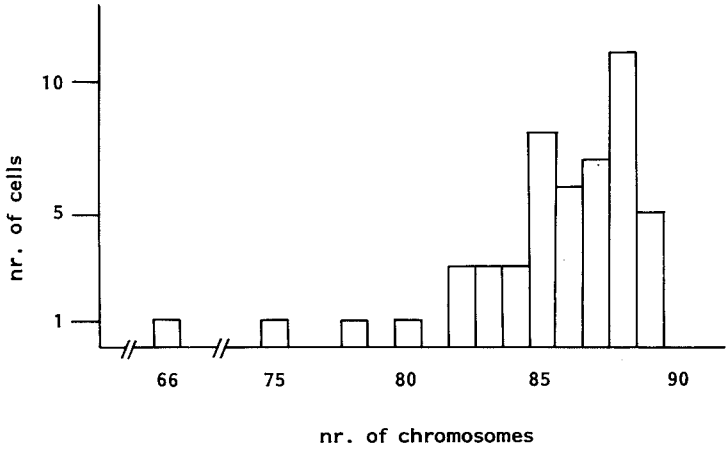

Figure 5 *Frequency diagram of the chromosome number in cells of PC-82 tissue of the 34th transplant generation. [From König et al.; reference 103]*

Androgen receptor. The androgen receptor in the PC-82 tumor tissue has recently been characterized by Brinkmann and co-workers [20]. Cytosol was prepared from tumor tissue grown in mice which were castrated 10 days before harvesting the tumor tissue. Scatchard plot analysis showed a binding protein with a Kd of 0.1 nM for R1881 (methyltrienolone) and a binding capacity of 120 fmol/mg protein. The receptor showed a high affinity for R1881, testosterone, and 5α-DHT respectively, whereas no or little affinity was found for progesterone and estradiol. Photoaffinity labeling of the [^{3}H]R1881-androgen receptor complex and subsequent analysis on SDS-polyacrylamide gels resulted in a covalently labeled protein with a molecular mass of approximately 50 kD. The androgen receptor has a sedimentation coefficient of 4S and a Stokes radius of 3.3 nm at high ionic strength (0.4 M NaCl). It was concluded that the PC-82 tumor contains a binding protein with the properties described for androgen receptors present in prostatic tissue.

Monoclonal antibodies. Monoclonal antibodies (McAbs) were raised against cells of the PC-82 tumor by Gallee [60]. Balb/c mice were immunized with PC-82 tumor cells derived from mouse-supported tumor tissue. McAbs were prepared by the fusion of spleen cells of these mice with a murine myeloma cell line. The resulting antibodies were screened and characterized further. Two McAbs, named ER-Pr 1 and ER-Pr 2, reacted exclusively with prostatic epithelium. Antibody affinity chromatography combined with SDS-PAGE showed that both antibodies were directed against a 35 kD protein. By using the technique of immuno-blotting it was found that the protein is identical with prostatic antigen (PA), originally described by Wang [227].

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CHAPTER 2

THE USE OF STEROID-CONTAINING SILASTIC IMPLANTS IN MALE NUDE MICE: PLASMA HORMONE LEVELS AND THE EFFECT OF IMPLANTATION ON THE WEIGHTS OF THE VENTRAL PROSTATE AND SEMINAL VESICLES

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Chapter 2

The Use of Steroid-Containing Silastic Implants in Male Nude Mice: Plasma Hormone Levels and the Effect of Implantation on the Weights of the Ventral Prostate and Seminal Vesicles

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The effects of implantation of steroid-containing capsules into male nude mice on steroid concentrations in plasma and weight of accessory sex organs were studied. Intact male nude mice had plasma levels of testosterone (T) of 8.0 ± 2.4 ng/ml, while T implantation (length 1.0 cm) of a group with castrated male mice resulted in a mean level of 8.1 ± 0.3 ng/ml. This level was reached within 2 days after implantation and lasted for at least 40 days. After longer periods of application (up to 75 days) physiological levels could still be attained. Treatment of intact male nude mice with estradiol (E2)-containing implants (length 0.5 cm) resulted in constant levels of plasma-E2 (250 pg/ml) also lasting for at least 32 days. This treatment resulted in a mean plasma-T level of 1.0 ± 0.2 ng/ml, which was still significantly higher than that obtained after castration (0.14 ± 0.03 ng/ml). Up to 16 days after implantation E2 did not cause a decrease of the weights of the accessory sex glands in intact male mice, while after 32 days a significant reduction (50% of the control animals) of the organ weights was observed. The present data obtained with T and E2 implantation show that this route of administration of hormones is also very applicable in the nude mouse model.

Key words: Silastic implant, nude mice, hormones, prostate, seminal vesicles

INTRODUCTION

Silicone rubber has been found to be useful as a material used for chronic implants because it does not cause foreign body reactions even after prolonged periods [1]. Silastic capsules have been shown to release testosterone (T) [2-4], estradiol (E2) [5,6], and various other steroids [7] at uniform rates in several species (eg, mouse, rat, and baboon). Especially because of the sustained and constant release of steroids from Silastic devices, this method of application is preferable to that of injection as an oil suspension, which results in fluctuating steroid concentrations in the blood following the rapid absorption of steroid from the injection site [8].

Plasma levels of T show extreme fluctuations in adult male mice of several strains, suggesting that T release is pulsatile in this species [9,10]. It was demonstrated by Rebar et al [11] that athymic nude mice had significantly reduced concentrations of serum gonadotropins and T compared to their heterozygous littermates. The poor

transplantability of hormone-dependent human tumors, such as breast and prostatic carcinomas, on nude mice might at least partly be related to the hormonal status of the host animal. It was shown by Rae-Venter and Reid [12] that the primary take of breast carcinomas on nude mice was improved by implantation of estradiol pellets simultaneously with tumor grafting.

The growth of the human prostatic tumor line (PC-82) in nude mice, which was developed in our own institution [13], was shown to be dependent on androgens [14]. In order to optimize the growth of PC-82 tumor tissue as well as to improve the primary take of newly transplanted prostatic carcinomas, T-containing Silastic implants were tested in the nude mouse model. Furthermore, for long-term estrogen treatment of this tumor, E2-containing implants might also be applicable as an alternative for daily injection of the mice.

Thus, time course experiments were carried out with T implants in castrated male mice and with E2 implants in intact male mice, while the effects of E2 implantation on the accessory glands were compared with that obtained by castration.

MATERIALS AND METHODS

Silastic Implants

Implants were prepared by cutting Silastic (polydimethylsiloxane) medical grade tubing (1.5 mm inner diameter, 2.1 mm outer diameter; Talas, Zwolle, The Netherlands) to the desired length and sealing one end with Silastic Medical Adhesive, Type A (Dow Corning, Midland, MI). After 24 hours the tubing was packed with crystalline steroid (Steraloids, Pawling, NY) and the open end sealed with adhesive, which was allowed to dry at least 24 hours before implantation. The purity of the steroids that were used (ie, testosterone and estradiol) was checked chromatografically. Capsule size is presented as the length of tubing effectively filled with steroid. Prior to implantation capsules were placed in absolute ethanol for 15 minutes to sterilize the capsule and to remove steroid that might be adhered to the outside of the implant.

Treatment of the Animals

Male nude mice of the Balb/c background, bred in our institute, were used for this study. The animals, housed separately, were maintained under controlled conditions of temperature (24-26°C) and humidity (60%). For these experiments mice at the age of 8-12 weeks and with a body weight between 20 and 30 g were used.

Through a small incision in the dorsal area Silastic implants were subcutaneously implanted on the abdominal wall as shown in Figure 1. Auto clips (Clay Adams, Parsippany, NJ) were applied to close the incision. Installation of implants was carried out under light ether anesthesia. Castration was carried out via the scrotal route under total anesthesia with chloralhydrate.

At different times (see Results) after implantation animals were exsanguinated from the orbital sinus under ether anesthesia. Plasma was harvested by centrifugation at 9000g for 3 minutes and stored at minus 20° until analyzed. After dissection, the weights of ventral prostate and seminal vesicles (including coagulation gland and seminal plasma) were determined.

Plasma Hormone Assays

Plasma levels of testosterone were estimated using the radioimmunological procedure described by Verjans et al [15]. Plasma levels of estradiol were estimated

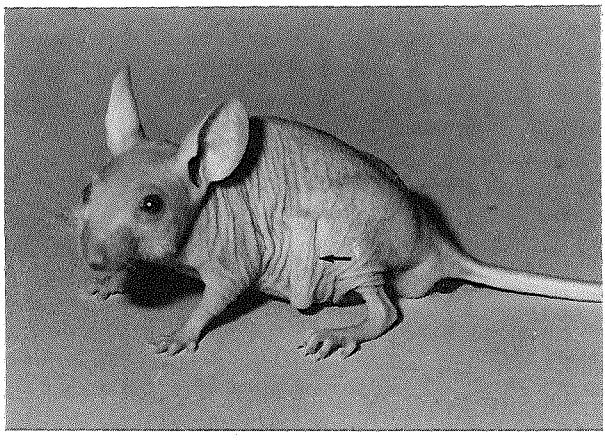

Fig. 1. Male nude mouse bearing a subcutaneously implanted Silastic capsule (arrow). Installation was carried out via an incision in the dorsal area of the mouse.

using the direct ^{125}I-estradiol radioimmunoassay kit supplied by EIR (Würenlingen, Switzerland).

Statistical Procedure

The significance of difference between values of different groups was calculated using two-tailed Student's T-tests. Differences were considered to be statistically significant when P was smaller than 0.05.

RESULTS

Testosterone Implantation

Time-course experiments were carried out to investigate the effectiveness of the implants and to determine the steroid plasma levels that can be attained by implantation.

Male nude mice, castrated 7 days before starting the experiment, were implanted with capsules (length 1.0 cm) containing approximately 10 mg T. Groups of animals were sacrified 0, 2, 6, 10, and 16 days following implantation. Constant levels of plasma-T were reached within 2 days after implantation and lasted for at least 40 days (Fig. 2). Since significant differences between the various time points were not observed, the mice in this experiment were considered to be one group (Table I). The mean level of plasma-T in this group of mice was similar to that in a group of intact male nude mice; the variation in the latter group, however, was much smaller compared to that observed in T-implanted mice (Table I, Fig. 2).

In the same group of mice implanted with T, a complete recovery of the weights of the ventral prostate (VP) and seminal vesicles (SV) was observed within 16 days after implantation (Fig. 3). In a subsequent experiment it was demonstrated that at 40 days after implantation the plasma level of T was similar to that observed in the 16-day experiment, while at 75 days after implantation plasma-T was decreased to a level of 5.4 ng/ml (Fig. 2). Lower levels of plasma-T were attained by using implants with a length of 0.25 cm (Table I).

In order to investigate the possibility of applying implants that were previously used in other animals, intact nude mice were implanted with T capsules (used for at

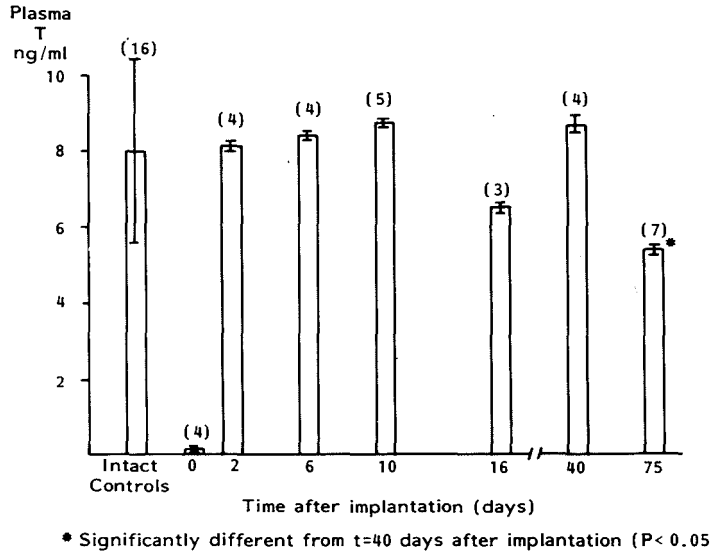

Fig. 2. Plasma levels of T in castrated male nude mice at different time points after implantation with capsules (length 1.0 cm) containing approximately 10 mg T. First bar represents the mean plasma level in a group of intact male mice. Values are expressed as mean ± SEM with a number of mice in parentheses.

TABLE I. Plasma Levels of Testosterone in Intact Control and T-Implanted, Castrated Male Nude Mice*

Group	N	Testosterone (ng/ml)[a]	Range
Intact mice	16	8.0 ± 2.4	0.5–24.7
Castrated mice			
T implant (1.0 cm)	16	8.1 ± 0.4	5.5–10.2
T implant (0.25)	4	2.2 ± 0.6[b]	0.9– 3.3

*The level of T-implanted mice represents the mean of the levels observed at 2–16 days after installation (compare Fig. 1).
[a]Values are expressed as mean ± SEM.
[b]Significantly different from T-implanted (1.0cm) animals ($P < 0.005$).

least 40 days). At 7 days after implantation the mean level of plasma-T in this group was 7.4 ± 0.5 ng/ml (N = 6), which was not different from that observed in the group of mice implanted with first-use implants.

Estradiol Implantation

A group of intact male nude mice was implanted with capsules (length 0.5 cm) containing approximately 5 mg E2. Groups of animals were sacrified at 0, 2, 6, 9, and 16 days following implantation. Plasma-E2 was estimated and the wet weight of VP and SV of individual mice was determined. After 2 days, plasma-E2 reached a level of 400 pg/ml which was also observed after 6 days. Hereafter the level decreased and remained at a value of 250 pg/ml (Fig. 4). In a subsequent experiment carried

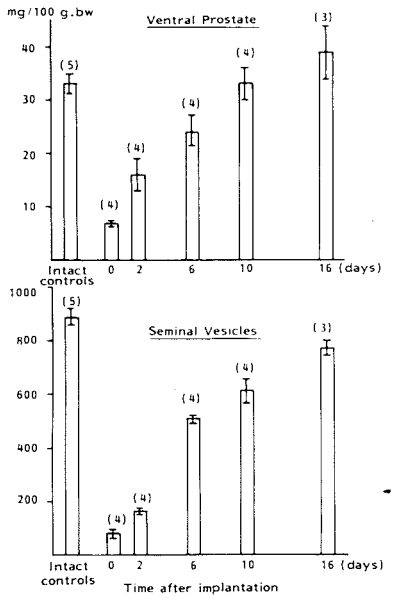

Fig. 3. Recovery of the weights of ventral prostate and seminal vesicles following T implantation in castrated male mice. The various time points correspond with those in Figure 2. Values are expressed as mean ± SEM with the number of mice in parentheses.

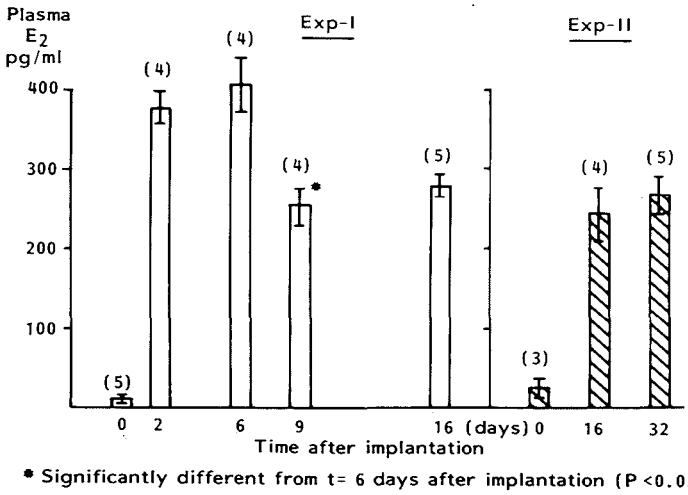

Fig. 4. Plasma levels of E2 in intact male mice at different time points after implantation with capsules (length 0.5 cm) containing approximately 5 mg E2. Experiments I and II were carried out in a similar way and were prolonged for 16 and 32 days, respectively. Values are expressed as mean ± SEM with the number of mice in parentheses.

out in a similar way, the result obtained after 16 days was confirmed, and it was shown that this level of plasma-E2 lasted for at least 32 days (Fig. 4).

During the course of the first experiment with E2 implantation the weights of the VP and SV were not significantly decreased and remained at the level observed in intact controls (Fig. 5, experiment I). In contrast it was shown that castration caused a gradual decrease of the weights of the VP and SV (respectively to 20 and 15% of the controls at 16 days after castration) in a group of intact male nude mice (Fig. 6). In order to explain these findings, the level of T in the plasma of E2-implanted and castrated mice was determined. Castration and implantation of E2 caused a decrease of plasma-T (Table II); while in E2-implanted mice plasma-T was significantly higher than that in the castrated group.

In the second experiment (Fig. 5) with E2 implants of the same length, it was observed that 32 days after implantation the weights of VP and SV were significantly decreased compared to those in the control group. The level reached at 16 days after castration, however, was not attained by using this type of implant (Fig. 5).

DISCUSSION

The athymic nude mouse is widely used for the heterotransplantation of human cancer tissues. Since it has been demonstrated by Rebar et al [11] that nude mice have reduced plasma concentrations of gonadotropins and testosterone, supportive treatment with androgens might improve the take and growth of androgen-dependent prostatic tumors on this animal. In some preliminary experiments steroid-containing

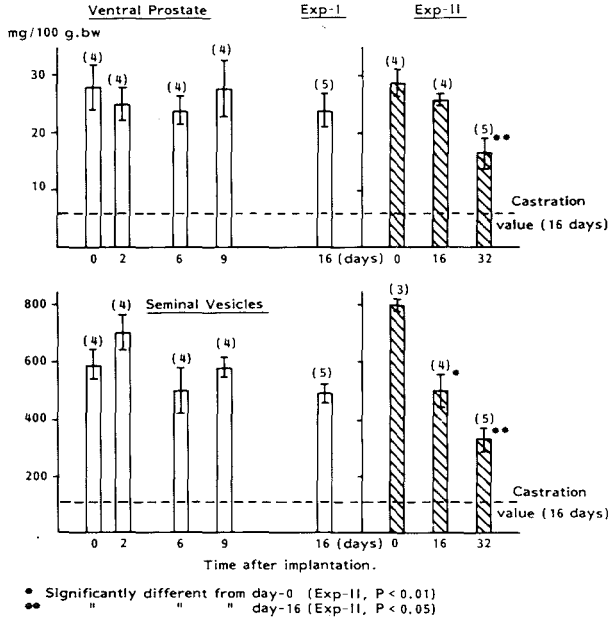

Fig. 5. Organ weights of ventral prostate and seminal vesicles after E2 implantation of intact male mice. The various time points correspond with those in Figure 4. The dotted lines represent the levels attained at 16 days after castration. Values are expressed as mean ± SEM, with the number of mice in parentheses.

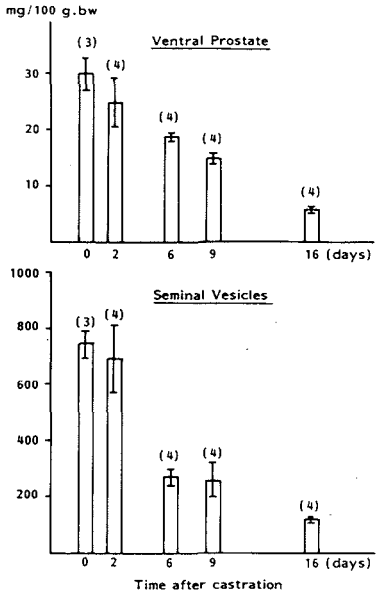

Fig. 6. Organ weights of ventral prostate and seminal vesicles after castration of intact male mice (compare Fig. 5). Values are expressed as mean ± SEM, with the number of mice in parentheses.

TABLE II. Plasma Levels of Testosterone in Control Male Nude Mice and in Male Mice After Castration and E2 implantation

Group	N	Testosterone (ng/ml)[a]	Range
Intact controls	16	8.0 ± 2.4	0.5 -24.7
Castration	16	0.14 ± 0.03[b]	0.03- 0.26
E2 implant (0.5 cm)	15	1.03 ± 0.24[b, c]	0.23- 3.9

[a]Values are expressed as mean ± SEM.
[b]Significantly different from control animals ($P < 0.01$).
[c]Significantly different from castrated animals ($P < 0.01$).

Silastic implants were shown to be an appropriate tool for long-term substitution of hormones in nude mice with the permanent prostatic tumor line PC-82 [16]. The present study was conducted to investigate the effects of Silastic capsule implantation on hormone levels in the nude mouse in more detail.

Levels of plasma-T within the physiological range (Table I) were attained by implantation of castrated male mice with T-containing capsules (length 1.0 cm) and could be maintained for at least 75 days (Fig. 2). Within 16 days after implantation the weights of the accessory sex organs in these animals had reached that of intact mice (Fig. 3), which also indicates that in the implanted animals plasma-T at least attained the physiological level. However, castrated mice implanted with T capsules showed less variation in circulating T levels than did intact animals (Fig. 2; Table I). The observation in the intact animals (eg, the wide range of plasma T levels) is in agreement with those of Bartke et al [9] showing that plasma T in male mice is highly fluctuating. Consequently peak plasma levels of T might also be included in the group

of intact male mice (Table I) resulting in a relatively high mean value in these animals. In general implantation will lead to T levels exceeding the low physiological levels in intact male nude mice. Therefore the use of T implants will be advantageous for the take and growth of hormone-dependent human cancers on nude mice.

Although the release of E2 from Silastic devices was shown to be slower than that of testosterone [17], this type of implant has also been applied in vivo [6, 18] as well as in vitro [7].

Pharmacological plasma levels of E2 (250-400 pg/ml) in intact male nude mice were attained by using 0.5-cm E2 implants (Fig. 4); these levels could be maintained for at least 32 days. This treatment resulted in a significant decrease of plasma-T compared to that in intact male mice (Table II). Plasma-T was not suppressed to the level reached after castration, however. This observation is in agreement with the absence of a significant reduction of the weights of the VP and SV following E2 implantation (Fig. 5), while castration resulted in a rapid decrease of the weight of both organs during the same period of time (Fig. 6). The pattern of involution of these organs, observed after castration, is identical to that obtained after androgen withdrawal in the rat [19].

In a similar experiment it was demonstrated that between 16 and 32 days after E2 implantation a significant reduction of the weights of both organs occurred, but not to the same extent as that reached after castration (Fig. 6).

It might be possible that, in spite of the low levels of plasma-T following E2 implantation, the VP and SV are able to take up a sufficient amount of androgen for maintenance of the organ weights for at least 14 days, although after this period a decrease of the weight of both organs occurred. The submaximal suppression of T following E2 implantation makes this type of treatment appropriate to obtain low levels of circulating T in the PC-82 nude mouse model. Besides this, using longer E2 implants with a larger diameter, plasma-E2 levels in the nanogram range could be attained, resulting in plasma-T levels near those obtained after castration (results not shown).

In contrast to the application in many other species, the use of Silastic implants in nude mice was reported in only a few cases. In a study of Drago et al [20] with the Dunning rat prostatic tumor grown on nude mice, T- and E2-containing capsules were applied; however, the levels of steroid attained following implantation were not determined. It is our opinion that, especially for the study of hormonal effects on human cancer tissues grown on nude mice, the application of steroid-containing implants requires monitoring the plasma steroid levels attained via this route of administration.

From the present results it was concluded that Silastic implants are very useful in nude mice, particularly for long-term treatment purposes.

ACKNOWLEDGMENTS

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CHAPTER 3

BIOLOGICAL EFFECTS OF HORMONAL TREATMENT REGIMENS ON A TRANSPLANTABLE HUMAN PROSTATIC TUMOR LINE (PC-82)

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CHAPTER 3

BIOLOGICAL EFFECTS OF HORMONAL TREATMENT REGIMENS ON A TRANSPLANTABLE HUMAN PROSTATIC TUMOR LINE (PC-82)

ABSTRACT

The effects of hormonal manipulation on the growth of a transplantable human prostatic carcinoma line (PC-82) were studied. The histological pattern of the PC-82 tumor, which still closely resembles the original tumor material, and the tumor growth rate did not change during the subsequent mouse passages. Growth of PC-82 tumor tissue on female and castrated male mice did not occur. Castration of tumor-bearing mice resulted in a cessation of tumor growth, after which the tumor volume decreased 50 ± 27 percent within 6 weeks after castration. Hormone-independent regrowth of the tumor tissue was not observed after long-term withdrawal of androgens. After a period of 10 weeks following tumor growth arrest, administration of testosterone almost directly resulted in regrowth of the tumor. Hormones, testosterone and estradiol, were administered by silastic implants. Intact male nude mice were shown to have highly fluctuating levels of testosterone. Implantation with testosterone resulted in constant levels of circulating testosterone, which could be maintained for at least 10 weeks, while the mean concentration of plasma testosterone was not different from that in control male mice. The doubling time of tumors grown on testosterone-substituted intact female and intact and castrated male mice was significantly shorter than that of tumors grown on intact male mice. Histologically the tumors grown on androgen-substituted mice were similar to those grown on untreated mice; the mitotic index, however, was much higher in the testosterone treated animals. Implantation of intact male mice with estradiol suppressed plasma testosterone to a mean level of 1 ng per ml and prevented the growth of PC-82 tumor tissue almost completely. Treatment of tumor-bearing mice with an estradiol implant following androgen withdrawal did not result in a further decrease of the tumor volume compared to the mice without additional estradiol implantation.

INTRODUCTION

Carcinoma of the prostate is one of the most common cancers in the male population. Many aspects of human prostatic carcinoma can be investigated properly only by the use of suitable model systems. In laboratory animals the incidence of prostatic carcinomas is

relatively low, although a number of cases have been described during the last few years (1). Perhaps the most important model systems now available have been developed from a spontaneous prostatic carcinoma in an aged Copenhagen rat (the R3327 Dunning tumor) (2). This tumor gave rise to a variety of lines and sublines with different and well-defined characteristics, such as hormone-sensitivity (3) and metastatic capacity (4). Other animal models that are currently being studied include the sex steroid-induced prostatic carcinoma in the Noble rat (5), which also has been studied after heterotransplantation on nude mice (6). Hormone dependent sublines of both the Dunning and the Nb-rat tumor ultimately showed a recurrence of growth after withdrawal of hormones. For this phenomenon, which is also generally observed in prostatic carcinoma in the clinical situation, different mechanisms were proposed (7).

A common disadvantage of the models mentioned above is their nonhuman origin. For that reason several attempts have been made to propagate human tumors in immune-deficient animals, such as the athymic nude mouse. In vitro cell lines, derived from human prostatic carcinoma, readily induce tumors after injection in nude mice. Thus tumors have been established from the cell lines EB-33 (8), DU-145 (9), PC-3 (10) and LNCaP (11). Heterotransplantation of human prostatic carcinomas on nude mice was shown to be successful in only a limited number of cases. Among these only a few appeared to be serially transplantable (12, 13). In our own institution the transplantable line PC-82 was developed (14). The properties of this tumor line, slow growth rate, hormone dependence and secretion of prostatic acid phosphatase (15), are comparable with those of prostatic cancer in the patient. Recently the influence of endocrine manipulation on the concentration of prostatic acid phosphatase in the PC-82 tumor tissue was studied (16).

In the present study we report the effects of castration and hormone substitution on PC-82 tumor growth. For long-term treatment purposes steroid-containing silastic implants (17) were also successfully applied to the PC-82 tumor model. Thus the influence of testosterone and estradiol implantation on the take and growth of PC-82 tumor tissue was studied.

MATERIALS AND METHODS

Tumor material

The original tumor, a moderately differentiated adenocarcinoma of the prostate, was removed from the patient by total perineal prostatectomy in July 1977. For initial and sequential grafting of the PC-82 tumor tissue, mice of the Balb/c background with an age of 6 to 12 weeks were used as recipients. Experiments were carried out with nude mice

transplanted with tumor fragments weighing about 50 mg. The tissue was grafted subcutaneously at each side of the shoulder through a single incision in the back of the mouse under light ether anesthesia. A representative fragment of each tumor that was used for serial transplantation was examined histologically.

Growth measurements

Tumor growth was monitored by (bi)weekly measurement of 2 perpendicular tumor diameters with calipers. The tumor volume was calculated from the formula:

$$V = \frac{\pi}{6} (d_1 \times d_2)^{3/2} .$$

Tumors reaching a volume of 50 mm^3 (TV-50) or more were considered as having positive tumor take on the host animal. The TV-50 as well as the tumor doubling time (Td) were estimated from a semilogarithmic plot of the tumor volume against the time after transplantation. Only exponentially growing tumors (between 50 and 500 mm^3) with a significant correlation between the measuring points were evaluated.

Treatment of the animals

Treatment (castration or hormone substitution) was started when tumors were in the exponential phase of growth. Castration of tumor-bearing mice was carried out via the scrotal route under total anesthesia with chloralhydrate. To avoid daily injection of the tumor-bearing nude mice, hormones (testosterone (T) and estradiol (E_2)) were administered by subcutaneous implantation of silastic implants packed with crystalline steroid. By using these implants, constant plasma steroid levels in nude mice were reached and could be maintained for an extensive period of time (2 to 3 months). Installation and removal of implants was carried out under light ether anesthesia.

Other procedures

Plasma levels of testosterone and estradiol were estimated using radioimmunoassay procedures. Statistical analysis of the data was performed by a nonparametric test (Wilcoxon's test) or Student's t test (when indicated). Differences were considered to be statistically significant when a p-value <0.05 was found.

RESULTS

Plasma hormone concentrations

In intact male nude mice a rather large variation of plasma-T levels was observed (table 1). Constant and physiological levels (8 ng per ml of T were attained after implantation of castrated male nude mice with T-implants (lenght 1.0 cm) (table 1). These levels were reached within 2 days after implantation and were maintained for at least 40 days. At 75 days after implantation, T was decreased but still at a constant level of 5.4 ± 1.0 ng per ml (n = 7). Implantation of castrated male mice with short T-implants (lenght 0.25 cm) resulted in plasma T-levels of approximately 2 ng per ml. E_2 implantation (length 0.5 cm) in a group of intact male mice resulted in a mean plasma concentration of E_2 of 250 ± 48 pg per ml (n = 15). In this group of mice plasma T was significantly suppressed compared to the intact controls (table 1). The level reached after E_2 implantation was not as low, however, as that after castration.

Growth characteristics of untreated PC tumors

As of May 1983 the PC-82 tumor was in the 22nd transplant generation. The histology of the tumor still closely resembled the original tumor, also showing the cribriform aspect with many acini. Figure 1 shows the histological pattern of a PC-82 tumor in the 15th passage, grown on an intact male nude mouse. The PC-82 tumor mainly consists of epithelial cells, with only small bundles of stroma tissue (less than 5 per cent of the total tumor), which was proved to be derived from the host animal. No major alterations in the histological pattern, which was monitored routinely, were observed during the subsequent mouse passages.

PC-82 tumor tissue was shown to have a slow growth rate. The tumor doubling time (Td), calculated from growth curves of 46 tumors (grown on intact male mice) in the 11 to 14th passage was 18 ± 5 days (mean ± S.D.). The time required to attain a tumor volume of 50 mm^3 (TV-50), in effect the tumor "lag-phase" which was also calculated from these curves, was 37 ± 16 days. No significant differences were observed between the tumor doubling times in the individual mouse passages (fig.2). This proved that the tumor growth rate did not change substantially during the subsequent passages.

The absence of growth on intact female and castrated male mice was shown in the 1st and several subsequent transplant generations, indicating the androgen dependence of this tumor line. Several experiments were carried out in order to characterize this important property further.

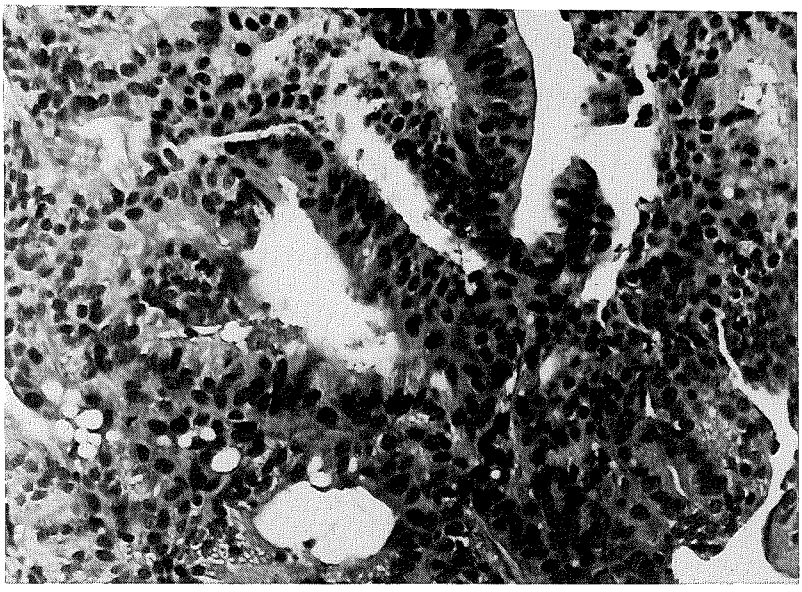

Figure 1 *Histological section of a PC-82 tumor in the 15th transplant generation. The tumor mainly consists of cuboidal epithelial cells; characteristics of the tissue are comparable with those of the tumor of origin. Small bundles of stroma tissue are derived from the host animal (magnification 125 x).*

Table 1. Plasma testosterone (T) levels in control and T-implanted male nude mice and in male mice after castration and estradiol (E_2) implantation.

Group	No.	Testosterone (ng/ml)*	Range
Intact controls	16	8.0 ± 9.7	0.5 - 24.7
T-implant (1.0 cm)	16	8.1 ± 1.4	5.5 - 10.2
Castration	16	0.14 ± 0.12	0.03 - 0.26
E_2-implant (0.5 cm)	15	1.03 ± 0.95**	0.23 - 3.9

* Values are expressed as mean ± S.D.

** Significantly different from castrated animals (Student's T-test; $p < 0.01$).

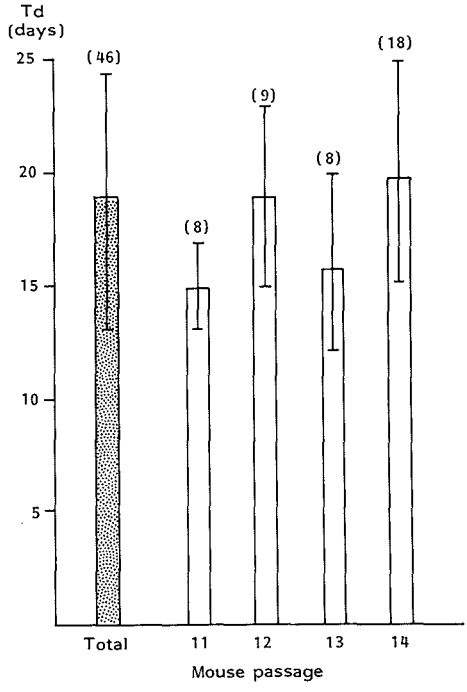

Figure 2 *Doubling time (Td) of tumors in the 11 - 14th passage. Td was calculated from a semilogarithmic plot of the tumor volume against the time after transplantation. For further details see section materials and methods.*

HORMONE-DEPENDENCE OF PC-82

Castration experiments

Castration of PC-82 tumor-bearing mice resulted in a cessation of tumor growth in all cases studied so far. In a group of 10 mice the tumor volume was decreased 57 ± 27 per cent (mean ± S.D.) within 6 weeks after castration. A rather extreme example of the response after castration is shown in figure 3A. After castration the volume of this tumor remained constant for a short period of time whereafter the tumor rapidly decreased 90 per cent at 40 days after castration. A histological section of this tumor (volume <10 mm^3) remaining at 86 days after castration (fig 3B) shows degeneration as indicated by hyperchromatic nuclei and clear cytoplasm of the cells. The main part of the tissue appeared to be fibrotic, while only a small part still had the original structure. In most cases, however, tumors did not

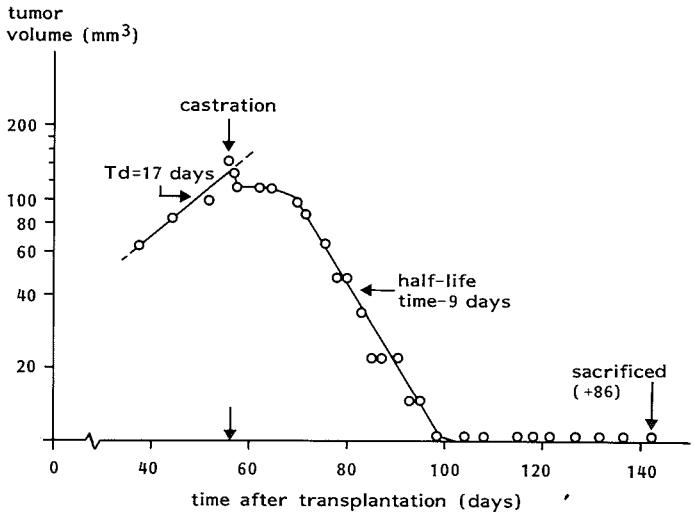

Figure 3A *Growth curves of a PC-82 tumor in the 11th passage and the response on castration of the host animal. Castration was carried out when the tumor had reached a volume of 140 mm^3, 56 days after transplantation.*

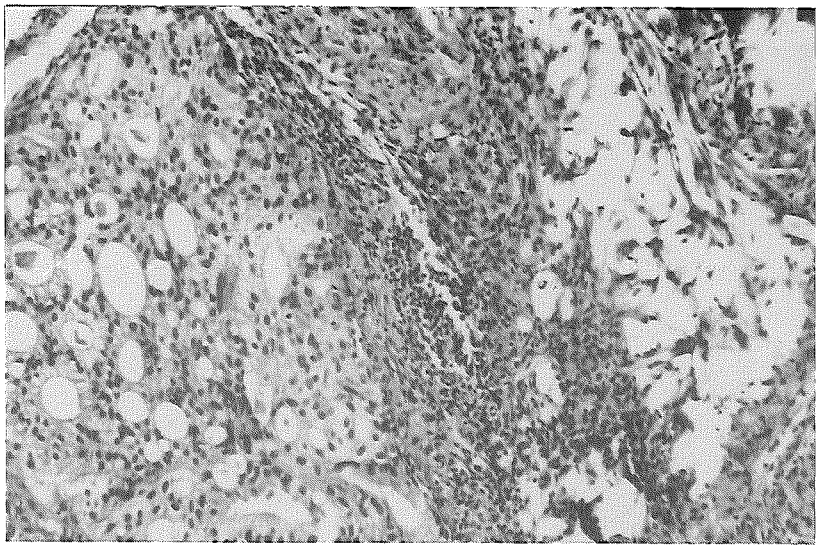

Figure 3B *Histology of this tumor that remained at 80 days after castration (A). Epithelial tissue with hyperchromatic nuclei and light and vacuolized cytoplasma. Right part: fibrotic tissue remained after disappearance of the epithelial part. Note the infiltration of leucocytes in the central part of this section (magnification 125 x).*

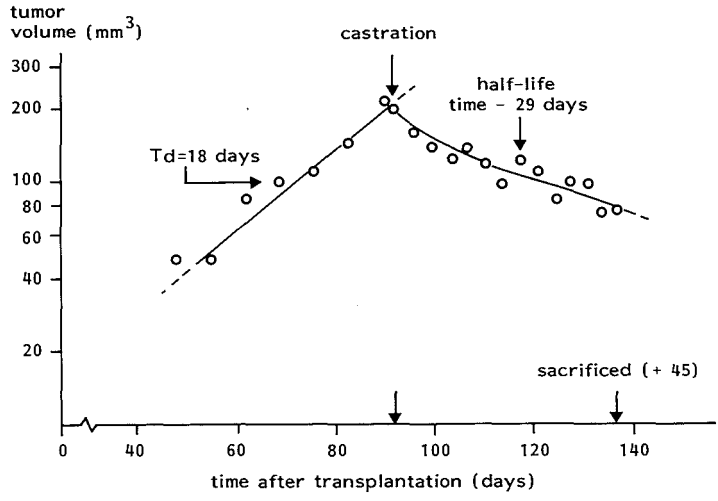

Figure 4A *Growth curve of the PC-82 tumor in the 12th mouse passage. Castration was carried out when the tumor had reached a volume of 200 mm^3, at 92 days after transplantation.*

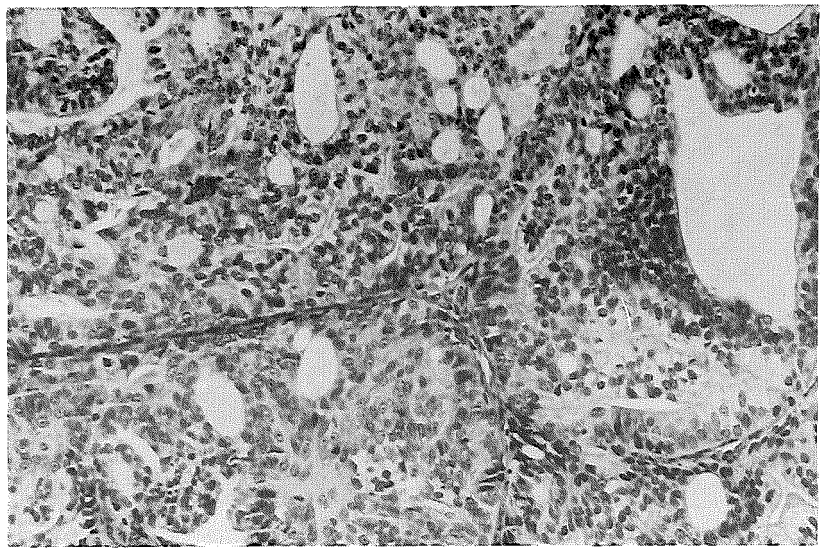

Figure 4B *Histological section of the tumor tissue that remained at 45 days after castration (A). Most of the epithelial cells appeared to be viable, only a minor part of the cells was vacuolized and in a degenerating stage.*

react so dramatically and in general a more gradual decline of the tumor volume was observed. Figure 4A shows the curve of a tumor with a less pronounced effect after androgen withdrawal. The volume of this tumor decreased 60 per cent at 45 days after castration. In contrast to the histology shown in figure 3B, the histology of this tumor (fig. 4B) mainly consists of vital cells with only a minor part that appeared to be degenerating. In none of the castration experiments performed up till now could spontaneous regrowth of the tumor be observed.

In an additional experiment, the effect of androgen administration was studied on a PC-82 tumor after castration-induced growth arrest. After a relatively long post-castration period of 79 days (with 25 per cent reduction of the tumor volume), administration of testosterone (via a silastic implant) resulted in regrowth of the tumor (fig. 5). The increase of the tumor volume, which started directly after testosterone stimulation, was exponential up to a volume of 1000 mm^3. The tumor doubling time (23 days) calculated from this part of the curve was considerably longer compared to that before castration (15 days).

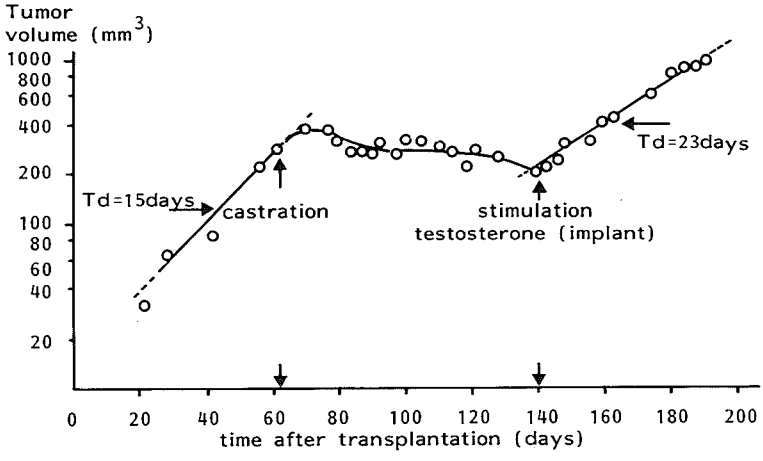

Figure 5 *Influence of castration and androgen stimulation of PC-82 tumor tissue grown on an intact male nude mouse. Testosterone was administered by using a silastic implant at 79 days after castration.*

Effect of testosterone substitution

As mentioned in the previous section, PC-82 tumor growth could not be demonstrated on female and castrated male mice. PC-82 tumor tissue grafted simultaneously with a T-implant and on intact or castrated male mice resulted in tumor growth in at least 80 per cent of the cases (table 2). This result was comparable to that obtained in sham-implanted intact

male mice, having a tumor take of 85 per cent. In the substituted groups the 50 mm^3 tumor volume was attained earlier than in the controls. These differences were statistically not significant, however (table 2).

Table 2. Tumor take and tumor doubling time of PC-82 tumor tissue grown on androgen-substituted castrated and intact male and intact female mice*

Mice	Implant	Number of Mice	Tumor take	TV-50** (Days)
Intact male	sham	11	85	32 ± 8
Intact male	T	7	100	23 ± 14
Castrated male	T	5	80	24 ± 6
Intact female	T	7	100	26 ± 11
Intact male	E_2	8	5***	> 65

* Values are expressed as mean ± S.D.

** TV-50: time required to attain a tumor volume of 50 mm^3

*** Representing one tumor (<200 mm^3) at 96 days after transplantation.

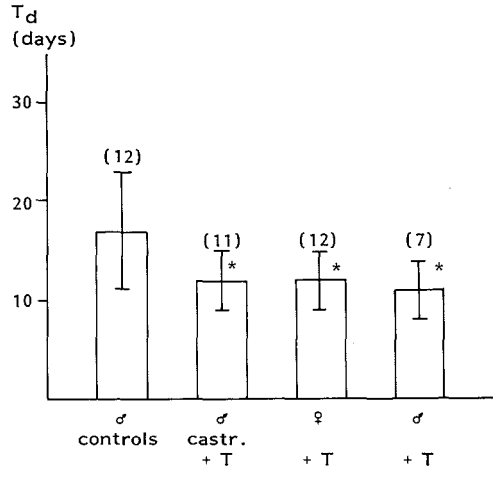

Figure 6 *Doubling times of tumors grown on control male mice and on T-substituted intact female and intact and castrated male mice. Values are expressed as means ± S.D., with the number of tumors between parentheses.*
**Significantly different from control group (p <0.05).*

Figure 6 shows the tumor doubling time in the groups with control and T-treated mice. The doubling times in the T-substituted intact male and female mice (11 ± 3 and 12 ± 3 days respectively) were significantly ($p < 0.05$) shorter than those of tumors grown on intact male mice (18 ± 5 days). In contrast to the other two T-substituted groups, the take and doubling time of tumors in the group of castrated male mice were not significantly different from those in the control mice. However, in an experiment with a group of 22 castrated male mice, T-substitution resulted in a tumor doubling time (12 ± 3 days) that was comparable with those in the intact male and female mice (fig.6). In a group of castrated male mice implanted with 0.25 cm T-implants, which resulted in low levels of circulating T, PC-82 tumor tissue also grew. The "lag-phase" of these tumors, however, was significantly longer compared to that of tumor grown on mice with 1.0 cm T-implants (preliminary observation). Generally the histological picture of all the tumors grown on androgen treated mice did not differ from that of tumors grown on intact male mice, except for the mitotic index which was much higher in tumors of T-treated mice.

Table 3. Effect of testosterone withdrawal and of estradiol treatment* on PC-82 tumor tissue.

Group		No.	Tv(+)/(-) (%)**	Range
I	Controls	7	+100 ± 28	52 - 134
II	"Castration"	6	-50 ± 7	45 - 63
III	"Castration" + E_2	7	-50 ± 10	35 - 65

* Treatment period: 25 days.

** Values are expressed as mean ± S.D.

Effect of estradiol implantation

Implantation of intact male mice with E_2-implants (lenght 0.5 cm, used in all further experiments) suppressed the plasma-T to a level of approximately 1 ng per ml (table 1).

E_2-implantation simultaneous with tumor grafting prevented the growth of PC-82 tumor tissue almost completely (table 2). Only 1 out of 16 tumor transplants resulted in a tumor that exceeded the volume of 50 mm^3. This volume was attained after a "lag-phase" of at least 65 days. In an additional experiment PC-82 tumor tissue was grown on T-substituted, castrated male mice. After the tumor reached the exponential phase of growth, the implants were removed ("castration-like" effect) and in a number of mice replaced by an E_2-implant.

During the treatment period of 25 days, androgen withdrawal resulted in a decrease of 50 per cent of the tumor volume (table 3), which was comparable with the result obtained after castration (see 1st part of this section). The same result was obtained with the group of mice with E_2-implantation. Control animals, from which the T-implant was not removed, showed an increase of the tumor volume of 100 per cent during the same treatment period. From these results it was concluded that estrogens, at the level reached with silastic implantation, only indirectly influenced the take and growth of PC-82 tumor tissue by the suppressive effect on plasma-T levels.

DISCUSSION

Several aspects (the slow growth rate, hormone-dependence and secretion of prostatic acid phosphatase) of the PC-82 tumor model make it very suitable for the study of human prostatic carcinoma. At the present time the histological pattern of the PC-82 tumor still closely resembles that of the original tumor (fig. 1), while the tumor growth rate on intact male mice did not change during the subsequent mouse passages (fig.2).

The absence of tumor growth on female and castrated male mice proved the androgen dependence of this tumor line. Tumor growth was achieved after castration of tumor-bearing mice. In most experiments androgen withdrawal resulted in a gradual decrease of the tumor volume (fig.4A), although in some cases tumors almost completely regressed to volumes that were hardly measurable (fig.3A). These results are in agreement with those obtained with the same tumor line by Hoehn et al (18). Since castration of tumor-bearing mice did not lead to a weight loss exceeding 10 per cent of the initial body weight (results not shown), we concluded that tumor growth arrest, achieved after androgen withdrawal, represents a high degree of androgen-dependence of the tumor and is not caused by a general anabolic effect of the host animal. In most cases castration did lead to histological changes of the tumor with a degeneration of the epithelial cells. It was also observed, however, that in spite of a substantial decrease of the tumor volume (fig.4A), the histology of the remaining part of the tumor closely resembled that of untreated tumors with epithelial cells that appeared to be vital (fig.4B).

Transplantation on intact male mice of tissue fragments derived from tumor residuals after long-term castration resulted in tumor growth. Moreover it was demonstrated that after castration administration of testosterone almost directly resulted in regrowth of the tumor tissue (fig.5). It was concluded from these observations that after androgen withdrawal at least a part of the tumor cells remained dormant with the potency to grow after stimulation with androgens. Also in the Nb-rat prostatic tumor such behavior was observed. Cells of estrogen-dependent tumors did not grow in males, but remained dormant and

could be stimulated after many months by estrogen treatment (5).

In none of the castration experiments with PC-82 performed until now could any hormone-independent tumor growth be observed. This might indicate that the PC-82 tumor line mainly (or completely) consists of a rather homogenous population of hormone-responsive cells. Regarding this point the PC-82 is different from the Nb-rat and the Dunning R3327 prostatic tumors, since complete withdrawals of hormones in these models ultimately resulted in autonomous tumor growth (2, 7, 19). However, since mitosis are sometimes seen in the histology of PC-82 tumors after withdrawal of androgens, the presence of hormone-unresponsive cells in the tumors cannot be excluded. In future experiments it will be necessary to extend the post-castration period in order to isolate the possible hormone-independent clones.

In the present study, particularly for long-term treatment purposes, steroid-containing silastic implants appeared to be a valuable tool. Testosterone (T) and estradiol (E_2) were released from the subcutaneously implanted vehicles for more than 10 weeks at a constant rate. Androgen substitution (via the silastic implants) in intact male and female mice resulted in tumor doubling times that were significantly shorter compared to those in the untreated control mice (fig.6). Although the histological picture of these tumors grown on androgen treated animals did not differ from that of tumors grown on intact male mice, the mitotic index of these tumors was much higher. It was concluded from these results that the growth of PC-82 tumor tissue was increased through the availability of constant levels of T, maintained by the silastic implants.

Plasma levels of T were shown to fluctuate in adult immune-competent male mice (20) as well as in male nude mice (21). Moreover, the concentrations of plasma-T were markedly reduced in the athymic male mice (21). Also in the Balb/c nude mice used for the present experiments with PC-82, rather low and fluctuating levels of plasma-T were found (table 1). Although the mean level of plasma-T in the T-implanted mice did not differ from that in the intact control mice, the variation of the levels in the implanted mice was much smaller. Therefore the T-implantation is currently used in order to improve the hormonal milieu of the mice receiving the PC-82 tumor, and thus increasing the take and the growth of the tumor. In addition it should be stressed that the plasma levels of T achieved after T-implantation are within the range found in normal human males (22), while in patients with prostatic carcinoma plasma-T was proved to be not significantly different from that found in control males (23).

Plasma levels of T were markedly suppressed in intact male mice (mean level 1 ng per ml) after implantation with E_2. The take and growth of PC-82 tumor tissue on intact male mice was almost completely prevented by the simultaneous administration of E_2 via silastic implants (table 2). It was concluded that circulating T-levels of 1 ng per ml were too low for the take and growth of tumor tissue, while a plasma concentration of 2 ng per ml (attained

via short T-implants) seems to be sufficient for PC-82 tumor growth. E_2 implantation of tumor-bearing nude mice following androgen withdrawal did not result in a further decrease of the tumor volume compared to that in the group of mice without additional E_2 implantation (table 3). It may be possible to conclude that the relatively low levels of E_2 reached with silastic implantation did not directly affect the tumor tissue, but only indirectly influenced the take and growth of the tissue through the suppression of T. It must however be remembered that the E_2 doses used do not completely suppress plasma-T and certainly are not in the pharmacological range. It will also be important to use higher doses of estrogens, as widely applied for the treatment of prostatic carcinoma, in the experiments with PC-82 in order to check whether this hormone also has a direct action on prostatic tumor cells.

Beside the additional investigation of estrogens the possible inhibitory action of antiandrogens and the effect of prolactin on PC-82 tumor tissue will be studied, while the combination of hormonal and chemotherapy will also be included in the subsequent experiments. Since PC-82 tumor tissue has been shown to contain androgen receptors (AR), it will be interesting to check whether possible alterations in the concentration of the AR correlated with the growth of PC-82 tumor tissue.

Recently experiments have been started to study the effect of hormonal treatment on the androgen receptor in the PC-82 tumor. The results of these experiments will be correlated to those obtained in the present study. Although hormone-independent tumor growth could not be observed until now, this important aspect will be emphasized in the further experimental approach. The results of the present experiments and those that will be performed in the near future might contribute to our knowledge of hormone-dependent tumor growth.

Overviewing the scarce literature about human prostatic tumor models on nude mice, PC-82 is the only tumor line that is completely androgen-dependent. This property together with the slow growth rate and the secretion of prostatic acid phosphatase (16) makes the PC-82 tumor model well-comparable to prostatic carcinoma in the clinical situation and is therefore very applicable.

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CHAPTER 4

EFFECT OF HORMONE TREATMENT ON PROSTATIC ACID PHOSPHATASE IN A SERIALLY TRANSPLANTABLE HUMAN PROSTATIC ADENOCARCINOMA (PC-82)

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CHAPTER 4

EFFECT OF HORMONE TREATMENT ON PROSTATIC ACID PHOSPHATASE IN SERIALLY TRANSPLANTABLE HUMAN PROSTATIC ADENOCARCINOMA (PC-82)

ABSTRACT

The influence of endocrine manipulation on the tissue concentration of prostatic acid phosphatase (PAP) was studied in the hormone dependent transplantable human prostatic tumor line PC-82. Tumor bearing nude mice were left intact, castrated or treated for a 5-day period with a subcutaneous implant containing testosterone or estradiol. The concentration of PAP in castrated mice was not different from that in the controls. The DNA content of PC-82 tumor tissue obtained from 5-day castrated animals was significantly lower than that of tissue from intact animals. Therefore the concentration of PAP in tissue from castrated mice was significantly elevated when expressed per mg of DNA ($p < 0.05$). Treatment of the mice with testosterone or estradiol did not affect the PAP concentration in the tumor tissue. A significant correlation was observed between the concentration of PAP in the serum and the tumor burden of the mice. Long-term withdrawal of androgens resulted in a decrease of the concentration of PAP in the serum, as well as in a decrease of the tumor burden. The concentration of PAP in the tumor tissue remaining after castration of these animals was not significantly different from that in controls. The present data from the tumor line PC-82 do not support the hypothesis that the concentration of PAP in prostatic tumor tissue is controlled by androgens, but are in agreement with the concept that the level of PAP in plasma is related to the tumor mass.

INTRODUCTION

The concentration of prostate-specific acid phosphatase (PAP) is often elevated in the serum of patients with prostatic carcinoma (1,2). It has been reported that the prognosis of disseminated prostatic carcinoma is better in patients with low PAP levels (3,4). Therapeutic measures which result in a decrease of circulating androgen and in a regression of the disease often also result in a decrease in the concentration of PAP in the circulation (5,6).

In human benign prostatic hyperplasia (BPH) the whole tissue concentration of PAP showed a positive correlation with those of tissue testosterone and androstenedione (7). In addition, significant correlations were noted between the concentrations of PAP and 5α-

dihydrotesterone, and PAP and 5α-androstane-3α, 17β-diol in isolated epithelium. On the basis of these results it was concluded that the synthesis of PAP in human BPH tissue is androgen dependent (7). From these observations it has been inferred that the secretion of PAP would be subjected to androgenic regulation. However, in *in vitro* studies which used permanent lines or short term cultures, controversial data have been obtained (8,9). The concentration of immunoreactive PAP in the seminal plasma of normogonadotropic subfertile men was not altered following clomiphene administration, despite significantly elevated circulating testosterone and estradiol concentrations (10). It can be concluded that the possible endocrine regulation of prostatic acid phosphatase is insufficiently known. PC-82 is a transplantable human prostatic carcinoma line which secretes PAP. PAP is detectable in the serum of tumor-bearing mice; tumor fluid, present in a minor part of the tumors, contains very high concentrations of PAP (11,12). This property makes the PC-82 tumor model very useful to study the effect of short-term endocrine manipulation *in vivo* on the concentration of PAP in human prostatic carcinoma tissue, and in peripheral blood of tumor-bearing animals.

MATERIALS AND METHODS

Tumor material

PC-82 tumor tissue was grafted on intact male nude mice, with the Balb/c background, as described by Romijn and associates (12,13). Tumor growth was monitored weekly by measurement of two perpendicular tumor diameters with calipers. Tumor volume was calculated from the formula:

$$V = \frac{\pi}{6} (d_1 \times d_2)^{3/2}.$$

For the present experiments, tumors from the 12-15th mouse transplant generation which were in the exponential phase of growth, were used.

Treatment of the animals

Animals were divided into 4 groups which were either left intact, castrated or treated with estradiol and testosterone respectively. Castration of tumor-bearing mice was carried out via the scrotal route under total anesthesia with chloralhydrate. Steroids were administered by subcutaneous implantation of Silastic implants (Talas, Zwolle, The Netherlands) packed with crystalline steroid (Steraloids, Pawling, New York).

Implantation of testosterone implants (1.0 cm. length) and estradiol implants (0.5 cm. length) resulted in circulating plasma levels of approximately 8 ng/ml and 250 pg/ml

respectively. These levels were reached within 2 days after implantation and were maintained for at least 16 days. During this period, estradiol implantation resulted in a mean level of circulating testosterone (1.0 ± 0.9 ng/ml, n = 15), which was significantly decreased ($p < 0.01$) compared to that in intact male nude mice (8.0 ± 9.7 ng/ml, n = 18). The level reached after estradiol implantation was not as low, however, as that after castration (0.14 ± 0.12 ng/ml, n = 16).

Tissue and serum specimens.

In order to study the effects on the tissue concentration of PAP, prior to the effect on the tumor tissue, a treatment period of 5 days was selected. After this period animals were exsanguinated from the orbital sinus under light ether anesthesia. Blood samples were allowed to clot at room temperature for 30 minutes and the serum was harvested by centrifugation at 9000 x g for 3 minutes. The samples were stored at -20°C until analyzed.

Tumor nodules were weighed and placed on ice immediately after excision. A representative part of each tumor weighing at least 60 mg was washed with saline and stored at -80 °C. For determination of PAP the tissue samples were thawed, minced on ice with chilled scissors and homogenized with a Potter-Elvehjem homogenizer. Extraction was carried out at 4°C in distilled water containing 0.1 per cent (v/v) Tween-80 as detergent, at a concentration of 200 mg tissue/ml (14).

Radioimmunoassay procedures

We used 2 modifications of the radioimmunoassay for serum PAP described by Vihko and associates (1). For routine assay, preincubation of the sample with antiserum during four hours and after the addition of tracer, incubation overnight were used. The PAP concentrations of the standards used were 0, 0.5, 1.5, 5, 10 and 30 μg per liter. In order to measure very low PAP concentrations, we used a radioimmunoassay with an overnight preincubation of antiserum with the samples, and an additional incubation of four hours after addition of tracer. PAP concentrations of the standards were 0.125, 0.5, 1.5 and 5 g per liter (15). Sera were diluted with PAP-free sheep serum; the first steps in the dilution of tissue extracts were carried out with buffer and the final step with sheep serum. The pH of sodium phosphate buffer (0.05 M Na_2HPO_4-$2H_2O$, 0.01 M EDTA, 0.05 per cent NaN_3, 0.1 per cent rabbit serum) was adjusted at 6.6. Tissue extracts were assayed in triplicate in dilutions of 1:5,000 to 1:15,000 (7).

Other procedures.

The protein concentrations of the remaining pellets were determined according to Bradford (16) using bovine serum albumin (Fluka, Buchs, Switzerland) as a standard. DNA

was measured according to the method of Giles and Myers (17) with calf thymus DNA (Sigma) as a standard. Statistical analysis of the data was performed by non-parametric tests (Wilcoxon's test, and Spearman's correlation test). Differences were considered statistically significant when $p < 0.05$.

RESULTS

PAP in serum of tumor-bearing and control animals

The concentrations of PAP in sera of control male nude mice and untreated mice bearing PC-82 tumors, are shown in table 1. The latter group included 17 mice, sacrificed at various points of time after transplantation and with different end volumes of the tumor nodules. The mean concentration of PAP in the serum of these mice was significantly elevated over that in the control group (table 1).

Table 1 Levels of human prostatic acid phosphatase (PAP) in sera of PC-82 tumor-bearing mice and control male nude mice

Group	n	Serum PAP (µg/l)*	Range (µg/l)	Tumor Volume (mm^3)*
Control male	11	0.5 ± 0.7	0.0 - 2.1	—
PC-82 tumor	17	73 ± 88**	4.0 - 320**	960 ± 888
PC-82 tumor after castration	10	6.4 ± 8.5***	0.5 - 28.0	201 ± 229

* Values are expressed as mean ± standard deviation.
** Significantly different from control group ($p < 0.005$).
*** Significantly different from tumor group ($p < 0.005$).

Castration of tumor-bearing animals in all cases caused a cessation of tumor growth followed by a decrease of the tumor volume. Figure 1 shows the effect of castration on tumor growth. In this example the decrease of the tumor volume was 43 per cent at 57 days after castration. The mean concentration of PAP in the serum of tumor-bearing mice after long post-castration periods (58 days on the average) was significantly lower than in untreated tumor-bearing animals (table 1). During this period, decline of the serum PAP

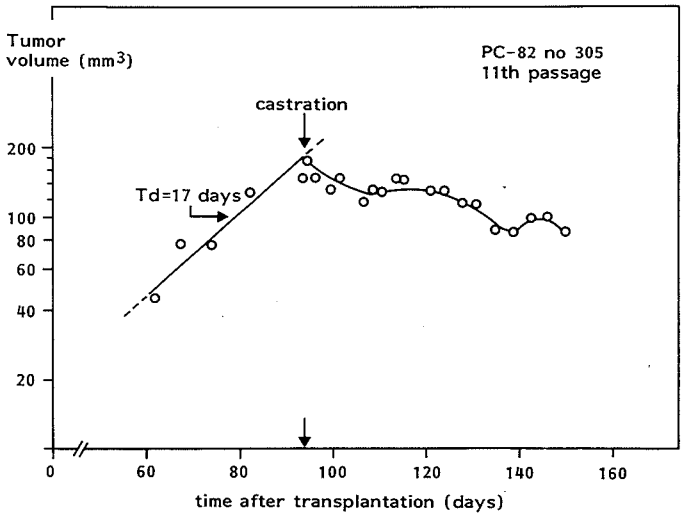

Figure 1 *Effects of castration on the growth of a PC-82 tumor. Tumor doubling time before castration: 17 days. Decrease of the tumor volume during the post castration period of 57 days: 43 per cent.*

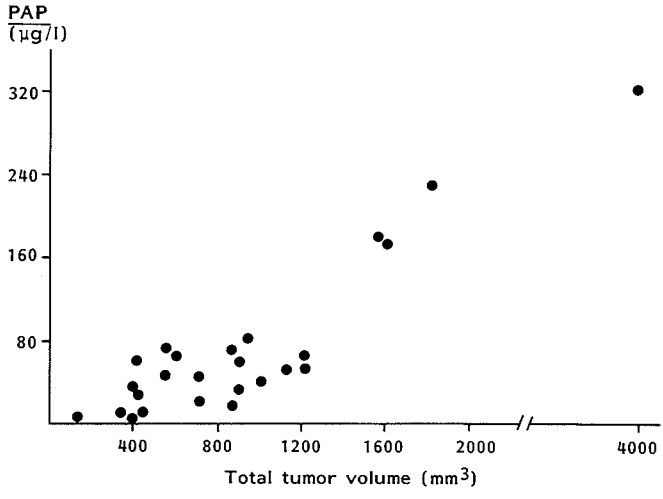

Figure 2 *Relationship between the total tumor volume and the serum concentration of human prostatic acid phosphatase in PC-82 tumor-bearing nude mice. (R=0.73; n=25 (p <0.001).*

concentration was accompanied by a 50 per cent decrease of the mean tumor volume.

To account for the large variation observed in the concentration of PAP in the sera of tumor-bearing mice (table 1), we studied the relation between PAP and the total tumor burden of the animals (fig.2). A significant correlation was observed between the total tumor burden and the concentration of PAP in the serum ($r = 0.73$, $n = 25$, $p < 0.001$).

Concentration of PAP in tissue extracts

To study the effect of hormonal manipulation on the tissue concentration of PAP in the absence of effects on the tumor size, a 5-day period of treatment was selected. During this period the tumor volume did not decrease (fig.1). Further growth of the tumor tissue following androgen administration was minimal since the doubling time of PC-82 tumor tissue is 18 days.

The results of short-term hormonal manipulation of tumor-bearing mice on the concentration of PAP in the tissue are given in table 2. In the group with castrated animals, tissue PAP, expressed per g tissue or per mg extracted protein, was not altered when compared with the control group. When PAP was expressed per mg DNA, a statistically significant elevation over the control group was observed. In the castrated group the DNA-content, expressed per mg tissue was significantly decreased when compared with the control group (table 3), and hence, the observed effect of castration on the concentration of PAP in the tissue was explained by the decrease in DNA-content. To investigate the long-term effects of castration, the concentration of PAP was estimated in a number of tissue samples, obtained at least 21 days after castration. The effects of castration on the tumor were described above (see also fig.1). The concentration in the tissue specimens remaining after long-term castration was not significantly different from the control group, irrespective whether it was expressed per g tissue, per mg DNA or per mg protein (table 2). It was concluded, that short-term and long-term androgen deprivation had no effect on the concentration of PAP in PC-82 prostatic carcinoma tissue. The concentration of PAP in the tumor tissues was not affected by short-term treatment of the mice with testosterone or estradiol (table 2).

As a control, the transplantable, non-prostatic human tumor NC-65 (18). was analyzed. In this tissue PAP was not detectable, indicating the high specificity of the antibody used for the determination of PAP in the PC-82 tumors.

Table 2 Concentration of human prostatic acid phosphatase in PC-82 tumor tissue after 5 days of endocrine treatment*

Treatment	No. Tumors	No. Animals	Human Prostatic Acid Phosphatase μ/g tissue	μ/mg DNA	μ/mg protein
Control	10	8	284 ± 115	26 ± 10	9 ± 3
Castration	9	5	269 ± 66	41 ± 9**	12 ± 4
Estradiol	8	5	318 ± 131	32 ± 10	10 ± 4
Testosterone	9	5	334 ± 143	35 ± 9	12 ± 4
Castration (long-term)***	4	3	293 ± 71	32 ± 2	14 ± 5

* Values are expressed as mean ± standard deviation.

** Significantly different from control group ($p < 0.05$).

*** PAP was determined in tissue remaining 21, 35 and 48 days after castration, respectively.

Table 3 DNA and protein content of PC-82 tumor tissue after 5 days of endocrine treatment*

Treatment	No. Tumors	No. Animals	Pellet DNA (μg/mg tissue)	Extracted Protein (μg/mg tissue)	μg Extracted protein (μg/ pellet DNA)
Control	10	8	10.9 ± 2.3	31.5 ± 5.0	3.0 ± 0.5
Castration	9	5	6.8 ± 1.8**	24.4 ± 7.3	3.7 ± 0.9
Estradiol	8	5	9.6 ± 2.5	31.0 ± 8.4	3.4 ± 0.8
Testosterone	9	5	9.4 ± 2.5	28.4 ± 5.0	3.2 ± 1.0
Castration (long-term)***	4	3	9.3 ± 2.2	25.9 ± 5.0	2.8 ± 0.6

* Values are expressed as mean ± standard deviation.

** Significantly different from control group ($p < 0.05$).

*** PAP was determined in tissue remaining 21, 35 and 49 days after castration, respectively.

Table 4 Levels of human prostatic acid phosphatase (PAP) in sera of PC-82 tumor-bearing mice after 5 days of endocrine treatment*

Treatment	No. Animals	Serum PAP (μg/l)	Total Tumor Volume (mm^3)	PAP/Tumor Volume
Control	8	54 ± 53	829 ± 474	59 ± 46
Castration	3	61 ± 82	769 ± 356	64 ± 80
Estradiol	5	16 ± 10	742 ± 278	23 ± 16
Testosterone	4	90 ± 31	998 ± 464	110 ± 58

* Values are expressed as mean ± standard deviation.
No significant differences were observed.

Influence of hormonal-manipulation on the concentration of PAP in the serum of tumor-bearing animals

The serum levels of PAP after 5 days of endocrine treatment of PC-82 tumor-bearing mice (compare tables 1 and 2) are shown in table 4. Although the serum levels were expressed as a function of the tumor burden of the host animals, rather large variations in the levels of each group were observed. The mean PAP concentration in serum of animals treated with estradiol was lower than that in animals treated with testosterone (table 4). These differences however, were not statistically significant.

DISCUSSION

The present study shows that castration of mice bearing the human prostatic tumor PC-82, did not affect the concentration of PAP in the tumor tissue based on tissue wet weight or on extracted protein (table 2). In this group the concentration of PAP was moderately but sugnificantly increased when expressed per mg DNA. This elevation could be attributed to a decrease of the DNA at 5 days after castration (table 3). We are unable to offer an explanation for this decrease in the content of DNA.

Long-term castration did not affect the concentration of PAP in the remaining tissue, regardless of the way in which the results were expressed (table 2). From these observations we concluded, that withdrawal of androgens did not result in a decline of the tissue concentration of PAP. No significant differences in tissue PAP were found between the

groups treated with testosterone or estradiol and the control group. The concentrations of PAP in PC-82 tumor tissue were considerably lower than those in tissue from human benign prostatic hyperplasia (data not shown). However, our results obtained with BPH tissue were comparable with published data (7).

Immunoreactive PAP was detectable (up to 320 μg/l) in the serum of tumor-bearing mice (table 1). This result is in sharp contrast with the results of Hoehn and associates (19). In their study with the same tumor line, PAP was hardly detectable in control male nude mice as well as in PC-82 tumor-bearing animals. The radioimmunologic assay of human PAP with the technique used in this study is highly specific (1, 20). No PAP was detectable in tissue from the non-prostatic tumor, NC-65.

A significant correlation between the serum level of PAP and the total tumor load in tumor-bearing mice was established (fig.2). Castration of tumor mice did not result in a decrease of PAP in the serum after 5 days (table 4), while after longer periods castration leads to a significant reduction of both the mean tumor volume and the concentration of PAP in the circulation (table 1). These results are consistent with the observations regarding the tissue concentrations. We concluded that serum levels of PAP reflect the total tumor burden, and follow the reduction of the tumor size after long-term castration. These findings are in agreement with clinical observations, that PAP in serum of patients which prostatic carcinoma correlates well with the extent of tumor involvement (5, 6). Furthermore PAP might be considered as a suitable biochemical marker to monitor the effect of therapeutic regimens in prostatic cancer (3).

It has been shown that castration of adult male rats causes the disappearance of secretory acid phosphatase (AP) activity in the prostate. This loss of enzyme activity could be prevented by the administration of androgens following castration (20, 21). It is not known whether the regulation of human secretory acid phosphatase follows the same pattern. Bolton and associates (7) demonstrated that in epithelium isolated from human BPH tissue a correlation exists between the concentration of PAP and 5α-dihydrotestosterone and 5α-androstane-3α, 17β-diol. It was concluded from this result that the synthesis of PAP in BPH tissue is androgen dependent. Most of the PAP appeared to be located extracellularly however, and the question remains, whether androgens influence in the synthesis or the release of PAP.

The observation of Geller and associates, (22) that PAP activity in flutamide treated patients with BPH was significantly higher than values in untreated BPH, is in contrast to our results. These data however, are based on the measurement of the katalytic activity of PAP, which might differ from the immunoreactive PAP measured in prostatic tissue. Katalytic activity of PAP is affected by several components, i.e., zinc and magnesium ions, as reported by Rönnberg and associates (10). In their study katalytic activity of PAP in seminal plasma was elevated after concomitant increase of plasma testosterone and estradiol. The

concentration of immunoreactive PAP, in contrast, was not altered. These data and the present results do not support the hypothesis, that the secretion of PAP in prostatic tissue is regulated by hormones. It was shown that the concentration of PAP in PC-82 tumor tissue is very insensitive to castration and treatment with testosterone and estradiol. However, the possibility cannot be ruled out that the release of PAP from the tumor tissue was influenced by the administration of these hormones. In the subsequent study this aspect will be investigated further.

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CHAPTER 5

TRANSPLANTABLE HUMAN PROSTATIC CARCINOMA (PC-82) IN ATHYMIC NUDE MICE: I. HORMONE-DEPENDENCE AND THE CONCENTRATION OF ANDROGENS IN PLASMA AND TUMOR TISSUE

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Chapter 5

Transplantable Human Prostatic Carcinoma (PC-82) in Athymic Nude Mice: I. Hormone Dependence and the Concentration of Androgens in Plasma and Tumor Tissue

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This paper is the first part of a series of three describing a number of observations made on the PC-82 human prostatic carcinoma, xenografted into nude mice. The previously described androgen-dependence, one of the main properties of this tumor, has been the subject of subsequent studies. The impact of hormonal manipulation on the growth of the tumor and on plasma and tissue concentrations of androgens is discussed in this first part of the series. The great variability of plasma testosterone (T) levels in intact male mice (range: 1–90 nmol/liter) has been levelled out by the use of T-containing Silastic implants, resulting in levels ranging from 18 to 35 nmol/liter. Moreover, this route of administration also facilitated hormonal manipulation of tumor bearing mice. Androgen withdrawal from male mice with growing PC-82 tumors caused 80% tumor regression at ten weeks after androgen deprivation; the decline of the tumor volume followed a biphasic course. Delayed androgen substitution in castrated male mice grafted with PC-82 30 days before resulted in growth of the tumor tissue. This indicates that cells do not die and keep the capability to respond to androgens. It was concluded that the growth of the PC-82 tumor is not compatible with plasma T levels lower than 1 nmol/liter. Variable concentrations of endogenous T and dihydrotestosterone (DHT) were detected in total homogenates of PC-82 tumor tissue. Androgen withdrawal from T-implanted, tumor-bearing female mice caused a rapid reduction (90% within one day) of the tissue-T and a slower decline (up to 90% within seven to ten days) of tissue-DHT concentrations.

Key words: prostate cancer, PC-82 tumor model, blood/tissue androgens

INTRODUCTION

Growth and function of the prostate are primarily dependent on androgenic stimuli [1]. In the early 1940s, the pioneering studies of Huggins and Hodges [2] delineated the role of testicular androgens in the regulation of the growth of prostatic carcinoma. These studies led to the introduction of castration and the administration of estrogens for treatment of the disease. Although castration leads to a 90% reduction of circulating androgens in men, this form of hormonal treatment of prostatic cancer does not completely eliminate plasma androgen concentrations, because the adrenal glands also secrete (precursors of) androgenic steroids [3,4]. The concept of total "androgen suppression," by means of testicular as well as adrenal androgen depletion

to improve clinical response of prostatic carcinoma, however, is still a matter of debate [5–7].

Measurement of intracellular levels of androgens in androgen target tissues is important for the elucidation of the mechanism of androgen action and for the understanding of pathogenesis of disease in such tissues. It is assumed that hormonal effects at the target tissue, ie, the prostate, depend on the conversion of testosterone (T) to 5α-dihydrotestosterone (DHT) [8,9] and on the binding of DHT to specific intracellular receptors [10]. In many clinical studies focussed on the role of androgens in the normal and pathological prostate only circulating plasma concentrations of androgens were considered [11–13]. A number of studies using the ventral prostate of the rat as a model focussed on the estimation of the tissue concentration of T and, possibly more important, DHT [14] and androgen receptors. Emphasis was also put on the relation between these parameters and the growth and function of the prostate [15].

Methods for extraction and determination of androgens in prostatic tissue have been described [16–18], and data have been reported both on androgen concentrations in human prostatic hyperplasia and carcinomatous tissues [19–21] and on the distribution of androgens over the different cellular compartments of the prostate [22]. Geller et al and Geller and Albert determined DHT in prostate tissue samples from prostatic carcinoma patients in an attempt to use these DHT levels as a parameter for prediction of the response of these patients to hormonal therapy [23,24]. In spite of the interesting findings of these studies many more data are required to determine the usefulness of this marker definitely.

In addition, studies are needed in which plasma and tissue levels of T and DHT content are measured as well as the concentration of these androgens and their specific receptor in the nuclear fraction of prostatic tissues during or after hormonal manipulation. Such studies, however, can only be accomplished by the use of suitable model systems originating from human prostate. As far as prostatic carcinoma is concerned some in vitro cell lines have been established [25–27], whereas a limited number of tumors were also described, which are transplantable in nude mice. Among these models the PC-82 [28], PC-EW [29], Honda [30], and TEN/12 tumor [31] were shown to be androgen-dependent, whereas the remaining tumors, PC-133 and PC-135 [32] and tumor-9479 [33], grew equally well in male and female nude mice, demonstrating the nonandrogen-dependent character of the latter tumors.

The PC-82 tumor model, which was established in our own institution [28], shares several properties with clinical prostatic carcinoma. In particular its androgen dependence, described before [34] and a subject of discussion in this paper, makes the PC-82 tumor an appropriate model with which to study the effects of hormonal treatment on human prostatic cancer tissue. This paper presents data on the impact of hormonal manipulation on androgen concentrations in both plasma and tumor tissue of nude mice bearing the PC-82 tumor. In addition, the role of the androgen receptor in the androgen-regulated growth of the PC-82 tumor was studied. The results of this study will be presented in the second paper of this series [35]. Finally, the effects of estrogens on the take, (ie the frequency of tumor development), growth rate, and some functional parameters of the PC-82 tumor tissue were investigated. The outcome of this part of the studies will be presented in a third paper [36].

MATERIALS AND METHODS

Tumor Material

The PC-82 tumor, a moderately differentiated adenocarcinoma of the prostate, initiated in the year 1977, has passed through 35 mouse generations so far and still possesses its original properties. Its origin and further characterization have been described extensively [28,37]. PC-82 tumor tissue was grown in nude mice of the Balb/c background obtained from our breeding facilities. The animals were housed separately in laminar flow cage racks and maintained under controlled conditions as described before [34]. The tumor tissue was grafted subcutaneously at both sides of the shoulders. Further details about the technique of transplantation and of monitoring tumor growth have been described in a previous paper [34]. The transplantable human prostate carcinomas PC-EW [29] and PC-135 [32] have been maintained in a similar way as described for the PC-82 tumor.

Hormonal Manipulation of the Nude Mice

PC-82 tumor tissue has been grown in intact male and T-supplemented male and female nude mice. Androgen substitution was carried out by subcutaneous implantation of Silastic implants (Talas, Zwolle, The Netherlands) packed with crystaline steroid (Steraloids, Pawling, New York). This technique, which proved to be suitable to maintain a constant level of the hormone for a long period of time (more than three months), was described in more detail elsewhere [38].

In the present study, mice receiving supplemental androgen were implanted with T-capsules 1.0 cm long at the time of grafting of the tumor inocolum. The Silastic devices were implanted in the left flank of the mice. Short-term effects of hormonal treatment on the PC-82 tissue were studied by using T-implanted, PC-82-transplanted female nude mice. The use of this model facilitates hormonal manipulation of the tumor by removal and reimplantation of the steroid-filled Silastic tubes. Installation and removal of implants were carried out under light ether anesthesia. Castration of mice was carried out via the scrotal route under total anesthesia with tribromoethanol (Aldrich, Beerse, Belgium).

Hormone Estimations in Plasma and Tissue Extracts

To determine endogenous concentrations of the androgens T and DHT in the PC-82 tumors, tissue (200 mg/ml) was placed in water containing 0.1% (v/v) of Tween-80 in glass tubes and homogenized with a Potter-Elvehjem homogenizer. Known amounts of [^{3}H]-T and [^{3}H]-DHT were added to each sample to monitor procedural losses. Steroids were extracted by addition of a threefold volume of acetone to the tissue homogenates. After centrifugation at 2,000g for ten minutes the supernatant was removed and the pellet was extracted 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 extract was evaporated and dissolved in 3% acetone and dichloromethane and T and DHT were separated on Silica columns (Silicagel-60; 70-230 mesh ASTM; Merck, West Germany), as described by Hämäläinen et al [39], After chromatography, part of each fraction was used for estimation of the recovery of the steroid. Plasma samples were extracted with n-hexane/ether; the extracts were chromatographed in the same way as described for tissue extracts.

T and DHT were estimated by radioimmunoassay, using the method and antiserum described by Verjans et al [40]. The inter- and intraassay coefficients of variation were 11.8 and 8.1%, respectively for T and 13.4 and 12.3% for DHT. Plasma levels of estradiol (E2) were estimated by the use of the direct ^{125}I-estradiol radioimmunoassay kit supplied by EIR (Würenlingen, Switzerland). The inter- and intraassay coefficients of variation were 18.5 and 8.2%, respectively.

Sex hormone binding globulin (SHBG) capacity of the plasma of nude mice was determined by using the method described by Hammond and Lähteenmäki [41] in which dextran-coated charcoal is used for the separation of high-affinity protein-steroid complexes from non-protein-bound steroid. Results were corrected for non-specific binding of steroid in low-affinity protein-steroid complexes. The inter- and intraassay coefficients of variation for this assay were 14.6 and 10.3%, respectively.

Measurement of 5α-Reductase

The activity of 5α-reductase was determined by measuring the conversion of radiolabeled T to DHT [42]. PC-82 tumor tissue was homogenized with a Micro-dismembrator (Brown Melsungen, West Germany). Protein concentration in the resulting samples was adjusted to 5–10 ng/ml and the suspensions were used without further centrifugation. Testosterone was used as the substrate in the presence of a NADPH-generating system. Samples were incubated in glass tubes for 60 minutes at 37°C. The formation of steroid metabolites was analyzed by thin-layer chromatography as described by Schweikert et al [43].

Miscellaneous

Protein was measured by the Bradford [44] method with the Bio-Rad assay kit (Bio-Rad Laboratories, Richmond, U.S.A.) with bovine serum albumin (Fluka, Buchs, Switzerland) as a standard. DNA was measured according to the method of Giles and Myers [45] with calf thymus DNA (Sigma) as a standard. The significance of difference between values of different groups was calculated by using two-tailed Student's T-tests. Differences were considered statistically significant when T was smaller than 0.05.

RESULTS

Hormone Dependence of PC-82

The absence of PC-82 tumor growth in intact female and castrated male nude mice, shown in several subsequent transplant generations (including the recent ones), indicated the absolute requirements of androgens for the growth of this tumor. Likewise, arrest of PC-82 tumor growth following androgen withdrawal has been observed in the recent mouse passages. In general castration of tumor-bearing mice led to a gradual, substantial reduction of the tumor volume (cf ref [34]). Since plasma-T in castrated male and female mice did not exceed the level of 1.0 nmol/liter (cf next paragraph) it is obvious that the PC-82 tumor does not proliferate in such a hormonal environment. Recently, the behavior of the PC-82 tumor under circumstances of long-term androgen ablation was also studied. This was carried out in groups of intact or T-implanted male mice that underwent castration and/or removal of the implant and which were grafted with tumor tissue of the 33rd-transplant generation. The castration-induced regression of the tumor resulted in a 80% reduction of the tumor volume

at 70 days after androgen withdrawal. The plot of the log of tumor volume vs days after castration (Fig. 1, inset) demonstrates a biphasic curve. An initial period of 14 days with a relatively rapid rate of regression is followed by a more gradual but also exponential rate of tumor reduction (times of half-life 15 and 32 days, respectively). The rate of regression was independent of the initial volume of the tumor (varying from approximately 300 to 1,300 mm^3) and of the hormonal status of the host animal (intact or T-implanted). In none of the animals did androgen deprivation lead to spontaneous regrowth, ie, androgen-independent growth, of the PC-82 tumor during the period of observation (at least 70 days).

T-substitution after castration-induced arrest of PC-82 tumor growth has been previously described [34] to result in a rapid proliferation of the tumor tissue, even after 80 days. In a similar way results of the present study demonstrate that delayed androgen substitution in castrated, PC-82-transplanted mice resulted in growth of the tumor tissue. The take (ie the development) of tumors in mice deprived of androgen for 30 days was identical to that of tumor grafted in animals receiving testosterone implants simultaneously with the PC-82 transplant (Table I). The increase of the

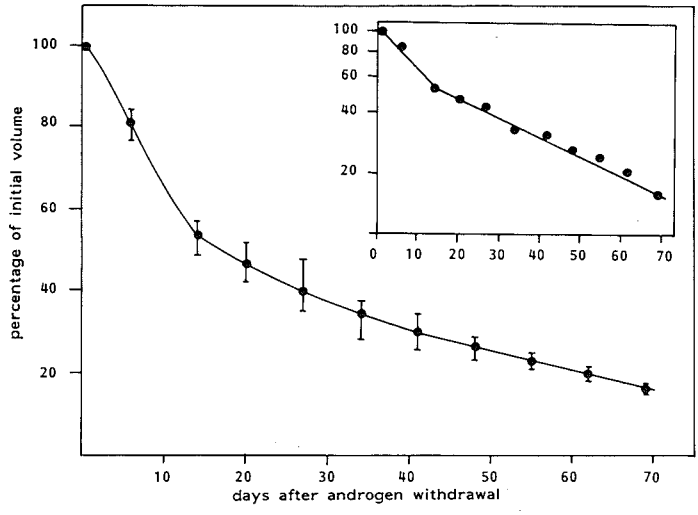

Fig. 1. The effect of castration and/or removal of T-implant or the PC-82 prostatic tumor grown in intact and T-implanted male nude mice, respectively. Values are expressed as percentage of the tumor volume at day 0 and presented as mean ± SEM (n=8). Inset shows the semilogarithmic plot of the data.

TABLE I. Effect of Delayed Androgen Substitution on the Growth of PC-82 Grafted in Castrated Male Nude Mice

Transplantation	T-implant (days after transplantation)	Tumor take at 40 days after implantation	Tv (+)[a] (%)
Castrated	0	5/6	303 ± 83
Male mice	30	5/6	244 ± 52

[a]Tv (+): increase of the tumor volume (mean ± SD) during a 35-day period after tumors had passed the volume of 50 mm^3.

tumor volume during a period of 35 days after the tumor had started to grow did not differ significantly in both groups of mice (Table I).

Plasma Concentrations of Hormones in Intact and T-Implanted Mice

In order to improve the take and growth of the PC-82 tumor and to facilitate hormonal manipulation in tumor-bearing nude mice T-implants were used. This type of implant resulted in an average plasma-T concentration of 25 nmol/liter. In contrast to the small variation of plasma-T levels in T-implanted PC-82-bearing male mice, intact male mice showed a wide range of circulating T-levels varying from less than 1.0 nmol/liter to levels exceeding 90 nmol/liter (Fig. 2). The fact that in these two groups the mice were not of similar age and their blood was taken at different times of the day might have contributed to the high variability of plasma-T found in the intact animals. However, the observations were confirmed in a group of intact male mice of similar age (15 weeks) whose blood was sampled at the same time of the day. It appeared that eight out of the 11 mice in this group had a relatively low (baseline) concentration of plasma-T (range 3–10 nmol/liter), whereas in the plasma of two of the remaining three animals extremely high concentrations (up to 90 nmol/liter) of T were found. In the T-implanted intact male mice of similar age a plasma-T concentration of 27.2 $\pm$ 4.7 nmol/liter (mean $\pm$ SD; n=10) was found.

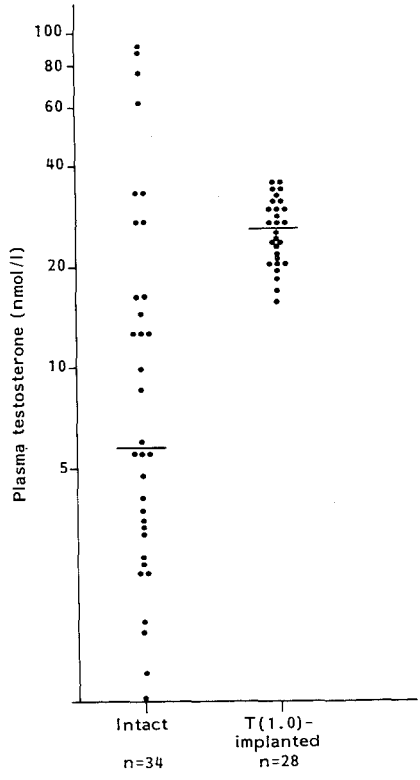

Fig. 2. Plasma levels of testosterone (T) in intact and T-implanted PC-82 tumor-bearing male nude mice.

As T-implanted and PC-82 tumor-bearing female mice were frequently used for studying effects of androgen deprivation on the tumor tissue (cf next paragraph), the concentrations of hormones in the plasma of these animals were also estimated. From Table II it was clear that in intact female mice plasma-T did not exceed 1.1 nmol/liter, and that levels in T-implanted female mice were not significantly different from those in T-implanted male mice (Table II, Fig. 2). Relatively low levels of estradiol (E2) were found in the plasma of intact female mice (Table II). T-implantation in these mice caused a significant suppression of plasma-E2. Prior to the study of short-term effects of androgen depletion and repletion in PC-82 tumor-bearing female mice, a time-course experiment was performed with intact female mice. It appeared that, starting from a control T-level of 0.39 ± 0.06 nmol/liter (n=3), the maximal level of 30 nmol/liter was already reached four hours after installation of the implant (Fig. 3).

Neither in the serum of intact male nor in that of T-implanted castrated male mice could significant levels of SHBG-binding of DHT be detected (results not shown).

TABLE II. Concentrations of Testosterone (T) and Estradiol in Plasma of Intact and T-Implanted Female Nude Mice*

Group	N	Testosterone (nmol/liter)	Range	Estradiol (pmol/liter)	Range
Females intact	9	0.9 ± 0.2	0.6–1.1	23 ± 15	4–50
Females +T (1.0) implant	11	22.0 ± 2.6[a]	18.0–25.5	6 ± 5[a]	0–16

*Mean ± SD.
[a]Significantly different from intact animals ($P < .01$).

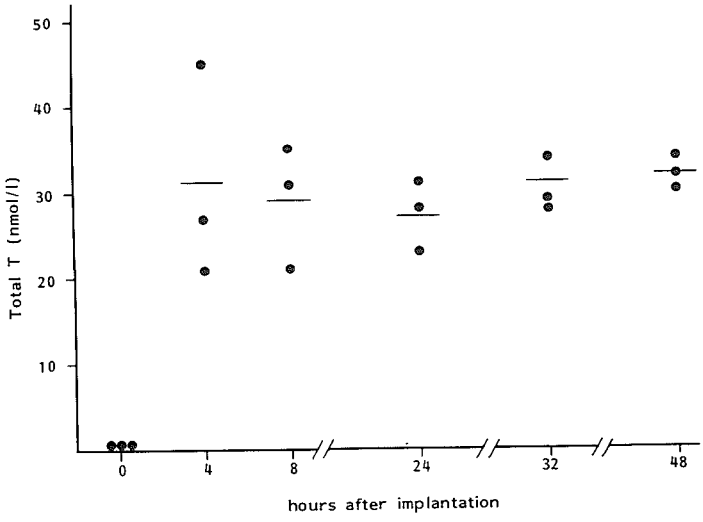

Fig. 3. Plasma level of testosterone (T) at several points of time after implantation of female nude mice with T-containing Silastic implants.

Tissue Concentrations of T and DHT

Methodology. Prior to the assay of androgens in the prostatic tissue the optimal method for tissue homogenization was determined. A comparison was made between solubilization of the tissue in NaOH and homogenization by using either a Micro-dismembrator or a glass-glass Potter. The highest recovery of steroid (62%) was obtained by using the Potter method in comparison to Micro-dismembrator and NaOH solubilization with recoveries of 51 and 38%, respectively.Considering the amount of protein and DNA obtained per gram of tissue the Potter method also appeared to be preferable and this method was used. The concentrations of steroids were expressed per g of tissue after correcting for the loss during homogenization.

Steroid concentrations. Figure 4 (first panel) shows the result of androgen determinations in tissue homogenates of PC-82 tumors grown in intact and T-implanted male mice. Under both experimental conditions T as well as DHT could be detected in the tumor tissue. Tumors grown in intact mice generally contained relatively low levels of T (< 12 pmol/g tissue) in comparison to those grown in T-implanted animals (> 18 pmol/g tissue). In contrast with this observation the concentrations of DHT in tumors grown under both experimental conditions were not different (Fig. 4).

No significant difference in tissue androgen concentrations between left and right tumor was observed (data not shown).

Additionally, T and DHT were estimated in a limited number of tissues derived from the transplantable tumors PC-EW and PC-135. Concentrations of T and DHT in the androgen-dependent PC-EW tumor were similar to those found in the PC-82 (Fig. 4, second panel). In the androgen-independent PC-135 tumor (shown to be

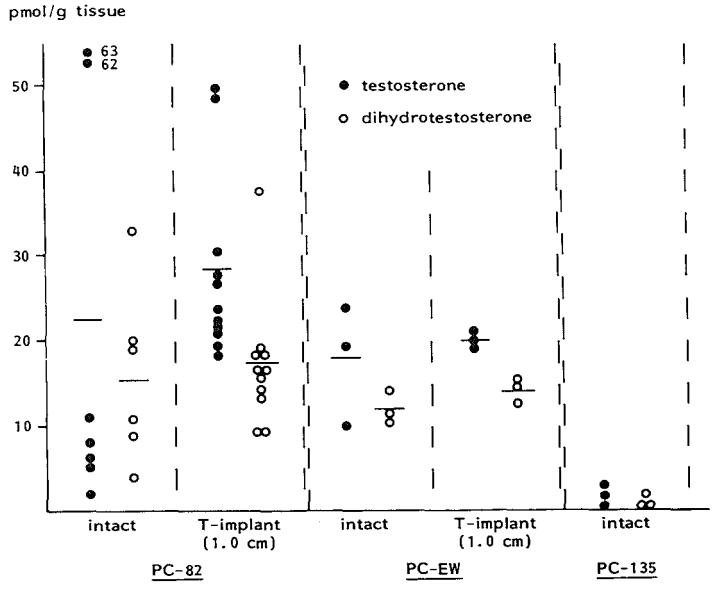

Fig. 4. Concentrations of testosterone (T) and dihydrotestosterone (DHT) in prostatic tumor tissues of the transplantable lines PC-82, PC-EW, and PC-135 grown in intact or in T-implanted male mice.

devoid of androgen receptors) both androgens were hardly detectable (Fig. 4, third panel).

Furthermore, T and DHT concentrations were determined in plasma and tumor tissue from male mice which were either left intact or which were injected with a high dosage of T (100 μg) one hour prior to sacrifice of the animals. In both groups of mice wide variations of T concentrations in plasma as well as tissue extracts were found (Fig. 5). Administration of the large dose of T resulted in extremely high concentrations of T in plasma and tumor tissue, whereas only slight increases of DHT were observed.

Time-Dependent Effects of Androgen Withdrawal and Resubstitution

Short-term effects of hormonal manipulation on levels of androgens in the PC-82 tumor tissue were studied according to a protocol of androgen depletion and repletion. Figure 6 comprises the data obtained in three separate time-course experiments. Removal of T-implants from PC-82 tumor-bearing female mice resulted in undetectable plasma levels of T and DHT (Table III) and in a drastic reduction of the tissue concentration of T within one day (Fig. 6). A more gradual decline of tissue-DHT concentrations was found. At ten days after androgen withdrawal neither T nor DHT was detectable in the tumor tissue. At this point of time, neither the volume nor the morphology of the PC-82 tumor showed discernible changes (not shown).

Resubstitution of T (again via T-implants) in the androgen-deprived tumor-bearing mice restored the androgen levels in the tumor tissue within one day.

Whereas in some mice plasma-T levels appeared to be extremely high ($>$ 50 nmol/liter), in all animals of these time-course experiments relatively low levels ($<$ 2 nmol/liter) of DHT were found (Table III). This was comparable with the result obtained in tumor-bearing intact mice (Fig. 5). Additionally, in the plasma of control, non-tumor-bearing, male nude mice previously described (preceding paragraph; Fig.

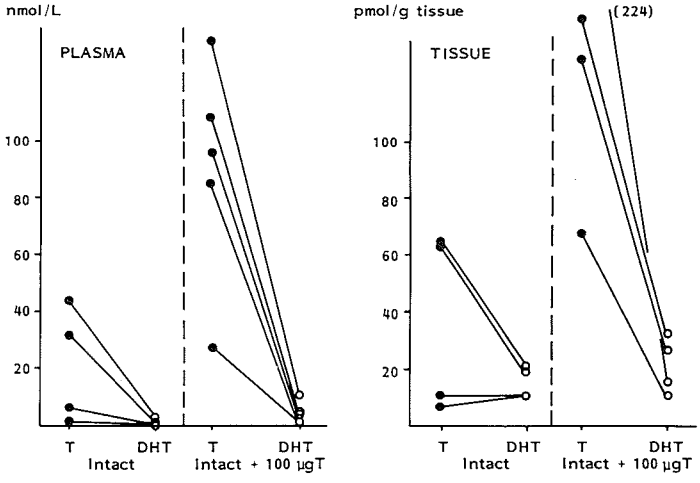

Fig. 5. Concentrations of testosterone (T) and dihydrotestosterone (DHT) in tumor tissue and plasma of PC-82-bearing male mice either left intact or one hour after a single injection of high dose of T. T and DHT levels of individual mice are connected by lines.

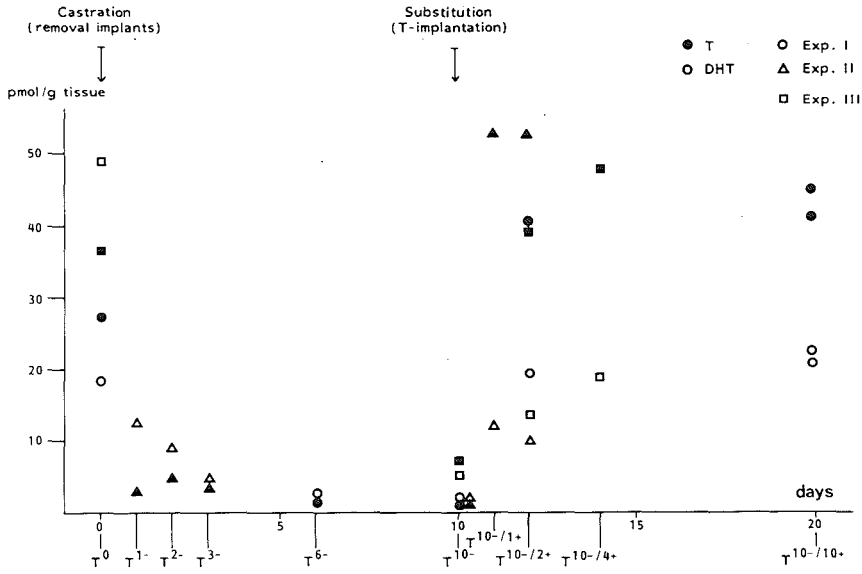

Fig. 6. Time-dependent effect of androgen withdrawal and subsequent resubstitition of testosterone (T) on the concentration of T and dihydrotestosterone (DHT) in tissue homogenates of PC-82 tumors grown in T-implanted female mice.

TABLE III. Plasma Levels of Testosterone (T) and Dihydrotestosterone (DHT) in Hormonally Manipulated PC-82 Tumor-Bearing Female and Intact Male Nude Mice*

Hormone status mice[a]	T (nmol/liter)	DHT (nmol/liter)	N
T0 controls	21.7 ± 3.6	1.3 ± 0.8	(3)
T1−/T2−/T3−	< 0.5	< 0.5	(3)
T6−	< 0.5	< 0.5	
T10−	< 0.5	< 0.5	(3)
T10−/2+	25.9 ± 2.3	0.6 ± 0.1	(3)
T10−/4+	17.7	1.1	(1)
T10−/10+	23.9	0.7	(1)
Intact male	8.5 ± 13.0	0.4 ± 0.2	(5)
Range	(1.0–31.6)	(0.25–0.8)	

*Mean ± SD.
[a]Compare Figure 6 endogenous concentrations of androgens at corresponding points of time.

2) variability of T-levels, and independent of this, a low concentration of plasma-DHT (< 1 nmol/liter), were measured as well (Table III).

The clearance of androgens from the PC-82 tissue, expressed as percentage of the control values at day 0, is shown in Figure 7. From these graphs it can be inferred that androgen withdrawal leads to a rapid (within one day) decrease of tissue-T concentrations, whereas DHT showed a more gradual pattern of decline. At ten days after androgen ablation 12 ± 10% and 8 ± 1% (mean ± SD) of the initial values of T and DHT were found, respectively.

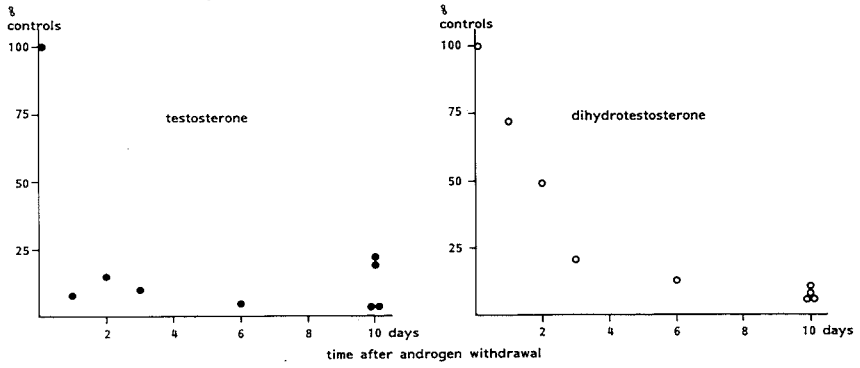

Fig. 7. Decline of androgen concentrations in PC-82 tumor tissue following androgen withdrawal. Values have been expressed as % of control values (T0) (compare Fig. 6 and Table III).

5α-Reductase

The tumor did not contain detectable amounts of 5α-reductase either when grown in intact or when grown in T-substituted male mice. The reliability of the assay was confirmed by the detection of the enzyme in homogenates of human BPH tissues.

DISCUSSION

Although some new models for human prostate cancer have been established during the last few years [29–33], the total number of tumor models demonstrating a hormone-dependent pattern of growth is still limited [29–31,34]. Careful and extensive characterization of such models is a prerequisite for their further application. In particular, the behavior under different endocrine circumstances must be considered. The human prostatic carcinoma PC-82 has been maintained in nude mice for almost ten years now and passed through 35 transplant generations. The hormone-dependence of the PC-82 tumor has been documented before [37], in particular with regard to the applications of steroid-containing Silastic implants to improve the androgen status of the host animal [38]. A standard T-implant (length 1.0 cm) was introduced to overcome the great variability of circulating T-levels in the plasma of male nude mice (Fig. 2), which was also observed by others [46]. It was concluded that in mice T is released in a pulsatile fashion, as is the case in rats [46]. On the one hand, the baseline level of plasma-T in male nude mice was shown to be reduced in comparison with their heterozygous (immunocompetent) littermates [47,48]. On the other hand, in the nude mice of the present study extremely high peak plasma levels of T (up to 90 nmol/liter) occurred, while in a population of intact male mice approximately 70% of the animals had a relatively low plasma-T level (< 10 nmol/liter). T-implantation resulted in fairly constant levels (20–15 nmol/liter) of plasma-T (Fig. 2, Table II) which are comparable to those found in patients with prostatic carcinoma [11]. However, in contrast to the human situation (cf ref [11]), the mouse plasma does not contain detectable amounts of SHBG. Thus, in mice the fraction of free steroid in the plasma is much higher than in men. Consequently, in the mouse tumor model the

accessibility of androgen to the tumor tissue is substantially better compared to the human situation.

Supplementary T treatment of PC-82-transplanted mice proved to be effective in enhancing the take and growth characteristics of the tumor [34] and also influenced the localization of the androgen receptor in this tumor [35].

In order to gain better insight into the process of the androgen-regulated growth of the PC-82 tumor, in the present study androgen concentrations were estimated in the PC-82 tissue. Concentration of T in the tumor tissue grown in intact male mice appeared to be variable, as was the case for plasma levels in these mice (cf Figs. 2,4). T-implantation resulted in more constant concentrations of tissue-T than was shown for tumors grown in intact male mice (Fig. 4). Although the variation of tissue-DHT levels in T-substituted mice was smaller than that for DHT in control intact mice, the difference between the mean tissue concentrations of DHT in both groups of animals was not statistically significant (Fig. 4). Apparently, no direct correlation exists between the concentration of T and DHT in the tumor tissue. Since no differences were found between androgen concentrations of tumors grown in both sides of the mice a direct accumulation of steroid from the Silastic tubing in the tumor can be excluded. The measurements of T and DHT in the PC-82 tumor are in agreement with those reported by Habib et al [19] and Ghanadian and Puah [20], who showed similar ranges of both androgens in series of prostatic cancer tissues obtained at operation. Geller et al [24] suggested that prostatic tissue with DHT levels below 6.5–8.5 pmol/g tissue were to be considered as nonandrogen target tissue; if DHT levels were larger than 6.5–8.5 pmol/g tissue the tissue should be considered as biochemically differentiated. Walsh et al [49] found that the DHT concentration in tissues of normal prostates obtained at surgery was similar to that of tissue from benign prostatic hyperplasia (approximately 18 pmol/g tissue in both types of tissue). Therefore, it should be considered that, in contrast to the relatively low levels of DHT (< 4 pmol/g tissue) measured in normal prostate obtained at autopsy [21,42,49], the normal values of DHT in human prostatic tissue are as high as 10 pmol/g tissue. Comparing these data with measurements in tissues derived from prostatic carcinoma it appeared that DHT levels in this type of tissue are not different from normal values. Likewise, in the majority of the androgen-dependent PC-82 tissues described in the present study the concentration of DHT was above 10 pmol/g tissue, whereas in the androgen-unresponsive PC-135 tumor the concentration of DHT (and also that of T) was far below this threshold (Fig. 4).

It is generally assumed that conversion of T to DHT (by the enzyme 5α-reductase) takes place at the target level, ie, in the prostate itself. However, the presence of this enzyme in the PC-82 tumor could not be demonstrated. The possible contribution of the stromal compartment, shown to be derived from the host animal, is limited as it is only a minor fraction (less than 10%) of the total tissue. In spite of this unexpected observation, DHT, which is assumed to be the major growth-stimulating androgen [8,9], could be detected in the PC-82 tumor also. Therefore, it was concluded from the present findings that DHT in the PC-82 tumor is derived from the relatively low levels of DHT in the plasma of the mice and thus from peripheral sources. On the other hand, involvement of T in the growth of the tumor can certainly not be excluded. Moreover, it was observed that under the influence of DHT-filled Silastic implants, PC-82 tumors had growth characteristics similar to those of tumors grown under conditions of supplementation with T-containing capsules (result not

shown). These observations do not conflict with the results of Andriole et al [50], who also used the PC-82 tumor model and demonstrated a castrationlike effect on the PC-82 tumor after the application of 17-β-N,N-diethylcarbamoyl-4-methyl-4-aza-5-α-androstan-3-one (4MA), a potent 5α-reductase inhibitor. However, as a similar inhibiting result of 4MA was seen in T- and DHT-treated mice, it was inferred that 4MA, or one of its metabolites, may exert an antiandrogenic effect by competing for intracellular androgen-receptor sites also. This possibility is supported by recent data of Brooks et al studying androgenic responses in the rat ventral prostate [51].

Absence of PC-82 tumor growth in intact female mice [cf 28,34] has also been found in the more recent transplant generations. As plasma-T in these animals did not exceed the level of 1.1 nmol/liter, it was concluded that the growth of PC-82 is not compatible with levels lower than 1 nmol/liter. However, it was also demonstrated that delayed androgen substitution in PC-82-transplanted, castrated male as well as intact female mice resulted in tumor growth (Table I). From these data it was concluded that androgen deprivation inhibited hormone-dependent growth of the PC-82 tumor by reversibly blocking cell proliferation rather than by a direct cytocidal effect. In both intact and T-implanted female mice relatively low levels of estradiol (E2) were found (Table II), which is in agreement with findings of Brünner et al [48]. As it was shown that such low levels of E2 did not affect the growth of the PC-82 tumor [cf 36], it was assumed that PC-82-transplanted T-supported female mice can be used as a model in the hormonal manipulation experiments, especially because T-implants caused a further suppression of E2 levels.

After removal of the T-implant from female mice bearing exponentially growing tumors, a rapid decrease of the tissue-T concentration and a more gradual decline of tissue-DHT were observed (Figs. 6,7]. At ten days after androgen deprivation the concentration of DHT in the tissue was below 2 pmol/g tissue. Under these circumstances the growth of the PC-82 tumor was arrested. Thereafter, the pattern of regression of the tumor was shown to be similar to that of tumors after castration of tumor-bearing intact male mice (Fig. 1). Regression of the tumor after androgen withdrawal follows a biphasic course. It must be recognized that the course of tumor regression after androgen withdrawal is the result of the decreased agonistic effect of androgen on prostatic cell proliferation as well as of the decreased antagonistic effect of androgen on prostatic cell death. It has been shown by Isaacs that the serum level of T required to inhibit prostatic cell death in the rat ventral prostate is different from that needed to stimulate prostatic cell proliferation [52]. In addition, it is known that in the nude mice xenograft system the host defense mechanism as well as local circumstances of the tumor influence the rate of tumor regression. The time course of androgen concentrations after removal of the implants agrees with observations of Gallee et al [53], who measured the expression of a cell-proliferation-associated nuclear antigen (detected by monoclonal antibody Ki-67) in PC-82 tissue by using a similar experimental approach. The number of positive nuclei in control tumors decreased from approximately 20% to almost 0% in tumors depleted of androgen for ten days [53].

In none of the long-term castration experiments, reaching postcastration periods over 100 days, was spontaneous regrowth of the PC-82 tumor observed. Therefore, it was concluded that the PC-82 tumor consists of a rather homogeneous population of hormone-responsive cells.

The continuing studies with the PC-82 tumor will include determination of endogenous androgen concentrations and tumor cell proliferation in tumors grown

under influence of low (near castrate) levels of circulating T. Preliminary results showed that the take and growth of PC-82 can still be more stimulated at a constant plasma-T concentration of approximately 2 nmol/liter. In order to establish the minimal dosage of circulating T required for take and growth of the PC-82 tumor, "hormonal titration" experiments [cf 54] are being performed at present.

It is concluded that the present findings with the PC-82 tumor model yield valuable information on the effects of hormonal manipulation on steroid levels in human prostatic carcinoma tissue.

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CHAPTER 6

TRANSPLANTABLE HUMAN PROSTATIC CARCINOMA (PC-82) IN ATHYMIC NUDE MICE: II. TUMOR GROWTH AND ANDROGEN-RECEPTORS

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CHAPTER 6

TRANSPLANTABLE HUMAN PROSTATIC CARCINOMA (PC-82) IN ATHYMIC NUDE MICE: II. TUMOR GROWTH AND ANDROGEN RECEPTORS

ABSTRACT

Androgen receptor levels were measured in PC-82 tumor tissue grown in hormonally manipulated nude mice. In the nuclei of tumor tissue from intact male mice a relatively low concentration (mean: 25 fmol/mg protein) of androgen receptors (ARn) was found, while no receptors for estrogens or progestins were detected. The total number of androgen receptors in the PC-82 tumor tissue (measured in the nuclei 1 h after injection of a single high dose of testosterone) was found to be 100 fmol/mg protein. The antiandrogen cyproterone-acetate, administered in combination with the high dose of T, significantly lowered the amount of ARn in the tumor tissue. In the nuclei of tumor tissue from intact tumor-bearing male mice with T-containing Silastic implants a 4-times higher amount of tightly associated AR was found. In addition, an increased growth rate of the tumor was observed following T-implantation. This finding suggests that the increased growth rate of the PC-82 tumor is associated with a continuous occupancy of AR in the nuclei of the tumor tissue. Castration of tumor-bearing male mice, which arrests the growth of this tumor, did not affect the concentration of ARn in the tissue compared to that of tissue in the intact control situation. In addition, the total amount of AR (ARnT) measured after T-injection was not affected by castration. Therefore, the availability of a sufficient and steady level of T in the plasma and consequently the duration of presence of AR in the nucleus of the PC-82 tumor tissue, rather than the total concentration of AR, appear to be the limiting factors in the modulation of hormonal responses in this androgen target tissue.

INTRODUCTION

The interaction of steroid hormones and their specific receptor proteins with the nucleus of target tissues is a prerequisite for achieving hormonal response in these tissues (see review 1). High affinity androgen receptors (AR) have been shown to be present in the majority of prostatic tissues derived either from normal or from hyperplastic and carcinomatous prostates (2-5). The presence of estrogen and progestin receptors in the prostate is a matter of controversy. If such receptors can be detected at all their concentration appears to be low

and thus their biological importance might be doubted (6-8).

By analogy with estrogen receptor content in breast cancer the AR content of prostatic tumors has been investigated as a means of predicting the response of the tumor to endocrine therapy (9-10). For breast cancer the concentration of cytosolic estrogen or progestin receptors provides a useful index for hormone dependence (11). In contrast, it has been shown by Trachtenberg and Walsh (12) and by Fentie *et al.* (13) that neither total nor cytosolic but only nuclear prostatic AR levels may predict response to hormonal therapy in patients with prostatic carcinoma. However, Brendler *et al.* (14) recognized that as a single parameter the AR is not sufficient for the prediction of hormone dependence. The use of an index based on multiple enzymatic activities and AR concentrations in the tumor tissue considerably improved the predictive value of single parameters (14). Likewise, van Aubel *et al.* (15) found no correlation between nuclear AR and the duration of response following orchiectomy in prostatic cancer patients.

AR have also been demonstrated to be present in experimental prostatic tumors such as the well-characterized Dunning R-3327 tumors in the rat (16). In this system it was shown that in the various sublines a relationship exists between the responsiveness to androgen and the total AR levels (17). Similarly, AR containing transplantable human prostatic carcinomas were described, which have an androgen-responsive growth pattern (18-21). In the first paper of this series (22) the PC-82 tumor model was described with respect to its androgen-dependency and the effect of hormonal manipulation on androgen levels in the plasma and in the tumor tissue. The PC-82 tumor contains an androgen binding protein with the characteristics described for AR present in prostate tissue (23). In the present study, the content of androgen receptors was estimated in nuclei of PC-82 tumor tissue grown in hormonally manipulated nude mice. Evidence is provided that the tumor growth rate is related to the level of nuclear AR (ARn), rather than to the amount of total AR in this tumor. Furthermore, it was investigated whether the synthesis of AR in PC-82 tissue is controlled by androgens, as is the case in the rat ventral prostate (24).

MATERIALS AND METHODS

Tumor material

The PC-82 tumor originated from a moderately differentiated adenocarcinoma of the prostate which was removed from a patient in July 1977. The tumor was maintained in Balb/c nude mice. Tumor growth was monitored by measurements of two perpendicular diameters using calipers. From the growth curves, which resulted from plots of the estimated tumor volumes and the time after transplantion, tumor doubling times were

calculated as described in more detail previously (18). Tissues used in the present study were derived from tumors of the 25th mouse generation and were harvested when they were in the exponential phase of growth. From each tumor nodule one or two pieces of tissue weighing 50-100 mg were stored at -80°C until processing.

Treatment of the animals

For long-term administration of testosterone (T) to the nude mice T-containing Silastic capsules were used. This method of steroid administration was described in more detail before (25). In the present study T-capsules of 0.25 and 1.0 cm length were applied. Data about hormone levels in plasma and tumor tissue of implanted and intact tumor-bearing mice were published in a previous paper (22). Some groups of PC-82 mice in the present study were injected subcutaneously with a high dose of T one h before sacrifice of the animals. Cyproterone acetate (CPA), a gift of Schering AG (Berlin, F.R.G.), was similarly administered at a dose of 10 mg. Both steroids were dissolved in benzylbenzoate which was subsequently mixed with arachid oil (3:2 v/v) to an injectable solution.

Conditions of androgen receptor measurements

Androgen receptors were always determined in the nuclear compartment. ARn (nuclear AR) denotes the amount of AR present in the nuclei of PC-82 tumor tissue without translocation of extra-nuclear AR. ARnT ("translocated" ARn) is the amount of AR estimated in the nuclei of tumor tissue after receptor translocation by administration of a single dose of 100 µg T 1 h prior to collection of tumor tissue. ARn was determined in tumors grown in intact, castrated or T-implanted male mice. ARnT concentrations were determined in tumor tissue grown in intact mice either implanted with T or treated by castration for a relatively short or longer period of time (6 and 28 days respectively).

Preparation of nuclear extracts

Prior to the receptor assay, a frozen section was made to assess the quality of the PC-82 specimens. Tissues consisting of more than 10 per cent necrosis were excluded. The remainder of each tissue (50-100 mg) was placed in a miniaturized steel grid device. This consisted of a 10 cm long stainless steel tube (8 mm in diameter) with a bottom of stainless steel gauze (80 mesh) and a pestle covered with the same gauze. After squeezing the tissue between the two layers of the gauze the nuclear pellet was extracted with heparin-containing buffer (26). The resulting extract was processed further as described below.

Assay of androgen receptors

The content of AR in the nuclear extracts was determined by an exchange assay which had originally been described by Foekens *et al.* (26). This assay was shown to be valid for small biopsy-size specimens as well (27). The nuclear extracts were incubated in siliconized glass tubes at 10°C in the dark, in the presence of 10^{-8} mol/liter ^{3}H-R1881 (methyltrienolone, specific activity 87 Ci/mmol; New England Nuclear; Dreieich, F.R.G.). Triamcinolone acetonide (5×10^{-6} mol/liter) was added to block the binding of ^{3}H-R1881 to progestin receptors possibly present in the nuclear extract. Correction for aspecific binding was made by a parallel incubation in the presence of a 200-fold excess unlabelled R-1881. The incubation volume was 40 µl. Androgen binding was quantified after protamine sulphate precipitation (26).

A statistically significant correlation ($Rs = 0.72$; $n = 74$; $P < 0.001$) was observed between the ARn content in the PC-82 tissues expressed per mg of DNA in the nuclear pellet and the ARn content expressed per mg of protein in the nuclear extract. This indicates the equivalence of the two methods for the expression of the results. No relationship ($Rs = 0.006$; $n = 61$) was found between the content of ARn expressed per mg protein and the amount of extracted nuclear protein. Therefore, the concentration of AR was expressed per amount of extracted protein. By estimating ARn concentrations in duplicate samples obtained from individual tumors ($n = 20$) the intra-tissue coefficient of variation was calculated to be 20 per cent.

Assay of estrogen and progestin receptors

In a limited number of PC-82 tumor specimens the presence of estrogen and progestin receptors was examined by application of a conventional biochemical receptor assay and by an immuno-cytochemical assay. The biochemical protocol involved pulverization of the tissue by the use of a Micro-dismembrator (B.Braun, Melsungen. F.R.G.), whereafter the tissue powder was extracted and processed further. The assay was performed in accordance with the guidelines of the EORTC Breast Cancer Cooperative Group (28) for measurement of ER and PR.

The immuno-cytochemical assay for visualisation of estrogen receptors consisted of a peroxidase-antiperoxidase technique based on the use of a monoclonal antibody as present in the ER-ICA kit provided by Abbott Laboratories, North Chicago, Ill.). This procedure was applied to frozen sections of PC-82 tumors stored at -70°C as described for prostatic tissue by Harper *et al.* (8).

Other procedures

The protein concentration of the nuclear extracts was determined according to Peterson (29). DNA was estimated in nuclear pellets using the method of Hinegardner (30). T was estimated by radioimmunoassay using the antiserum as described by Verjans *et al.* (31).

The significance of differences between values of different groups was calculated using two-tailed Student's T-tests. Differences were considered statistically significant when P was smaller than 0.05.

RESULTS

Plasma concentrations of T in hormonally manipulated mice

Plasma levels of T in intact male mice fluctuated considerably (Table I; ref 22). In order to provide a more constant hormonal milieu for the transplanted tumor tissue, T-containing Silastic implants were used as exogenous sources of androgen. In the T-implanted mice, PC-82 tumor tissue was found to have substantially better growth properties (see below). In mice which received a single dose of 100 μg T, administered 1 h prior to sacrifice, supra-physiological concentrations of plasma-T were found (Table I).

Table I Plasma levels of testosterone (T) in intact male mice and in mice receiving supplementary T by Silastic implantation or by injection of a single high dose.

Group	N	Plasma-T (nmol/l)	Range
Intact	16	27.7 ± 33.3	1.7 - 85.6
T-implant (1.0 cm)	16	28.1 ± 5.5	19.1 - 35.4
T-implant (0.25 cm)	4	7.6 ± 4.2	3.1 - 11.4
single dose of 100 μg T*	6	140 ± 65	73 - 260

* the blood of these animals was collected 1 h after a subcutaneous injection of the hormone.

Values are expressed as means ± S.D.

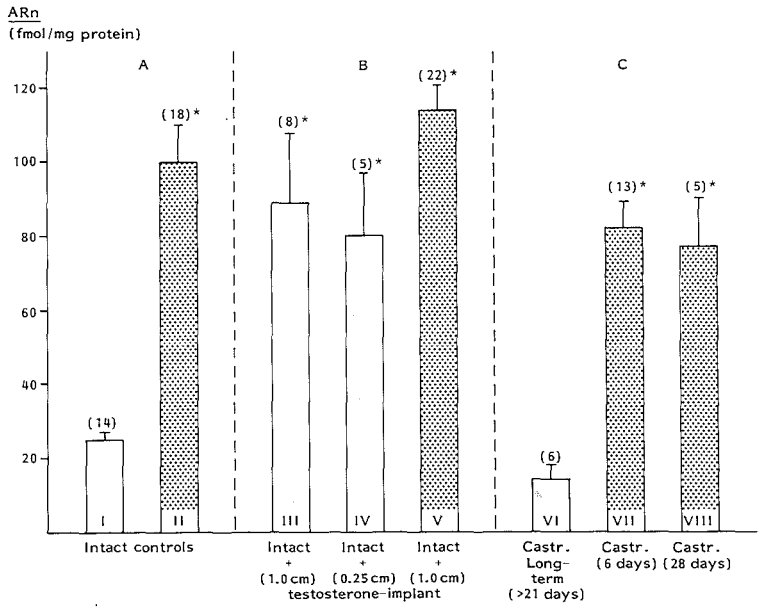

Figure 1 *Nuclear androgen receptors (ARn) in PC-82 tumor tissue grown in control and in hormonally manipulated male nude mice. ARn was determined in tissue of intact (A), T-implanted (B) and castrated (C) male mice.* ☐ *:ARn measured under standard conditions,* ⊡ *:ARnT measured under conditions of receptor translocation, i.e. 1 h after injection of 100 µg T.*

* *Significantly different from group I ($P < 0.005$). Values represent mean ± SEM of the number of observations (in parentheses).*

Nuclear androgen receptors in PC-82 tumor tissue

The concentration of ARn in PC-82 tissue grown in intact male mice turned out to be relatively low compared to the amount of receptor measured after a single dose of 100 µg T, administered 1 h prior to sacrifice (Figure 1A; Group II versus I). To further investigate the ARn concentration after administration of this high dose of T, an experiment with the antiandrogen cyproterone acetate (CPA) was carried out. ARn concentrations were measured after injection of T (100 µg), CPA (10 mg) or a combination of both compounds in tumor-bearing mice 1 h before sacrifice of the animals. The PC-82 tumor tissue was grown in T-implanted, castrated male mice. The T-capsules were removed two days before injection, resulting in a depletion of ARn. In animals receiving a single CPA injection the

concentration of ARn was found to be as low as 20 fmol/mg protein (Figure 2). This was similar to the concentration of AR in tissue after long-term castration (see Figure 1; Group VI). As shown before (Figure 1A) the single high dose of T increased the concentration of ARn to a level of about 100 fmol/mg protein, whereas administration of both CPA and T resulted in a partial, statistically significant decrease of the ARn content compared to that of the group of mice injected with T only (Figure 2).

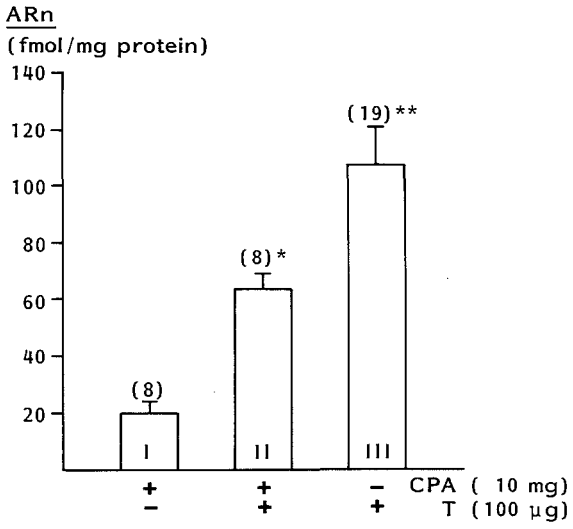

Figure 2 *Nuclear androgen receptor (ARn) in PC-82 tumor tissue grown in androgen deprived male nude mice treated with a single high dose of T only or in combination with a single dosage of the anti-androgen cyproterone acetate (CPA).*

* Significantly different from group I ($P < 0.002$)

** " " " " II ($P < 0.002$)

The more constant circulating levels of T obtained after T-implantation (Table I) resulted in a higher take rate and in a shorter lag-phase (i.e. the time required to obtain palpable tumors). Furthermore, the mean tumor doubling time calculated from growth curves of tumors grown in mice receiving supplementary T was significantly shorter than that of tumors grown in intact animals (Figure 3). ARn levels in tumors grown in T-implanted intact mice were significantly higher than those in non T-implanted control tumors (Figure 1, Groups III & IV vs Group I), but were similar to the levels of ARnT in tumor tissue derived from non T-implanted animals receiving a single high dose of T (Figure 1, Groups III & IV vs Group II). Administration of a single dose of T did not lead to a further increase of nuclear AR levels (Figure 1, Groups III & IV vs Group V).

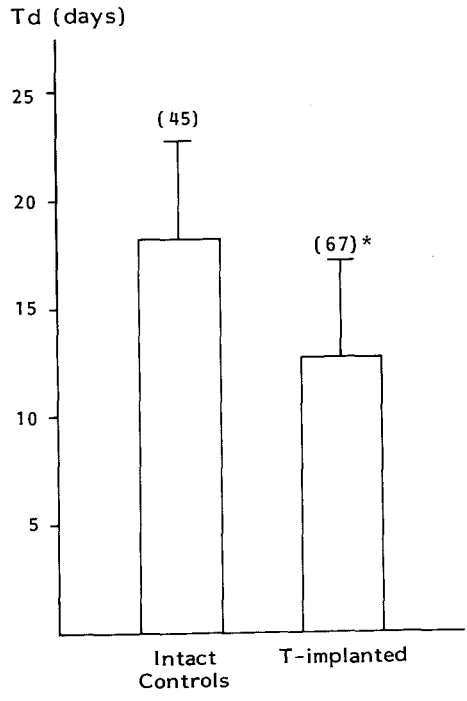

Figure 3 *Doubling-time (Td) of PC-82 tumor tissue grown in intact male mice and in mice substituted with testosterone (T).*

* *Significantly different from intact controls ($P < 0.001$). Values represent the mean ± S.D. of the number of observations (in parentheses).*

Effect of androgen withdrawal

Changes in the tumor volume could not be detected after a relatively short post-castration period (6 days) in tumor-bearing mice, whereas 28 days after androgen withdrawal a decline of the tumor volume of 40 ± 15 per cent (mean ± S.D., n = 8) was observed. The concentration of ARn was determined in tumor tissue derived from mice sacrificed at both points of time after androgen withdrawal. Like the control intact mice (Figure 1A; Group II) these animals received the single high dose of T prior to collection of the tumor tissue. In comparison with the intact controls only a small, statistically non-significant decrease (mean: 20%) of the concentration of ARnT was found in tissues either after the short or after the longer period of time (cf. Figure 1; Group VII & VIII vs Group II). Furthermore, receptor content was determined in tissues deprived of androgen for at least 21 days

without previous administration of T to the host animal. In these tissues the concentration of ARn was relatively low, and not significantly different from the level of ARn in tissue of untreated intact control mice (Figure 1; Group VI vs Group I).

Estrogen and progestin receptors

Estrogen and progestin receptors were determined in PC-82 tissues. Neither of the receptors could be detected in any of the five tissues which were assayed. The immunocytochemical assay of estrogen receptors confirmed this negative result, whereas in parallel incubations some human breast carcinoma tissues were found to be positive for this receptor.

DISCUSSION

The aim of androgen receptor measurements in the study of human prostatic carcinoma has always been to identify correlations between receptor levels in prostate cancer and the biological behavior of tumors in response to androgen ablation. The present investigation provides evidence that the growth of the PC-82 tumor is determined mainly by the continuous presence of androgen above the castrate level rather than by the total concentration of androgen receptors in the tumor tissue. In tumor-bearing male mice treated by castration for relatively long periods of time the concentration of nuclear androgen receptors (ARn) appeared to be low but not significantly different from the content of ARn in tissue grown in intact male mice (Figure 1A and C). Yet, a low amount of occupied receptors together with the physiological plasma-T levels of the intact mice led to the growth of the PC-82 tumor, whereas in castrated animals growth of the tumor was completely impaired (cf. ref. 18, 22).

Administration of a single high dose of T to tumor-bearing intact or castrated male mice resulted in an increase of the amount of ARn in the tumor tissue (Figure 1 A, C). The 1 h time interval between T administration and sacrifice of the animals was chosen in order to exclude possible *de novo* synthesis of receptor protein as a result of the high concentration of androgen. Apparently, this single injection of a high dose of T leads to an almost maximal translocation of AR in the PC-82 tumor tissue, whereas in tissue from intact male mice only 25 per cent was present in the nucleus (Fig. 1). The concept to determine unoccupied receptors through translocation of AR to the nucleus by a high dose of T, was based on observations in the ventral prostate of the rat (32). In these studies it was demonstrated that the distribution of AR over the nuclear and cytoplasmic compartments depends on the concentration of androgens. King *et al.* (33) had already shown by using a rat mammary

tumor model that 1 h after administration of radiolabeled estradiol, 90% of the radioactivity was recovered from the nuclei. Likewise, studies on the rabbit liver by Sheets *et al.* (34) demonstrated that 1 h after administration of a high dosage of androgen almost all of the AR was detected in liver nuclei. We considered this concept to be valid for human prostatic tissue as well. The assumption that unoccupied receptors are not localized in the nuclear compartment was the starting point of the present study of the PC-82 tumor. Accordingly, the degree of receptor occupation determines the presence of the AR in the nucleus. This contrasts with the findings of King and Green (35) and Welshons *et al.* (36), who showed that under all circumstances the estrogen receptor is localized in the nucleus. Since the localization of the androgen receptor system has not been definitely established yet, the interpretation of the present findings is based on the "classical" model of distribution of androgens over the cytosolic and nuclear compartments.

Cyproterone acetate (CPA), a compound which can prevent translocation of the AR to the nucleus of androgen target tissues (37, 38) was shown to exert this effect in the PC-82 tumor as well (Figure 2). The amount of CPA was based on reports in the literature describing that a 100-fold excess of the anti-androgen over the amount of T causes anti-androgenic effects (37, 39). Although the dose of CPA applied was not effective in completely blocking the translocation of the AR in the PC-82 tissue in comparison with the control tumors from mice receiving T only, tissue from CPA and T treated mice contained a 40 per cent lower amount of ARnT. It was inferred from this finding that the concept of the administeration of a high dose of T in order to obtain androgen binding to unoccupied receptors, actually involves receptor translocation. This observation is in agreement with observations by Brinkmann *et al.* (37) who studied the AR in the rat prostate.

The administration of a high dose of T to tumor-bearing mice, which had been castrated for a relatively short (6 days) or longer (28 days) period of time, led to an increase of the total amount of receptor (ARnT) to a mean concentration of 80 fmol/mg protein (Figure 1C). As this concentration of ARn did not significantly differ from that found in T-treated intact control animals (Figure 1A), it was concluded that in the PC-82 tumor the concentration of unoccupied AR is not influenced by androgen withdrawal. It is assumed that differences in ARn concentrations found in PC-82 tissues under various endocrine circumstances in the host animal reflect differences in the degree of receptor occupancy rather than differences in the total amount of receptor. In contrast, it has already been shown that in the regressed prostate of 7-day castrated rats only 25% of nuclear receptor is replenished through translocation of existing cytoplasmic receptor (24, 38). Administration of androgen in such rats resulted in a rapid (between 6 and 48 h) increase of AR in the ventral prostate of the animals (24).

The use of T-containing Silastic implants in PC-82 transplanted mice was shown to enhance the growth rate of tumor tissue in comparison with that of tissue grown in intact

male mice (Figure 3). Administration of T via these implants during the entire period of tumor growth, resulted in a higher degree of receptor occupancy, and thus in a more tight association of AR with the nucleus, compared to ARn in tissue of intact control animals (Figure 1A and B). The increase appeared to be independent of the levels of circulating androgen concerned (obtained by two different lengths of implants). It can be inferred from these findings that the accelerated growth of the PC-82 tumor following T-implantation is associated with a prolonged duration of receptor occupancy and therefore with a less variable presence of occupied AR in the nucleus. Therefore, the availability of a sufficient and steady level of T in the plasma, and consequently the duration of presence of AR in the nucleus of the tumor tissue, rather than the total concentration of AR, must be considered as the limiting factors in the modulation of hormonal responses in this androgen target tissue.

In spite of the occasional presence of peak plasma-T levels (above 50 nmol/l) in intact male mice (22) the mean level of T in these animals is relatively low (Table I). Under these conditions the content of AR is at a low level as well (Figure 1A). Apparently, under these circumstances the AR has not been translocated to the nucleus entirely (i.e. is not occupied by its ligand) and only approximately 25 per cent of AR in the PC-82 tumor tissue is tightly associated with the nucleus. It had previously been shown that the content of T and not that of dihydrotestosterone in the PC-82 tissue is significantly higher in tumors of T-implanted mice as compared to those in intact animals (cf. ref. 22). Therefore it is tempting to speculate that this higher concentration of T in the tissue is responsible for the higher content of ARn in the PC-82 tumor and thus for the accelerated growth of the tumor tissue described above. The injection of the high dose of T in T-implanted tumor-bearing mice did not further increase the concentration of ARn (above the level of 100 fmol/mg protein). Obviously, the maximal amount of AR in the PC-82 tumor tissue was recovered from the nucleus under both these conditions.

Using the tritiated ligand binding assay neither estrogen nor progestin receptors (ER and PR respectively) could be demonstrated in the PC-82 tumor specimens tested. Likewise, ER was not detected using the ER-ICA in tissue sections of the tumor tissue. ER or PgR are apparently not present in the PC-82 prostate tumor in concentrations comparable to those detectable in breast tumors. The wide range of ER concentrations reported in the literature (6,7) in prostatic tissue probably relates to methodological differences or to overestimation of receptors due to measurement of other binding proteins. The functional importance of low amounts of ER found by most investigators (cf. ref. 40, 41) is uncertain. Effects of physiological levels of estrogens might result from mechanisms not involving the receptor system. Such possible estrogenic effects on prostatic tissue have also been studied in the PC-82 tumor model (42).

Finally, it must be kept in mind that the present findings with the PC-82 tumor model

only represent the situation of one single (human) prostatic carcinoma. The outcome of receptor measurements of clinical samples represents an average of receptor levels of all (androgen-dependent and androgen-insensitive) cells in a tumor specimen. A large variation in receptor values found by van Aubel *et al.* (15) in multiple biopsies taken from individual tumors confirmed the existence of a large tumor cell heterogeneity. The 20% intra-tissue coefficient of variation found for the measurements of AR in the PC-82 tumor indicates that this tumor consists of a rather homogeneous population of (hormone-dependent) cells (cf. 18, 22). On the one hand, this makes the receptor data of the present study applicable only to this type of tumor cells. On the other hand, the androgen-dependence of the growth of the PC-82 tumor and the presence of androgen receptors make this model suitable for studying the putative relation between these two parameters as well as for studying the possible mechanism responsible for the control of the growth of human prostatic carcinomas.

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CHAPTER 7

TRANSPLANTABLE HUMAN PROSTATIC CARCINOMA (PC-82) IN ATHYMIC NUDE MICE: III. EFFECTS OF ESTROGENS ON THE GROWTH OF THE TUMOR

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CHAPTER 7

TRANSPLANTABLE HUMAN PROSTATIC CARCINOMA (PC-82) IN ATHYMIC NUDE MICE: III. EFFECTS OF ESTROGENS ON THE GROWTH OF THE TUMOR TISSUE

ABSTRACT

The transplantable human prostatic tumor model (PC-82) in nude mice was used to evaluate the indirect and possibly direct effects of estrogens on the growth of prostatic tumor tissue. High (pharmalogical) doses of plasma-estradiol (E_2) were achieved in tumor-bearing mice by using E_2-containing Silastic implants of different lengths. In comparison with the situation in men, in mice much higher concentrations of circulating E_2 (exceeding 3 nmol/l) were necessary to attain (near)-castrate levels of plasma-testosterone (T). Treatment of tumor-bearing mice with a high dose of E_2 resulted in tumor growth arrest and a subsequent decline of the tumor volume which equals the effects of castration. No evidence was found that either of the two doses of E_2 applied had any additive inhibitory effect on tumor growth when compared to castration alone. It was inferred from these findings that in the PC-82 tumor model estrogens, rather than having a direct effect on the tumor tissue, mainly act indirectly by their suppressive effect on T secretion in the host animal. A different and unexpected result was obtained in castrated tumor-bearing mice treated with a combination of E_2 and T. With both doses of E_2 this type of treatment led to a smaller increase of the tumor volume in comparison with that in mice receiving T only, the result of the high dose being statistically significant. This antagonistic effect of the two steroids on the PC-82 tissue was paradoxically associated with a sharp increase of nuclear androgen receptor levels and a higher concentration of dihydrotestosterone in the tumor tissue. Plasma and tissue concentrations of T appeared not to be altered. The present study of the PC-82 prostate tumor shows that only by careful monitoring of plasma-steroid levels in tumor-bearing mice can conclusions about the effectiveness of hormonal treatment regimens, such as estrogen therapy, be drawn.

INTRODUCTION

Hormonal therapy has been used for treatment of adenocarcinoma of the prostate since the benefit of endocrine manipulations for prostate cancer patients was described (1,2). Thus

for 45 years orchiectomy and/or administration of exogenous estrogens has been standard therapy for this disease. Estrogenic action is considered to primarily mediate inhibition of testicular testosterone secretion not only by inhibiting the hypophyseal gonadotropin release, but also by its direct effect on the testes (3). The ability of estrogenic substances, such as the synthetic estrogen diethylstilbestrol (DES), to reduce plasma testosterone to castrate levels in humans has been well documented (4). Apart from reducing plasma androgen levels estrogen treatment also induces an increase in the plasma concentration of sex hormone-binding globulin (SHBG) (5,6). Since protein-bound steroid is biologically inactive (7), the concentration of free androgen at the tissue level will be decreased even more than indicated by the decrease of total androgen concentrations.

A limited number of investigators could demonstrate a direct action of estrogens at the cellular level in the prostatic tissue. Pharmacological dosages of an estrogen-conjugate directly affected proliferation and differentation of prostatic epithelium in the dog (8) and high dose estrogen treatment (the natural estrogen estradiol-17β (E_2) as well as DES) modified androgen metabolism in human prostatic tissues (9,10). In addition, direct cytotoxic effects of DES on prostatic carcinoma cells have also been demonstrated (11,12). By using hormone-independent and/or metastasizing variants of the Dunning R3327 rat prostatic tumor Lazan *et al.* showed a dose-dependent inhibition of the primary (R3327 MAT-Lu) tumor as well as a reduction of the formation of lung metastases of this tumor (12). In spite of the possibly direct effects of estrogen treatment in addition to the castration-like reduction of circulating levels of testosterone, the combined treatment of prostate cancer patients with orchiectomy and estrogens appeared not to improve survival or death rates when compared to either treatment alone. (13,14).

Besides the above-mentioned therapeutical approaches there is also an increasing interest in the effects of combined treatment with androgens and estrogens on the hormone-induced growth of male sex accessory tissues. Interactions of these two steroids, either synergistically or antagonistically, have been shown in the reproductive tract and kidney of male rats (15,16), in the prostate of the dog (17), as well as in the human prostate (18). Studies of the normal prostate in the rat demonstrated that testosterone had a stimulating effect on the prostatic blood flow, whereas this effect is reversed by concomitant treatment with estradiol or DES (19). Treatment of castrated rats bearing the androgen-dependent Dunning R3327H prostatic carcinoma with E_2 and T combined produced an arrest of tumor growth. This suggests a direct inhibiting effect on the T-stimulated growth of this tumor (20).

Permanent transplantable human tumors in nude mice offer the possibility of studying the impact of different (endocrine) treatment regimens, such as estrogenic substances on human cancer tissues. In clinical practice the greater part of such investigations is hampered by ethical problems. The human prostatic tumor model (PC-82), which is transplantable in

nude mice, was described in the two preceeding papers of this series with respect to its hormone-dependence (21) and androgen-receptor characteristics (22). In addition, the effects of different dosages of estrogen (E_2) on this prostatic tumor were studied. This was conducted by using three different approaches: 1) estrogen treatment in the intact animals; 2) estrogen administration in addition to orchiectomy and 3) combined treatment with testosterone and estradiol in castrated animals.

MATERIAL AND METHODS

PC-82 tumor

The transplantable human prostate carcinoma, PC-82, was maintained in Balb/c nude mice from our own breeding colony. Details about the technique of transplantation and about the main characteristic of this tumor, i.e. the hormone dependence, have been described previously (23). PC-82 tumor tissue was grown either in intact or in testosterone (T)-supplemented castrated male nude mice. The present experiments were carried out with tumors of the 20-25th transplant generation. For light microscopic studies a tissue specimen of at least one tumor of individual mice was fixed in 10 per cent formaldehyde solution, embedded in paraffin and stained with hematoxylin-eosin.

Treatment of the animals

Treatment of the PC-82 tumor bearing mice was started when tumors were in the exponential phase of growth. Castration was carried out via the scrotal route under total anaesthesia with tribromoethanol (Aldrich, Beerse, Belgium). Testosterone and estradiol (Steraloids, Pawling, NY), were administered by the use of subcutaneously implanted Silastic devices to avoid daily injection of the tumor-bearing nude mice. The implants were prepared in our own laboratory as described in previous papers (24,23). Mice receiving supplemental androgens were implanted with testosterone (T)-capsules of 1.0 cm length at the time of grafting the tumor inoculum in castrated male mice. Data about hormone levels in plasma and tumor tissue of tumor-bearing mice implanted with this standard T-implant were reported in the first paper of this series (21). Relatively low and high circulating levels of plasma-estradiol (E_2) in tumor-bearing mice were attained by the application of a single E_2-implant of 0.5 cm and by a double implant of 2.0 cm length respectively. The 1.0 cm T-implant and 0.5 cm E_2 implant were made of medical grade silastic tubing (Talas, Zwolle, The Netherlands) of 1.5 inner and 2.1 mm outer diameter and were implanted under the skin of the left (T) or right (E_2) dorso-lateral wall of the mice. The 2.0 cm E_2-implants consisted of tubing with 2.0 mm inner and 2.8 mm outer diameter and were installed as a

pair of vehicles at the ventral side of the abdomen. Installation and removal of Silastic devices was carried out under light ether anesthesia.

Experimental protocols

Experiment 1. This experiment was performed to investigate the possible effect of estrogens on the PC-82 tumor in addition to that of castration alone. Tumor tissue was grown in castrated male mice which received supplementary T (via T-implants) and were grafted with tumor simultaneously. At the time treatment was started (cf. next paragraph) the tumor-bearing mice were depleted of androgen by removal of their T-implants. Part of the animals were implanted with a 0.5 cm E_2-capsule (low-dose) in addition to withdrawal of androgen. In a similar way the high dose of E_2 was tested. However, this experiment was carried out in PC-82 transplanted male mice which were treated either by castration or by castration in combination with E_2.

Experiment 2. The effectiveness of the high dose of E_2 to suppress plasma-T concentrations to castrate level and thus to inhibit the growth of the PC-82 tumor was tested in tumor transplanted intact male mice.

Experiment 3. The possible interaction of T and E_2 on the growth of the PC-82 tumor has also been studied by growing tumor tissue in T-implanted castrated male mice. When tumors were in the exponential phase of growth (cf. next paragraph) part of the mice were additionally implanted with either the low or the high dose of E_2, whereas the remaining (sham-operated) animals served as controls.

Growth measurements

Tumor growth was monitored by measurements of two perpendicular diameters using calipers twice a week. The tumor volume was calculated and growth curves were constructed as described previously (23). Tumors that reached a volume of at least 50 mm^3 were considered to have a positive take in the host animal. The majority of tumors showed an exponential phase of growth within the range of 50 to 800 mm^3 which corresponds to four tumor doublings. When the maximal tumor take (approximately 80 per cent) in a series of mice had been reached the animals were distributed over the different treatment groups in such a way, that an equal distribution of tumor sizes was obtained within each of the groups. Tumors with a volume of 500 mm^3 or more were excluded from the studies. In all three experimental protocols the growth of the tumors was expressed as the percentage increase or decrease of the tumor volume during the period of treatment. This was calculated from the final tumor volume and the volume at the start of the treatment.

Hormone estimations in plasma and tissue extracts

Endogenous concentrations of T and DHT were determined in some tumor tissue samples of mice in experiment 3. After homogenization of the tissues and subsequent extraction of the steroids T and DHT were separated on silica columns. The concentrations of both androgens in the tissue extracts and in the plasma of the mice were estimated by radioimmunoassay, by using the method and antiserum described by Verjans *et al.* (25). Plasma levels of E_2 were estimated by the use of the direct ^{125}I-estradiol radioimmunoassay supplied by EIR (Würenlingen, Switzerland). Inter and intra-assay coefficients of variation of both assays were included in the method section of the first paper of this series (21).

Androgen receptor measurements

A limited number of samples (weighing 50-100 mg each) derived from tumors of mice in experiments 1 and 3 were frozen for androgen receptor (AR) measurements. Nuclear AR (ARn), the amount of AR present in the nuclei of PC-82 tumor tissue as measured under the (hormonal) condition under which the tumor has grown in the host animal until sacrifice, was determined in tissues derived from mice of experiment 3. Tissue for receptor determinations in experiment 1 were harvested from mice which were injected with a high dose (100 μg) of T 1 h prior to sacrifice of the animals. This concept was previously applied to the PC-82 model and has been described in more detail in the second paper of this series (22). The content of AR in the nuclei of the tumor tissue was estimated by an exchange assay originally described by Foekens *et al.* (26).

Statistics

Values are expressed as means $\pm$ S.D. The significance of differences between values of different groups was calculated using two-tailed Student's T-tests. Differences were considered statistically significant when P was smaller than 0.05.

RESULTS

Dosage of estradiol

Table I shows the plasma-estradiol (E_2) concentrations in intact and castrated male nude mice which in both groups of mice were at a level of approximately 40 pmol/l. By the use of the two lengths of E_2-implants (as described in more detail in the method section) relatively high, pharmacological levels (up to 5000 pmol/l) of plasma-E_2 were obtained (Table I). Both dosages of E_2 caused a suppression of the testosterone (T)-secretion in the implanted

mice, resulting in significantly lower levels of plasma-T in comparison with those in intact controls (Table I). However, in both groups of E_2-implanted mice plasma-T was still above the castrate level. On the other hand, the high dose of E_2 was associated with circulating T-levels very near to those found in castrated animals.

Estradiol treatment of intact and castrated PC-82 mice

Experiment 1. In PC-82 tissue grown in T-supplemented mice removal of the T-implant resulted in a "castration-like" effect i.e. a reduction of the tumor volume with 50 per cent after the 25 day period of treatment (Figure 1). The same result was obtained when androgen withdrawal was combined with the administration of the low dose of E_2 (Figure 1; left panel). Control animals, keeping their T-implant, showed an increase of the tumor volume of 100 per cent during the same period of time. Likewise, treatment of intact tumor-bearing mice by castration concomitantly with the high dose of E_2 did not influence the pattern of tumor regression to a greater extent than observed after castration alone (Figure 1; right panel).

Similar amounts of total androgen receptors (77 ± 24 (n=5) and 66 ± 24 (n=8) fmol/mg protein; mean $\pm$ S.D.), as measured after T-injection (see method section), were found in tissue of mice treated by castration or by castration combined with the high estrogen dose respectively.

Experiment 2. In addition to the experiment described above a comparison was made between the effects of the high dose of E_2 and of castration in a group of intact male mice. The result (shown in Figure 2) confirmed that, as was found after castration, treatment with this dose of E_2 effectively arrested the growth of the PC-82 tumor tissue and resulted in a decrease of the tumor volume of approximately 40 per cent. In non-treated control animals the tumor volume increased by 100 per cent during the same period of time. The mean concentrations of T in the plasma of these three groups of mice, which were collected at the end of the period of treatment, are shown in Table II. In comparison with the plasma-T concentration of intact male mice shown in Table I the intact male mice of this experiment had considerably lower levels of plasma-T. Although the mean concentration of plasma-T in the E_2-treated mice (approximately 1 nmol/l; compare Table I) was significantly lower than that in intact controls, the difference between these two groups was rather small. No statistically significant difference was found between plasma-T levels in the E_2-implanted and castrated (tumor-bearing) mice of this experiment.

Table I Plasma concentration of estradiol (E_2) and testosterone (T) in control, E_2- implanted or castrated male nude mice*.

Group	N	Plasma-E_2 (pmol/l)	Plasma-T (nmol/l)
intact controls	11	42 ± 15	11.1 ± 9.8
E_2-implant (0.5 cm)	9	946 ± 211	2.5 ± 1.0[a,b]
E_2-implant (2x2.0 cm)	8	4442 ± 962	1.0 ± 0.2[a,b]
castration	8	40 ± 13	0.4 ± 0.3[a]

* Blood of the mice was collected at 7 days after implantation; Values expressed as means ± S.D.

a) Significantly different from control group ($P < 0.001$)

b) Significantly different from castrated group ($P < 0.001$)

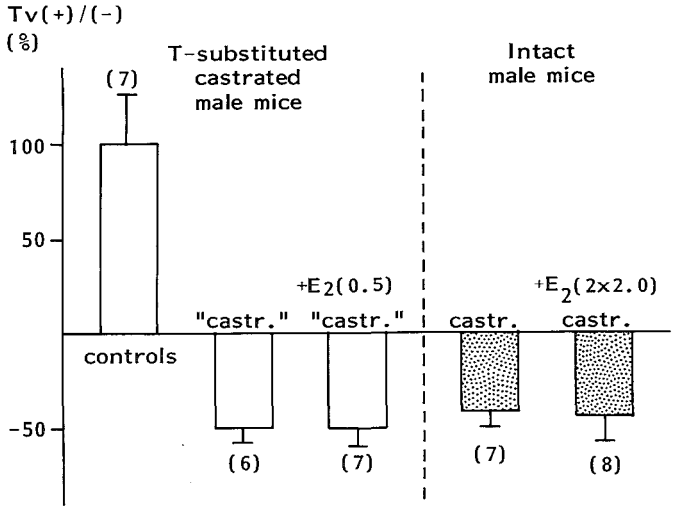

Figure 1 *Effect of androgen withdrawal with or without additional treatment with estradiol (E_2) on PC-82 tumor tissue grown in male nude mice. Tv (+/-): percentage increase or decrease of the tumor volume during the period of treament (25 and 28 days respectively).*
Values are expressed as means ± S.D.; with the number of mice in parentheses.

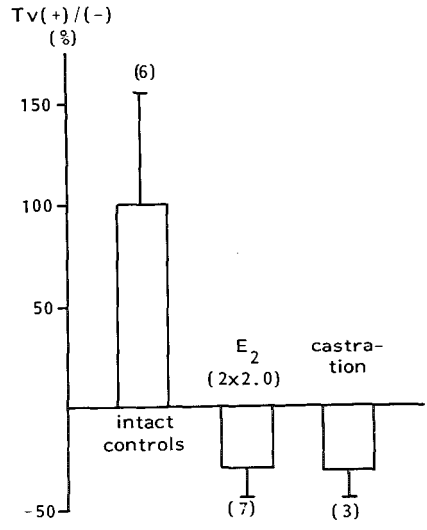

Figure 2 *Effect of estradiol (E_2) implantation or castration on the growth of PC-82 tumor tissue grown in intact male nude mice.*
Tv(+/-): percentage increase or decrease of the tumor volume during the period of treatment (42 days).
Values are expressed as means ± S.D.; with the number of mice in parentheses.

Table II Plasma levels of testosterone (T) in PC-82 tumor-bearing male nude mice at 42 days after castration or treatment with high dose estradiol*.

Group	N	Plasma-T (nmol/l)	Range (nmol/l)
intact controls	6	2.72 ± 2.29	1.25 - 7.39
E_2-implant (2x2.0 cm)	7	0.83 ± 0.49**	0.31 - 1.53
castration	3	0.31**	—

* Values are expressed as means ± S.D.
** Significantly different from control group ($P < 0.02$)

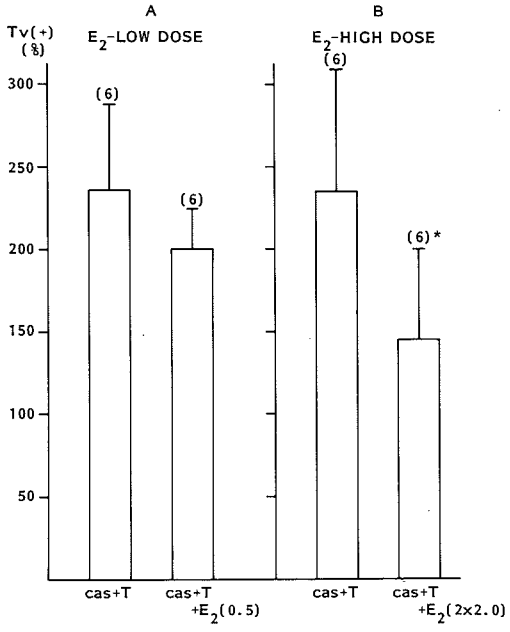

Figure 3 *Effect of administration of different amounts of estradiol (E_2) on the growth of PC-82 tumor tissue transplanted in castrated male nude mice substituted with testosterone (1.0 cm T-implant). A: low dose of E_2; B: high dose of E_2. Tv(+): percentage increase of the tumor volume during the period of treatment (28 days).*
**Significantly different from control group ($P < 0.05$)*

Combined administration of testosterone and estradiol

Experiment 3. In the presence of a constant level of circulating T the additional treatment (for 28 days) of tumor-bearing mice with the low dose of E_2 did not significantly affect the growth of the PC-82 tumor tissue (Figure 3A). Administration of the high dose of E_2 for the same period of time resulted in a retardation of the growth of the tumor tissue. In the group of mice treated with the combination of T and (high dose) E_2 a statistically significant ($P < 0.05$) smaller increase (by almost 30 per cent) of the tumor volume was found in comparison with the mice receiving T only (figure 3B). This experiment was repeated and a similar result was obtained (Table III). The initial tumor volume in both groups of tumor-bearing mice was identical. Like in the previous experiment the increase of the tumor volume in mice treated with both T and E_2 was diminished by 35 per cent as compared with

the control animals. However, the tumor-doubling time, calculated from tumor measurements during the 28 days period of time, was found to be similar for tumors in both groups of mice (Table III). From this table it can also be seen that treatment with the high dose of E_2 led to a statistically significant decline of the weights of the seminal vesicles, whereas E_2 did not affect the final body weight of the animals. In both groups of mice a loss of body weight of approximately 10 per cent was observed. The circulating levels of T and E_2 in these mice are presented in Table IV. After a period of 28 days the plasma concentrations of E_2 were substantially lower than found in mice implanted for 7 days (compare table I). Still, in the implanted animals circulating E_2-levels above 1 nmol/l were found. This high concentration of E_2 did not influence the concentration of circulating-T, since in both groups of mice plasma-T levels of approximately 25 nmol/l were found (Table IV).

Table III Effect of testosterone (T) and estradiol (E_2) on the weights of seminal vesicles and the growth of the PC-82 tumor in castrated male mice.

Treatment	Body weight change (%)	Seminal vesicles weight (mg)	Initial tumor volume (mm^3)	Percent change in tumor volume
Castr + T (controls)	- 8.9 ± 4.4 (10)	114 ± 10 (10)	185 ± 76 (9)	200 ± 50 (9)
Castr + T + E_2 (2x2.0 cm)	-12.0 ± 6.0 (10)	96 ± 6* (10)	190 ± 78 (10)	133 ± 27* (10)

Values are expressed as means ± SD
*Significantly different from controls ($P < 0.005$).

In PC-82 tumor tissue grown in mice implanted with E_2 simultaneously with T a considerably higher amount of nuclear ARn was found compared to tissue grown in mice receiving T-implants only (Table IV). Likewise, in tissue specimens of the same tumors the concentrations of T and dihydrotestosterone (DHT, the major metabolite of T) were estimated. In tissues derived from both groups of mice similar concentrations (25-30 pmol/g tissue) of T were found (Figure 4). In contrast to this, tissues grown in mice implanted with T as well as E_2 contained slightly, but statistically significantly, higher concentrations of DHT than tissues grown in T-treated mice.

Table IV Plasma hormone levels and nuclear androgen receptor (ARn) concentrations in PC-82 Tumor tissue grown in castrated male mice supplemented with testosterone (T) and/or estradiol (E_2)

Treatment	Plasma-E_2 (pmol/l)	Plasma-T (nmol/l)	ARn (fmol/mg protein)
Castr + T (controls)	6 ± 6 (9)	25.5 ± 5.7 (9)	89 ± 54 (8)
Castr + T + E_2 (2x2.0 cm)	1631 ± 397 (9)	23.8 ± 2.8 (9)	314 ± 110* (10)

Values are expressed as means ± SD.
*Significantly different from controls ($P < 0.001$).

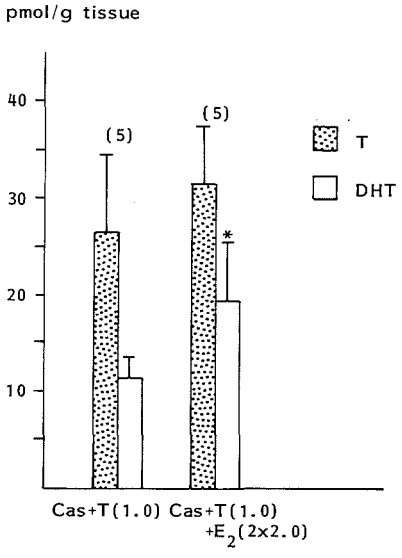

Figure 4 *Concentrations of testosterone (T) and dihydrotestosterone (DHT) in PC-82 tumor tissue grown in T-implanted castrated male mice treated with high dose of estradiol (E_2).*
**Significantly different from control group ($P < 0.05$).*

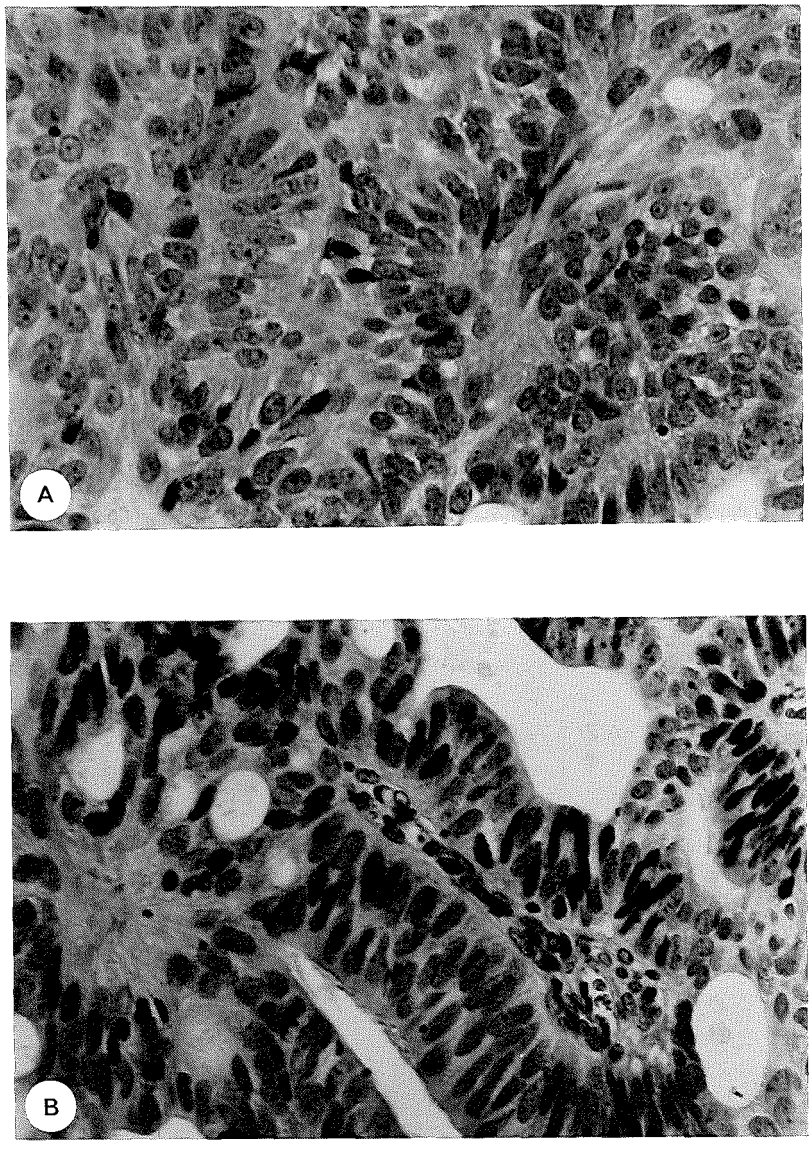

Figure 5 *Histological sections of PC-82 tumor tissues from intact male mice treated for 28 days with castration (A) or with castration plus (high dose) estradiol (B). The tumor consists of a cribriform adenocarcinoma with papillary formations and small strands of connective tissue derived from the host animal. Necrotic areas were not seen. Magnifications: x 380.*

Histology

The histological appearance of the PC-82 tumor tissue was that of a cribriform adenocarcinoma of the prostate with papillary formations and with the presence of distended acini. Secretory activity of the cells could be observed. The overall feature of the tumors grown in T-implanted mice (experiments 1 and 3) was similar to that of tumors grown in intact animals (experiments 1 and 2); only in the T-substituted mice mitoses were found more frequently. Androgen withdrawal (by castration or by removal of T-implants) from tumor-bearing mice (experiments 1 and 2) resulted in a decline of the mitotic activity, whereas further histological alterations were not found. Likewise, no apparent differences could be observed between tissues of mice treated by castration or of mice treated by castration in combination with either dose of E_2 (experiment 1). Representative histological pictures of tumors in this experiment are shown in figure 5.

In experiment 2 the tumors from mice treated with the high dose of E_2 had a similar histological appearance compared to those of mice treated by castration; in comparison with the control tissues only the mitotic activity was substantially reduced. Neither the low nor the high dose of E_2 induced remarkable changes in the overall histological picture of the tissues derived from the T-implanted mice of experiment 3. In general, if necrotic areas (covering maximally 20 per cent of the total tumor) were present at all, the occurence appeared to be related to the size of the tumor rather than to any type of treatment.

DISCUSSION

Like orchiectomy of prostatic carcinoma patients, estrogen treatment is known to be effective in controlling this disease through its ability to lower circulating levels of testosterone (T). A direct influence of estrogens on tissues of the normal and pathological prostate has been reported (9,11,12,27), but the question whether estrogen treatment has any synergistic or additive effect to that of orchiectomy alone has not been answered convincingly. In the present study the indirect and possibly direct effects of two dosages of estradiol-17β (E_2) on the transplantable human prostatic carcinoma PC-82 were evaluated.

Total concentrations of plasma-E_2 of 50-150 pmol/l were reported for the human male (6,28). Control, intact male nude mice had plasma-E_2 levels of approximately 40 pmol/l (Table I), which is comparable to values in nude mice published by others (29,30). Since mice do not have SHBG-binding in their plasma the concentration of "free" E_2 will be similar to, or possibly higher than, the concentration in the human male situation. High levels of circulating-E_2 were obtained in the tumor-bearing mice by the use of E_2-filled

Silastic implants, which also made frequent injection of the animals unnecessary. Both concentrations of estrogen in the plasma of the mice released from the two lengths of implants (Table I) must be considered as pharmacological dosages. Nevertheless, the plasma-T concentration in intact male mice implanted with 0.5 cm E_2 was not decreased to the castrate level, whereas by the administration of the high dose of E_2 near-castrate levels of plasma-T were obtained. By contrast, a standard therapy in patients with prostate carcinoma receiving estrogens (ethinyl-estradiol and polyestradiolphosphate) resulted in plasma E_2 levels of 400 pmol/l which were associated with plasma-T concentrations (approximately 3 nmol/l) similar to those found after orchiectomy (6). Treatment of the mice with high dosages of E_2 will certainly lead to an increase of the secretion of prolactin in the animals. As it was previously shown that the PC-82 tumor is devoid of prolactin receptors (M.A. Blankenstein, unpublished results), a possible effect of prolactin on the growth of the PC-82 tumor tissue is not likely.

In spite of the incomplete suppression of plasma-T in the E_2 (0.5 cm)-implanted mice it was previously shown that administration of this (relatively) low dose of E_2 simultaneously with tumor grafting almost completely prevented the take of PC-82 tumor tissue in intact male mice (23). However, tumors once growing and subsequently treated with this type of E_2-implant were not affected by this dose of estrogen (result not shown). In addition, it was demonstrated that implantation of tumor-bearing intact male mice with the high dose of E_2 was as effective as castration in producing a tumor growth arrest and a gradual decrease of the tumor volume (Figure 2). Since plasma-T in the E_2-treated and castrated mice did not exceed the concentration of 1.5 nmol/l (Tabel II), it can be inferred from these findings that circulating levels of T lower than 1.5 nmol/l in the host animal are not compatible with growth of the PC-82 prostatic tumor. The growth of two other androgen-sensitive human prostate tumor systems in nude mice (the Honda and LNCaP tumor) could not be inhibited effectively with estradiol (31,32). The lack of data on circulating levels of E_2, and more important of T, in these studies does not allow definite conclusions about the effectiveness of this type of treatment, in particular since the level of T (either suppressed or not) which is still able to stimulate the growth of these tumor tissues is not known. Therefore, careful monitoring of plasma-steroid levels must be included in all hormonal manipulation studies with experimental prostate tumors.

In the present study with the PC-82 tumor no evidence was found that either of the two dosages of E_2 applied had any additive inhibitory effect on the growth of the tumor when compared to the effect of castration alone (Figure 1). It was concluded from the results in intact and castrated tumor-bearing mice that in the PC-82 tumor model estrogens mainly act indirectly by their suppressive effect on plasma-T rather than directly. The present findings contradict those of Hoehn and coworkers (33), who also studied the effects of E_2 in the PC-82 tumor model. These investigators claimed an additional inhibitory effect of E_2 on

the pattern of castration-induced regression of the PC-82 tumor tissue. However, in their study 21 days after castration only a 10% reduction of the mean tumor volume was found, whereas treatment of mice with castration and E_2 resulted in a 30 per cent decline of the tumor volume. The latter result was similar to that found for both treatment groups in the present study (figure 1). It should also be noted that the daily single injections of E_2 in the study of Hoehn will lead to substantially different profiles of plasma-E_2 as compared to those found after administration of the hormone via Silastic implants in the present study.

The present result of the PC-82 tumor in castrated mice does not confirm findings with the Dunning R3327 prostatic tumor in the rat. Shessel *et al.*. (34) and, more recently, Daehlin *et al.* (27) found that treatment of androgen-dependent sublines of this model system with E_2 or DES in combination with castration was a more effective means of suppressing tumor growth than castration alone. In addition, a direct effect of estrogens on prostatic cancer tissue appeared to be likely since some studies demonstrated that the growth of the hormone-independent R3327AT subline as well as the metastatic spread of the R3327MAT-Lu tumor of the Dunning model system were suppressed by high dose DES treatment (11,12). These effects appeared not to be mediated through any steroid receptor system, since in contrast to the hormone-dependent R3327H tumor, the forementioned sublines were shown to be devoid of androgen and estrogen receptors (35,36). However, it must be considered that these results were obtained by administration of extremely high doses of DES to tumor-bearing rats. By treatment of rats bearing the hormone-independent Dunning-AT tumor with a 200-fold lower dose of DES, which is comparable to the 5 mg dose applied in the clinical situation (13,14), the above-mentioned results could not be confirmed (37). Treatment of patients with orchiectomy plus estrogens (DES) did not offer an advantage (in terms of successfully changing survival or death rates) over either treatment alone (13,14). Moreover, the use of estrogen in dosages producing a "castration-like" effect on plasma-T levels is hampered by considerable side effects such as cardiovascular complications (38).

Robaire *et al.* (15) demonstrated that in the adult male rat T and E_2, applied by silastic implantation, interact with each other. At relatively high doses of both steroids direct competition at the target level was observed resulting in a decrease of the weights of the accessory sex organs. Likewise, in the present study with the PC-82 tumor the T-stimulated growth of the tumor tissue in castrated male mice was significantly retarded by the high dose of E_2 (Figure 3; Table III). It is suggested that estrogens are able to influence the balance between cell proliferation and cell death which has also been shown for the prostate in the dog (17). Similar results were obtained in the above-mentioned hormone-dependent subline of the Dunning prostate tumor in the rat. It was demonstrated that treatment of castrated rats with E_2 in combination with T resulted in an arrest of tumor growth (20,27), but also in an increase of the blood flow in this tumor (19,20).

In the present experiments with the PC-82 tumor in the presence of supplementary T the high dose of E_2 induces a sharp increase of androgen receptor (AR) concentrations without affecting the level of circulating-T (Table-IV). In agreement with this a significantly higher concentration of dihydrotestosterone was found in the PC-82 tissue from E_2-treated mice (Figure 4). Evidence that estrogens affect prostatic tissue by increasing the amount of AR was also obtained by other studies in experimental prostatic (tumor) systems (39,40) as well as in human prostatic tissues (41). From the present data it may seem that estrogens, by increasing the AR level, paradoxically decrease the sensitivity of the prostate to androgenic stimulation. In the second paper of this series the absence of an estrogen receptor (ER) in the PC-82 tumor was reported (22). Therefore, the assumption that the forementioned increase of AR is mediated by the ER as suggested to be the case for the rat prostate (39), does not apply to the PC-82 tumor. The mechanism underlying this phenomenon remains unclear. In castrated mice substituted with T the response of the pituitary to estrogen, i.e a decreased LH secretion, will not affect plasma-T. Hence, E_2 must be able to directly counteract the effect of T on the PC-82 tumor in the high dose applied.

In conclusion, the present study shows that in the intact male mice high doses of E_2 inhibit the growth of the transplantable PC-82 tumor only through suppression of plasma-T. No evidence was provided that either of the estrogen doses applied to castrated, tumor-bearing mice has an additional effect on the regressing tumor. The findings that pharmacological doses of estrogens counteract the action of androgen in T-substituted mice and concomitantly increase the concentration of androgen receptors in this tumor need further investigation.

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CHAPTER 8

ADMINISTRATION OF AN LHRH-ANTAGONIST TO MALE MICE: EFFECTS ON IN VIVO SECRETION OF HORMONES AND ON THE GROWTH OF A TRANSPLANTABLE HUMAN PROSTATIC CARCINOMA

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CHAPTER 8

ADMINISTRATION OF AN LHRH-ANTAGONIST TO MALE MICE: EFFECTS ON IN VIVO SECRETION OF HORMONES AND ON THE GROWTH OF A TRANSPLANTABLE HUMAN PROSTATIC CARCINOMA

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Summary

The potent luteinizing hormone releasing hormone (LHRH) antagonist |N-Ac-D-p-Cl-Phe[1,2],D-Trp[3],D-Arg[6],D-Ala[10]|-LHRH was chronically administered to male nude mice bearing the transplantable human hormone-dependent prostatic adenocarcinoma PC-82. Treatment of tumor-bearing male mice with a daily dose of 100 µg (4 mg/kg b.w.) for 21 days did not significantly affect the growth of the PC-82 tumor tissue, or the weights of ventral prostate, seminal vesicles and testes. At 24 hours after the last dose of the antagonist the mean plasma-testosterone (T) value in these animals was not different from the control level. Administration of similar doses of the antagonist to intact normal immunocompetent male mice significantly reduced plasma LH concentrations and suppressed plasma-T to near-castrate levels, when blood was taken 2 hours after the last injection. At 24 hours after the last dose, however, plasma concentrations of LH and T had returned to control levels. This time-dependent pattern of T suppression by the antagonist was confirmed by a time-course experiment in animals receiving a single dose of the compound. These data demonstrate that a daily high dose of this antagonist cannot effectively suppress plasma-T in male mice. Therefore, the mouse may not be a suitable model for the investigation of the "castration-like" effect of LHRH-antagonists on androgen-dependent prostate xenografts.

Chronic administration of superactive analogues of luteinizing hormone releasing hormone (LHRH) results in a paradoxical inhibition of the pituitary-gonadal axis characterized by a decrease of the levels of sex steroids and the atrophy of accessory sex organs (for reviews see 1,2). This effectiveness of analogues of LHRH in suppressing gonadal steroid production makes these compounds suitable for treatment of hormone-dependent cancers such as breast and prostatic carcinomas. A large number of analogues with agonistic or antagonistic properties were extensively studied in experimental models (3-5) as well as in clinical trials (6,7). Among the agonists (D-Trp)[6]-LHRH (7,8) and HOE-766 (Buserelin)(9-11) have been widely applied in the management of clinical prostate cancer.

The use of antagonistic analogues of LHRH avoids the initial stimulation of the release of gonadotropins and sex steroids that occurs during the first period of treatment with the LHRH-agonists, before the inhibitory effects eventually take over. One of these antagonists, N-Ac-D-p-Cl-Phe[1,2],D-Trp[3],D-Arg[6], D-Ala[10]-LHRH (Org-30276), which is considerably more potent and has better solubility properties (12) than the previously tested antagonists, was shown to

be effective in inhibiting the growth of the Dunning rat prostate tumor (3). In mice the growth of the MXT estrogen-dependent mammary tumor was suppressed by the chronic treatment with this antagonist (13).

In our institution the human prostatic tumor line PC-82 was developed (14), which shares several properties with clinical prostatic cancer. The growth of this tumor, which is serially transplantable in nude mice, is completely dependent on androgens (15). Because Bex et al. (16) and Wang et al. (17) reported that mice are insensitive to the antitesticular effects of LHRH-agonists, the present study was undertaken to investigate the effect of chronic administration of an antagonist, Org-30276 in nude mice, bearing the human PC-82 prostatic tumor. In addition, the effects of this compound on the weights of reproductive organs and on the pulsatile testosterone and gonadotropin secretion in (immunocompetent) heterozygous nude mice was studied in more detail. The results of a related study concerning the in vitro gonadotropin secretion from pituitaries after in vivo treatment with the antagonist are reported in a separate paper (18).

Material and Methods

Peptide analogue of LHRH. The antagonist N-Ac-D-p-Cl-Phe^{1,2}-D-Trp^{3}-D-Arg^{6}, D-Ala^{10}-LHRH, Org-30276 (12) was synthesized and supplied by Organon International BV (Oss, The Netherlands) and dissolved in saline containing 5% mannitol at a concentration of 1 mg/ml.

PC-82 tumor growth. The PC-82 tumor originated from a moderately differentiated adenocarcinoma of the prostate, which had been removed from a patient by total perineal prostatectomy in July 1977 (14). PC-82 tumor tissue was grown in male nude mice of the Balb/c background obtained from our breeding facilities. The animals were housed separately in laminal flow cage racks and maintained at controlled conditions as described before (15). Tumor growth was monitored by weekly measurement of perpendicular tumor diameters by the use of calipers, growth data were further analyzed as described earlier (15).

Some additional experiments were carried out using normal immunocompetent mice from same breeding colony and thus with a similar genetical background as the PC-82 transplanted nude mice.

Treatment of the animals. PC-82 tumor tissue was grown in male nude mice. The subcutaneous administration of Org-30276 (once daily for 21 days at a dose of 100 µg/day (4 mg/kg b.w.)) was started when tumors were in the exponential phase of growth, i.e. at 69 days after tumor inoculation. Control tumor-bearing mice received injections of 100 µl vehicle. Castration of one group of animals was carried out via the scrotal route under total anaesthesia with tribromoethanol (Aldrich, Beerse, Belgium).

Normal immunocompetent (NIC) heterozygous nude mice were also treated with the same dose of Org-30276 for 21 days. Subsequently, animals were sacrificed at different time points (2 or 24 hrs) after the last injection. In addition, the response of circulating levels of plasma-T, LH and FSH to a single (100 µg) dose of the antagonist was studied in the NIC mice at the forementioned points of time.

Finally, the time-dependent decline of plasma-T levels after a single injection of Org-30276 was studied in NIC mice. Animals were sacrificed at 2,8, 16 and 24 h after administration of the compound.

At the end of the experiments the mice were exsanguinated from the orbital sinus under ether anaesthesia. The blood was centrifuged and the plasma was stored at -20°C for hormone determinations. Various organs were dissected and weighed after removing the adherent connective tissue.

Hormone estimations. The concentrations of FSH and LH in the plasma of the mice were measured using the radioimmunoassay technique described by Welschen et al. (19). Details about the assay, intra- and interassay variations are given in the accompanying paper (18). All results are expressed in terms of NIADDK-rat-FSH RP-1 and NIADDK-rat-LH RP-1 per litre plasma. Testosterone in the plasma was estimated by the radioimmunoassay technique described by Verjans et al. (20).

Statistical procedures. The significance of differences between the results of different treatment groups was assessed using Student's t-test or Wilcoxon's test (when indicated). Differences were considered to be significant when P-values smaller than 0.05 were found.

Results

Effect of LHRH-antagonist on growth of the PC-82 tumor, hormone levels and organ weights in nude mice. Figure 1 shows the response of the PC-82 tumor grown in male nude mice which had been treated with the LHRH-antagonist or orchiectomized for a period of 21 days. In control mice the PC-82 tumor volume increased by almost 100%, whereas the increase in the group of antagonist-treated mice was 70% (Fig. 1). The difference between the two groups was statistically not significant. The findings of an additional experiment showed that castration leads to cessation of tumor growth and to a reduction of the tumor volume with 40% after 21 days.

Body and organ weight of the antagonist-treated control tumor-bearing nude mice have been listed in Table I. Small statistically not significant declines of the weights of ventral prostate and seminal vesicles were observed after 21 days of antagonist treatment. Administration of LHRH-antagonist did not significantly affect body or testes weights (Table I).

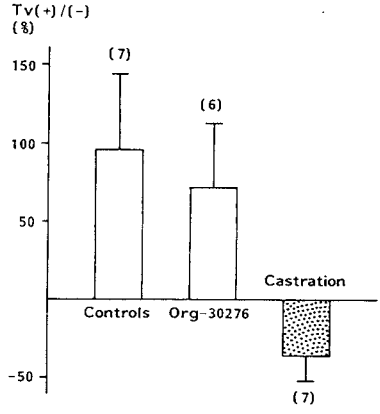

FIG. 1

Effect of castration and treatment with LHRH-antagonist (Org-30276; 4 mg/kg b.w.) on PC-82 tumor tissue grown in male nude mice. $T_V(+)/(-)$: percentage increase or decrease of the tumor volume during the period of treatment (21 days). Values are expressed as means ± S.D., with the number of mice in parentheses.

At 24 hours after the last injection the mean levels of testosterone (T) in the plasma of control mice and in the plasma of both mice treated with the antagonist and which have been castrated, were below the level of 2 nmol/l (Table II). Although the antagonist suppressed the mean level of plasma-T compared to that of mice in the control group, the difference between these two groups was statistically not significant. Moreover the plasma levels of T in the antagonist-treated nude mice were significantly higher than those in castrated nude mice after the same period of time.

TABLE I

Organ	Organ weights (mg)[a]	
	Controls	Antagonist[b]
Ventral prostate	7.2 ± 2.4 (7)	5.5 ± 1.4 (6)
Seminal vesicles	163 ± 51 (7)	116 ± 40 (6)
Pituitary	2.0 ± 0.6 (7)	1.5 ± 0.5 (6)
Testes	179 ± 28 (7)	175 ± 20 (5)
Final body weight	21.0 ± 4.3 (7)	23.0 ± 4.6 (6)

a) Values are expressed as means ± S.D. with the number of mice in parentheses

b) Mice were treated for 3 weeks with a daily injection of 100 µg (4 mg/kg b.w.) Org-30276.

Effects of LHRH-Antagonist (Org-30276) on the Weight of the Accessory Sex Glands in Male Nude Mice bearing PC-82 Tumors.

TABLE II

Group	n	Testosterone* (nmol/l)	Range
Controls	7	1.93 ± 1.59	(0.94 - 4.26)
Org-30276	6	0.82 ± 0.33	(0.34 - 1.32)
Castration	7	0.24 ± 0.06	(0.16 - 0.31)

* Values are expressed as means ± S.D.

Plasma Levels of Testosterone in Male Nude Mice bearing PC-82 Tumors at 21 days after Castration or Treatment with an Antagonist of LHRH (Org-30276; 4 mg/kg b.w.).

Effects of LHRH-antagonist on hormone secretion and weights of accessory sex glands in immunocompetent mice. Intact (NIC) male mice treated either with a single dose or for 21 days with the antagonist showed similar patterns of circulating gonadotropins and testosterone. Mice sacrificed at 2 h after the last dose of Org-30276 had significantly declined plasma levels of LH and T as compared to control animals receiving vehicle injections (Table III and Figure 2 respectively). In animals sacrificed at 24 h after the last injection plasma concentrations of LH and T had returned to levels found in control mice. The changes in plasma-FSH appeared to be opposite to those of LH: only in the mice sacrificed at 24 h after the last dose the suppression of FSH was statistically significant.

In order to give a better illustration of the secretory pattern of T in these mice after long-term treatment with the antagonist, the individual values of plasma-T are outlined in Fig. 2. The large inter-individual differences of plasma-T indicate that control mice have a pulsatile T-secretory pattern, with levels varying from lower than 5 to higher than 100 nmol/l. In Fig. 2 it is clearly demonstrated that at 2 h after administration of Org-30276 the peak plasma levels of T were not found, whereas at 24 h a similar distribution of T-levels was found compared to that in control mice sacrificed at both points of time. Furthermore, the mean level of T at 2 h after injection was slightly but significantly higher compared to that of male mice treated by orchiectomy (results not shown). In spite of the incomplete suppression of circulating T-levels a significant decrease (by 38 and 54 percent respectively) of the weights of V.P. and S.V. in antagonist-treated mice was observed (Table IV). The weights of testes had not changed after prolonged administration of the antagonist.

TABLE III

	LH (ng/ml)		FSH (µg/ml)	
Treatment/ Sampling time*	Control	Antagonist	Control	Antagonist
Single dose (2 h)	67.2 ± 18.4 (6)	9.0 ± 2.3** (6)	3.0 ± 0.1 (3)	1.7 ± 0.4 (3)
21 days (2 h)	150 ± 39 (4)	7.5 ± 0.9** (4)	3.7 ± 1.1 (3)	0.8 ± 0.4 (3)
21 days (24 h)	120 ± 46 (6)	107 ± 42 (6)	4.1 ± 1.0 (4)	1.3 ± 0.1** (4)

Values are expressed as means ± S.E.M., with the number of mice in parentheses.
*Time (h) of sacrifice after the last dose.
**$p < 0.05$ compared to appropriate controls, according to Wilcoxon's test.

Plasma Levels of Gonadotropins and Testosterone in immunocompetent Male Mice, treated by a Single Dose or during 21 Days of LHRH-Antagonist, Org-30276.

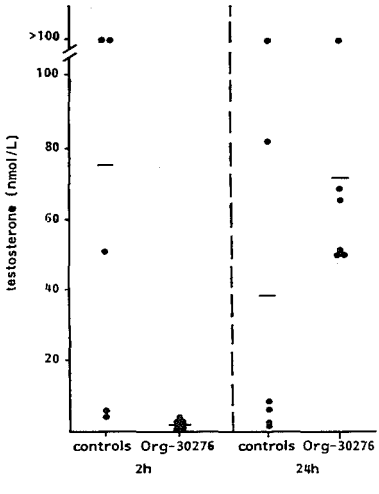

FIG. 2

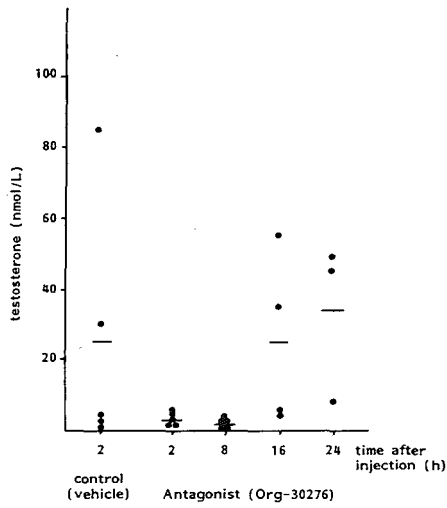

FIG. 3

Figure 2. Plasma levels of testosterone after 3 weeks of antagonist or placebo treatment of mice sacrificed at 2 or 24 h after the last dose. Horizontal lines represent means.

Figure 3. Plasma levels of testosterone in mice at various times after administration of a single dose of Org-30276 (100 µg). Horizontal lines represent means.

TABLE IV

Organ	Organ Weights (mg)[a]	
	Controls	Antagonist[b]
Ventral prostate	12.8 ± 4.1 (11)	7.9 ± 1.9 (11)[c]
Seminal vesicles	296 ± 56 (11)	137 ± 63 (11)[c]
Testes	224 ± 21 (11)	208 ± 21 (11)
Final body weight	31.9 ± 3.3 (11)	31.1 ± 1.8 (11)

a) Values are expressed as means ± S.D. with the number of mice in parentheses.
b) Mice were treated for 3 weeks by a daily injection of 100 µg (4 mg/kg b.w.) Org-30276.
c) Significantly different from controls ($p < 0.005$).

Effects of LHRH-Antagonist (Org-30276) on the Weights of the Accessory Sex Glands in Normal Immunocompetent Male Mice.

Time-dependent suppression of plasma-T secretion by the antagonist. A group of intact (NIC) male mice was injected with a single dose of 100 µg Org-30276. Animals were sacrificed at 2,8,16 and 24 h after administration of the compound. Plasma-T levels decreased rapidly within 2 h after injection and remained below the level of 2 nmol/l for 8 h (Fig. 3). After this time, however, the concentration of T in the plasma started to rise, resulting in pre-treatment (control) levels at 16 h after administration of the antagonist.

Discussion

Analogues of LHRH with an agonistic action are successfully applied in the treatment of patients with prostatic as well as breast carcinomas. In contrast to these agonists LHRH-analogues with an antagonistic action do not lead to the initial rise of plasma-T concentrations in patients with prostate cancer, which consequently results in the well-known flare-up of the disease at the start of this therapy. The present data demonstrate that 21 days of treatment with a high dose of the LHRH-antagonist N-Ac-D-p-Cl-Phe^{1,2},D-Trp^{3},D-Arg^{6}-D-Ala^{10}-LHRH was not effective in significantly inhibiting the growth of the hormone-dependent human prostatic tumor (PC-82) grafted in male nude mice (Fig. 1). Nevertheless, the relatively high dose (4 mg/kg b.w.) of the antagonist used in this study is a 20-fold excess of the dose necessary to achieve a chemical castration effect in the rat model (3). Additionally, the antagonist (Org-30276) did not significantly affect the weights of the accessory sex organs in these tumor-bearing animals (Table I). This finding can be explained by an incomplete suppression of T-secretion since at 24 h after the last treatment the circulating levels of T in the antagonist-treated mice were significantly above the castrate level (Table II). During the same period of time the tumor volume after orchiectomy had decreased by 40%, as reported also in earlier experiments with this tumor (15).

In contrast to the result in nude mice, the same dose of the antagonist (4 mg/kg b.w.) was effective in significantly decreasing the weights of ventral prostate and seminal vesicles in immunocompetent (heterozygous/nude) mice (Table IV). Rebar et al. (21) indicated that nude mice had reduced concentrations of circulating androgens and gonadotropins as compared to their heterozygous littermates. In the present study also lower levels of plasma-T were found in control nude mice (Table II) compared with normal mice (Table III, Fig. 2), which was also reflected by decreased weights of androgen target organs in the nude mice (Table I versus IV). The difference in responsiveness to the antagonist might at least be partly attributed to the different endocrine status of both types of mice.

In immunocompetent mice, plasma levels of LH and T were diminished at 2 h after the last treatment (Table III, Fig. 2). However, Fig. 2 demonstrates that at 24 h after the last dose another group of these animals had levels of circulating T which were not different from control levels. This important observation, which was confirmed by an additional time-course experiment in mice receiving a single dose of Org-30276 (Fig. 3), showed the limited duration of action of this compound in mice. Apparently, even at high daily dosage of 100 µg, the antagonist was not effective in depressing T-pulses for up to 24 h. Plasma-T concentration above 50 nmol/l could already be recorded at 16 h after a single dose of the antagonist.

These data concerning plasma levels of T in control animals were confirmed by observations made in a group of male nude mice of similar age of which the blood was sampled at the same time of the day (G.J. van Steenbrugge and F.H. de Jong, unpublished results). It appeared that 8 out of the 11 mice had a relatively low (baseline) range (3-10 nmol/l) of plasma-T, whereas in the remaining animals this range was considerably exceeded. Two out of these mice had extremely high concentrations (even up to 90 nmol/l) of T in their plasma.

In contrast to several other studies showing that in rats, rabitts and mice plasma FSH was less responsive to the inhibitory effects of antagonists than LH, the present study demonstrates a prolonged suppression of plasma FSH after administration of Org-30276 (Table III). At this point of time plasma LH had already returned to the control level. This is in agreement with data of in vitro studies which show that the pituitary content of FSH was significantly suppressed after long-term treatment with the LHRH-antagonist, while pituitary LH did not significantly differ from the controls (18).

Complete or partial insensitivity of mice to the antitesticular effects of LHRH-agonists was reported by Bex et al. (16) and Wang et al. (17). Using an antagonist of LHRH, Thau et al. (22) demonstrated suppression of plasma-T levels for 24 h in the rat, whereas in the rabbit, mouse and monkey a similar dose did not result in prolonged suppression. Differences among species in the rate of inactivation of the antagonist might play a role in the different sensitivity of the pituitary to the antagonist. However, the data on this subject are still not in agreement with each other and they also strongly depend on the analogue and dosage schedule used (23).

It is concluded that, in spite of promising results obtained with the LHRH-antagonist Org-30276 in the androgen-dependent Dunning rat tumor model (13) and in the estrogen-dependent MXT mammary tumor in female mice (24), the male nude mice is not a suitable model to study the "castration-like" effects of LHRH-antagonists on androgen-dependent prostate xenografts. From our previous experiments (4) and from the present data it became clear that, in order to obtain inhibition of PC-82 tumor growth, castrate levels of T (less than 0.5 nmol/l) must be reached in the host animal. In order to attain this level in the mice and thus to obtain a longer lasting suppression of plasma-T twice or even thrice daily injection of tumor-bearing animals appears to be necessary. It must be emphasized, however, that frequent injection is not tolerated by the host animal. An alternative route of administration might be the sustained-release form of the antagonist. The application of the microcapsule formulation of this kind of analogue, which became available recently, provides a more constant release of the compound and consequently better results with the antagonist in the PC-82 nude mice model might be obtained.

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ANDROGENS AND PROLIFERATIVE ACTIVITY OF HUMAN PROSTATIC TISSUE IN NUDE MICE

CHAPTER 9

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INTRODUCTION

Since the discovery of Huggins and Hodges that prostatic cancer could be made to regress by either orchiectomy or by the administration of exogenous estrogens, the treatment of advanced prostatic cancer has been dependent upon suppression of the circulating levels of androgens (1). Nonetheless, the majority of patients with advanced prostate cancer relapse and die after an initial period of remission (2). The standard forms of endocrine management do not completely eliminate plasma androgens. Some investigators suggest that a more complete form of androgen withdrawal, in which the very low levels of non-testicular androgens are counteracted (e.g. by simultaneous treatment with an anti-androgen) might be more effective than castration alone (3,4). In the view of these investigators, relapse from androgen ablation therapy must be considered as a manifestation of inadequate androgen suppression. The validity of this suggestion strongly depends on the answer to the question what minimal amount of androgen is necessary to support tumor growth. Using the hormone-dependent subline of the Dunning R3327 prostatic tumor in the rat Trachtenberg (5) tried to determine the optimal concentration of circulating testosterone (T) which should be reached in the treatment of prostatic cancer. The approach used in such studies can be designated as *in vivo* hormonal titration.

Ellis and Isaacs (6) ascertained by using the same Dunning R3327 system that plasma levels of T must be maintained below 1.7 nmol/l but do not have to be completely eliminated to produce the maximal therapeutic response. Recently, this group of investigators confirmed these findings by determining the biological significance of measurable levels of androgen remaining following castration, on cell number and rate of DNA synthesis of the ventral prostate in the rat (7). These observations support the predominant view held today that prostate cancer is composed of different clones of cells with different degrees of sensitivity to or dependency on androgens (8).

In order to gain insight into the effect of varying concentrations of circulating-T on the growth of human prostatic cancer tissue the serially transplantable PC-82 tumor of human prostatic adenocarcinoma has been used as a model. This tumor model, derived from a primary adenocarcinoma of the prostate (9), mimics many of the important properties of clinical prostate cancer (10). We have succeeded in maintaining constant levels of plasma-T in nude mice (11,12) as well as in administering pharmacological doses of estradiol to

tumor-bearing mice (13) by using Silastic implants filled with testosterone and estradiol respectively. However, the release rate of steroids from these implants appeared to be too high to obtain low (near-castrate) levels of circulating androgen. Therefore, in the present study with the PC-82 tumor various low levels of plasma-T were maintained by implantation of Silastic capsules containing different proportions of T mixed with cholesterol (14).

The use of a suitable marker, which recognizes patients who will benefit from hormonal therapy and patients who will not, might prevent the latter group from having to undergo unnecessary and often drastic forms of hormonal treatment. The lack of such a marker as well as its application for monitoring the response of prostatic cancer patients to either hormonal or any other kind of treatment provides a challenging problem for investigation.

In experimental and clinical human prostatic cancer tissues mitotic figures are occasionally seen. This precludes the use of this feature to assess the tumor cell proliferative fraction of such tissues (15). Recently, a monoclonal antibody Ki-67 was described (16) which identifies a proliferation associated nuclear antigen. Although the precise nature of this antigen is still unknown, it was demonstrated to be expressed in the G_1, S, G_2 and M-phase of all human continuously cycling cells, but to be absent in G_0 cells (17). As a marker this antigen was shown to be suitable to detect early responses of the PC-82 tumor to hormonal manipulation (18). This chapter describes the use of this proliferation marker to monitor time-dependent effects of hormonal manipulation on the PC-82 tumor. This includes preliminary results obtained by application of the Ki-67 antibody to fine-needle aspiration material derived from individual tumors. Furthermore, it was investigated whether the expression of the proliferation-associated antigen in the PC-82 tumor can be used to monitor tumor growth in addition to measuring tumor volume changes. For this reason the marker was applied to the hormonal titration experiments with the PC-82 tumor. Finally, we tried to correlate tissue androgen levels with the percentage of Ki-67 positive cells in tissue specimens of the same tumor.

MATERIALS AND METHODS

PC-82 tumor

The PC-82 tumor was maintained in Balb/c nude mice as described in chapters 5 and 6 (ref.10). The experiments described in this chapter were performed with mice bearing exponentially growing tumors of the 30-35th transplant generation.

Hormonal manipulation

Castration of tumor-bearing mice was carried out via the scrotal route under total anesthe-

sia with tribromoethanol (Aldrich, Beerse, Belgium). Testosterone (Steraloids, Pawling, NY) was administered by using subcutaneously implanted Silastic capsules, releasing the steroid at a constant rate, as described before (11, 12). To obtain low levels of circulating testosterone (T) in the nude mice Silastic implants were prepared containing different proportions of T mixed with cholesterol. For these "hormonal-titration" experiments the implants were made of Silastic tubing (Talas, Zwolle, The Netherlands) of 1.5 mm inner and 2.1 mm outer diameter. The implants had a length of 1.0 cm of which 0.6 cm tubing was effectively filled with steroid. Installation of the implants was carried out under light ether anesthesia.

Experimental protocols

The low-dose T-substitution experiments were performed in male mice which had been castrated and had received a T-implant 7 days prior to transplantation of PC-82 tumor tissue. The size of the tumors, once palpable, was measured by means of calipers. Tumor tissue was considered having a positive "take" when tumors did reach a volume of at least 100 mm^3. Short-term effects of hormonal manipulation on concentrations of androgens in the PC-82 tumor tissue were studied according to a protocol of androgen depletion and repletion. This type of experiment has been carried out in male mice and in T-implanted female mice as described in more detail in chapter 5. In an attempt to correlate these hormone levels (presented in chapter 5) with the proliferative activity of the tumor tissue, the present study focusses on proliferation monitoring by means of flow cytometric DNA-analysis and by the Ki-67 and the bromodeoxyuridine (BrdUrd) antibody methods.

Flow cytometry

Samples for flow cytometric DNA-analysis were prepared of PC-82 tumor tissue (200-300 mg) derived from tumor-bearing mice. The tissue was minced, enzymatically dispersed using a solution of 200-400 U collagenase/ml at 37°C (Worthington, Freehold, NJ) and filtered through a 40 μm nylon filter to prepare a suspension of single cells. The cell suspension was adjusted to a concentration of 4.10^6 cells/ml and stored in polypropylene tubes (NUNC, Roskilde, Denmark) in liquid nitrogen as described by Vindeløv *et al.* (19). Before having been analysed the samples were rapidly thawed in a waterbath of 37°C, processed further and stained with propidium iodine (Sigma) according to the protocol of Vindeløv *et al.* (20). Prior to the staining procedure chicken red blood cells (CRBC) were added to the tumor samples as internal standard and normal human fibroblasts were used as external standard. The samples were processed using a FACS-2 cell sorter (Becton Dickinson, Sunnyvale, Calif.) at an excitation wavelength of 514 nm between 0.5 and 3 h after staining.

Immunocytochemistry

The immunocytochemical procedures with the Ki-67 and the anti-BrdUrd antibodies were performed either on cryostat sections (5 μm thickness) or on fine-needle aspiration material. The tissue sections were made of (small) pieces of tumor frozen with isopentane in liquid nitrogen and stored at -80°C. Fine-needle aspiration biopsies were taken from tumor-bearing mice without anesthesia by using a 20-ml disposable syringe in a special holder (Cameco, 20 ml, Precision Dynamics, Burbank, CA). Smears, which had been made directly after taking the aspirate, were subsequently air-dried (1 h), fixed in acetone for 10 min, wrapped in tinfoil and stored at -20°C. After having been thawn these preparations were rehydrated and stained as described below.

The BrdUrd-antibody method was applied to cryostat sections of tumors harvested from mice which were intraperitoneally injected with 10 mg/kg BrdUrd 1 h before sacrifice of the animals. To visualize incorporated BrdUrd a murine McAb (Becton and Dickinson, The Netherlands) diluted 1 : 10 in PBS was used in a similar way. Treatment of slides prior to application of the anti-BrdUrd antibody was performed as described by Schutte *et al.* (22); enzymatic treatment of the frozen slides with pronase was omitted. The murine monoclonal antibody (McAb) Ki-67 (Dakopatts, Denmark) was applied in a dilution of 1:10 in phosphate buffered saline (PBS) containing 0.01% gelatine and 0.1% sodium-azide. A peroxidase-conjugated polyclonal rabbit anti-mouse antibody (Dakopatts, Denmark) was used as a second step reagent in the indirect conjugated peroxidase assay for detection of both the antibodies. The further procedure was similar to that described by Gallee *et al.* (18). In each staining session instead of with the primary antibody a tissue section was incubated with PBS as aspecific control. The percentage of antibody-stained nuclei in the preparates was determined at x 400 magnitude counting 500-600 cells in non-adjacent randomly distributed fields. Counting was facilitated by using a grid inserted in one of the ocular tubes of the microscope.

Miscellaneous

At the end of the hormone-titration experiment the remaining tumor-bearing mice were exsanguinated from the orbital sinus under ether anaesthesia. Plasma was obtained by centrifugation of the heparinized blood at 9000 g for 3 min and stored at -20°C until analysis. The concentration of T in the plasma of the mice was determined by radioimmunoassay by using the method and antiserum described by Verjans *et al.* (23). The significance of differences between values of different groups was calculated using two-tailed Student's T-tests or Chi-squared test (where indicated).

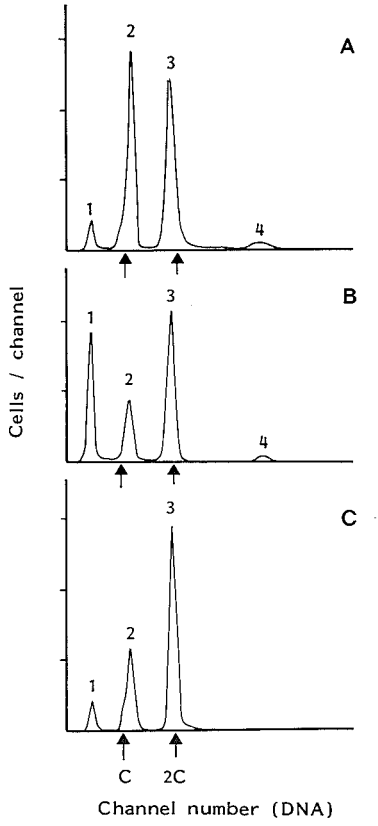

Figure 1 *Flow cytometric DNA distribution of the human PC-82 prostatic tumor grown in T-implanted female mice. The tissues were derived from a control (T0) animal (A), from mice at 2 days (B) and at 10 days (C) following androgen-withdrawal. 1. CRBC-peak; 2. mouse (diploid) cells; 3. G_0/G_1 (hypotetraploid) cells of the PC-82 tumor (DI:1.9); 4. G_2/M cells of the cells indicated under peak 3. Arrows indicate the positions of the C and 2C peaks of external diploid reference cells (normal human fibroblasts).*

RESULTS

Time-dependent effects of hormonal manipulation on tissue proliferation

The DNA histogram obtained by flow cytometric (FCM) determination of a control tumor, grown in a T-implanted female mouse, is shown in Figure 1A. Analysis of the DNA

histogram indicated that the PC-82 tumor consists of aneuploid cells with a DNA-index (DI) of approximately 2.0 as compared to normal human diploid cells. It was calculated that between 85 and 90 per cent of the cells in the PC-82 tumor are in G_0/G_1 phases. The fraction of cells in G_2/M was found to be approximately 8 per cent. The present histograms of the PC-82 did not allow accurate calculation of the fraction of cells in the S-phase. However, this fraction was estimated not to exceed 5 per cent. Androgen withdrawal, carried out by removal of the T-implant from tumor-bearing female mice, caused changes in the pattern of DNA distribution of the PC-82 tumors (Figure 1B and C), comprising an increase in the fraction of G_0/G_1 cells accompanied by a decrease in the fractions of S-phase and G_2/M cells. Ten days after androgen-withdrawal the fraction of G_2/M cells appeared to be undetectable (less than 1 per cent); the DNA histogram only showed a G_0/G_1 peak (Figure 1C).

Table I The effect of hormonal manipulation on the proliferative activity of PC-82 tumor tissue grown in testosterone (T)-substituted female mice as determined by application of the Ki-67 and BrdUrd antibodies respectively (from Gallee *et al.*; reference 18).

Animal	L/R tumor[a]	Endocrine state[b]	Ki-67 positive nuclei(%)	BrdUrd positive nuclei(%)
1	L	T^0	16.3	11.2
	R	T^0	18.6	7.4
2	L	T^{10-}	1.0	0.4
	R	T^{10-}	1.0	0.4
3	L	$T^{10-/4+}$	19.3	10.2
	R	$T^{10-/4+}$	27.6	16.0

a) sample derived from tumor at left (L) or right (R) flank of the mice respectively.
b) T^0: control tumor; (-): duration in days of T withdrawal; (+): duration in days of T resubstitution.

These findings were confirmed by application of the Ki-67 antibody to cryostat sections of specimens derived from tumors in a similar experiment. The percentage of Ki-67 positive cells, i.e. the total fraction of cells in G_1, S, G_2 and M phases of the cell cycle, decreased from around 17 per cent in control tissues to 1.0 per cent in tissue harvested at 10 days after androgen withdrawal (Table I).

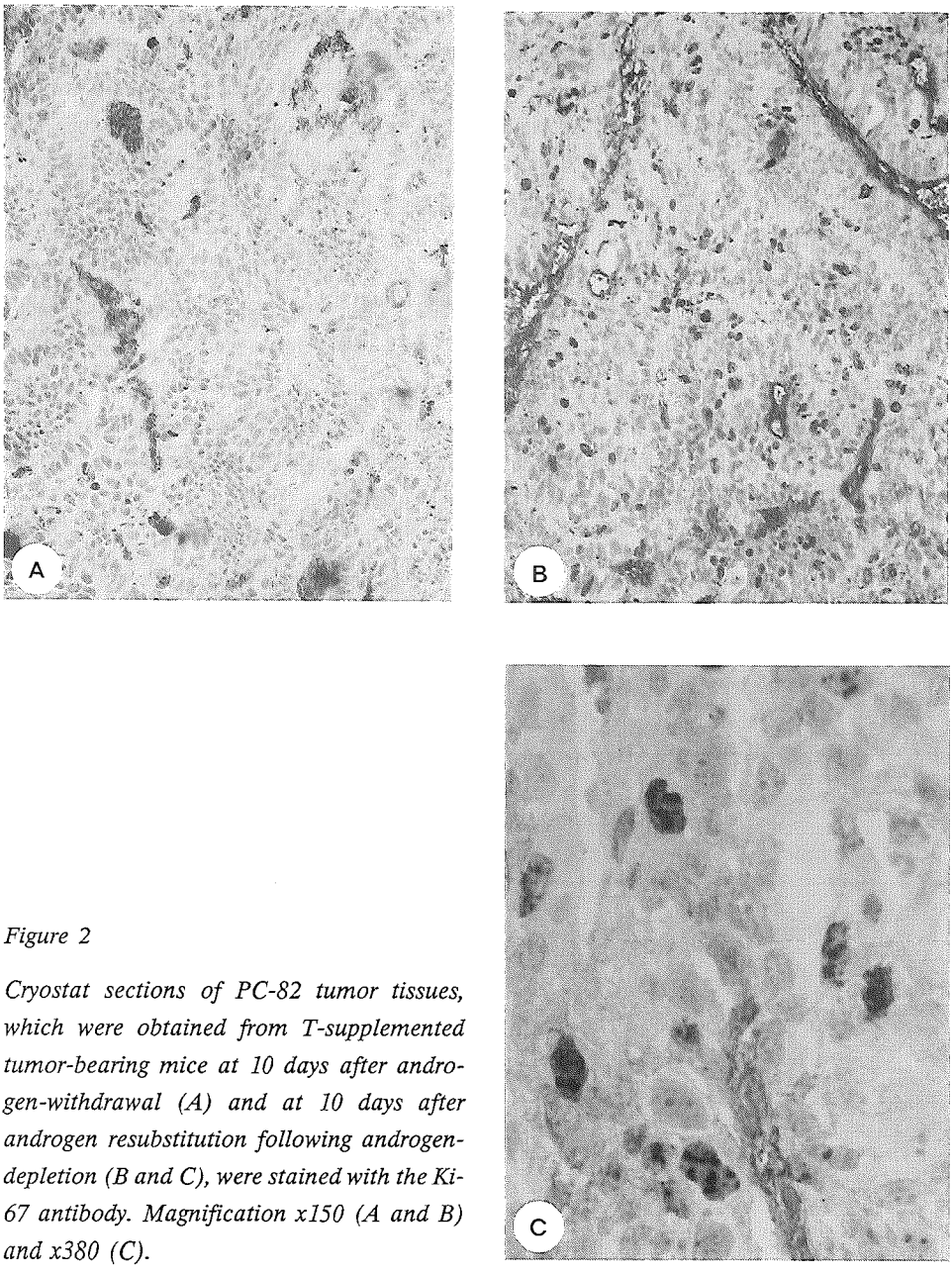

Figure 2

Cryostat sections of PC-82 tumor tissues, which were obtained from T-supplemented tumor-bearing mice at 10 days after androgen-withdrawal (A) and at 10 days after androgen resubstitution following androgen-depletion (B and C), were stained with the Ki-67 antibody. Magnification x150 (A and B) and x380 (C).

Resubstitution of T (via Silastic implantation) in tumor-bearing mice depleted of androgen resulted in a relatively rapid return to the control situation (Tabel I). Four days after resubstitution of androgen the G_2/M-fraction was detectable again in the FCM DNA histogram (result not shown) and the percentage of Ki-67 positive nuclei had increased to the control value of approximately 20 per cent (Table I). Figure 2 shows the histological appearance of Ki-67 stained tissue sections of a tumor at 10 days after androgen-withdrawal (A) and of a tumor at 10 days after androgen-supplementation following this androgen-withdrawal (B).

The pattern of response following hormonal manipulation found after application of the BrdUrd-antibody method, which is used to detect cells in the S-phase of the cell cycle, closely resembled those obtained with the Ki-67 antibody (Table I).

The rapid changes of the tumor cell proliferative fraction in the PC-82 tumor following hormonal manipulation, as detectable by the two antibody methods and by flow cytometric DNA analysis, preceded the changes of the tumor volume, which in general can only be determined by (long-term) consecutive measurements of the tumor size.

Table II The effect of hormonal manipulation on the expression of a proliferation-associated antigen in cytological specimens obtained by sequential aspiration of one individual PC-82 tumor grown in an intact male mouse*

Sample	Total number of nuclei counted	Number of Ki-67 positive nuclei(%)
T^0 (control)	511	69 (13.5)
T^{2-}	500	11 (2.2)
T^{9-}	500	0 (0)
$T^{9-/2+}$	535	15 (2.8)
$T^{9-/12+}$	510	67 (13.1)

* The animal was hormonally manipulated by castration and testosterone(T)-substitution respectively (see also the legend to table I).

The technique of performing fine-needle biopsies from tumors appeared to be applicable to the PC-82 tumor in nude mice as well. Accordingly, the short-term effects of hormonal manipulation on an individual tumor could be monitored by application of the Ki-67

antibody to cytological specimens of sequentially performed biopsies. An example of such an experiment with a tumor-bearing male mouse is shown in Table II. A similar pattern of response to castration and T-resubstitution was found as compared with experiments making use of a series of PC-82 bearing mice (compare Table I and II).

Relationship between tissue androgens and proliferative activity

Short-term effects of hormonal manipulation on levels of the androgens T and dihydrotestosterone (DHT) in the PC-82 tumor tissue were studied according to a protocol of androgen depletion and repletion. Tissue sections derived from the same tumors in which the concentrations of T and DHT had been determined (results presented in chapter 5) were stained with the Ki-67 antibody. Figure 3 illustrates that the relation between the concentration of DHT and the percentage of Ki-67 positive cells has the feature of an "S-shaped" curve. Clearly two groups of tissues can be distinguished. Below the concentration of 15 pmol/g tissue the fraction of Ki-67 positive cells did not exceed 5 per cent, whereas above this concentration at least 14 per cent of the cells expressed the proliferation antigen. When the concentration of T instead of DHT was related to the Ki-67 expression the separation of two groups of tissues with low and relatively high proliferative activity was not complete (result not shown).

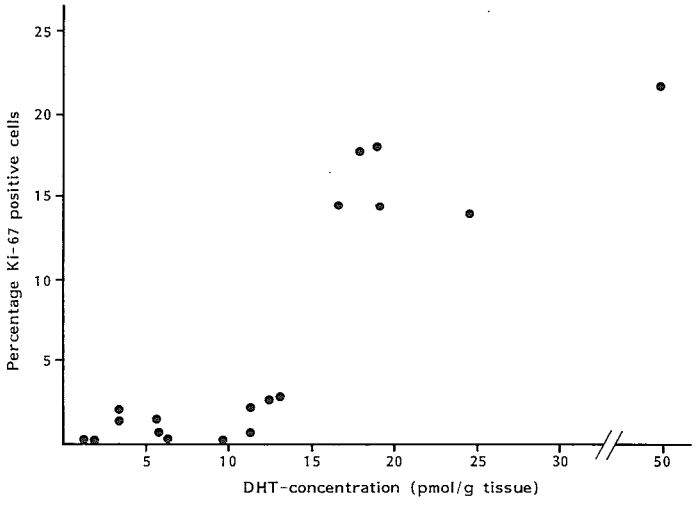

Figure 3 *The relationship between the tumor cell proliferative fraction determined by the Ki-67 antibody method and the concentration of DHT in PC-82 tissue grown in T-implanted hormonally manipulated female mice.*

Growth-stimulating effects of different levels of circulating androgen

Prior to application in tumor-transplanted castrated male mice the implants used for "hormonal-titration" experiments were tested in intact female mice. The implants with different proportions of T resulted in mean plasma-T levels of 0.2 - 19.4 nmol/l (Table III). Initially, four types of T-implants (0, 10, 25 and 100%), covering the entire range of the fore-mentioned plasma-T levels, were applied to determine their ability to stimulate the growth of the tumor. Except for the 100%-T animals the final concentrations of T in the other three groups of mice (Table IV), which were sacrificed 120 days after tumor transplantation, were not different from the levels found in female mice receiving an implant for 7 days (Table III). The take rate (i.e. the percentage of tumors reaching the volume of 100 mm^3) in mice supported with 25% T-implants was found to be similar to that of tumors in mice receiving 100% T-implants. Tumor tissue transplanted in the 10%-T group had a significantly lower take (40%) in comparison with the take (79%) in 100% T-implanted mice (79%), and finally in the control mice receiving implants with 100% cholesterol no tumor growth was observed. The mean volume reached at 90 days after transplantation of tumors in the 10 and 25% T-implanted mice was significantly smaller in comparison with tumors in the 100% T-supported animals. The difference between the tumor volume in the 10% and 25%- implanted mice was not statistically significant (Table IV).

Table III Plasma-testosterone (T) concentrations in female nude mice supplemented by different doses of T*

Proportion of T in the implant(%)	Plasma level T (nmol/l)**	
0	< 0.2	(7)
1	0.3 ± 0.08	(5)
5	1.2 ± 0.30	(5)
10	1.5 ± 0.9	(7)
25	2.2 ± 0.6	(9)
100	19.4 ± 5.9	(7)

* T was administered by Silastic implants containing different proportions of T and cholesterol. Blood was taken from the animals at 7 days after implantation

** means ± S.D.

Table IV Growth properties of PC-82 tumor tissue transplanted in castrated male mice supplemented with different doses of testosterone (T)*

Proportion of T in the implant (%)	Final T-level (nmol/l)	Tumor take (%)	Tv (mm^3)	Td (days)
0	0.7 ± 0.4 (4)	0 (0/8)	—	— (8)
10	1.8 ± 0.3 (3)	40 (4/10)[a]	127 ± 53 (4)[b]	18.1 ± 6.9 (4)
25	4.0 ± 0.7 (4)	70 (10/14)	239 ± 126 (9)[c]	15.8 ± 4.7 (7)
100	7.0 (2)	79 (11/14)	742 ± 436 (11)	19.1 ± 5.2 (8)

* Values represent the mean ± S.D. of the number of observations (in parentheses). The take (the point tumors had reached the volume of 100 mm^3) and tumor volume (Tv) of the tumors were determined at 90 days after transplantation. The tumor doubling time (Td) was calculatedfrom the exponential phase of growth.
Significantly different from 100%-T group; a) P <0.02, Chi-squared test; b) P <0.01 and c) P <0.02, Student's T-test.

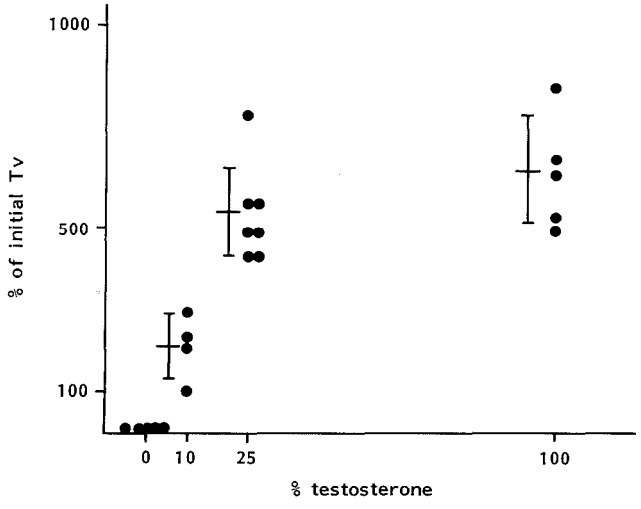

Figure 4 *Growth of PC-82 tumor tissue under influence of several plasma levels of testosterone (T). The (relatively low) circulating levels of plasma-T were achieved by the use of Silastic implants filled with different proportions of T mixed with cholesterol implanted in castrated male nude mice.*

Figure 4 also illustrates that the growth in the 10% T-implanted mice (with circulating plasma-T levels of 1.5 nmol/l) is not maximally stimulated. However, as far as the exponential phase of growth was concerned the mean doubling time (varying from 15 - 20 days) of tumors in the three groups of mice turned out to be similar.

An additional experimental set up with T-implants in the lower range (0 - 10%), resulting in T concentrations of 0.2 - 1.5 nmol/l in the circulation, had an almost completely negative result. After a period of 135 days in the group of 5% and in the group of 10% T-implanted mice only one animal bearing a small (histologically proven) PC-82 tumor (volume < 100 mm^3) was found. In these groups of mice, including the group of 10%-T, plasma T did not exceed the level of 1 nmol/l.

DISCUSSION

This chapter comprises a description of the application of methods to determine changes of proliferative activity in tissue of the transplantable human prostate tumor (PC-82) during hormonal manipulation. Furthermore, the growth properties of the PC-82 tumor tissue under conditions of low circulating levels of T were studied. By flow cytometric (FCM) DNA analysis of control tumor tissue (see Figure 1A) it was demonstrated that a fraction of approximately 90 per cent of the PC-82 tumor consists of G_0/G_1 cells. The fractions of S-phase and G_2/M cells did not exceed 5 and 10 per cent respectively. Application of the Ki-67 monoclonal antibody and the BrdUrd labeling technique to cryostat sections of PC-82 tumors yielded additional information about the cell kinetic properties of this tumor. Preliminary results obtained by staining of tumor tissue with anti-BrdUrd after *in vivo* BrdUrd labeling showed that control (androgen-supported) tumors contained 7 - 16 per cent cells in S-phase (Table I). The fact that FCM-analysis and the BrdUrd technique were not applied to the same PC-82 tumor specimen as well as the generally inaccurate calculation of S-phase from FCM histograms possibly contribute to the dissimilarity of the data obtained by both techniques.

With respect to the number of dividing cells well-differentiated human (prostatic) adenocarcinoma contains only a minimal number of mitotic figures (15). In addition, compared to other malignant neoplasms the low thymidine labeling indices of prostatic carcinoma (PC) indicate that a large proportion of cells present are either quiescent with respect to cell cycle or are progressing through it very slowly with long intermitotic intervals (24). From the present findings with the PC-82 tumor it can be inferred that also in this experimental model the main fraction of tumor cells is in a resting (G_0) stage. In particular, by the application of the Ki-67 antibody method to the PC-82 tumor it was demonstrated that maximally 30 per cent of the cells expressed the human nuclear proliferation antigen

which is recognized by Ki-67. It was shown by Gerdes *et al.* (17) that this antigen is expressed in G_1, S, G_2 and M-phase of all human continuously cycling cells but is absent in G_0 cells. Therefore, the Ki-67 positive cells in PC-82 tissues are to be considered as the fraction of cycling cells of this tumor. In spite of its relatively low expression in control tumors, grown in T-supported animals, this antibody might also be suitable to detect changes in proliferative activity in the PC-82 tumor following hormonal-manipulation. It was shown that androgen-withdrawal from T-implanted tumor-bearing female mice did indeed result in a decline of the percentage of Ki-67 positive cells in the tumor to a value of about one per cent at 10 days after androgen-depletion (Table I). Resubstitution of T (via T-implants) in androgen-depleted tumor-bearing mice resulted in a rapid recovery of proliferation and control values of 20-30 per cent Ki-67 positive cells were reached within four days after T-implantation (Table I). A similar pattern of response following hormonal manipulation was found when the BrdUrd labeling technique was applied in parallel with the Ki-67 method (Table I).

Recently, the comparable effects of hormonal manipulation on proliferative activity were reported by others performing similar experiments with the use of the rat ventral prostate (25) and the Dunning rat prostate adenocarcinoma (26). In the hormone-dependent R3327-H tumor it was shown that after a post-castration period of 12 days androgen-repletion resulted in a rapid increase (from 2 - 10 per cent) of cells in S-phase. A peak of reactivity exceeding the pre-castration value was reached at 72 h after androgen administration, whereafter a decline to control levels was observed.

In addition, the feasibility of sequential fine-needle aspiration biopsies taken from individual PC-82 tumors potentiates the applicability of the Ki-67 technique in the clinical situation. This possibility should be further investigated since changes in proliferative activity during hormonal manipulation in the PC-82 tumor could indeed be monitored by consecutive biopsies (Table II).

As the BrdUrd labeling technique requires previous administration of BrdUrd *in situ*, its application will be restricted to experimental model systems. By contrast, the Ki-67 antibody method directly detects a proliferation-associated antigen in human cycling cells. Moreover, contrary to the technique of FCM, which is applied to a suspension of tumor cell nuclei, the Ki-67 antibody method can be applied to histological or cytological specimens, in this way permitting discrimination between neoplastic and non-neoplastic cells. Thus, this antibody method can be easily incorporated into the routine protocol of cytological diagnoses of prostatic cancer patients by transrectal aspiration biopsies (27, 28). The Ki-67 antibody had already been successfully applied to clinical human breast cancer. In a recent study of Gerdes *et al.* (29), a significant correlation was found between the histological grade and the tumor growth fraction, as determined by Ki-67, in mammary carcinomas. The outcome of preliminary experiments dealing with application of the Ki-67 antibody to the

PC-82 prostatic tumor in nude mice, as described in this chapter as well as by Gallee *et al.* (18), proved that the Ki-67 marker provides a reliable method to estimate the proliferative fraction of hormone-responsive prostate cancer.

By relating the expression Ki-67 and the concentration of androgen (DHT) in the same tissue specimens which were obtained from tumor-bearing mice during hormonal manipulation a critical threshold (approximately 15 pmol/g) of DHT in the tissue was found (Figure 3). Above this concentration at least 14 per cent of the cells expressed the proliferation antigen. Remarkably, 2 out of the 12 tissues with a low DHT concentration and a relatively low proliferative fraction contained high levels of T. On the basis of this observation it may be tempting to speculate that the presence of a certain amount of DHT, produced either by local or peripheral conversion of T, is obligatory for stimulating the growth of the PC-82 prostate cancer tissue. To confirm these preliminary findings more investigations, including substitution experiments with low androgen concentrations, must be carried out.

By using Silastic implants, containing different amounts of T mixed with cholesterol, it turned out to be possible to create circulating-T levels in castrated male mice varying from above the physiological level (20 nmol/l) down to near-castrate levels (lower than 1 nmol/l; Table III). The first series of so-called hormonal titration experiments, covering the entire range of 0 - 100 % T-implants, was carried out in PC-82 transplanted castrated male mice. It was found that a plasma-T level between 1 and 2 nmol/l (obtained by 10% T-implants) is the threshold below which growth of the PC-82 tumor is no longer stimulated (Table IV). In spite of the results depicted in Figure 4 no differences were found with respect to the calculated tumor doubling time (Td) of tumors in the different groups of mice (Table IV). However, the Td was calculated from the exponential phase of growth of tumors (between 50 and 500 mm^3) only when a significant correlation between the measuring points was found. It may be possible that in some cases the increase of the tumor volume (Figure 4) was calculated at a point of time the tumor had passed the exponential phase of growth. This possibly explains the discrepancy between the different data on tumor growth kinetics. Differences in the growth stimulatory property of the 10 per cent implant, as found in the consecutive experiments, might indicate small differences in the degree of androgen-sensitivity of the PC-82 tumor. Earlier observations in estradiol treated tumor-bearing mice had also shown that plasma-T concentrations must be decreased to levels around 1.5 nmol/l to cause tumor growth arrest (results described in chapter 7).

The present results with the PC-82 human prostate model agreed fairly well with recent observations made with the Dunning R3327 prostate tumor in the rat. By using hormone-dependent sublines of this model, and also by application of the technique of Silastic implantation, it was shown that plasma-T levels must be kept below 1.7 nmol/l but do not have to be suppressed to levels lower than 1 nmol/l in order to obtain a maximal suppressive

effect on the growth of the tumor (5,6). In addition, it was found by Kyprianou and Isaacs that depletion of DHT levels in rat ventral prostate to approximately 15% of intact values (i.e. from 2.9 to 0.4 ng/10^8 cells) by means of surgical castration maximally suppressed prostatic cell proliferation and maximally activated prostatic cell death (7). All in all, the observations in the normal and malignant rat prostate as well as those in the human PC-82 tumor support the conclusion that relatively low androgen levels remaining after castration-induced androgen deprivation do not have any biological significance with regard to prostatic growth. Thus, from the studies of experimental prostatic tumors no evidence has been obtained sofar that the approach of total androgen blockade, indicating simultaneous testicular and adrenal suppression (3), is preferable to the concept of partial androgen-withdrawal which e.g. can be achieved by castration alone (6, 7, 30).

The continuing experiments with low-dose androgen substitution in the PC-82 and other hormone-dependent human tumors in nude mice will include concomitant measurements of endogenous androgen levels and the proliferative fraction of the tissues. Such studies will yield valuable information on the relationship between androgens and growth of prostatic cancer tissue.

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CHAPTER 10

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

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The answer to many fundamental questions with respect to the nature and treatment of prostatic carcinoma can be obtained only by the use of appropriate animal models and *in vitro* systems. Much knowledge of prostatic cancer has been gathered from the studies of the two well-known rat prostate tumor models, the Dunning R3327 and the Noble prostate tumor systems [4]. Besides the applicability of these rat tumors, the nude mice xenograft model offers the possibility of studying human prostatic carcinoma under *in vivo* circumstances i.e. in laboratory animals [16]. The number of tumors which are permanently transplantable in nude mice is limited, however (chapter 1, section 5.2). The PC-82 tumor model, the subject of this thesis, was extensively characterized and a substantial number of other aspects of this tumor were investigated further. In each of the preceding chapters the results of the various investigations into the PC-82 tumor have been discussed. Therefore, in conclusion, the applicability and future perspectives of this model will be reviewed briefly in this final chapter.

The significance of the PC-82 tumor xenograft in nude mice as a model for the study of human prostatic cancer is determined by its characteristics and by its biological behavior i.e. the pattern of growth and the function of the tumor tissue maintained in the host animal. The characteristics of the PC-82 tumor (cf. chapter 1, section 6.3) meet most of the requirements for an ideal animal model of prostatic carcinoma (cf. 1.4). Especially its histological features, its relatively slow growth rate, its androgen-dependence and the presence of androgen receptors make this tumor well comparable with prostatic cancer in the clinical situation. However, a number of differences between the human xenograft system and the tumor in the patient on the one hand, and between mouse and man on the other hand, limit the applicability of the nude mice model. In particular, the endocrinological distinctions between mice and men must be recognized, because one of the major properties of the PC-82 tumor concerns its hormone-dependence.

The following limitations of the prostatic tumor xenograft model in general, and the PC-82 tumor in particular, can be distinguished:

(1) Differences of growth rate between the xenograft and the tumor in the patient.
(2) The PC-82 tumor does not contain the stromal elements which are usually present in prostatic tissues.

(3) Absence of the 'relapse phenomenon', i.e. no occurrence of androgen-independent tumor growth.
(4) The subcutaneously growing PC-82 tumor does not metastasize.
(5) Endocrine differences between the (nude) mice and the human situation.

These points are discussed in more detail below:

1) Although the PC-82 tumor has a relatively slow growth rate, the tumor doubling time of this tumor, which varies from 10-20 days, is still considerably shorter than the estimated doubling time of tumors in the clinical situation.
2) With respect to permanent growth, it can be remarked that the successful transplantation of human tumor tissues in athymic nude mice is limited to tumor cells [15]. Thus, the non-malignant stromal elements cannot be maintained in the host animal, in contrast to the carcinomatous epithelial tissue in the original PC-82 transplant. Instead of this, connective tissue of murine origin supports the growth of the carcinoma tissue [11].
3) After hormonal manipulation, e.g. long-term androgen ablation therapy, of the PC-82 tumor androgen-independent tumor growth was not observed (chapter 5). Apparently, the PC-82 tumor consists of a homogeneous population of hormone-responsive cells and so far, this tumor has not shown the clinically well-known relapse phenomenon. In this respect, the behavior of the PC-82 tumor differs from that of the Dunning (R3327) prostate tumors in the rat [10]. The pre-existing androgen-independent cell population in the R3327-H tumor continues to proliferate after androgen withdrawal which leads to a relapse of the tumor to an androgen-unresponsive state (cf. 1.3.3 and 1.4.2). Evidence has been provided that the prostate contains a population of immortalized cells. These so-called stem cells are supposed to be protected from the growth inhibiting effects of androgen withdrawal and may be considered androgen-independent. If one presumes that the PC-82 tumor also contains a limited number of stem cells, then it can be expected that after androgen-withdrawal this cell population eventually will cause regrowth of the tumor. As a consequence, the post-castration periods of the studies with the PC-82 tumor performed so far (chapters 3 & 5) have possibly been too short to detect any androgen-independent regrowth. Therefore, future androgen ablation experiments with this tumor should focus on this important aspect of prostatic tumor cell biology.
4) Human prostatic tumor xenografts metastasize very infrequently [12], whereas in patients with advanced prostatic carcinoma metastatic lesions can often be detected [13]. Likewise, in none of the subsequent transplant generations of the subcutaneously growing PC-82 tumor metastases were observed. The limited ability of the tumor to

invade the adjacent tissues and the substantial activity of Natural-Killer (NK) cells, which is generally found in nude mice [9], could possibly contribute to the low metastatic capacity of the majority of human tumor xenografts in nude mice.

5) The baseline level of plasma-T in male athymic nude mice was shown to be reduced in comparison with their heterozygous littermates [3]. Nevertheless, in the present study of the PC-82 tumor extremely high peak plasma levels of T also occurred in the nude mice (chapter 5). It was concluded that in mice T is released in a pulsatile fashion, as is the case in rats [1]. We succeeded in maintaining constant levels of plasma-T in our studies of nude mice (chapter 2) as well as in administering pharmacological doses of estradiol (E_2) to PC-82 tumor-bearing mice (chapter 7) by using Silastic implants filled with T or E_2 respectively. The resulting peripheral levels of T (20-25 nmol/l), which were found in the plasma of the mice receiving a standard (1.0 cm) T-implant, are comparable to those found in patients with prostatic carcinoma [5]. However, in contrast to man, the plasma in the mouse does not contain detectable amounts of sex hormone-binding globulin (SHBG). Consequently, in the mouse the fraction of free steroid in the plasma is higher and the accessibility of androgens to the tumor tissue is likely to be substantially better compared to the human situation.
   Human adrenals have a relatively high secretion rate of steroids which can be converted to potent androgens and estrogens in peripheral tissues. It has been reported that the adrenals of the mouse also convert pregnenolone into C-19 steroids, such as dehydroepiandrosterone (DHEA) and androstenedione (A-4) [6], but the concentration of C-19 steroids from adrenal origin in mouse plasma is far less important than in the human [2]. It has been suggested that in castrated men androgens from the adrenal gland may stimulate prostatic cancer growth (cf. 1.3.4). However, the present studies of the PC-82 tumor model in nude mice have shown that it is obvious that the very low concentrations of adrenal androgens, which were shown to remain in circulation after castration of mice [2], do not have the potency to stimulate the growth of the PC-82 prostatic carcinoma tissue (chapter 5 & 9).

The limitations of the xenograft nude mouse model, which are discussed in the preceding paragraph, and the endocrine differences between mouse and man, must be taken into consideration when comparing the behavior of the PC-82 tumor with that of clinical prostatic carcinoma under endocrine ablation therapy. The nude mouse system, however, allows the study of the effects of endocrine therapy in a physiological environment with controlled drug pharmacokinetics and metabolism. Thus, in spite of the fore-mentioned endocrine differences between mouse and men (cf. also chapter 8), the circumstances in such a model system may be more closely related to the clinical situation than those which are achieved in *in vitro* experimental systems. Moreover, the stabile phenotype of the PC-82

carcinoma itself and the possibility of studying human prostatic carcinoma in laboratory animals strengthen the suitability of this model.

In summary, the the following facts underline the applicability of PC-82 as a model for human prostatic carcinoma:

(1) The PC-82 cell line enables the study of *human* prostatic carcinoma in an animal model.
(2) Through its permanent character the xenografted PC-82 tumor forms an abundant source of tumor tissue outside the human body.
(3) The main characteristics of the PC-82 tumor: its androgen-dependence, the histological pattern, the secretion of prostate-specific acid phosphatase and prostatic antigen and the presence of androgen receptors, are well comparable with clinical prostate cancer.
(4) All studied properties of this tumor appeared to have been conserved fairly well during its 10-year maintainance in its host animal.
(5) Likewise, in contrast to many *in vitro* cell lines, the karyotype of the PC-82 tumor remained essentially unchanged at the passages studied [11].
(6) The investigations of a nearly homogeneous and constant population of hormone-responsive cells of the PC-82 tumor allow conclusions on this particular counterpart of prostatic carcinoma in the patient.

The most important aim of the studies of the PC-82 tumor model was to investigate the role androgens play in the growth of this tumor. The outcome of these studies was discussed extensively in chapters 3, 5, 6, 7 and finally, chapter 9, in which conclusions were drawn with respect to the tumor cell kinetics and the effects of hormonal manipulation on tumor cell proliferation. The main findings with respect to androgen action in the PC-82 tumor are presented in the summary.

The preliminary results obtained with low-dose T-substitution of PC-82 tumor-bearing mice confirmed that a plasma-T level between 1 and 2 nmol/l is the threshold below which growth of the PC-82 is no longer stimulated (chapters 5 & 9). The continuing experiments with low-dose androgen substitution in the PC-82 and other hormone-dependent human tumors in nude mice will include concomitant measurements of tissue androgen levels and the proliferative fraction of the tissues. Such studies will yield valuable information on the interrelationships between peripheral and tissue androgen levels and growth of prostatic cancer tissue. Further attention will be paid to the promising results obtained by applying the Ki-67 antibody to the PC-82 model [chapter 9; ref. 7]. The application of this antibody to the routine cytological diagnosis of prostate cancer patients will elicit whether it is of

potential value as a proliferation marker in hormone-responsive carcinomas.

Recently, investigations into exciting new areas of fundamental tumor biology, involving oncogenes and growth factors, were started using the PC-82 and other prostatic tumor models [14,17]. The recognition that autocrine polypeptides can play an additional regulatory role in the hormonal control of cancer cell proliferation [8], stimulates the study of the involvement of growth factors in the growth of the PC-82 and other *in vivo* cell lines.

Finally, it must be kept in mind that the present findings with the PC-82 tumor model only represent the situation of one single (human) prostatic carcinoma. Therefore, the answer to many questions concerning fundamental problems of prostatic tumor cell biology and to issues related to the clinic of prostate cancer should come from comparative studies using a panel of androgen-dependent and -independent models *in vivo*. It can finally be stated that the outcome of the studies of experiments with the PC-82 tumor, presented in this thesis, has been the incentive for a comprehensive study of a number of different prostate tumor model systems (cf. chapter 1, section 5.2) available at the Urological Department in Rotterdam.

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SUMMARY

This thesis describes the characterization and further application of a model system for prostate cancer, the human prostatic adenocarcinoma PC-82 which is transplantable in athymic nude mice.

The mortality rate of patients suffering from prostatic carcinoma is high, in spite of the high response rates which are initially achieved with hormonal treatment of these patients. Growth and function of the prostate are primarily dependent on androgenic stimuli. Hormonal treatment of prostatic carcinoma is based upon the suppression of the testicular production of androgens. This can be achieved directly by surgical removal of the testes or indirectly by inhibiting the hypophyseal gonadotropin release through treatment with estrogens or analogues of luteinizing hormone-releasing hormone (LHRH). However, in the majority of patients relapse of the tumor occurs following a favorable response to androgen-ablation therapy. Progression is caused mainly by a loss of androgen-sensitivity of the tumor.

Since many investigations relevant to prostate cancer cannot be performed in patients, there is a great need for well-characterized model systems which reflect the properties of clinical prostate cancer. Relevant aspects of the prostate in general and of prostatic carcinoma in particular are described in *chapter 1*, which also contains a summary of the current knowledge of androgen action in the prostate and information on the available model systems for prostatic cancer.

Chapter 2 describes the manner in which hormones were administered to the nude mice of our studies. It was demonstrated that steroid-containing Silastic implants are appropriate for the purpose of testosterone-(T) substitution in tumor-bearing nude mice. This method also provides a means of administering pharmacological dosages of estradiol (E_2) to these animals.

Chapter 3 contains the results which were initially obtained by application of steroids in Silastic implants to the PC-82 tumor model. The constant levels of circulating-T appeared to fasten the growth rate of the tumor tissue. The absence of tumor growth in female and castrated male mice as well as the inhibition of tumor growth achieved after castration of tumor-bearing mice demonstrated the androgen-dependence of this tumor. The castration-induced regression of the tumor resulted in an 80 per cent reduction of the tumor load during a period of 10 weeks, as described in *chapter 5*. Experiments with T-substitution after long-term androgen-withdrawal in tumor-bearing mice and with delayed androgen substitution in PC-82 grafted castrated male mice demonstrated that under circumstances of

androgen deprivation part of the cells kept the potency to proliferate when restimulated (*chapters 3 & 5*). In none of the many castration experiments with the PC-82 tumor performed so far could any spontaneous, i.e. androgen-independent, regrowth be observed.

The concentration of prostate-specific acid phosphatase (PAP) is often elevated in the serum of patients with prostatic carcinoma. *Chapter 4* comprises the results of PAP measurements, carried out by a radioimmunological method, in tumor-bearing mice. Immunoreactive PAP was detectable in PC-82 tumor tissue and in the serum of tumor-bearing mice. Short-term hormonal manipulation of tumor-bearing mice did not affect the concentration of PAP in the tumor tissue. The decline of PAP after long-term androgen-withdrawal of tumor-bearing mice parallels the reduction of the tumor volume. Both these observations did not support the hypothesis that the production of PAP in prostatic tumor tissue is controlled by androgens, but they are in agreement with the concept that the level op PAP in the plasma is related to the total tumor load.

The principal aim of our studies of the PC-82 tumor was to investigate the role androgens and their specific receptors play in the growth of this tumor. The outcome of these studies is extensively discussed in *chapters 5, 6, and 9*. In the last-mentioned chapter conclusions are drawn with respect to the tumor cell kinetics and the effects of hormonal manipulation on tumor cell proliferation. In combination with chapters *5 & 6, chapter 7* evaluates the results obtained with estrogen treatment of the PC-82 tumor.

The following conclusions with respect to the androgen-regulated growth of the tumor were drawn:

* Evidence was provided that the growth of the PC-82 tumor is determined mainly by the continuous presence of androgen above the castrate level rather than by the total concentration of androgen receptors (AR) in the tumor tissue (*chapters 5 & 6*). The AR is not involved as a critical factor in the mechanism of androgen action in this prostatic tumor tissue. The availability of a sufficient and steady level of T in the plasma and, consequently, the duration of presence of AR in the nuclei of the tumor tissue must be considered the limiting factors in the modulation of hormonal responses in this androgen target tissue.

* No direct relationship between the concentration of T and that of DHT in the tumor tissue was found. As no significant activity of 5α-reductase could be demonstrated in the tumor tissue, it is assumed that the concentration of DHT in the tumors originates from the relatively low levels of DHT in the plasma of the mice and thus that DHT stems from peripheral sources (*chapter 5*). It should be stressed, however, that involvement of T in the growth of the tumor cannot be excluded.

* The absence of PC-82 tumor growth in female mice and the results obtained with a sub-maximal suppression of T in estradiol-treated mice showed that plasma-T must be decreased to levels around 1.5 nmol/l to cause growth arrest (*chapters 3 & 5*). The preliminary results obtained with low-dose T-substitution of tumor-bearing mice, which are described in *chapter 9*, confirmed that a plasma-T level between 1 and 2 nmol/l is the threshold below which growth of the PC-82 tumor is not stimulated.

* In the PC-82 model estrogens mainly act indirectly by their suppressive effect on the secretion of T rather than directly. Specific estrogen-receptors were not detectable in the tumor tissue (*chapter 6*). Nevertheless, pharmacological doses of estrogens possibly counteract the action of androgens in T-substituted tumor-bearing mice (*chapter 7*). The finding that such estrogen dosages concomitantly increase the concentration of AR in this tumor is of interest and needs further investigation.

Chapter 8 deals with the administration of a potent LHRH-antagonist to intact normal immunocompetent male mice and to nude mice which were transplanted with the PC-82 tumor. It was demonstrated that a daily high dose of this antagonist cannot effectively suppress plasma-T in male mice. Therefore, it was inferred that the mouse may not be a suitable model to investigate the "castration-like" effect of LHRH-antagonist on androgen-dependent prostate tumor xenografts.

Finally, in *chapter 9* the monoclonal antibody, Ki-67, was shown to be a very suitable marker to monitor short-term effects of hormonal treatment of the PC-82 tissue. It appeared to be feasible taking sequential fine-needle aspiration biopsies from individual tumors. The preliminary results obtained when applying the Ki-67 antibody to such biopsy specimens potentiate the applicability of the Ki-67 marker in the clinical situation.

All in all, it is concluded from the results presented in this thesis that the transplantable PC-82 tumor is a suitable model for the study of human prostatic carcinoma. The outcome of the continuing studies with the PC-82 tumor, and with other models developed during the last decade, will contribute to our knowledge of hormone-dependent prostate cancer.

SAMENVATTING

Dit proefschrift beschrijft de karakterisering en de toepassing als model van de prostaatcarcinoomlijn PC-82. Deze tumor, welke afkomstig is van een menselijke prostaattumor, groeit in de immuundeficiënte, naakte muis.

Het sterftecijfer van patiënten die lijden aan prostaatkanker is hoog, ondanks de aanvankelijk goede respons op hormonale behandeling, welke bij de meeste van deze patiënten kan worden waargenomen. Groei en functie van de prostaat is hoofdzakelijk afhankelijk van het mannelijk geslachtshormoon, testosteron. Dit steroïdhormoon wordt voor het overgrote deel geproduceerd door de testes. De hormonale behandeling van het klinisch prostaatcarcinoom is dan ook gebaseerd op de onderdrukking van de androgeenproduktie. De meest eenvoudige doch ingrijpende wijze waarop androgenen kunnen worden uitgeschakeld, is castratie, de chirurgische verwijdering van de testikels. Aangezien de produktie van testosteron in de testis wordt gereguleerd door het luteiniserend hormoon (LH), kan uitschakeling van de testosteronproduktie eveneens plaats vinden door middel van remming van de secretie van LH door de hypofyse. Deze indirekte wijze van testiculaire androgeensuppressie kan worden bereikt door behandeling met oestrogenen, of met analoga van het "LH-releasing" hormoon (LHRH). De goede respons op de hormonale behandeling wordt (na kortere of langere tijd) bij de meeste patiënten gevolgd door een hergroei van hun prostaattumor. Dit proces, tumorprogressie wordt hoofdzakelijk veroorzaakt door het verlies van hormonale gevoeligheid van de tumor.

Aangezien een groot gedeelte van het onderzoek op het gebied van prostaatkanker niet bij patiënten kan worden uitgevoerd, is de ontwikkeling van goed gekarakteriseerde modelsystemen noodzakelijk. Een dergelijk model is slechts dan goed toepasbaar indien de eigenschappen ervan die van het klinisch prostaatcarcinoom goed weerspiegelen. Een aantal relevante aspecten van de prostaat in het algemeen, en van het prostaatcarcinoom in het bijzonder, worden beschreven in *hoofdstuk 1.* Dit hoofdstuk omvat eveneens een samenvatting van de beschikbare kennis omtrent de wijze waarop androgenen hun werking in de prostaat uitoefenen, alsmede een overzicht van de modelsystemen die thans ter beschikking staan.

Hoofdstuk 2 beschrijft de wijze waarop in onze studies de hormonen werden toegediend aan de muizen. De toepasbaarheid van onderhuids geïmplanteerde, met steroidhormoon gevulde, siliconenslang voor de konstante toediening van testosteron (T) aan tumordragende muizen, werd aangetoond. Door gebruik te maken van deze implantaten werden

meer constante concentraties testosteron in het plasma van de muizen verkregen in vergelijking met die in intakte muizen. Deze methode bleek tevens geschikt voor de toediening van hoge, farmacologische doseringen van oestradiol (E_2) aan de dieren.

In *hoofdstuk 3* worden de eerste resultaten beschreven die werden verkregen bij de toepassing van de steroid-bevattende implantaten in het PC-82 model. Deze wijze van toediening, die het frequent injecteren van de muizen overbodig maakte, bleek de groei-eigenschappen van de tumor te verbeteren.

De androgeenafhankelijkheid van de PC-82 tumor blijkt uit de observaties dat noch in vrouwelijke noch in gecastreerde mannelijke muizen groei optreedt en dat castratie van reeds tumordragende muizen leidt tot onderdrukking van tumorgroei. De op castratie volgende regressie van de tumor resulteerde in een afname van de hoeveelheid tumor met ongeveer 80 procent gedurende een periode van ongeveer 10 weken, zoals beschreven in *hoofdstuk 5*. Na langere perioden van androgeendeprivatie bij tumordragende muizen, bleek dat een deel van de tumorcellen hun vermogen tot proliferatie had behouden. Dit werd onder meer aangetoond door transplantatie van tumorweefsel in gecastreerde mannelijke muizen die pas in latere instantie een implantaat met T kregen. In geen van de vele castratie experimenten met tumor-dragende muizen die tot nu toe werden uitgevoerd kon enige spontane, androgeen-onafhankelijke, groei worden waargenomen.

In het serum van patiënten met een prostaatcarcinoom wordt vaak een verhoogde concentratie van het prostaatspecifieke enzym, zure fosfatase (PAP) gevonden. *Hoofdstuk 4* omvat de resultaten verkregen door na het, langs radioimmunologische weg, meten van PAP in muizen met groeiende PC-82 tumoren. Het PAP was zowel aantoonbaar in PC-82 tumorweefsel als in serum van tumordragende dieren. Er werd aangetoond dat hormonale manipulatie, zoals castratie, van tumordragende muizen geen invloed heeft op de concentratie van PAP in het tumorweefsel. Aangezien na androgeen onttrekking, de afname van PAP in het serum van tumordragende muizen evenredig was aan de afname van het tumorvolume, werd geconcludeerd dat het niveau van PAP in het serum van een muis gerelateerd is aan de totale hoeveelheid tumor in dat dier.

Het hoofddoel van onze studies met de PC-82 tumor, was de bestudering van de rol welke androgenen, en hun specifieke receptoren, spelen bij de groei van deze tumor. In de *hoofdstukken 5, 6, en 9* worden de uitkomsten van deze studies uitgebreid geëvalueerd. In *hoofdstuk 9* worden conclusies getrokken ten aanzien van tumorcelkinetiek en effecten op de tumorcel proliferatie, die werden waargenomen na hormonale behandeling van tumordragende muizen. In relatie tot de *hoofdstukken 5 & 6*, worden in *hoofdstuk 7* de resultaten geëvalueerd verkregen na oestrogeen behandeling van de PC-82 tumor.

Ten aanzien van de door androgenen gereguleerde groei van de PC-82 tumor zijn de volgende conclusies getrokken:

* Duidelijk is aangetoond dat niet de totale concentratie van androgeen receptoren (AR), maar de permanente aanwezigheid in het tumorweefsel van een concentratie androgenen boven het castratieniveau bepalend is voor de groei van de PC-82 tumor. De AR zelf is geen kritische factor in het mechanisme van androgeenwerking in dit prostaatweefsel. De beschikbaarheid van een voldoende hoog en constant niveau van T in het plasma en, als gevolg daarvan, de duur van de aanwezigheid van de AR in de celkern van het tumorweefsel, moeten worden beschouwd als bepalende factoren in de regulering van de hormonale respons in dit doelwitweefsel voor androgenen.

* Er is geen directe relatie tussen de concentraties van T en die van 5α-dihydrotestosteron in het tumorweefsel. Aangezien er geen duidelijke activiteit van 5α-reductase in het tumorweefsel kon worden aangetoond, wordt aangenomen dat het in het weefsel aanwezige DHT afkomstig is van de relatief lage concentratie DHT in het plasma van de muizen (*hoofdstuk 5*). De betrokkenheid van T in de groei van de tumor kan echter geenszins worden uitgesloten.

* De observatie dat er geen tumorgroei in vrouwelijke muizen plaatsvindt en de resultaten verkregen met sub-maximale onderdrukking van T in oestradiol behandelde muizen, tonen aan dat voor het compleet stoppen van de tumorgroei de concentratie van plasma-T moet worden verlaagd tot 1,5 nmol/l (*hoofdstuk 3 & 5*). De preliminaire resultaten verkregen door middel van experimenten met lage-doses T-substitutie in getransplanteerde muizen, zoals beschreven in *hoofdstuk 9*, bevestigen dat de drempelwaarde van plasma-T, waaronder de groei van de PC-82 tumor niet meer wordt gestimuleerd, tussen de 1 en de 2 nmol/l ligt.

* In het PC-82 model oefenen oestrogenen hun invloed op de tumor uitsluitend uit via hun onderdrukkende werking op de androgeen produktie van de muis. De aanwezigheid van specifieke receptoren voor oestrogenen kon niet in het tumorweefsel worden aangetoond (*hoofdstuk 6*). Toch zijn er aanwijzingen gevonden dat farmacologische doseringen van oestrogenen de werking van androgenen in het tumor weefsel in T-gesubstitueerde muizen remmen (*hoofdstuk 7*). De bevinding dat dergelijke hoge doseringen tegelijkertijd de concentratie van de AR in deze tumor verhogen, lijkt van belang en vraagt derhalve om verder onderzoek.

In *hoofdstuk 8* worden de resultaten behandeld van experimenten waarbij een krachtig werkende antagonist van LHRH werd toegediend aan intakte, normaal immuuncompe-

tente, mannelijke muizen en aan naakte muizen die met de PC-82 tumor werden getransplanteerd. Deze experimenten tonen aan dat een dagelijks toegediende, hoge dosis van deze antagonist de concentratie van T in het plasma van de muizen niet effectief onderdrukt. Uit deze resultaten wordt daarom de conclusie getrokken dat het muizenmodel niet geschikt is voor onderzoek naar de op castratie gelijkende effecten van LHRH antagonisten op androgeenafhankelijke prostaattumor transplantaten.

Tenslotte wordt in *hoofdstuk 9* beschreven dat een peroxidasekleuring met behulp van het monoclonale antilichaam Ki-67 een geschikte methode blijkt te zijn voor het vervolgen van (korte termijn) effecten van hormonale behandeling op PC-82 weefsel. Daarbij bleek het mogelijk om via de dunne naald aspiratie, individuele PC-82 tumoren te biopteren. De resultaten verkregen bij toepassing van het Ki-67 antilichaam op deze biopsiën, brengen de mogelijke klinische toepassing van deze methode dichterbij.

Concluderend kan worden gesteld dat de PC-82 prostaattumor, zoals beschreven in dit proefschrift, de eigenschappen van het klinisch prostaatcarcinoom goed weerspiegelt en derhalve hier een goed model voor is. Samen met andere tumormodellen kunnen de studies beschreven in dit proefschrift, een bijdrage leveren tot onze kennis omtrent de hormoonafhankelijkheid van prostaatkanker.

LIST OF OTHER PUBLICATIONS

Besides the papers which form part of this thesis, the following publications resulted from the studies described.

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NAWOORD

De vermelding van slechts mijn naam op de omslag van dit proefschrift wekt ten onrechte de indruk dat de totstandkoming ervan het werk is van maar één persoon. Graag wil ik dan ook een woord van dank richten aan allen die op enigerlei wijze een bijdrage hebben geleverd aan het onderzoek en aan de verschijning van dit boekwerk. Zonder te suggereren compleet te zijn wil ik met name noemen:

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Behoudens de eerder gepubliceerde hoofdstukken 2, 3 en 4, werd het Engels van het gehele manuscript door Liset van Steenbrugge gecontroleerd. Met jouw consciëntieuze wijze van corrigeren heb jij ook een belangrijke bijdrage aan de totstandkoming van het proefschrift geleverd. De discussies met jou over soms slechts een enkel woord zal ik nooit vergeten.

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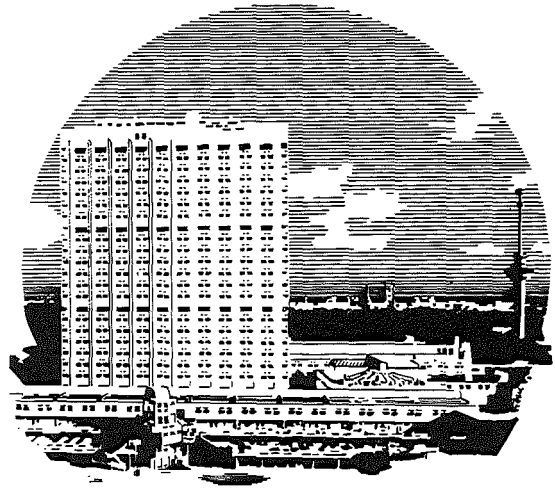
CURRICULUM VITAE

Gert Jan van Steenbrugge werd geboren op 13 september 1953 te Voorburg. In 1971 behaalde hij zijn diploma HBS-B aan het Christelijk Lyceum te Voorburg. In datzelfde jaar begon hij met de studie Biologie aan de Rijks Universiteit te Leiden. Het kandidaatsexamen "B4" (studierichting Biologie met tweede hoofdvak Scheikunde) werd in januari 1976 behaald. Zijn doctoraalexamen (januari 1979) omvatte het hoofdvak Biochemie (Prof. Dr. L. Bosch) en de bijvakken Immunohaematologie (Prof. Dr. J.J. van Rood) en Stralengenetica (Prof. Dr. F.H. Sobels).

Sinds april 1979 is de schrijver van dit proefschrift werkzaam aan de Erasmus Universiteit te Rotterdam, Medische Faculteit, afdeling Urologie (Prof. Dr. F.H. Schröder). Binnen de "tumor-research groep" van de afdeling Urologie verricht hij onderzoek in het kader van het onderzoeksprogramma "Modelsystemen, Endocrinologisch en Genetisch Onderzoek van het Menselijk Prostaatcarcinoom". Binnen dit programma werd in 1980 het onderzoek aangevangen dat tot deze dissertatie heeft geleid.



COLOFON

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