ANDROGEN RECEPTOR EXPRESSION IN HUMAN PROSTATE CANCER

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ANDROGEN RECEPTOR EXPRESSION IN HUMAN PROSTATE CANCER

Androgeen Receptor Expressie in het Humane Prostaatcarcinoom

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aan de Erasmus Universiteit Rotterdam

op gezag van de rector magnificus

Prof. Dr. P.W.C. Akkermans M. Lit

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Ter nagedachtenis van mijn vader
Aan mijn familie
Voor Astrid en Nienke

Contents

Abbreviations

ACTH	adrenocorticotrophic hormone
AIS	androgen insensitivity syndrome
(h)AR	(human) androgen receptor
ARE	androgen response element
ARTIS	androgen receptor transcription initiation site
bcl-2	B-cell lymphoma associated gene
bp	base pair
BPH	benign prostatic hyperplasia
BRdU	bromodeoxyuridine
Casodex	ICI 176334 (anti-androgen)
cDNA	complementary deoxyribonucleic acid
COS	monkey kidney cell line
CPA	cyproterone acetate
(k)Da	(kilo)Dalton, molecular mass
DAB	3,3'-diaminobenzidine
DHEA	dehydroepiandrosterone
DHT	5α-dihydrotestosterone
(h)EF	(human) elongation factor 1-alpha
(h)ER	(human) estrogen receptor
ELISA	enzyme-linked immunosorbent assay
EP-cell	endocrine/paracrine cell
ERE	estrogen response element
(h)GR	(human) glucocorticoid receptor
GRE	glucocorticoid response element
HR	hormone refractory
HRE	hormone response element
hsp	90 kDa heat shock protein
LH	luteinizing hormone
LHRH	luteinizing hormone-releasing hormone
LNCaP	human prostate cancer cell line
MAb	monoclonal antibody
MCF-7	human breast cancer cell line
MR	mineralocorticoid receptor
MRE	mineralocorticoid response element
mRNA	messenger ribonucleic acid
NHIK	human cervix carcinoma cell line
PAb	polyclonal antibody
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
(h)PR	(human) progesterone receptor
PRE	progesterone response element
PSA	prostate-specific antigen
R1881	methyltrienolone (synthetic androgen)
R5020	promegestone (synthetic progestin)
S	Svedberg unit (sedimentation coefficient)
SDS	sodium dodecyl sulfate
SSCP	single strand conformation polymorphism
T	testosterone
T47D	human breast cancer cell line
TAF	transcription activation function
TAU	transactivation unit
TeBG	testosterone-binding globulin
TURP	transurethral resection of the prostate

Scope of the thesis

In 1991, prostate cancer was the second leading cause of cancer mortality in men in the Netherlands. Some 40% of the patients diagnosed with prostate cancer have metastatic disease, that will be treated by hormonal therapy. However, initially 20% of the patients do not respond to endocrine therapy, and eventually all patients will progress to hormone-refractory cancer, the median time to progression being 12-18 months.

Hormone independence has been extensively studied in tumor model systems. Prostate tumors, transplantable in mice and rats, are in part androgen-dependent, as are their sublines, but some display androgen-independent growth. In these model systems hormonal dependence and the biochemically assayed androgen receptor (AR) content are generally correlated. However, in patient management, lack of correlation between hormonal dependence and the biochemically assayed AR content limits the use of androgen receptor data to predict the interval between the start of therapy and the occurrence of tumor progression. The lack of a positive correlation between androgen dependence in human tumors and the presence of AR has been attributed to tissue heterogeneity; that is, contamination of the prostate cancer specimen by non-malignant prostatic tissue. Furthermore, the heterogeneous composition of the tumor samples analyzed for AR content may also be important in this respect, the tumor being a mixture of androgen-dependent, presumably AR-positive, and androgen-independent, i.e. AR-negative, cells. Studies on tissue distribution of the AR performed thus far have made use of the hormone binding properties of these receptors. A drawback of these ligand-binding techniques, based on tissue homogenates, is that they do not provide information about cellular and subcellular distribution of the receptor. Therefore, it was anticipated that an immunohistochemical technique, directly visualizing the receptor, could contribute to a more detailed understanding of AR distribution in tissues.

The cloning and sequencing of the human AR (hAR) cDNA, the subsequent elucidation of the hAR protein structure and the synthesis of oligopeptides permitted the generation of anti-hAR antibodies. The availability of these hAR-specific antibodies is essential for the development of immunohistochemical techniques for direct visualization of AR expression at the cellular level.

The two appendix papers of this thesis describe the generation and biochemical characterization of poly- and monoclonal anti-hAR antibodies (Chapters 10,11). The immunohistochemically determined pattern of reactivity of a monoclonal anti-hAR antibody (F39.4) is given in Chapter 5. Chapters 4,6 and 7 describe the application of a polyclonal antibody (Sp061) and monoclonal antibody F39.4 in studies on the hAR distribution in hormone-dependent and hormone-refractory prostate cancer. Furthermore, a subset of androgen-independent cancers was screened for mutations in the hAR gene, because hormone independence may also be the consequence of receptor malfunction due to structural aberrations of the receptor protein. In a subset of these tumors hormone-binding capacity of

the immunohistochemically detected hAR was assessed by an androgen-binding assay (Chapter 7). The effects of endocrine manipulation on hAR mRNA and protein expression, as well as on proliferative activity of strictly androgen dependent growing tumor cells, were studied in the human prostatic carcinoma tumor line PC-82 (Chapter 8).

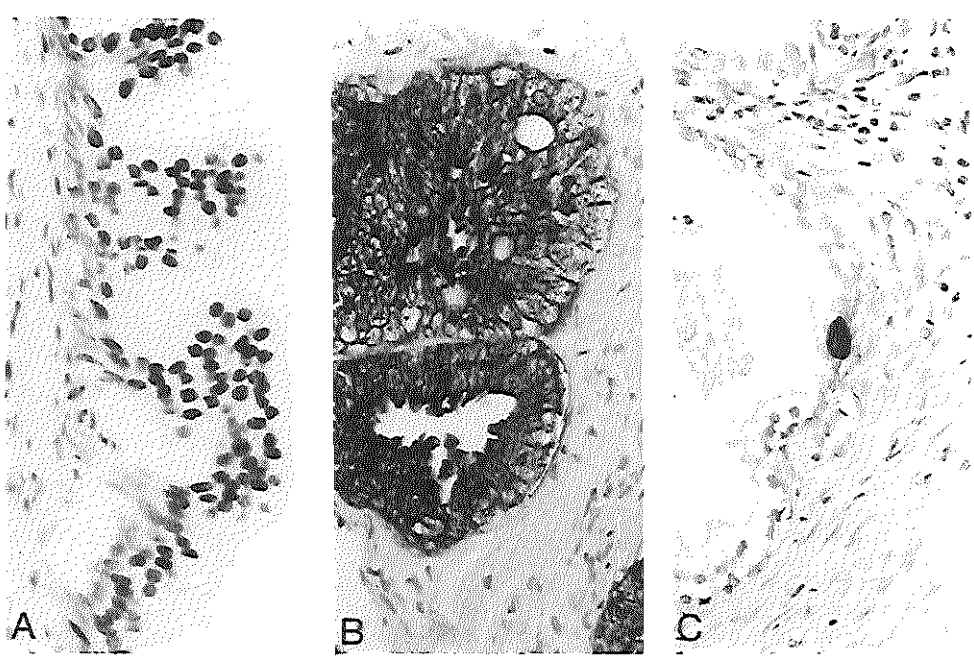
Chapter 1

Prostate cancer: clinicopathological aspects

1.1 The human prostate gland

The prostate is a retroperitoneal organ, encircling the neck of the bladder and the urethra. The shape of the human prostate resembles a horse chestnut, with the length of the anterior aspect being between 3 and 4 cm and width between 3.5 and 5 cm. In a normal adult the prostate weighs approximately 20 g. According to current opinion, the prostatic tissue is anatomically organized in concentric zones; that is a peripheral zone which constitutes 70% of the normal glandular prostate, a small transition zone and a central zone making up 25% of the prostate gland. These zones appear to differ with regard to morphology, function and predisposition to pathological changes. Almost all adenocarcinomas arise in the peripheral zone, whereas the prostatic tissue of the transition zone is apt to develop prostatic hyperplasia.

Histologically the prostate is a tubulo-alveolar gland, consisting of glandular structures supported by a stroma of fibromuscular connective tissue, bloodvessels, lymphatics and nerves. The glands are lined by two layers of cells: a basal layer of flattened to cuboidal cells and a covering layer of mucus-secreting epithelium. Neuroendocrine cells also reside among the more numerous secretory epithelial cells (Figure 1.1).



The peripheral prostatic ducts are lined by cuboidal epithelium, which merges with the transitional cell epithelium of the prostatic urethra.

The human prostate gland secretes three major proteins: prostate-specific antigen (PSA), prostatic acid phosphatase (PAP) and prostate-specific protein (PSP_{94} or ß-inhibin) (1).

1.2 Prostate cancer: epidemiology

In 1991, prostate cancer was the most common cancer diagnosed in American men and was the second leading cause of cancer mortality in these men (2) as it was in the Netherlands (3). From 1973 to 1985 the age-adjusted prostate cancer incidence increased from 68.4/100,000 to 83.4/100,000 men (a 22% increase) among American males. Age-adjusted mortality rates from prostate cancer in this period increased slightly (7%) from 20.1/100,000 to 21.5/100,000 men among American white males (4). In the Netherlands the age-adjusted mortality rate from prostate cancer increased from 19.5/100,000 men in 1970 to 24.4/100,000 men in 1990 (5). The absolute number of deaths from prostate cancer in the Netherlands in 1991 was 2108 (3).

Many epidemiologic studies have been aimed at identifying etiologic and predisposing factors for prostate cancer (1,6). Remarkably, countries with widely different clinical frequencies of prostate cancer have similar frequencies of the incidental finding of carcinoma of the prostate at autopsy (7-9). It has therefore been proposed that factors responsible for the initiation of prostate cancer may be similar throughout the world and that differing rates of progression to clinically overt cancer arise from differences in unidentified environmental promoting factors. No consistent evidence exists for a substantial role of potential risk factors associated with diet, occupation, socioeconomic status, infectious disease history, sexual practices, habitus or hormonal factors. Genetic factors are probably involved in prostate cancer, as familial aggregation of prostate cancer has been observed (1,6,10).

Figure 1.1. Hyperplastic prostatic glands surrounded by stromal cells. A: Nuclei of secretory epithelial cells and stromal cell nuclei are reactive with the monoclonal anti- androgen receptor antibody F39.4. No nuclear counterstaining. B: PSA expression, visualized by ER-PR8 immunoreactivity of the cytoplasm of prostatic secretory epithelial cells. Nuclear counterstaining with Mayer's haematoxylin. C: Neuroendocrine cell in a benign prostatic gland, as expressed by immunoreactivity for chromogranin A. Nuclear counterstaining with Mayer's haematoxylin. Original magnification 400x.

1.3 Histologic typing of prostate cancer

The primary malignant tumors occurring in the prostate may originate in the epithelial and in the stromal compartments. The classification of tumors of the prostate according to the World Health Organization (11) is shown in Table 1.1.

Table 1.1. Histological classification of prostate tumors*

I. Epithelial tumors		
A. Benign		
B. Malignant	1. Adenocarcinoma	a. small acinar
		b. large acinar
		c. cribriform
		d. solid/trabecular
		e. others
	2. Transitional cell carcinoma	
	3. Squamous cell carcinoma	
	4. Undifferentiated carcinoma	
II. Non-epithelial tumors		
A. Benign		
B. Malignant	1. Rhabdomyosarcoma	
	2. Leiomyosarcoma	
	3. Others	
III. Miscellaneous tumors	1. Neuroendocrine tumors	
	2. Carcinosarcomas	
	3. Others	
IV. Secondary tumors		
V. Unclassified tumors		
VI. Tumor-like lesions and epithelial abnormalities.		

* Modified from Mostofi (Ref 11).

Over 95% of prostate cancers are adenocarcinomas arising from the epithelium of the prostatic acini. The remaining malignant epithelial and stromal neoplasms are beyond the scope of this thesis.

1.4 Grading of prostate cancer

Grading systems are developed to predict recurrent disease, metastasis and patient survival. The recommended or widely used grading systems for adenocarcinoma of the prostate use architectural and/or nuclear features in assessing biological behaviour of the tumor (6). The grading systems most often used for adenocarcinoma of the prostate are the Gleason grading system (12) and the grading systems according to Brawn *et al.* (M.D. Anderson) (13), Gaeta *et al.* (14) and Mostofi-(Schröder) *et al.* (15,16).

Table 1.2. Tumor grading protocols*

Gleason (Ref 12)**	Brawn (Ref 13)
Pattern 1: Closely packed, single, separate, round, uniform glands; well defined tumor margin.	Grade I: 75%-100% of tumor glands (predominantly cribriform-papillary pattern excepted).
Pattern 2: Single, separate, round, less uniform glands separated by stroma up to one gland diameter; tumor margin less well defined.	Grade II: 50%-75% of tumor forms glands (includes cribriform-papillary tumors); tumors >50% of which are cribriform-papillary pattern.
Pattern 3: Single, separate, irregular glands of variable size; enlarged masses with cribriform or papillary pattern; poorly defined tumor margin.	Grade III: 25%-50% of tumor forms glands.
Pattern 4: Fused glands in mass with infiltrating cords, small glands with papillary, cribriform or solid patterns; cells small, dark, or hypernephroid (clear cells).	Grade IV: 0%-25% of tumor forms glands.
Pattern 5: Few or no glands in background of masses with comedo pattern or sheets of tumor cells infiltrating stroma.	

* Modified from Petersen (Ref 6).
** Gleason tumor grade = dominant pattern + secondary pattern.

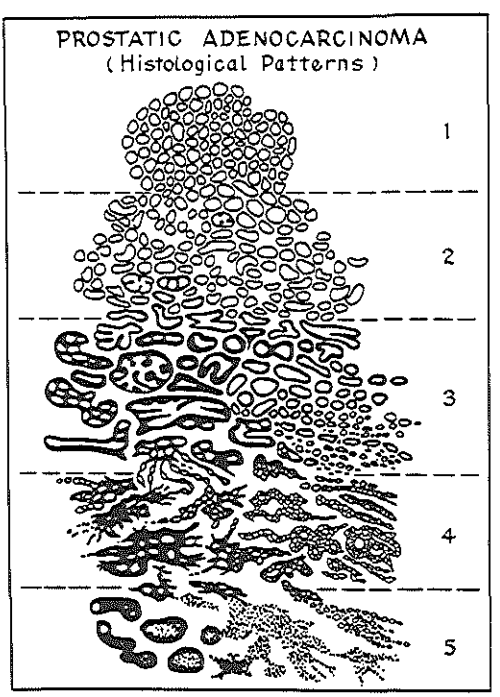


Figure 1.2: Simplified drawing of histologic patterns, emphasizing degree of glandular differentiation and relation to stroma. All black in the drawing represents tumor tissue and glands with all cytologic detail obscured except in right side of pattern 4 where tiny open structures are intended to suggest the "hypernephroid" pattern. (Adapted from Tannenbaum, M (ed.): Urologic pathology: the prostate. Philadelphia, Lea & Febiger 1977).

The gains and the limitations of these grading systems and/or the prognostic significance of tumor grade are beyond the scope of this thesis. In our studies the grading systems according to Brawn and Gleason were used, since nuclear features — both part of the Mostofi-(Schröder) and Gaeta grading systems — could not be determined reliably in frozen sections, which we had to use for the immunohistochemical studies. The criteria of the grading systems used are shown in Figure 1.2 and Table 1.2.

1.5 Patterns of dissemination of prostatic cancer

Local extension: Adenocarcinomas of the prostate may spread by local invasion or penetration of lymphatics and/or blood vessels (17). Capsule penetration is a very common phenomenon. Perineural invasion frequently occurs and facilitates invasion through the capsule. Seminal vesicle invasion almost always results from direct spread of the tumor in the ejaculatory duct wall inside the prostate (1). Ultimately, there is urethral obstruction in approximately 50% of patients (18). Ureteral obstruction from prostate cancer occurs in 10-35% of patients. Posterior extension into the rectum is an unusual event (17).
Lymphatic metastasis: Invasion of cancer cells into prostatic lymphatics is responsible for the occurrence of lymph node metastasis. Metastasis of prostate cancer to the lymph nodes most frequently involves the internal iliac (hypogastric), the external iliac, the periaortic and the pre-sacral lymph nodes (18).
Hematogenous metastasis: The most common form of hematogenous metastasis from prostate cancer is skeletal metastasis, which is observed in up to 85% of patients dying of prostatic carcinoma (19). The high frequency of bone metastasis of prostate cancer is a remarkable observation. Prostate cancer selectively spreads to the axial skeleton. The composition of the microenvironment of the bones may be of importance in accelerating the growth of prostate cancer. Stimulation of prostate cancer growth by factors derived from bone fibroblasts has been described in an *in vivo* tumor model (20). The most common sites of visceral and soft-tissue metastases are the lungs (25-38%), liver, pleura, adrenals and kidneys, but almost any organ may be involved (18).

1.6 Staging of prostate cancer

Tumor size/volume and extent, the malignant potential of the tumor and the likely response to therapy are critical parameters in the planning of treatment for prostatic adenocarcinoma. The stage of a malignant neoplasm reflects the extent of spread of a cancer within the patient and is used as a parameter of the clinical gravity of disease. Two commonly used staging systems for prostate cancer are the Whitmore-Jewett classification (21) and the tumor/ node/

Table 1.3. TNM classification of prostate cancer*(1992)

T1	Clinical inapparent tumor not palpable or visible by imaging
	T1a ≤ 5%
	T1b > 5%
	T1c Needle biopsy
T2	Tumor confined within the prostate
	T2a ≤ ½ of a lobe
	T2b > ½ of a lobe
	T2c Both lobes
T3	Through the prostate capsule
	T3a Unilateral
	T3b Bilateral
	T3c Seminal vesicle(s)
T4	Fixed or invades adjacent structures other than seminal vesicles
	T4a Bladder neck and/or external sphincter/ rectum
	T4b levator muscles and/or is fixed to the pelvic wall
N1	Metastasis in a single regional lymph node 2 cm or less in greatest dimension
N2	Single > 2 cm to 5 cm, multiple ≤ 5 cm
N3	Regional lymph node > 5 cm
M1a	Non-regional lymph node(s)
M1b	Bone(s)
M1c	Other site(s)

* Adapted from Schröder (Ref 22).

metastasis (TNM) system of staging (UICC 1992) (Table 1.3) (22). The clinical stage is determined by a staging work-up (Table 1.4) (23).

In a recent study 24% of 654 patients analyzed had hematogenous metastases at presentation, 17% were in clinical stage T1 (TA), 32% were in clinical stage T2 (TB) and 49% were in clinical stage T3-4 (TC) (24). It is generally accepted that 40-50% of prostate cancer patients have metastatic disease at the time of diagnosis (25). It is estimated that 30% of patients with clinical stage T1 and T2 have pelvic lymph node metastasis (26-28). Fifty percent of patients with T3-4 cancer will have positive lymph nodes (26,29). Extrapolating these lymph node metastasis data to the study by Johansson mentioned above, 261 of 654 patients, that is 40%, would probably have metastatic disease at presentation. These figures illustrate the need for adequate therapeutic modalities in the treatment of metastatic prostate cancer.

1.7 Treatment of prostate cancer

The treatment modality chosen depends on the results of the staging work-up (Table 1.4).

Table 1.4. Staging work-up*

1. clinical diagnostic work-up:
   a. digital rectal examination
   b. transrectal ultrasound
   c. radiographic survey: chest films and skeletal films CT-scan abdomen and pelvis
   d. radio-isotopic survey: bone scan
   e. laboratory studies: serum acid phosphatase; serum prostatic acid phosphatase; serum prostate-specific antigen
2. surgical evaluative work-up:
   a. cystoscopy
   b. exploration of primary tumor and/or lymph nodes
   c. biopsies of primary tumor and/or lymph nodes
3. pathologic staging work-up:
   a. determination of tumor extension in prostate gland and lymph nodes

* Modified from Keller, McCune, Sahasrabudhe (Ref 23).

Surgery and radiotherapy are most suited for treatment of patients with localized disease (confined to the prostate). Patients with metastatic disease are treated preferentially by endocrine therapy. Chemotherapy is generally ineffective in hormone refractory metastatic prostate cancer.

The dependence upon androgens of many prostatic carcinomas for their growth is undoubted. The rationale of androgen ablation/withdrawal treatment is to lower circulating androgen levels and/or to counteract the biological effects of the hormone at the cellular level. Endocrine therapy of human prostate cancer was introduced in 1941 by Huggins *et al.* (30). Surgical or pharmacological androgen deprivation in patients with metastatic carcinoma leads to a rapid reduction of symptoms. Originally, it was assumed that this palliation was also associated with increased survival. Response rates of hormonally treated patients amount to 30-40%, and another 40% of the patients demonstrate stable disease. Median time to failure of endocrine management of metastatic carcinoma of the prostate is 12-18 months and median survival is 24-30 months (31). When compared to survival data from times that hormonal therapy for metastasized prostate cancer had not yet been introduced (1925-1940), hormonal therapy resulted in little improvement in terms of survival. In his review on the natural history of prostate cancer Bumpus (1926) reported that 66% of patients with metastatic disease (243 patients) died within 9 months (32). Nesbit and Plumb (1946) also used a historical control group in their studies on the effects of endocrine therapy. In this control group 82% of 260 patients died within 2 years, the 5-year survival being only 6% (33).

Eventually all androgen dependent tumors progress to a state of autonomous growth and then are no longer sensitive to androgen withdrawal. Progression to endocrine therapy resistance may become manifest by an increase in the size of the primary tumor (local progression), by the occurrence of new soft tissue lesions, new hot spots on a bone scan, new pulmonary or liver metastases, or by the increase of any measurable metastatic lesion (objective criteria for progression of the EORTC GU Group (European Organization of Research on Treatment of Cancer, Genitourinary group) (34).

Studies on AR expression and function in hormone dependent and hormone independent prostate cancer may increase our knowledge on its role in progression to androgen insensitivity. Information on this subject is highly relevant, since all tumors eventually progress to a state of androgen independence. A large group of patients — either with metastatic disease or with localized disease that will progress to metastatic disease (30-50% of stage A, B and C patients develops metastases) — depend on the effects of hormonal therapy.

Strategies of first-line endocrine management of metastatic prostate cancer and their mode of action (35,36) will be discussed briefly.

Androgen deprivation:

Surgical castration:

Bilateral orchiectomy removes the primary source of testosterone (T) and is a direct method for lowering plasma T levels.

Medical castration:

a: Prevention of testicular androgen production can be induced by chronic administration of luteinizing hormone-releasing hormone (LHRH) agonists (Buserelin, Leuprolide, Decapeptyl, Goserelin), agents that suppress pituitary luteinizing hormone (LH) secretion by down-regulation of LHRH receptors at the pituitary level.

b: Estrogens (diethylstilbestrol) also reliably reduce serum T levels into the range observed in castrated males. Estrogens act by the suppression of LH release from the pituitary, and they also decrease the amount of available T by increasing the synthesis of testosterone-binding globulin (TeBG) by the liver.

Androgen blockade:

Nonsteroidal anti-androgens:

Pure anti-androgens (Flutamide, Nilutamide, ICI 176,334) are AR antagonists that act by inhibiting the binding of T and DHT to the AR in target tissues.

Steroidal anti-androgen:

Cyproterone acetate (CPA) is a steroidal anti-androgen that interferes with androgen synthesis and action. Next to AR blocking activity, the progestagenic and glucocorticoid properties of CPA partially suppress LH secretion.

Unfortunately, at present no clear-cut and well established strategy for the endocrine management of hormone-refractory prostate cancer (second-line endocrine therapy) is at hand. Irrespective of the treatment regimen there is at best some subjective improvement lasting for approximately 6 months, but most patients will have died within one year (37).

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Chapter 2

The human androgen receptor

2.1 Androgen synthesis, secretion and effects

The major steroidal androgens, testosterone (T) and dihydrotestosterone (DHT), are directly involved in the development and differentiation of the genital tract from an ambisexual stage to the male phenotype (1,2). Prostate development from the urogenital sinus, as well as growth and differentiation in adult life, depends on the presence of androgen (1,3,4). Moreover, androgens exert a variety of functions in non-reproductive tissues (5). The androgen dependence of prostate cancer was first reported by Huggins *et al* (6).

The Leydig cells of the testis synthesize T and are the primary source of androgens (95%). Circulating T is largely bound to plasma proteins. Non-bound T enters cells by diffusion and acts as a pro-hormone, in that the most active hormone in most but not all tissues is its 5α-reduced metabolite DHT (2). The adrenals account for 5% of androgen production by synthesis and secretion of dehydroepiandrosterone (DHEA), DHEA-sulfate and androstenedione. Although not potent androgens themselves, they are capable of maintaining low DHT levels in prostatic tissues (7,8). Both Leydig cell and adrenocortical function are controlled by negative feedback at hypothalamic and pituitary level (9,10).

In the nuclear and cytosol fractions of target cells, the hAR binds androgen with high affinity and specificity. Tissue-specific effects of androgens on growth and function are considered to be the consequence of differential expression of gene networks. Activation or repression of gene transcription of most eukaryotic genes is mediated by multiple gene regulatory proteins, including both general transcription factors binding to a promoter region near the transcription start site, and enhancer elements acting at a distance (11,12). The hAR, as a member of the steroid hormone receptor family (13), is a ligand-dependent transcription activator. After binding of androgen, the receptor structure is altered to a transcription modulating state, resulting in the production of mRNA and protein synthesis from androgen-regulated genes (12).

2.2 Structural organization of the hAR gene

The hAR cDNA has been cloned and sequenced and its protein structure elucidated (14-18). A schematic diagram of the hAR gene is shown in Figure 2.1. The hAR gene has a length of over 90 kb and is located in the q11-12 region of the X-chromosome. The hAR gene is organized in 8 exons and 7 introns (16,19,20). The sequence encoding the N-terminal region is present in one large exon (17). The DNA binding domain is encoded by exons 2 and 3. The remaining exons (4-8) encode the hormone binding domain (19). The open reading frame of the hAR mRNA consists of 2751 nucleotides (2.7 kb) (21). The 5'- and 3'- untranslated regions of the hAR mRNA have a length of 1.1 kb and 6.8 kb respectively (22,23).

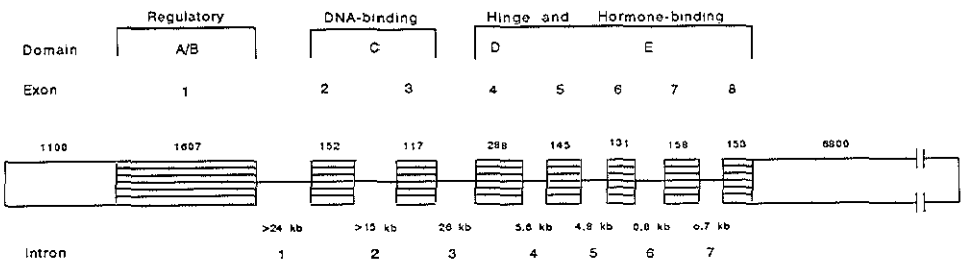

Figure 2.1: Organization of the human androgen receptor gene. The eight exons of the gene (1-8) are depicted by boxed areas. The seven introns are shown by thin lines. The lengths of the exons (in nucleotides) are shown above the boxes, whereas the approximate sizes of the intronic sequences in kilobases (kb) are indicated below the thin lines. (Adapted from Janne AO and Shan L: Structure and function of the androgen receptor. Ann NY Acad Sci 626: 81-91, 1991) (Ref 19,20,22,23).

2.3 Structure of the hAR protein

The hAR protein encompasses 910-919 amino acid residues, with an approximate molecular mass of 99-110 kDa (14-17,21). Figure 2.2 and 2.4 depict the hAR protein structure. Sequence comparisons of the hAR with the structure of other steroid receptors demonstrate a highly conserved DNA-binding domain (amino acids 557-621)(12,24). Regions in the COOH-terminus of the protein reveal significant homology (amino acids 704-746 and 790-812)(12,16,21,24). The N-terminal region of the hAR is a region with low homology with other steroid receptors. Structural homology among the hAR, progesterone receptor (PR), glucocorticoid receptor (GR), mineralocorticoid receptor (MR) and estrogen receptor (ER) is depicted in Figure 2.3. (21).

2.4 Functional organization of the hAR protein

Steroid hormones modulate transcription of target genes by interaction of the DNA-binding domain of the receptor with target gene sequences, known as hormone-responsive

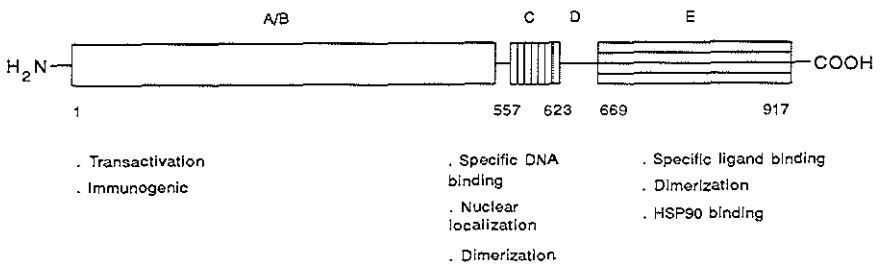

Figure 2.2: Schematic illustration of the structure/function organization of the androgen receptor. Regions A-E indicate the different domains of the androgen receptor. Domain functions are depicted below the scheme (see text). (Adapted from ref 13,27-31).

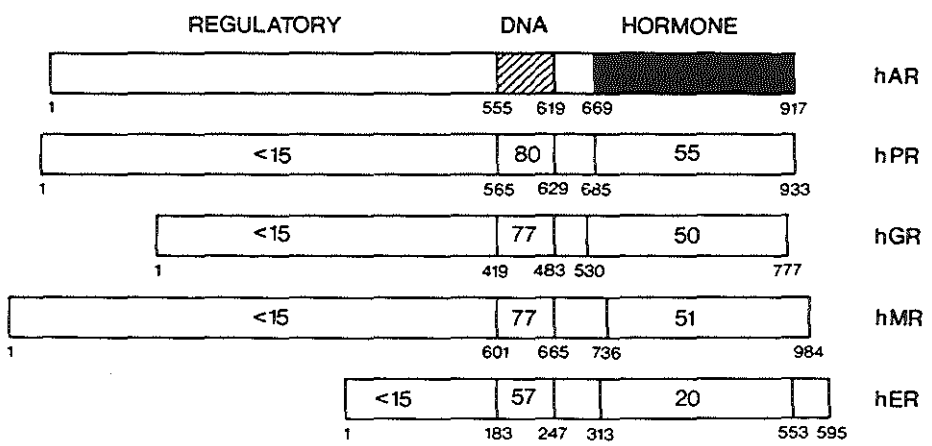

Figure 2.3: Structural homology among the human androgen receptor, progesterone receptor, glucocorticoid receptor, mineralocorticoid receptor and estrogen receptor. (Adapted from Carlstedt-Duke J, Eriksson H and Gustafsson OÅ (eds.): The steroid/ thyroid hormone receptor family and gene regulation. Basel, Birkhäuser congress reports. Life sciences 1989, 4: 170-181).

elements (HREs). The GRE, PRE, MRE and ARE consensus sequence is GGTACA-XXX-TGTTCT (12).

The DNA-binding domain of the hAR can be separated into two regions. These regions contain respectively 4 and 5 perfectly conserved cysteine residues, constituting two so-called zinc fingers, involved in receptor dimerization and the binding of the receptor to

DNA (25-27). Nuclear localization of steroid receptors occurs by translocation signals present in the receptor protein. In the hAR, a bipartite nuclear-localization signal is situated in the C-terminal site of the DNA-binding domain (28-30).

The C-terminal domain of steroid receptors is involved in hormone binding and in the interaction with various non-receptor proteins (Figure 2.2.). The hormone-binding site consists of a hydrophobic pocket of some 200-250 amino acid residues. Mutations in this region of the protein abolish steroid binding (12,28).

The interaction of steroid hormone receptors with non-receptor proteins is exemplified by receptor interaction with heat shock protein 90 (hsp90). Such an association has also been described for the hormone free hAR (31). The hsp90-steroid receptor complex is considered to maintain the receptor in an inactive form. The presence of hsp90 either maintains the receptor in a conformation unable to undergo tight nuclear binding in the nucleus or masks the DNA binding domain (32). Upon hormone binding, the hsp90-receptor complex dissociates and the hormone-receptor complex can regulate target gene expression.

Transcription activation regions (trans-activation functions, TAFs) have been located both in the N-terminal and in the C-terminal domain of steroid receptors (12). The N-terminus of the hAR has also been reported to contain transactivation function (Figure 2.2.) (28-30). Transcription activation by the TAFs is considered to be mediated by the interaction with other (tissue-specific) transcription factors (12). Thus, complexing of steroid hormone receptors and their respective ligands results in receptor activation (transformation) by dissociation of receptor-associated proteins (e.g. hsp90), by receptor dimerization, DNA binding and activation of transcription.

Changes in the androgen transduction cascade may be responsible for the development of hormone independence. Hormone independence of tissues may be associated with either absence of receptors or with loss of receptor function. Partial or complete deletion of the AR gene and/or (point)mutations in the receptor gene may result in a functionally defective receptor, as has been reported in the androgen insensitivity syndrome (33). Loss of steroid responsiveness may be due to partial deletions, which activate transcription irrespective of the presence of ligand (truncated receptor).

2.5 AR immunohistochemistry

Demonstration of the hAR has thus far relied on ligand-binding properties of this receptor, either in tissue homogenates (34) or by use of autoradiography in tissue sections (35). In the search for a less laborious technique to study the cellular and subcellular distribution of the hAR, efforts have been focussed recently on the generation of hAR specific poly- and monoclonal antibodies (Chapters 10,11) (36-40). The cloning of the hAR cDNA and the

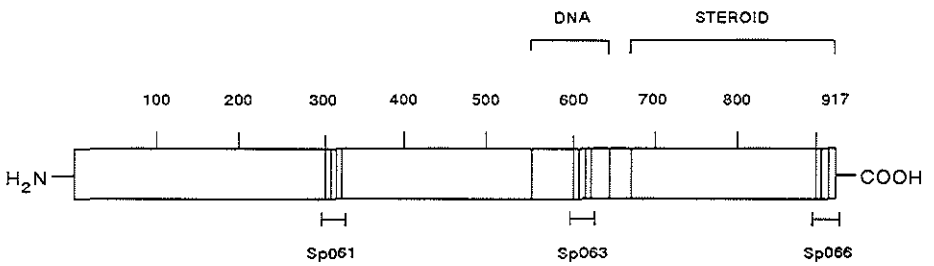

Figure 2.4: Schematic representation of the primary structure of the human androgen receptor. The diagram illustrates the location of the functional domains and the location of the peptides (Sp061, Sp063 and Sp066) that were used for immunizations. Numbers indicate the amino acid residues.

elucidation of the protein structure have provided tools to generate hAR specific antibodies using synthetic peptides for immunization. The polyclonal and monoclonal antibodies used in this thesis were generated against synthetic peptides corresponding to amino acid sequences of the N-terminal hAR domain (Sp61, F39.4), of the DNA-binding domain (F52.24.4), or of the C-terminal region of the receptor (Sp66) (Figure 2.4.).

Immunoprecipitation and Western blots of LNCaP nuclear extracts verified the interaction of polyclonal antibody Sp61 and monoclonal antibody F39.4 with the hAR. Both these antibodies were raised against synthetic peptide Sp061 (amino acids 300-320) and interacted with the intact and denatured hAR (Chapters 5,10,11) (39-41). Monoclonal antibody F52.24.4, raised to synthetic peptide Sp063 (amino acids 600-619), recognized the hAR in immunoprecipitation and immunohistochemical assays, but demonstrated considerable cross-reactivity with GR, ER and PR (31). Polyclonal antibody Sp66, developed against an epitope in the C-terminal domain (amino acids 899-917), recognized the 110-112 kDa hAR on Western immunoblots but was unable to interact with the native hAR complexed with radioactive ligand in solution (Chapter 8) (42).

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Chapter 3

Tumor cell heterogeneity, androgen receptor expression and androgen independence

3.1 Tumor cell heterogeneity and tumor progression

Malignant neoplasms are known to be composed of cell populations that are heterogeneous for a wide variety of characteristics, including genetic, biochemical and biological properties. Despite this heterogeneity, neoplasms generally develop from a single transformed cell. Tumor progression is probably the consequence of diversification of tumor cells in their unique environments, and variants with altered properties arise in the tumor cell population, causing the observed tumor cell heterogeneity. In time, more stable variant tumor cells, with divergent properties and enhanced survival, growth, autonomy and malignant characteristics may become dominant in the cellular composition of the tumor. During progression of tumors, neoplastic cells are thought to accumulate increasing genetic alterations, which are generated by random somatic mutational events. In concert with such "genotypic instability" of progressing tumors, host selection pressures tend to enrich tumors with cell subpopulations that are more adapted to survival and growth. However, genotypic instability and selection alone cannot explain the rapid rates of tumor cell phenotypic variation. Epigenetic (gene regulation) and microenvironmental changes also contribute to phenotypic diversity of tumor cells (1).

3.2 Theories on the development of androgen independence

The eventual occurrence of androgen independence in all initially androgen-sensitive prostate cancers treated by androgen withdrawal may be considered as the consequence of tumor progression. This phenomenon of tumor relapse, that is progression during conventional hormonal therapy, is still not understood. Tumors and their sublines may display various types of biological responses to hormonal manipulation. "Androgen dependence" is the term to indicate an absolute requirement for androgen, whereas "androgen responsiveness" indicates altered proliferation above a basal growth rate. Tumor growth in the absence of androgen is designated as "androgen insensitivity".
Concepts on the development of androgen independence have been derived from observations on human prostate cancer and in both *in vivo* and *in vitro* experimental tumor model systems.

Hypothesis based on the evolution of human prostatic carcinoma: castration effects on normal prostatic glandular tissue

The concept of the normal prostate as a steady-state self-renewing system (Figure 3.1) (2,3) is an attractive option to explain the progression of prostate cancer to androgen independence (4). The steady-state self-renewing of the prostate may be considered to be organized

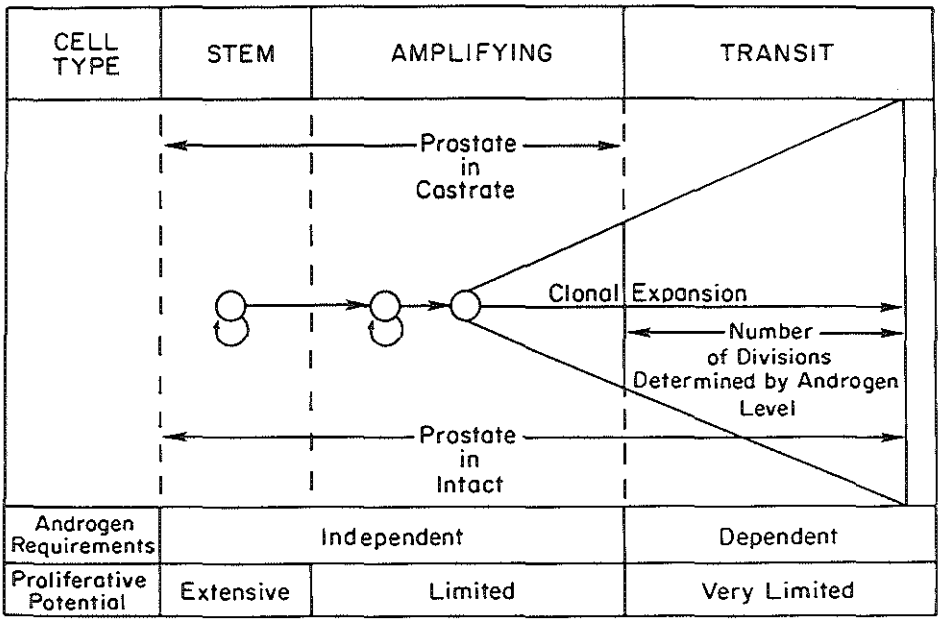

Figure 3.1. Stem cell model for the organization of the prostate (see text). (Adapted with permission from Ackermann, R. and Schröder, F.H. (eds.): Prostatic hyperplasia: etiology, surgical and conservative treatment. New developments in biosciences. Berlin. Walter de Gruyter 1989).

according to the "diminishing stemness spiral" model (5). According to this model the prostatic tissue is composed of actual stem cells, mature cells and transit cells. The transit cells are a transition population of proliferating cells situated between the stem cells and the mature, functioning compartment, sharing properties both with stem cells and with mature cells. Cell division potential decreases with increasing differentiation and maturation (5). The concept of the prostate as a self-renewing system is based on the effects of androgen withdrawal upon cell death, and cell regeneration after androgen resubstitution in rat ventral prostate. The observation that involution and regeneration of the prostate can be repeated several times, with no suggestion of exhaustion of proliferative potential of surviving cells, provides the strongest piece of evidence that the prostate contains a population of self-replicating cells. Next to stem cells, a subclass of amplifying cells — cells capable of limited proliferation — was postulated, because stem cells alone — with limited life span and number of population doublings — cannot account for the vigorous ability of prostatic cells to restore their total cell number after androgen withdrawal. The bulk of the prostatic glandular tissue is composed of differentiated epithelial cells, which are liable to die after androgen withdrawal. The viability of the stem cell and amplifying cell population is not influenced by androgen withdrawal and is therefore considered androgen independent (2). A

similar selection proces may be involved in the progression from androgen dependence to androgen independence after androgen withdrawal (castration), eradicating the androgen-dependent cells, resulting in a selection of androgen-independent cells.

Hypothesis based on the evolution of human prostatic carcinoma: castration-induced effects on neuroendocrine prostatic cells

Another explanation for the development of androgen-independent prostate cancers originates from the observations on neuroendocrine features in prostatic carcinomas (6). Neuroendocrine differentiation in prostatic adenocarcinoma is a rather frequent finding (8-32.5%). However, carcinomas of the prostate composed entirely of neuroendocrine cells are distinctly rare. It is generally accepted that the prognosis of small cell (neuroendocrine) carcinomas of the prostate is far worse than that of conventional adenocarcinomas (7). In an overview of 20 patients with small cell carcinoma of the prostate, 8 patients presenting with conventional adenocarcinoma developed a small cell carcinoma during endocrine treatment. Most progressive prostate carcinomas show a concomittant increase in the number of neuroendocrine cells (8). A prostatic adenocarcinoma evolving into a carcinoid has been described by Stratton *et al.* (9). It was suggested by these authors that conventional adenocarcinomas were sensitive to hormonal manipulations used in treatment, but that the originally sparse neuroendocrine components were resistant to therapy and hence became the predominant tumor cell population. Despite the attractiveness of the notion that the small cell component might be therapy resistant and might cause tumor progression, only a minor percentage of progressing prostatic carcinomas are completely composed of (neuroendocrine) small cells.

Experimental *in vivo* and *in vitro* models of the development of androgen independence of prostate cancer

Various hypotheses have been put forward to explain the development of hormone unresponsiveness. Based on experimental studies both environmental selection and environmental adaptation have been proposed to explain the development of androgen independence.
The environmental selection model is based on prostate cancer heterogeneity with regard to its androgen requirements for growth. According to this selection model prostate cancers are thought to be composed of pre-existing clones of androgen-dependent and androgen-independent cells. Androgen ablation selects for androgen-independent tumor cells, which progressively expand and produce the relapse phenomenon (10). An alternative explanation

for the development of androgen independence in originally androgen-dependent tumors is the environmental adaptation model (10), which implies that these tumors are initially composed of tumor cells that are homogeneous at least with regard to their requirement of androgenic stimulation for their growth. Under environmental pressure, that is androgen ablation, androgen-dependent cells adapt to an androgen-independent state.

The selection theory is based on experiments with the Dunning R3327 rat prostatic adenocarcinoma system, a well differentiated, slowly growing, androgen-dependent tumor, claimed to be a tumor subline of the original Dunning prostatic adenocarcinoma which originated in a Copenhagen rat. This tumor, transplanted in intact and castrated male syngeneic rats, in time progresses to autonomy. The heterogeneous nature of the Dunning tumor as to the property of androgen dependence was demonstrated by the occurrence of androgen-independent tumor sublines either spontaneously or after castration, and by the dissimilar fluctuation in time required for trocar pieces of tumor to grow to 1 cm^3 in castrate rats as compared to tumor cell homogenates. Androgen ablation creates a host environment in which the preexisting clones of androgen-independent tumor cells have a highly selective growth advantage over the androgen-dependent cells (environmental selection model). With time the continuous proliferative growth of these androgen-independent tumor cells, at the expense of androgen-dependent cells, leads to the "escape phenomenon" (11).

The potential validity of the environmental clonal selection model was underlined by studies on the Shionogi mouse mammary adenocarcinoma, an androgen-dependent transplantable tumor line, which may be passaged in syngeneic DD/S mice or cultured *in vitro*. Mixed tumors, composed of SC-115 (androgen-dependent) and CS-2 (androgen-independent) tumor cells transplanted into male and female mice, showed a selective growth advantage of CS-2 tumor cells in female mice. The predominant outgrowth of CS-2 tumor cells was considered to be the consequence of differences in angiogenesis (12).

Recently, the stem cell composition of the androgen-dependent Shionogi SC-115 carcinoma was studied to test the validity of the stem cell theory, which may also be considered a variant of the environmental selection model. In these experiments the proportions of androgen-dependent and androgen-independent tumorigenic (stem) cells in parent, regressing and recurrent tumors were compared using a limiting dilution assay. Decreasing numbers of tumor cells were injected into male and female mice and tumor take was recorded. Despite a striking increase in the number of androgen-independent stem cells between parent and recurrent tumors, no enrichment of androgen-independent stem cells was noticed in regressing tumors. These data seem to imply that the androgen-independent tumor cells, which survive androgen withdrawal, do not result from the environmental selection of androgen-independent stem cells, but from the ability of a small number of androgen-dependent cells to adapt to an altered hormone environment (13).

The environmental adaptation model was validated in cultured Shionogi S115 cells (14). S115 cells cultured in the continuous absence of androgen lose their responsiveness after 3-4 weeks of culture. The spontaneous mutation rate in S115 cells could not explain the rapid loss of response to androgen, which the authors consequently consider to represent a phenotypic rather than a genotypic change.
Another variant of the environmental adaptation model was proposed by Labrie, who studied the adaptation of androgen-dependent cultured Shionogi tumor cells to an environment (almost) devoid of androgen. The effect of decreasing concentration of DHT on DNA content/dish and cell growth was examined in androgen-dependent cells. The analyzed tumor clones showed marked heterogeneity with regard to basal growth in the absence of androgens, maximal response to DHT and sensitivity to DHT action (15). Moreover, exposure of clones, derived from the androgen-sensitive clone (SEM-1) of the Shionogi mammary carcinoma cell line, to a dose as low as 0.3 nM DHT for 67 days facilitated the development of androgen-hypersensitive clones (16). Those cell lines, having a high degree of sensitivity to DHT (designated androgen-hypersensitive), were considered to continue their growth even in the presence of castration levels of androgen.

3.3 Androgen binding in prostate tumor/cell lines

Although tumor cell diversity may be generated in many ways this section will be devoted to AR expression in androgen-dependent, androgen-responsive and androgen-independent carcinomas. The usefulness of quantitative data on steroid hormone receptors in tumor tissue to predict patient response on hormonal therapy originated from the observation in human breast cancer that estrogen receptor-negative tumors were mostly unresponsive to hormonal therapy (17). Generally, loss of receptor was thought to be accompanied by loss of response to the specific ligand. Likewise, it was postulated that AR content in prostate cancer might reflect its degree of responsiveness to androgen withdrawal. It was hypothesized that androgen-independent cells had few or no AR, whereas tumors containing AR were androgen responsive. Subsequently, characterization of androgen binding and quantitation of AR were tackled with much effort.
Until recently the correlation between androgen responsiveness and AR content was analyzed by [^{3}H]-ligand binding assays. A selection of AR data, gathered by this technique, from experimental tumor systems is shown in Tables 3.1 and 3.2.
All androgen-dependent experimental prostatic tumors express AR. Most androgen-independent prostatic tumor lines do not contain detectable AR. Exceptions are the androgen-unresponsive Dunning subline HIF, the Shionogi subline CS-2 and the LNCaP-r tumor line, which contain AR. In general, the androgen responsiveness of a given tumor subline

Table 3.1. Androgen receptor (AR) in human prostate tumor cell lines and transplanted tumors

	Androgen dependent (sensitive)	Androgen[1] binding	AR[2] mRNA	AR[3] protein	Ref
DU-145	-	-	-	-	18
PC-3	-	-	-	-	18
LNCaP	+	+	+	+	18,19
TSU-PR1	-	-	ND	ND	20
PC-82	+	+	+	ND	21
HONDA	+	+	ND	ND	22
PC-EW	+	ND	+	ND	19
PC-133	-	ND	-	ND	19
PC-135	-	ND	-	ND	19
UCRU-PR2	-	-	ND	ND	23

[1] Androgen binding assay
[2] Northern blot analysis
[3] Western blot analysis
ND Not determined

Table 3.2 Androgen receptor (AR) in rodent androgen (un)responsive tumor models

	Androgen dependent (sensitive)	Androgen[1] binding	AR[2] mRNA	AR[3] protein	Ref
Dunning	+	+	+	+	24-26
	-	+	+	+	24-26
	-	-	-	-	24-26
Noble	+	+	ND	ND	27
	-	±	ND	ND	27
	-	±	+*	ND	28,29
Pollard	-	ND	ND	ND	30
Shionogi	+	+	ND	ND	14,31
	+	+	+	+**	32
	-	-	ND	ND	31
	-	+	+	+**	32

[1] Androgen binding assay
[2] Northern blot analysis or *in situ* hybridization*
[3] Immunohistochemistry or chloramphenicol acetyltransferase assay**
± Testosterone-induced androgen binding

correlates well with the level of AR. Many attempts have been made in clinical studies on prostate cancer to relate AR status, as determined by [^{3}H]-ligand binding, to the response rate to endocrine treatment (Table 3.3). However, neither cytosol AR nor nuclear AR content

Table 3.3. Attempts to relate androgen receptor status[1] to response of prostate cancer to endocrine treatment

No. of Pts[2]	Fraction	Relationship	Ref
19	Cytosol	no	33
11	Cytosol	yes	34
11	Cytosol	no	35
23	Cytosol	yes	36
10	Cytosol	yes	37
15	Cytosol	yes	38
43	Cytosol	no	39
30	Nuclear	yes	40
26	Cytosol	yes	41
23	Nuclear	yes	42
	Cytosol	no	
16	Nuclear	yes	43
	Cytosol	no	
13[3]	Nuclear	yes	44
	Cytosol	no	
12[3]	Nuclear	yes	45
	Cytosol	yes	
30	Nuclear	no	46
	Cytosol	no	
37	Nuclear	yes	47
	Cytosol	no	
37	Nuclear	no	48

1 Androgen receptor status determined by androgen binding assay
2 All patients without previous therapy
3 Studies based on the same patient material

could accurately predict patient response to endocrine therapy (49). Methodological aspects of the [^{3}H]-ligand binding assay have been indicated as an explanation of the discrepancy between AR detection and androgen responsiveness. Lack of detectable receptors using radiolabelled ligands also prompts the question whether lack of binding indicates receptor absence or loss of binding function. In the latter situation, receptors may lack a hormone-binding domain and still be transcriptionally active (constitutively active) (50). On the other hand, [^{3}H]-ligand binding does not distinguish an intact receptor protein from a receptor that has lost function but retained ligand binding. Since androgen responsiveness seems to be linked to AR expression it is important to define events resulting in loss of receptor function by studying AR gene structure, messenger RNA, protein structure and the occurrence of post-translational modifications of the receptor protein. Failure to predict patient response to hormone therapy by biochemically determined nuclear hAR content has been attributed, next to the shortcomings of ligand binding assays as mentioned above, to tissue heterogeneity; that is, the contamination of the prostate cancer specimens by non-malignant prostate tissue.

The cloning of cDNAs, encoding the mouse, rat and human AR has provided a new

approach for the study of AR gene structure and AR expression in prostate cancer. Moreover, the elucidation of the primary protein structure of the hAR and the generation of antibodies against hAR fusion proteins and against synthetic peptides, corresponding to parts of the AR protein, have provided new tools to study functional receptor characteristics and receptor distribution in tissues, addressing the issue of heterogeneous AR expression.

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Androgen Receptor Heterogeneity in Human Prostatic Carcinomas visualized by Immunohistochemistry

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ANDROGEN RECEPTOR HETEROGENEITY IN HUMAN PROSTATIC CARCINOMAS VISUALIZED BY IMMUNOHISTOCHEMISTRY

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SUMMARY

Expression of the human androgen receptor was examined in 26 primary prostatic carcinomas by immunohistochemical staining with a polyclonal antibody reactive with the N-terminal domain of the human androgen receptor. Eighteen carcinomas showed homogeneous staining for the androgen receptor, whereas in seven cases a considerable heterogeneity in expression of the receptor was found. In one case, only a very limited number of immunoreactive tumour cells were detected. Comparison of androgen receptor expression with the tumour grading score, according to the MD Anderson grading system, revealed that the proportion of immunostained tumour cells and—to a lesser extent—the intensity of immunostaining were decreased in the more aggressive (grade III) tumours. The use of immunohistochemistry for detection of expression of androgen receptor in prostatic carcinomas may become a new and sensitive method for predicting prostatic tumour behaviour under hormonal therapy and prognosis.

KEY WORDS—Human androgen receptor, prostatic carcinoma, immunohistochemistry, tumour grading.

INTRODUCTION

Initial regression of prostatic carcinoma in response to endocrine treatment has become a well-established phenomenon. However, approximately 20–25 per cent of patients with prostatic carcinoma are unresponsive to endocrine therapy and eventually all prostatic carcinomas relapse to a status of androgen independence.[1,2]

Earlier studies on prostatic carcinomas showed that increased tumour grade and diminished responsiveness to hormonal therapy correlated with a decreased tissue level of androgen receptor.[3,4] In recent studies using a biochemical assay[5] or autoradiography,[6] this association was, however, disputed.

Admixture of benign prostatic glands in cancerous tissue as well as heterogeneity[7] of prostatic carcinoma may require assessment of androgen receptor expression at the microscopical level, using immunocytochemical techniques, as is now increasingly used for oestrogen receptor determination in breast cancer.[8]

The recent cloning and sequencing of the human androgen receptor cDNA permitted the synthesis of oligopeptides which were used to raise human androgen receptor specific antisera.[9,10] Here we describe the application of an antiserum reactive with the N-terminal domain of the human androgen receptor for detection of androgen receptor expression in human prostatic carcinoma.

METHODS

Samples of prostatic carcinoma removed from prostatectomy specimens immediately after surgery were rapidly frozen in chilled isopentane and stored in liquid nitrogen until use. Prostatectomy was performed only if the prostatic cancer was confined

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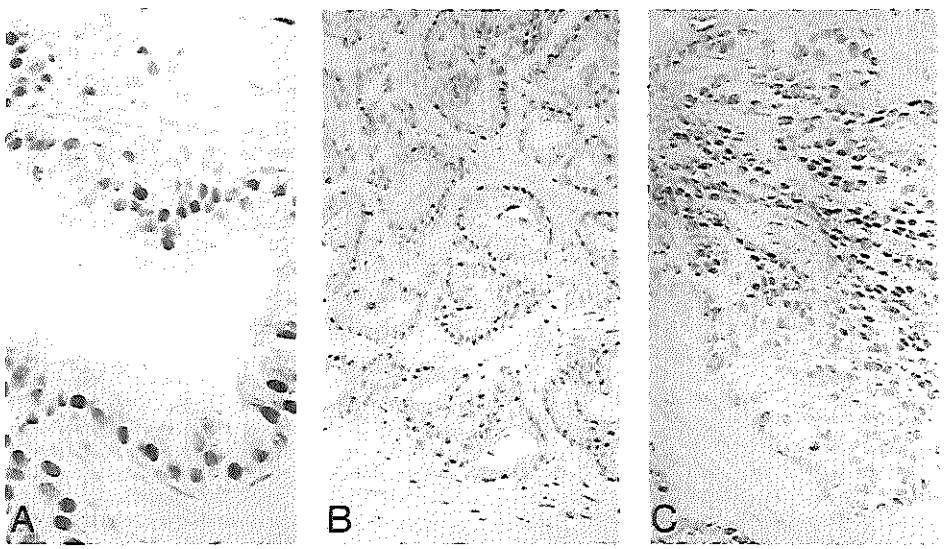

Fig. 1—(a) Hyperplastic prostatic glands surrounded by stromal cells. Nuclei of secretory epithelial cells and some stromal nuclei are reactive with the polyclonal anti-androgen receptor antibody Sp061. No nuclear counterstaining. (b) Well-differentiated adenocarcinoma of the prostate. The majority of tumour cells contain nuclei stained with Sp061. (c) Adenocarcinoma of the prostate with cribriform growth pattern showing considerable heterogeneity in androgen receptor expression

within the prostate (i.e., < stage T4). Tumour grading according to the MD Anderson grading system[11] was performed on haematoxylin–azophloxin stained cryostat sections adjacent to sections used for immunohistochemistry. Benign hyperplastic prostatic tissue was also derived from prostatectomy specimens.

The polyclonal antibody designated Sp061 used in this study was raised in rabbits hyperimmunized with a synthetic oligopeptide conjugated to keyhole limpet haemocyanin.[9] The synthetic oligopeptide represented a unique amino acid sequence of the N-terminal domain of the human androgen receptor, as indicated by analysis of cDNA coding for the human androgen receptor.[12,13] Sp061 contained high titre anti-androgen receptor antibodies directed against a single determinant, as previously established with sucrose density analysis, immunoprecipitation, and Western blotting. Immunoprecipitation studies with Sp061 showed no significant cross-reactivity with human progesterone, oestrogen, or glucocorticoid receptors.[9]

Immunocytochemistry was performed on 5 μm cryostat sections, air-dried for 30 min and subsequently fixed in buffered 4 per cent formalin for 10 min. Slides were then processed as described for detection of the oestrogen receptor.[8] Incubation with Sp061 diluted 1:500 in PBS (pH 7·8) was performed overnight at 4°C. Slides were washed again in two changes of PBS and reactivity was visualized using an indirect conjugated two-step immunoperoxidase method. Horse-radish peroxidase conjugated swine anti-rabbit immunoglobulin (DAKO, Denmark) diluted 1:100 in PBS containing 5 per cent non-immune human serum and 5 per cent non-immune swine serum was used as the conjugate. Diaminobenzidine was used as the substrate. Control slides incubated with the same dilution of the preimmune rabbit serum did not reveal reactivity.

For each tumour 200 cells were counted by two independent observers (JARdW and THvdK). The data of one observer (THvdK) have been given. In the tumours with areas showing different proportions of immunopositive tumour cells, each of these areas was counted separately. The average intensity of staining was estimated visually on an arbitrary scale of 0 (no staining) to 3 (intense staining). Data from both observers were analysed with the two-tailed Mann–Whitney *U*-test and reached the same level of significance.

Table I—Occurrence of androgen-receptor-positive cells in prostatic carcinomas

Grade	Specimen	Age	% AR-positive cells	Intensity
I	65–9	—	95	2
	175–14	44	88	3
	73–2	65	89	3
	144–10	65	82	2
	104–13	66	87	1
	132–13	62	5/52*	1
	135–5	51	94	1
II	96–7	63	82	2
	96–11	59	96	3
	70–12	53	88	2
	70–14	61	29/72*	1
	96–13	57	92	2
	73–7	59	83	2
	175–3	67	88	2
	175–9	59	90	2
	182–8	61	90	3
	132–4	67	86	2
	144–4	66	97	2
III	70–5	65	9	1
	144–1	60	84	2
	135–7	65	27	1
	135–13	59	0/67*	1
	70–7	67	49	2
	104–3	67	54	1
	135–9	59	44	1
	144–3	66	91	2

*The figures represent percentages of androgen receptor-positive tumour cells in two areas with clearly different proportions of immunopositive cells within the same section.

RESULTS

In hyperplastic prostatic tissues, Sp061 consistently stained the nuclei of the secretory epithelial cells of the prostatic glands. Notably, nuclei of the basal cells surrounding these secretory cells remained unstained. Furthermore, nuclei of a variable number of stromal cells were immunoreactive (Fig. 1a).

In 18 out of 26 primary prostatic carcinomas, more than 80 per cent of tumour cells (see, for example, Fig. 1b) revealed nuclear staining. In four tumours, a relatively high proportion of unstained tumour cells were intermingled with positive tumour cells (Fig. 1c) and in three cases largely unstained neoplastic glands, strands, or solid areas were adjacent to immunopositive tumour areas. In one case (specimen 70-5) of a largely undifferentiated carcinoma, only 9 per cent of androgen receptor-positive tumour cells was detected (Table I).

Solid tumour areas and tumour strands without glandular lumen formation displayed less reactivity with Sp061, while areas with a cribriform growth pattern contained variable proportions of immunopositive tumour cells. Only in one case were glandular areas immunonegative (specimen 132-13). It was noticed that the latter case showed considerable nuclear atypia.

Histological grading using the MD Anderson grading system revealed that 16 out of 18 grade I and II tumours contained over 80 per cent of immunopositive tumour cells, whereas 6 out of 8 grade III tumours showed considerable heterogeneity with a relatively large number of tumour cells lacking detectable androgen receptor expression. Statistical analysis showed that the high-grade tumours contained significantly lower percentages of androgen receptor-positive cells as compared with grade I and II tumours ($P = 0{\cdot}0133$).

DISCUSSION

This study shows that the antibody Sp061 specific for the N-terminal domain of the human androgen receptor can be used for immunohistochemistry on formalin-fixed frozen sections of prostatic tissues. The nuclear localization of the androgen receptor antigen in secretory epithelial cells fits well with data obtained earlier with other antisera or with autoradiography,[6,10] but contrasts with the reported cytoplasmic staining obtained with a monoclonal antibody raised against a partially purified androgen receptor protein.[14]

Our results demonstrate that the majority of primary prostatic carcinomas found in prostatectomy specimens largely consists of androgen receptor-positive tumour cells. These data are consistent with the well-known initial high responsiveness of most prostatic carcinomas to hormonal therapy.[1] On the other hand, particularly in the high-grade prostatic carcinomas (MD Anderson grade III), a variable percentage of tumour cells remain unstained after immunohistochemistry with Sp061, whereas only 2 out of 16 lower grade tumours showed substantial heterogeneity with respect to androgen receptor expression by tumour cells. This correlates well with the more aggressive behaviour of grade III carcinomas.

In conclusion, immunohistochemical determination of the androgen receptor status of primary and metastatic prostatic carcinomas has now become feasible and may provide a useful adjunct in the set-up of trials on the management of prostatic carcinoma. Issues such as timing of androgen ablation and chemotherapy may be addressed in patient groups with a different receptor status. The development of monoclonal antibodies specific for various domains of the androgen receptor[15] may be of additional help.

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Androgen Receptor Expression in Human Tissues: An Immunohistochemical Study

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Original Article

Androgen Receptor Expression in Human Tissues: An Immunohistochemical Study

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The cellular localization of the human androgen receptor was visualized immunohistochemically using a mouse monoclonal antibody (MAb) F39.4, directed against a fragment of the N-terminal domain of the androgen receptor. The nuclear immunoreactivity of various human tissues with F39.4 was generally consistent with earlier biochemical and autoradiographic data. However, previously suggested androgen receptor expression in thyroid, pancreatic, gastrointestinal, and bladder tissues was not confirmed immunohistochemically. Stratified squamous epithelia of vagina and cervix showed selective immunostaining of the basal cell layer, whereas in the preputial epithelium the intensity of immunoreactivity decreased gradually with maturation. In contrast, glandular epithelia of the sweat glands, male accessory sex organs, and female breast showed nearly exclusive F39.4 staining of the inner cylindric layer. In the testis, Sertoli cells, peritubular myoid cells, and interstitial cells were immunoreactive with MAb F39.4. Expression of the androgen receptor by smooth muscle tissue was largely confined to the male reproductive organs. The specificity and sensitivity of this simple and rapidly performed immunohistochemical technique in the detection of the human androgen receptor at the cellular and subcellular level makes it worthwhile to study tissue androgen receptor expression by immunohistochemistry in physiological and pathological states. (*J Histochem Cytochem 39:927–936, 1991*)

KEY WORDS: Human androgen receptor; Immunohistochemistry; Human tissues; Male accessory sex glands.

Introduction

The development and physiological function of male accessory sex organs are dependent on androgens. Similarly, androgens play an important role in the functioning of several other organs and tissues. Androgen action in these organs and tissues is believed to be mediated either directly by the androgen receptor or, after aromatization, via the estrogen receptor (25).

The androgen receptor belongs to the superfamily of ligand-responsive transcription regulators, which includes the retinoic acid receptors, the thyroid hormone receptors, and several steroid hormone receptors (7,12). Androgen receptors are nuclear proteins which are expressed at variable, relatively low levels in a number of tissues.

Studies on tissue distribution of the androgen receptor performed thus far made use of the ligand binding properties of these receptors, either in tissue homogenates or in tissue sections, by use of autoradiography. Biochemical exchange assays generated quantitative data on the androgen receptor content of tissue homogenates but did not provide information about cellular and subcellular distribution of this receptor. [^{3}H]-steroid autoradiography is a laborious and time-consuming procedure and does not always lead to detailed cellular localization of steroid receptors in tissues owing to background labeling. Moreover, fluorescein-conjugated androgens are of no use in the detection of androgen receptors (4). Therefore, it was anticipated that an easily performed immunohistochemical technique, being more sensitive and specific than autoradiography, could contribute to understanding of the detailed cellular and subcellular distribution of the androgen receptor.

The isolation and characterization of the human androgen receptor cDNA permitted the establishment of the amino acid sequence of the androgen receptor protein (9,14,22,37). Although all receptors belonging to the thyroid hormone/retinoic acid/steroid receptor superfamily exhibit a similar functional domain structure, the N-terminal transactivation domain of the androgen receptor displays a low degree of homology with the other steroid hormone receptors (5,12,13). After identification of unique immunogenic sequences in the N-terminal domain of the androgen receptor, we prepared androgen receptor-specific polyclonal and monoclonal antibodies (39,46). Monoclonal antibody (MAb) F39.4 proved to be very effective in demonstrating the androgen receptor, both in immunohistochemistry on formalin-fixed frozen sections and in several biochemical assays with high specificity and affinity (38,46).

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Since we were interested in the immunohistochemical characteristics of MAb F39.4 with regard to detection of the androgen receptor in non-reproductive and reproductive tissues at the cellular and subcellular level, we undertook a body survey with this MAb.

Materials and Methods

Antibodies. MAb F39.4 was prepared and characterized as described previously (38,46). In brief, Balb/c mice were immunized with a synthetic peptide conjugate corresponding to amino acids 301–320 of the N-terminal domain of the human androgen receptor conjugated to keyhole limpet hemocyanin. Murine spleen cells were fused with Sp2/0 myeloma cells. Hybridomas were screened for specific antibody production in an ELISA. Supernatants selected by ELISA were subsequently screened in the rabbit anti-mouse immunoglobulin (RαM) agarose screening assay for their ability to adsorb [^{3}H]-R1881–androgen receptor complexes from nuclear extracts of the androgen receptor-positive human prostate carcinoma cell line LNCaP (16). Hybridoma F39.4 was selected on the basis of its strong reactivity with radioactively labeled ligand-bound nuclear extract from the LNCaP cells in a sucrose gradient density centrifugation assay and its good performance in immunohistochemistry (46). Western blot analysis showed that F39.4 is reactive with a protein with an apparent size of 110 KD in a lysate from the LNCaP cell line (38). Specificity of F39.4 for the human androgen receptor was established both in immunoprecipitation assays (46) and in a goat anti-mouse immunoglobulin (GαM) agarose test using nuclear extracts from LNCaP (androgen receptor), NHIK (glucocorticoid receptor), MCF7 (estrogen receptor), and T47D (progesterone receptor), and radiolabeled R1881, dexamethasone, estradiol, R5020, respectively, as ligand (Figure 1). Slight crossreactivity with estrogen receptors was seen only at a very high antibody concentration.

Tissue Sources. Fresh human tissues were collected from patients undergoing surgery. One cryptorchid testis was included in the study. Non-diseased testis and epididymis were obtained from autopsy cases. These autopsies were performed within 6 hr after decease of the patient. The tissues investigated are listed in Table 1. Tissue specimens were transported to the laboratory within 15 min after surgery, and were stored in liquid nitrogen until further use.

Figure 1:
Cross reactivity of F39.4
in GaM agarose assay.

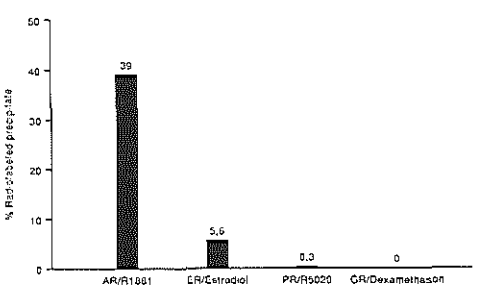


Figure 1. Specificity of F39.4 for the androgen receptor tested in a GαM agarose assay using nuclear extracts from LNCaP, MCF7, T47D, and NHIK as a source for androgen receptor, estrogen receptor, progesterone receptor, and glucocorticoid receptor, respectively.

Immunohistochemical Techniques. Frozen tissues were mounted on cryostat chucks in Tissue-Tek II OCT compound obtained from Miles Laboratories (Naperville, IL). Air-dried 6-μm thick cryostat sections of fresh frozen tissues were fixed in 4% formalin (10 min, 4°C) and dehydrated in chilled methanol (4 min, −20°C) and acetone (2 min, −20°C). Preliminary experiments indicated that the epitope of androgen receptor defined by F39.4 is sensitive to formalin fixation, which prohibits its use on routinely fixed paraffin-embedded material. Formalin fixation lasting for more than 10 min or omission of methanol in the fixation procedure led to deterioration of staining. To minimize nonspecific binding of reagents in subsequent steps, sections were first treated with normal rabbit serum diluted 1:10 in PBS (pH 7.4) for 15 min at room temperature. Incubation with MAb F39.4 (hybridoma culture supernatant), diluted 1:100 in PBS containing 0.5% BSA and 0.1% sodium azide (pH 7.8), was performed overnight at 4°C. After washing in PBS for 10 min, sections were incubated with horseradish peroxidase-conjugated RαM IgG (Dakopatts; Glostrup, Denmark) diluted 1:100 in PBS, for 30 min at 37°C. Alternatively, the indirect unconjugated peroxidase-antiperoxidase complex method (PAP) was used employing unconjugated RαM (Dakopatts) as linking reagent and PAP complexes (Sigma; Munchen, FRG) as tertiary reagent (20). Both reagents were diluted 1:100 in PBS. Incubations with RαM and PAP complexes were performed at 37°C for 30 min. Because the PAP method led to significantly better staining results, the indirect conjugated method was employed only for staining of male accessory sex glands, as they appeared to have a high androgen receptor content.

After rinsing of the sections in PBS, they were incubated with 3,3'-diaminobenzidine (DAB; Sigma) containing 0.03% hydrogen peroxide for 7 min at room temperature. Nuclear staining intensity of the prostate secretory epithelial cells in glandular hyperplasia was considered high and was used for comparison with staining intensity found in other organs or tissues. Sections of lymph nodes incubated with the primary antibody were used as negative control for F39.4.

To confirm androgen receptor expression by smooth muscle cells in male reproductive organs, a double immunoenzymatic staining on androgen receptor and desmin was performed sequentially using horseradish peroxidase and alkaline phosphatase as enzymatic labels (27). After overnight incubation with F39.4 and immunostaining with the indirect conjugated peroxidase method, slides were subsequently incubated with monoclonal anti-desmin antibody (Sanbio; Uden, The Netherlands) diluted 1:20 in PBS

Table 1. *Human tissues examined*[a,b]

Tissue	M	F	Tissue	M	F
Myocardium	2	2	Skeletal muscle	3	2
Lung	2	0	Prostate	2	-
Esophagus	1	1	Seminal vesicle	2	-
Stomach	1	1	Vas deferens	2	-
Colon	1	2	Epididymis	3	-
Salivary gland	1	3	Testis	3	-
Liver	2	2	Prepuce	2	-
Pancreas	0	2	Skin	2	3
Thyroid gland	2	0	Vagina	-	1
Adrenal gland	2	0	Cervix	-	4
Kidney	1	2	Uterus	-	4
Ureter	0	2	Mammary gland	0	3
Bladder	2	0	Sweat gland	1	3
Thymus	0	3	Sebaceous gland	2	2
Lymph node	1	1			

[a] M, male; F, female.
[b] Figures represent the number of cases investigated.

for 30 min at 37°C. Visualization of desmin was performed with the indirect conjugated alkaline phosphatase method (Dakopatts), using a substrate yielding an alcohol-resistant red precipitate (Vector; Burlingame, CA).

Results

Reproductive Tissues

MAb F39.4 yielded an intense staining reaction in all male reproductive organs, excluding the testis (Table 2). In general, the exclusive nuclear staining reaction was observed in the columnar epithelial cells of the prostate, the seminal vesicle, and the epididymis, whereas the basal epithelial cells of these organs did not show detectable androgen receptor expression (Figure 2). In the vas deferens, occasional basal cells showed an intense nuclear staining reaction. Stromal cells of the prostate, seminal vesicle, and epididymis displayed variable immunoreactivity. Immunoenzymatic double staining showed that a substantial proportion of (desmin-positive) smooth muscle cells of these male accessory sex organs were reactive with F39.4 (Figure 2A).

In both normal and cryptorchid testis, F39.4 gave a moderate staining reaction with the nuclei of a circle of cells located close to the spermatogonia. This localization of the immunoreactive nuclei within the transversely cut seminiferous tubules indicates that the stained cells are Sertoli cells (34). F39.4 also stained nuclei of peritubular myoid cells and testicular fibroblasts. Leydig cell androgen receptor expression was variable in normal testis but uniform in cryptorchid testis. Spermatogonia, spermatids, and spermatozoa did not stain with MAb F39.4 (Figure 2D).

In the stratified squamous epithelium of the penile prepuce, highest reactivity with F39.4 was in the basal cell layers, gradually diminishing in the more superficial layers (Figure 3A). In the vagina and cervix, F39.4 yielded detectable reactivity only in the basal cell layer of the stratified squamous epithelium (Table 2). Stromal fibroblasts of preputium, cervix, and vagina also displayed variable and moderate reactivity (Figures 3C and 3D). No immunostaining was observed in the reserve cells and columnar cells lining the cervical glands. No significant staining with F39.4 was observed in the uteri examined.

Skin, Its Appendages, and Mammary Gland

Although in non-genital skin the epidermis and the hair follicles did not reveal detectable nuclear staining with MAb F39.4 (Figure 3B), sebaceous glands, sweat glands, and ducts were immunopositive (Figures 4A and 4B; Table 2). In sebaceous glands most nuclei were positive, whereas in sweat glands and ducts only a proportion of cells were stained. Notably, the myoepithelial cells did not show detectable F39.4 reactivity. Expression was not detectable in dermal or in subcutaneous mesenchymal cells.

Immunostaining of the mammary skin and associated sebaceous glands did not differ widely from skin staining patterns previously mentioned (Table 2). In one of three mammary gland specimens studied, the nuclei of mammary acinar cells were labeled with F39.4. Some of the nuclei of the inner ductal epithelial lining cells showed a moderate staining reaction. Myoepithelial cells were not stained (Figure 4C).

Digestive, Respiratory, and Urinary Tracts

No staining with F39.4 was found in any of the tissue constituents of the salivary glands, esophagus, stomach, colon, pancreas, bronchi, alveoli, kidney, or bladder. In the liver the comparatively weak reactivity was confined to the nuclei of the hepatocytes (Table 2).

Muscle

In myocardial biopsy specimens from two male patients, low-intensity nuclear androgen receptor expression was found, which contrasts with the negative staining results obtained on myocardial biopsy specimens from the two female patients (Figure 5A). The androgen receptor was not detectable in skeletal muscle cell nuclei (Figure 5B). Smooth muscle of bronchi, intestines, and bladder lacked detectable immunoreactivity.

Other Tissues

Lymph nodes, thymus, thyroid gland, parathyroid gland, and adrenal gland revealed no immunoreactivity with F39.4 (Table 2). Similarly, no staining of peripheral neural tissue, including ganglion cells, was found in the tissues examined.

Table 2. *Androgen receptor immunoreactivity*[a]

Testis		Thymus	−
Sertoli cells	+	Lymph nodes	−
Germ cells	−		
Myoid cells	+ / −	Skeletal muscle	−
Leydig cells	+	Cardiac muscle	+
Fibroblasts	+ / −		
		Skin	
Prostate, seminal vesicle,		Keratinocytes	−
vas deferens,[b] epididymis		Fibroblasts	−
Secretory cells	+ +	Sebaceous glands	+ +
Basal cells	−	Sweat glands	+ + / −
Fibroblasts	+ + / −		
Smooth muscle cells	+ + / −		
		Respiratory tract	−
Foreskin			
Keratinocytes[c]	+ + / +	Urinary tract	−
Fibroblasts	+ / −		
		Gastrointestinal tract	−
Ectocervix, vagina			
Squamous cells[d]	+ / −		
Fibroblasts	+ / −	Salivary glands	−
		Hepatocytes	+
Endocervix	−	Bile ducts	−
		Exocrine pancreas	−
Uterus	−		
Mammary gland			
Ducts	+ + / −	Thyroid	−
Acini	+ + / −	Pancreatic islets	−
Myoepithelium	−	Adrenal gland	−

[a] Signs designate high (+ +), moderate (+), no (−) immunoreactivity or variable (+ + / −, + / −) immunoreactivity.
[b] A significant proportion of basal cells in the vas deferens showed intense nuclear staining.
[c] Androgen receptor expression both in the basal and in the more superficial cell layers.
[d] Only the basal cell layer is reactive with F39.4.

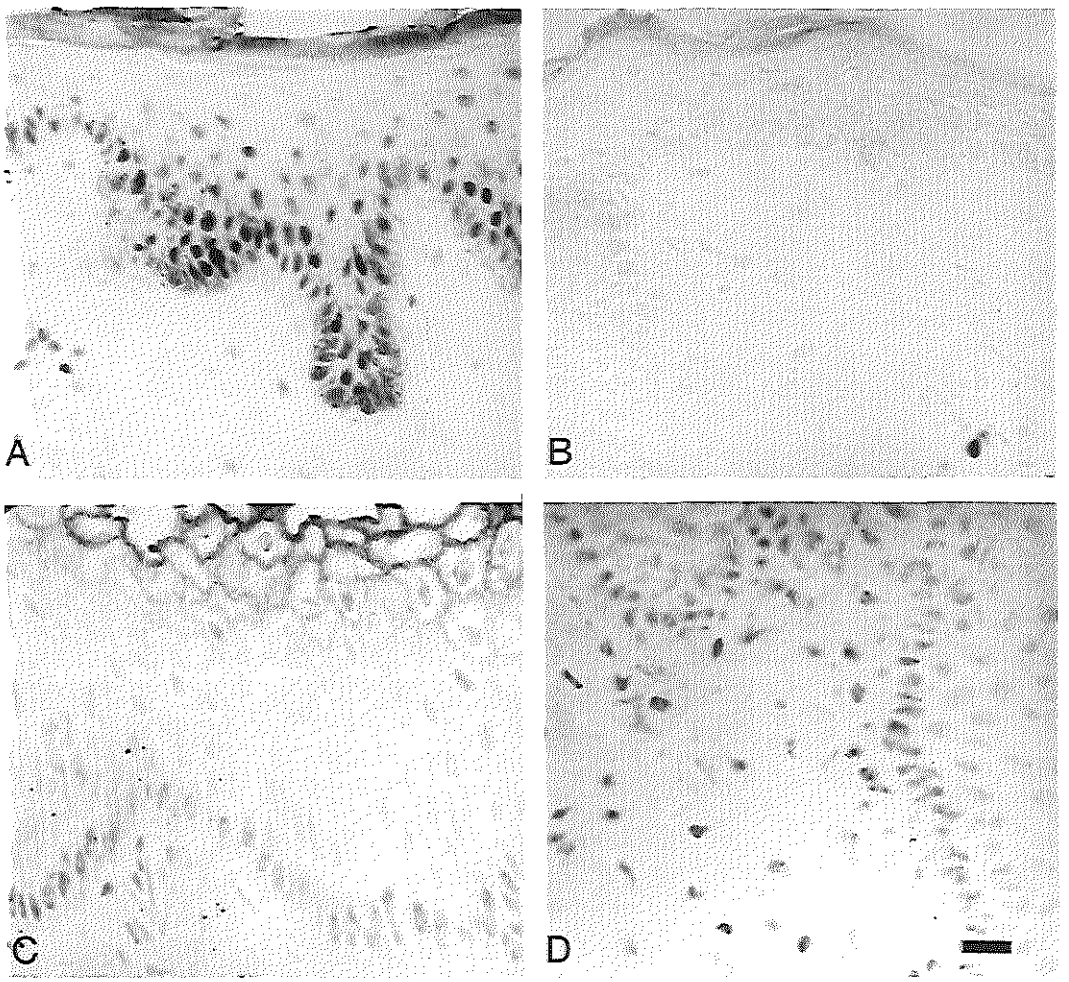
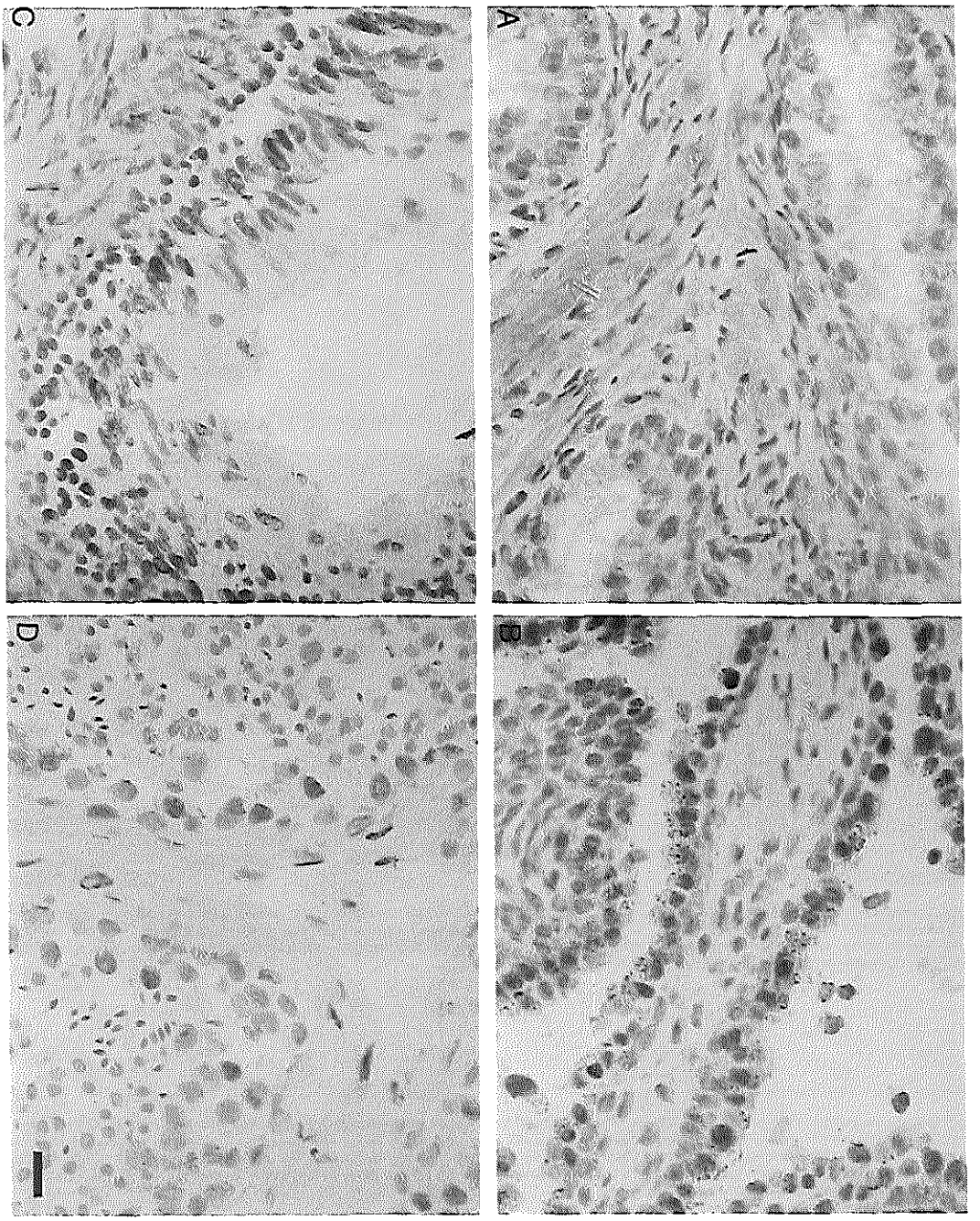


Figure 3. Immunostaining of stratified squamous epithelia with F39.4 visualized with the indirect PAP technique. (A) Immunoreactivity of the basal and more superficial epithelial cells of the foreskin and variable staining of preputial fibroblasts. (B) Epidermis without detectable reactivity. (C) Ectocervix with faint reactivity of the basal cell layer and nonspecific membrane staining of superficial cells. (D) Vagina with faint reactivity of the basal cells and rather intense labeling of stromal fibroblasts. No nuclear counterstaining. Original magnification × 310. Bar = 20 μm.

Figure 2. Immunostaining of male sex organs for androgen receptor expression with F39.4 visualized with (A) the indirect PAP technique or with (B–D) the indirect conjugated peroxidase technique. (A) Prostatic hyperplastic tissue stained with immunoperoxidase for F39.4 reactivity and with immunoalkaline phosphatase for expression of desmin. Both the inner layer of secretory epithelial cells and desmin-positive cells are reactive with F39.4. (B) Vesicula seminalis with nuclear staining of the inner layer. (C) Epididymis with selective nuclear staining of the cylindric cells. (D) Testis in which Sertoli cells and peritubular myoid cells are stained. Nuclear counterstaining with Mayer's hematoxylin. Original magnification × 310. Bar = 20 μm.


A
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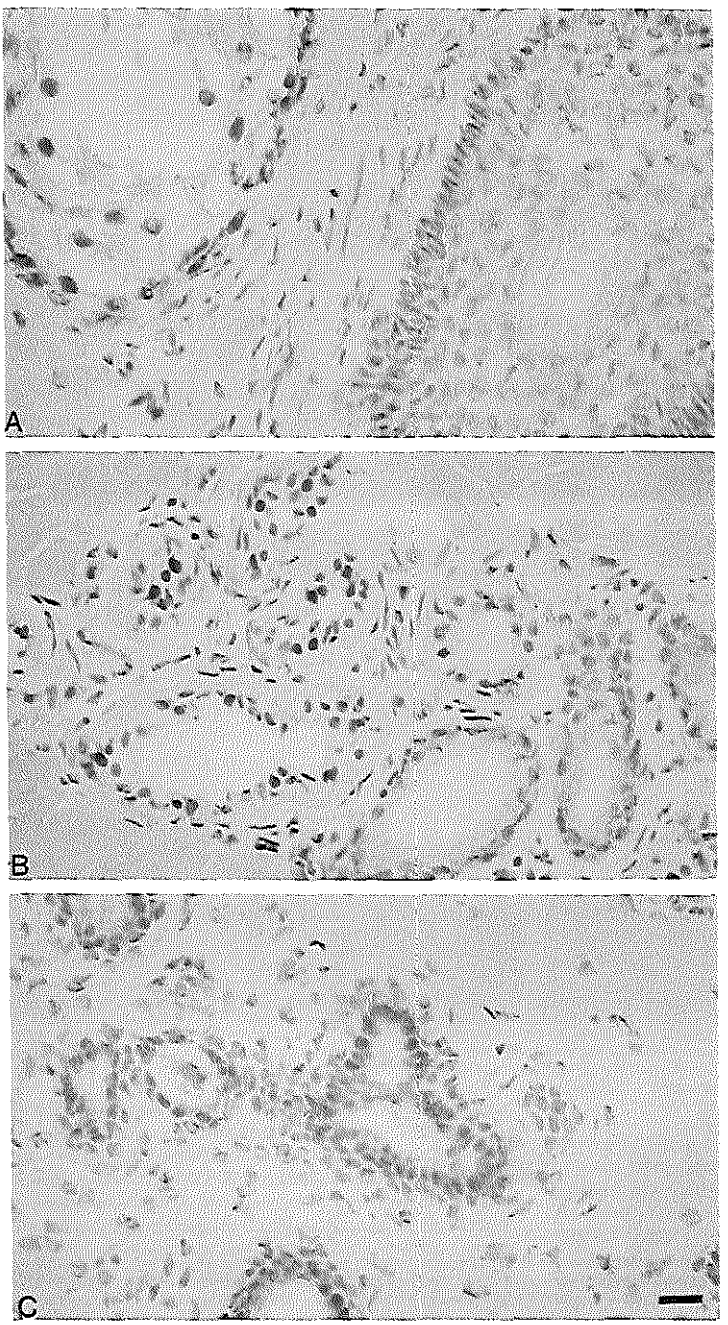

Figure 4. Immunostaining of skin appendages and mammary gland with F39.4 visualized with the indirect PAP technique. (A) Intense staining of sebaceous gland and no reactivity with hair follicle. (B) Variable but intense staining of sweat glands. (C) Mammary acini with staining of the inner cells. Nuclear counterstaining with Mayer's hematoxylin. Original magnification × 310. Bar = 20 µm.

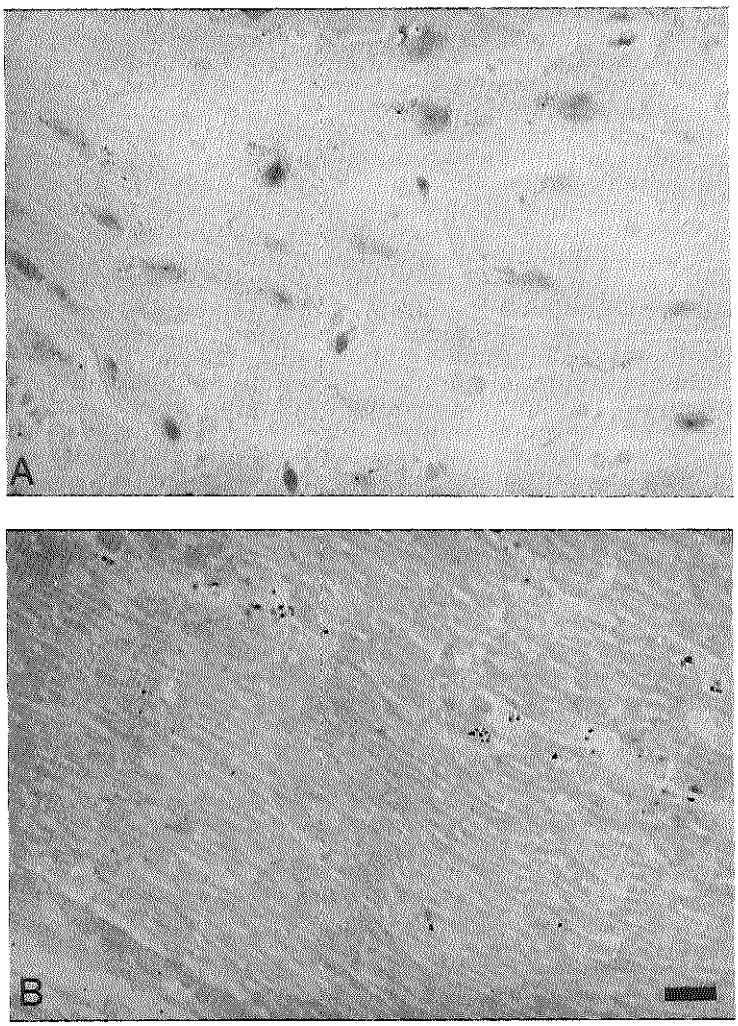

Figure 5. Immunostaining of (A) cardiac muscle and (B) skeletal muscle with F39.4, visualized with the indirect PAP technique. Reactivity is found only in cardiac muscle. No nuclear counterstaining. Original magnification × 310. Bar = 20 μm.

Discussion

The immunohistochemical data here reported on androgen receptor localization in human tissues largely confirm those obtained previously with biochemical ligand binding assays and autoradiography (1–3,17,29,31,33,45). They extend the observations made on tissue homogenates in that the exact cellular localization within several tissues and organs has now been visualized (Table 2).

In all tissues examined immunohistochemically the selective nuclear localization of the androgen receptor has now been established. The nuclear localization of androgen receptor was previously described in tissues of various species by autoradiography with radiolabeled androgens (3,17,24,26,29,35,36,40–44). Using well-defined polyclonal antibodies and a monoclonal antibody to the androgen receptor, other authors similarly found an exclusively nuclear localization in the human prostate secretory epithelial cells (8,18,23). This subcellular localization of the androgen receptor is in line with the reported predominantly nuclear localization of other sex steroid receptors, in both the presence and the absence of the specific ligand (30).

The absence of immunostaining of the thyroid gland, pancreas,

salivary gland, and interstitial tissues of the gastrointestinal and urinary tract with our MAb F39.4 is in contrast to positive findings in these organs with ligand binding assays on tissue homogenates or with autoradiography (10,11,26,36,40,43). Although species differences may in part explain these discrepancies, the inability to detect the androgen receptor immunohistochemically in human pancreatic and renal tissue may be attributed to the very low androgen receptor content of these tissues. Alternatively, binding of radioactively labeled ligands to non-receptor proteins may have led to false-positive labeling of tissue sections. Labeling of both thyroid follicle lining epithelial cells and colloid in baboon thyroid glands further suggests that nonspecific binding of tritiated androgens to proteins unrelated to the androgen receptor may occur (36). Similarly, it was reported that high nonspecific background labeling of hepatic tissue by radiolabeled dihydrotestosterone precluded the unequivocal identification of the androgen receptor in this organ (43).

Since endogenous androgens interfere with autoradiographic examination of androgen receptor expression, these studies were performed only on adrenalectomized and castrated animals. Immunohistochemistry with F39.4 made it possible to study androgen receptor expression in adrenal gland and testis as well. The presence of androgen receptor in Sertoli cells and interstitial Leydig cells is consistent with data from cultures of purified Sertoli cells and interstitial Leydig cells (1). The immunohistochemically detectable expression of 5α-reductase in adult rat germ cells has led some authors (32) to suggest a direct influence of testosterone in promoting and controlling the activity of initiation factors for germ cell protein synthesis. The observed androgen receptor expression in Sertoli cells and lack of its expression in germ cells indicate that androgens mediate their effect on spermatogenesis indirectly.

Thus far, we have not found conclusive evidence for sexual dimorphism in the expression of the androgen receptor in various tissues. Although in the four specimens of myocardial tissue tested it was noted that only the two specimens from male patients demonstrated detectable androgen receptor expression, this seeming sex difference could also be attributed to the presence of myocardial hypertrophy in these two specimens. Earlier autoradiographic and biochemical ligand binding data on both baboon and Rhesus monkey cardiac muscle demonstrated androgen receptor expression in both male and female animals (24,35). Immunoenzymatic double labeling experiments revealed that high-level immunostaining of smooth muscle was largely confined to the male reproductive organs. Expression of the androgen receptor in prostate fibroblasts and smooth muscle cells is consistent with the known androgen dependence of fibromuscular hyperplasia of the prostate.

The selective expression of androgen receptor in the basal cell layer of the stratified squamous epithelia of cervix and vagina and the declining staining intensity for androgen receptor in the more superficial epidermis of the penile foreskin suggest a role of androgen receptor in the differentiation of the epithelial cells of these organs. As no immunostaining was observed in the reserve cells of the glandular cervical epithelium, whereas the latter cells do have the potential to differentiate into squamous epithelium, an involvement of androgen receptor can be postulated in squamous metaplasia of the transition zone of the cervix.

The highly variable androgen receptor expression of the subepithelial foreskin fibroblasts contrasts not only with the reported androgen receptor content of these cells but also with the high level of androgen receptor expression by the squamous epithelial cells of the penile foreskin (6,31). Selection of preputial fibroblasts with a high content of the androgen receptor owing to in vitro conditions may explain the suitability of cultured foreskin fibroblasts in the in vitro study of androgen receptor properties in cases of androgen insensitivity syndrome. However, the high androgen receptor levels in keratinocytes suggests the use of these cells rather than fibroblasts in such studies.

The demonstration of androgen receptor in sebaceous glands of male and female skin confirms previous data from biochemical ligand binding studies (33). Studies performed recently on androgen receptor content of the pilosebaceous unit of scalp, beard region, and pubic skin revealed immunostaining with F39.4 in the dermal papilla of the hair follicle (15). It is obvious that further studies are required on androgen receptor expression in cells of the pilosebaceous unit to evaluate its role in androgen-dependent processes such as alopecia and acne.

Most striking was the detection of androgen receptor in variable proportions of the inner lining epithelial cells of mammary ducts and acini of females and in sweat glands of both males and females. The presence of androgen receptor in these structures suggests a functional role of androgens that is hitherto unknown. Furthermore, androgen receptor may be involved in the genesis of benign (19) and malignant neoplasms of the breast. A substantial proportion of breast carcinomas do indeed express androgen receptor (21). Similarly, a role of androgen receptor has been suggested for the genesis of hepatocellular carcinomas, as males are disproportionally inflicted by this latter malignancy (28).

In conclusion, the availability of a highly specific monoclonal antibody directed against the human androgen receptor will enable the study of androgen receptor expression at the cellular level in both physiological and pathological processes of human organs by application of a simple and rapid immunohistochemical technique.

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Chapter 6

Androgen Receptors in Endocrine-Therapy-Resistant Human Prostate Cancer

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ANDROGEN RECEPTORS IN ENDOCRINE-THERAPY-RESISTANT HUMAN PROSTATE CANCER

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Despite the initial androgen-dependent growth of most human prostate cancers, eventually all prostate cancers become androgen-independent at varying intervals after androgen ablation or anti-androgen therapy. In order to gain more insight into the role of the androgen receptor (AR) in this process, AR and prostate-specific antigen (PA) expression was evaluated immunohistochemically in prostatic tumour tissues from patients who developed urinary flow obstruction between 4 and 107 months after onset of treatment. AR expression was evaluated with a monoclonal antibody (MAb) specific for the N-terminal domain of the human AR. To substantiate the progressive tumour growth, proliferative activity was assessed immunohistochemically by staining with MAb Ki-67. Ki-67-defined tumour-growth fractions varied from 0.8–64.7%. In 13 of the 17 examined tumours over 80% of the tumour cells were AR-positive, 3 tumours showed a considerable heterogeneity in AR expression and in 1 tumour almost all tumour cells seemed to be AR-negative. Two-thirds of the examined tumours contained variable proportions of PA-positive tumour areas. These observations contrast with the view that androgen ablation induces a preferential outgrowth of receptor-negative tumour cells.

In hormonal therapy of steroid sensitive cancers, the relapse of disease in virtually all patients is a generally recognized problem. Human prostate cancer is no exception to this rule, though initially a high percentage of tumours is androgen-sensitive (Lepor *et al.*, 1982). The mechanism of the development of steroid hormone unresponsiveness remains unclear. Experimental studies on prostate and breast cancer cell lines have indicated that after steroid depletion, hormone-insensitive tumours can develop from tumours composed of responsive and non-responsive subpopulations. Most of these non-responsive tumour cell lines expressed decreased levels of ligand bindings as compared with the parental cell line (Isaacs *et al.*, 1982; Sluyser *et al.*, 1981; Diamond and Barrack, 1984). These findings suggest that steroid depletion of initially steroid-dependent cancers may result in the selective outgrowth of tumour cells lacking these receptors.

Particularly in human breast cancer, data are accumulating to show that steroid-insensitive tumours may still express oestrogen receptors. A considerable percentage of oestrogen-receptor-positive breast cancers does not respond to hormonal therapy and a number of oestrogen-insensitive human breast cancer cell lines were developed from responsive parental lines that do express oestrogen receptors (Clarke *et al.*, 1989). Similarly, sublines of the androgen-dependent breast cancer cell line Shionogi 115 generated by long-term depletion of androgens both *in vivo* and *in vitro* do contain unchanged levels of androgen receptors (AR) (Nonomura *et al.*, 1988). This suggests that in human cancers initially responsive to endocrine manipulation, the transition to steroid insensitivity does not need to be accompanied by a loss of these receptors.

Recently, analysis of AR expression by Northern blotting and by immunohistochemistry with polyclonal antibodies was performed on androgen-sensitive and -insensitive Dunning rat prostate carcinoma cell lines. Six out of the 7 tested androgen-insensitive sublines were lacking AR at both the mRNA and protein levels (Quarmby *et al.*, 1990*a*). Besides, human prostate tumour cell lines which are androgen-unresponsive do not contain AR mRNA (Trapman *et al.*, 1990). To our knowledge, no ligand binding or immunohistochemical data are yet available on the expression of steroid receptors in human prostate cancers at the time of relapse after long-term steroid depletion.

More particularly in prostate cancers, ligand binding assays tend to yield spurious results due to tumour heterogeneity and admixture of AR-positive benign prostatic epithelial and stromal cells (Gorelic *et al.*, 1987). The recent availability of well-characterized antibodies specific for the N-terminal domain of the human AR has permitted the direct visualization of this receptor in prostatic tissues (Lubahn *et al.*, 1988; Ruizeveld de Winter *et al.*, 1990; Zegers *et al.*, 1991). Previously, we demonstrated in an immunohistochemical study that at diagnosis most primary prostatic carcinomas display detectable AR expression in the majority of the tumour cells (Ruizeveld de Winter *et al.*, 1990). The purpose of the present immunohistochemical investigation is to examine AR expression in prostatic carcinomas during local relapse under endocrine treatment using an MAb specific for a unique epitope in the N-terminal domain of the human AR (Zegers *et al.*, 1991). Local relapse is a not infrequent complication of prostatic carcinoma under endocrine treatment leading to urinary flow obstruction. Tumour growth at the time of transurethral resection during androgen ablation therapy was confirmed in our study by determination of the cell growth fraction with the Ki-67 MAb. This antibody is directed against a proliferation-associated nuclear antigen (Gerdes *et al.*, 1984). Since prostate-specific antigen (PA) is increasingly used as a tumour marker for early detection of relapse of prostatic carcinoma (Kuriyama *et al.*, 1981) we also evaluated its expression in tumour specimens obtained from patients showing tumour progression during endocrine manipulation.

MATERIAL AND METHODS

Tumour specimens

From 17 patients with a histologically proven adenocarcinoma of the prostate, 11 were treated with subcapsular orchiectomy, 3 with (ethyl-)oestradiol and 3 more patients, respectively with cyproterone-acetate (CPA), LHRH agonists or a combination of ethyl-oestradiol and CPA (Tables I and II). At varying intervals after initiation of therapy, the patients underwent transurethral resection of the prostate for relief of progressive urine flow obstruction. Histology of transurethrally resected prostatic tissues revealed in all cases that they were diffusely infiltrated by prostatic carcinoma accounting for urinary obstruction. Transurethrally resected prostatic tissues were freshly frozen immediately after surgery and stored in

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TABLE I – EXPRESSION OF AR AND PA IN PROSTATIC CARCINOMAS WITH PROGRESSION AFTER ORCHIECTOMY

Case	Months after orchiectomy	Tumour grade[1]	% AR pos. tumour area[2]	% AR pos. tumour cells[3]	Ki-67 index	% PA pos. area[2]
1	10	3	100	93	6.3	60
2	12	1	100	87	0.8	0
3	14	3	100	95	13.0	60
4	14	2	100	55	8.0	0
5	14	3	97	99	26.3	80
6	15	3	100	96	7.3	80
7	18	2	95	76	8.8	70
8	21	3	2	98	64.7	2
9	21	2	100	92	40.0	70
10	48	2	100	99	30.8	nd
11	60	3	100	89	7.8	40

[1]Tumour grade at time of transurethral resection due to local progression.–[2]Figures reflect the percentage of the tumour area with immunoreactivity for F39.4 (AR-positive) and ER-PR1 (PA-positive).–[3]Figures represent the percentage of F39.4 immunopositive tumour cells within the immunoreactive areas.

TABLE II – EXPRESSION OF AR IN PROSTATIC CARCINOMAS WITH PROGRESSION DURING ENDOCRINE THERAPY

Case	Treatment	Months of therapy	Tumour grade[1]	% AR pos. tumour area[2]	% AR pos. tumour cells[3]	Ki-67 index
12	Estradiol	36	3	100	98	14.3
13	Estradiol	56	3	100	95	30.8
14	Estradiol	65	2	100	98	10.0
15	CPA	4	3	40	75	2.5
16	LHRH agonist	60	3	100	93	8.3
17	Estradiol/CPA	107	3	100	81	18.0

[1]Tumour grade at time of transurethral resection due to local progression.–[2]Figures reflect the percentage of tumour area reactive with F39.4.–[3]Figures represent the percentage of F39.4 immunoreactive (AR-positive) tumour cells within the immunopositive tumour areas.

liquid nitrogen until further processing. The presence of prostatic carcinoma in frozen sections used for immunohistochemistry was confirmed on adjacent sections stained with haematoxylin and azophloxin. Grading on paraffin-embedded specimens of the corresponding cases of orchiectomized patients was performed using the criteria of the (modified) Anderson system (Ruizeveld de Winter *et al.*, 1990). As compared with the grade of the original tumour at diagnosis in 4 cases (cases 8, 11, 12 and 15) an increase in tumour grade was noted (Table I and II).

Antibodies

Mouse MAb F39.4 was raised against a synthetic oligopeptide corresponding to a unique epitope situated at the N-terminal domain of the human AR. F39.4 is specifically reactive with the human AR in immunoprecipitation studies, Western blot and sucrose gradient analysis without showing cross-reactivity with other steroid receptors (Zegers *et al.*, 1991). The antibody precipitates a 100-kDa protein from the human prostatic cancer cell line LNCaP, which is in agreement with the molecular weight of the human AR. In benign prostatic tissue the antibody stains the nuclei of secretory epithelial cells and of a proportion of the stromal cells (Zegers *et al.*, 1991). MAb ER-PR1 is specifically reactive with a 34-kDa protein present in prostate lysates which corresponds with the molecular weight of prostatic antigen (PA). In immunohistochemistry ER-PR1 is exclusively reactive with prostatic secretory epithelial cells and prostatic carcinomas (Gallee *et al.*, 1986). Ki-67 (DAKO, Copenhagen, Denmark) defines a proliferation-associated nuclear antigen which is present during most of the G_1-phase, the S-, G_2- and M-phase of the cell cycle (Gerdes *et al.*, 1984).

Immunohistochemistry

Immunostaining with F39.4 for AR and ER-PR1 for PA was performed on cryostat sections 5 μm thick which were allowed to air-dry for at least 30 min before being fixed for 10 min in 4% buffered formalin (pH 7.4). Subsequently, slides were rinsed in phosphate-buffered saline for 15 min, incubated in pure methanol at −20°C for 5 min and further dehydrated in acetone at −20°C for 4 min. Pre-incubation with 5% non-immune rabbit serum was followed by overnight incubation with F39.4 at 4°C or by incubation for 1 hr with ER-PR1. Reactivity was visualized with the indirect peroxidase anti-peroxidase complex (PAP) procedure. MAb Ki-67 was applied on air-dried cryostat sections and reactivity was visualized with an indirect conjugated peroxidase method (Gallee *et al.*, 1987). Peroxidase conjugated rabbit anti-mouse immunoglobulin (DAKO) was used as secondary antibody and mouse monoclonal PAP-complexes (DAKO) as third step reagent.

Quantification of immunoreactivity

The proportion of tumour areas reactive with F39.4 or ER-PR1 was assessed by measurements of the immunopositive areas and negative tumour areas in an entire section using an X-Y tablet linked to an interactive image analysis system (IBAS 2000, Zeiss, Oberkochen, Germany). The immunoreactive area is expressed as a percentage of the total tumour area in the examined section. The percentage of Ki-67 (Ki-67 index) and F39.4-positive tumour cells was calculated after counting the number of immunopositive tumour cells in immunoreactive tumour areas on a total of 400 tumour cells. For counting of Ki-67-positive cells, areas with the highest proportions of labelled cells were selected visually (Gallee *et al.*, 1987).

RESULTS

Androgen receptor expression

All immunopositive tumour areas showed an intense staining reaction with typical nuclear localization. In a few tumours a variable degree of cytoplasmic staining was present. Nuclear

staining was unequivocal in all cases, permitting an easy distinction from immuno-negative cells (Fig. 1*a* and 1*b*). Only one out of the 17 examined tumours was largely negative for AR, with about 2% of the tumour area positive (Fig. 1*c*). Histologically, the latter case (case 8) had the features of an undifferentiated small-cell carcinoma. In 3 cases (case 4, 7 and 15) a considerable heterogeneity in AR staining was observed, both area to area and amongst tumour cells within one tumour field (Tables I and II). In the remaining 13 cases, tumours were almost entirely composed of AR-positive tumour cells. Especially in tumour areas with a cribriform growth pattern, some heterogeneity occurred: the peripherally localized tumour cells generally showed an intense expression of AR, while more centrally localized tumour cells were in part immuno-negative. The small numbers of patients treated by hormone administration (n = 6) obviously precludes a good comparison with the results obtained on the material of orchiectomized patients (n = 11). Nevertheless, with respect to AR expression the data do not point to a striking difference between the 2 groups of patients.

Proliferative activity

To verify the progressive growth of the tumours obtained, proliferative activity was assessed by immunohistochemical staining with MAb Ki-67. A strong area-to-area variation of density of Ki-67 labelled cells was found (Fig. 1*d*). In one case it was noted that several mitotic figures remained unstained (case 3). In this case the frequency of Ki-67-positive tumour cells may not be a proper reflection of the actual growth fraction of the tumour. The Ki-67 score of the different tumour specimens varied from 0.8 to 64.7%. An extremely high Ki-67 index (*i.e.* 64.7%) was found in the case largely consisting of tumour tissue with the morphology of undifferentiated small-cell carcinoma (case 8). The interval between initiation of therapy and time of transurethral resection did not influence the Ki-67 score. A relationship between tumour heterogeneity for AR and growth fraction was not found (Tables I and II).

Expression of prostatic antigen

Tumours examined for PA expression displayed a great variability in staining reactivity. Seven of the 11 tumour specimens of orchiectomized patients contained PA-positive tumour areas. A considerable heterogeneity in PA expression was present within most tumours (Table I). Examination of adjacent sections stained for AR and PA respectively revealed that AR-positive tumour areas may or may not express PA. In the case largely composed of AR-negative tumour cells (case 8), PA expression was only observed in the tumour area (2%) which showed reactivity with F39.4. Expression of PA did not depend on duration of androgen ablation or proliferative activity of a tumour.

DISCUSSION

This study was designed to investigate whether available

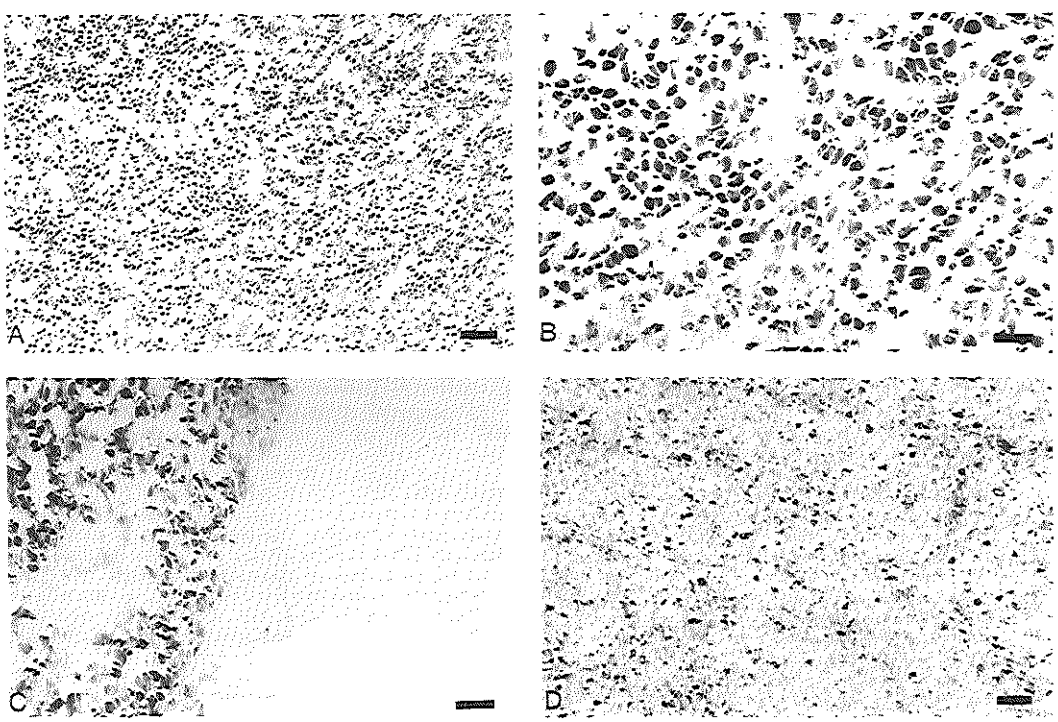

FIGURE 1 – *(a)* Solid tumour areas with an intense expression of AR detected by immunostaining with F39.4 (bar = 5 μm). *(b)* Tumour with heterogeneous expression of AR (bar = 2 μm). *(c)* Largely immunonegative tumour with a small area of AR-positive tumour cells (bar = 5 μm). *(d)* Visualization of proliferative activity by immunostaining with Ki-67 (bar = 5 μm).

data can support the hypothesis that the transition from androgen-dependent to androgen-independent growth of prostate cancer is associated with the preferential outgrowth of AR-negative tumour cells. The latter view can be based on the following observations: (1) AR-negative human prostatic tumour cell lines are not androgen-dependent for their growth, in contrast to AR-positive tumour cell lines (Trapman *et al.*, 1990); (2) in a heterogeneous Dunning rat prostatic carcinoma cell line composed of androgen-dependent and -independent tumour populations, selection of the androgen-independent tumour population occurs after castration of the host (Isaacs *et al.*, 1982) and (3) in the Dunning R-3327 rat prostatic carcinoma model, transition to androgen independence is generally associated with loss of ARs (Diamond and Barrack, 1984). By immunohistochemical application of MAb F39.4 directed against the N-terminal domain of the human AR (Zegers *et al.*, 1991) we unequivocally demonstrated that, in most human prostatic adenocarcinomas treated with androgen ablation or oestrogens, no preferential increase in AR-negative tumour cells takes place. In fact, even at long intervals (up to 100 months) after onset of therapy, an intense AR expression was found in over 80% of tumour cells in the majority of the prostate cancers investigated. This high level of AR expression in these tumours suggests that, in several tumours, expression of AR results in a growth advantage over AR-negative tumour cells. These observations imply that data on AR expression obtained with the Dunning rat prostatic carcinoma model do not hold true for human prostate cancer.

Several mutually non-exclusive mechanisms may lead to the activation of an AR-mediated circuit even at a very low level of circulating androgen. Firstly, increased numbers of structurally intact ARs could be present. This upregulation of AR mRNA expression under low androgen conditions has been described for the human prostatic carcinoma cell line LNCaP and rat ventral prostate tissue (Trapman *et al.*, 1990; Quarmby *et al.*, 1990*b*). Enhanced or prolonged binding to hormone-responsive elements of small numbers of activated ARs could then lead to a stimulation of the AR circuit. Secondly, a truncated AR may show constitutive transcriptional activation (Rundlett *et al.*, 1990), as suggested for other steroid receptors. The production of a set of antibodies specific for the N-terminal, the DNA-binding region and the steroid binding domain of ARs will provide the appropriate tools for demonstrating the presence of truncated ARs in prostatic carcinomas. Thirdly, steroid receptors with a mutation in the steroid binding region may have a changed affinity for steroids. This can be accompanied by loss of specificity of binding. The recently reported point mutation in the steroid binding domain of LNCaP tumour cells (Trapman *et al.*, 1990), in association with changes in affinity of AR for other steroids (Veldscholte *et al.*, 1990) adds further weight to the latter hypothesis.

Alternatively, ARs, although present in most but not all tumours, may not be activated at all in a proportion of the investigated tumours and the AR-dependent circuit may be completely bypassed by an androgen-independent circuit, *i.e.*, the autocrine hypothesis (Lippman and Dickson, 1989). The demonstration that the growth of LNCaP cells could be enhanced by exogenous addition of epidermal growth factor is in line with the supposed existence of an autocrine circuit in prostatic cells (Schuurmans *et al.*, 1988).

In previous studies on the human prostatic carcinoma cell line PC-82, we showed that Ki-67 determined proliferative activity falls considerably within a few days after androgen withdrawal (Gallee *et al.*, 1987). In fine-needle aspiration smears of responsive patients with prostate cancer, the percentage of Ki-67-positive tumour cells also drops to below 10% of the level present at time of diagnosis within 2 months after onset of endocrine therapy (Oomens *et al.*, 1991). Ki-67 may therefore serve as a sensitive parameter for therapeutic monitoring, since the comparatively high Ki-67 scores, as observed in our patient material, seem to reflect accurately the tumour's escape from androgen-controlled growth.

It has been shown by Northern blot analysis that in LNCaP cells the synthesis of PA mRNA is regulated by androgens (Trapman *et al.*, 1988). Furthermore, a hormone-responsive element has been identified in the promoter region of the PA gene (Riegman *et al.*, 1989). The observed PA expression, in association with a high level of ARs in the majority of progressive prostate cancers even at long intervals after androgen ablation therapy, supports the notion that ARs still play an active role in some of these tumours.

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Chapter 7

Androgen Receptor Status in Localized and Locally Progressive, Hormone Refractory Human Prostate Cancer

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Androgen Receptor Status in Localized and Locally Progressive Hormone Refractory Human Prostate Cancer

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Heterogeneity in human androgen receptor (hAR) expression in prostate cancer is considered to be implicated in tumor progression. hAR expression was therefore studied immunohistochemically in localized and locally progressive, hormone refractory (HR) prostate cancer. Because altered functional activity of the hAR may be due to changes in the structural integrity of the hAR gene, exons 2 to 8 of the hAR gene were assessed for mutations by single-strand conformation polymorphism (SSCP) analysis and exon 1 was analyzed for the size of the CAG repeat. The hormone binding capacity, a prerequisite for ligand-regulated receptor function, was determined by a ligand binding assay. Coexpression of the hAR and prostate-specific antigen (PSA) was studied by a sequential double immunoenzymatic staining to verify whether PSA expression is a parameter of hAR function. Almost all human prostatic carcinomas revealed heterogeneous hAR expression, regardless of tumor differentiation and progression. Putative predominance of hAR-negative tumor areas in HR prostate cancer was not observed. No hAR gene mutations or major changes in the CAG repeat were found in the 18 HR carcinomas or in the 9 control samples. Moreover, all selected hAR-expressing cancers were able to bind the synthetic androgen methyltrienolone (R1881). Immunoenzymatic double staining revealed even PSA expression in hAR-negative tumor areas. PSA immunohistochemistry in human prostatic carcinomas therefore is of no use in determining hAR functional activity. Thus, most prostatic carcinomas, even when progressed to a state of hormone insensitivity, contain a structurally intact hAR gene, heterogeneously expressed with retained androgen binding capacity. (Am J Pathol 1994, 144:735–746)

For over 50 years, androgen ablation has been the standard treatment for metastasized prostate cancer.[1,2] The occurrence of hormone resistance has been a central issue in the treatment of this disease ever since.[3]

The cellular composition of the rat ventral prostate has been shown to be heterogeneous with respect to androgen dependence: castration favors the growth of androgen-independent stem cells, whereas selectively eliminating the nonstem cell androgen-dependent compartment.[4] The androgen-dependent Dunning R3327-H rat prostatic adenocarcinoma only temporarily ceases growth after castration. The androgen-independent growth of this tumor after hormone withdrawal is a consequence of the presence of androgen-dependent and androgen-independent cells in the original tumor.[5] The androgen-dependent tumor was subsequently shown to contain androgen receptor (AR) mRNA and protein. The androgen-independent tumor sublines revealed very low AR mRNA levels and lacked AR expression immunohistochemically.[6] Absence of hAR expression and the

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corresponding mRNA has also been shown in androgen-independent growing human prostate tumors/cell lines.[7]

Because of the androgen dependence of most but not all prostate cancers and the variable response to androgen ablation,[8] the correlation of the human androgen receptor (hAR) content of prostatic carcinoma and the degree of responsiveness to hormonal therapy has been studied extensively using ligand binding assays.[9–12] The predictive value of androgen binding data of nuclear extracts is still a matter of debate.[9–12] Failure to predict individual patient response to hormonal therapy by the biochemically determined nuclear hAR content has been attributed to tissue heterogeneity, ie, contamination of the prostate cancer specimen by nonmalignant prostatic tissue. Differences in hAR assay methodology or use of distinct response criteria may explain discrepancies in the outcome of the different studies.

The application of [^{3}H]steroid autoradiography and fluorescent ligand histochemistry to address the heterogeneity of hAR expression in prostate cancer provided little information[13] or conflicting results.[14]

Until now, few reports have been published on hAR expression in prostate cancer using immunohistochemistry.[15–20] With the monoclonal anti-hAR antibody F39.4[21,22] we studied tissue and cellular hAR heterogeneity in prostate cancer samples at various stages of disease progression, comprising prostatectomy specimens and transurethrally resected tissues (TUR) of patients with locally progressive disease.

The immunohistochemical detection of the hAR protein in prostatic tissue samples does not imply the presence of a structurally intact, functionally active hormone binding receptor. Altered androgen response in the androgen insensitivity syndrome is reported to be the consequence of structural aberrations of the hAR.[23,24] In addition, some sporadic hAR gene mutations have been described in human prostate cancer cells.[25–27] Therefore, we examined the TUR specimens of patients with carcinomas that no longer responded to hormonal therapy for mutations in exons 2 to 8 of the hAR gene. In a subset of these endocrine therapy-resistant prostatic carcinomas, hormone binding capacity of the immunohistochemically detected hAR was assessed by a ligand binding assay. Finally, we conducted a study on the immunohistochemical coexpression of hAR and prostate-specific antigen (PSA) because PSA expression is known to be an androgen-regulated process.[28,29] Consistent coexpression of hAR and PSA at the cellular level may indicate the presence of a functional hAR circuit.

Materials and Methods

Human Tissue Sources

Human prostatic tissue was collected from patients at Dijkzigt Hospital Rotterdam and from the cooperative prostate tissue network Zuidwest-Nederland. Prostatectomy specimens were obtained from patients with localized prostate cancer. The majority of the prostatic carcinomas, resected transurethrally before hormonal therapy (7/10), and all TUR specimens (20) resected during endocrine therapy were obtained from different patients with metastasized prostate cancer (Table 1). Tissues were snap-frozen within 15 minutes after surgery. The specimens were stored in liquid nitrogen until further use.

The presence of prostatic carcinoma in the frozen sections used for hAR and PSA immunohistochemistry was always confirmed on adjacent sections stained with hematoxylin-azophloxin. Tumors were graded according to the Gleason grading system.[30] Separate tumor areas with a different histological pattern (Gleason grade) were identified in each sample and the grades of the two dominant histological patterns in tissue samples of individual patients were added, resulting in a Gleason score ranging from 3 to 10.

Total numbers of patients and tissue samples analyzed, histological patterns discerned, and number of hAR values determined are listed in Table 1. In 173 tissue blocks of 49 patients, specimens of 21 patients displayed only 1 Gleason growth pattern (Gleason grade), specimens of 24 patients contained 2 Gleason grades, and specimens of 4 patients displayed 3 Gleason grades. hAR expression was determined in each Gleason grade and scored as high (>70%), intermediate (30 < x < 70%), or low level (<30%) immunoreactivity. Using these arbitrarily set limits, 29 hAR values were obtained in 21 patients with 1 Gleason grade, 64 hAR values in 24 patients with 2 Glea-

Table 1. *Patient Material Studied for hAR and PSA Expression*

Specimen	Patients	Tissue Blocks	Histological Patterns*	hAR†
Radical prostatectomy	49	173	81	108
TUR, no prior hormonal therapy	10	45	16	20
TUR, after hormonal therapy	20	90	30	40

* Number of separate tumor areas with a different histological pattern according to Gleason (Gleason grade).
† Total number of hAR determinations.

son grades, and 15 hAR values in 4 patients with 3 Gleason grades. Thus, 108 hAR values were determined in 173 tissue blocks of 49 patients (Table 1). Likewise, 20 hAR values were determined in 16 Gleason grades, discerned in 45 TUR specimens of 10 nontreated patients with locally progressive disease. In 90 tissue blocks of 20 patients with local progression under hormonal therapy, 40 hAR values were determined in 30 Gleason grades (Table 1).

Antibodies

Monoclonal anti-hAR antibody F39.4 was prepared and characterized as described previously.[21] Conjugates of synthetic peptides corresponding to the amino acid residues 301–320 (Sp061) of the NH_2-terminal domain of the hAR were used for immunization of Balb/c mice. Monoclonal antibody F39.4 (IgG1 k) is not only specifically reactive with the hAR in immunoprecipitation studies, sucrose gradient analysis, and Western blot (21), but this antibody also proved to be an excellent antibody for immunohistochemical applications.[22]

Monoclonal antibody ER-PR1 is specifically reactive with a 34-kd protein in prostatic tissue lysates, which corresponds with the molecular weight of PSA. Moreover, this antibody is exclusively reactive with prostatic secretory epithelial cells.[31]

Ki-67 (DAKO, Denmark) defines a proliferation-associated nuclear antigen that is present during most of the G1 phase, S phase, G2 phase, and M phase of the cell cycle.[32]

Immunohistochemistry

Immunostaining with F39.4 for hAR and with ER-PR1 for PSA was performed on air-dried cryostat sections of 5-µm thickness fixed for 10 minutes in 4% buffered formalin (pH 7.4). Subsequently, slides were rinsed in phosphate-buffered saline for 15 minutes, incubated in pure methanol at –20 C for 5 minutes, and further dehydrated in acetone at –20 C for 4 minutes. Preincubation with 5% nonimmune rabbit serum was followed by overnight incubation with F39.4 at 4 C or by incubation for 1 hour with ER-PR1. A low titer of ER-PR1 was used to detect even low concentrations of PSA in the tumor cells. hAR immunoreactivity was visualized using rabbit anti-mouse immunoglobulin (DAKO) as secondary antibody and mouse monoclonal PAP complexes (DAKO) as third-step reagent. Visualization of PSA was performed with the indirect-conjugated alkaline phosphatase method (DAKO) using a substrate yielding an alcohol-resistant red precipitate (Vector Laboratories, Burlingame, CA).

To confirm the coexpression of the hAR and PSA, a sequential double immunoenzymatic staining was performed using horseradish peroxidase and alkaline phosphatase as respective enzymatic labels.[33]

Monoclonal antibody Ki-67 was applied on air-dried cryostat sections and reactivity was visualized with an indirect-conjugated peroxidase method.[34]

Quantification of Immunoreactivity

At low power view, hAR immunoreactivity in each histological pattern was subdivided in three categories, ie, high, moderate, or low, as described above. Subsequently, the hAR immunoscores were determined in these fields by counting three clusters of 100 cells using a Zeiss bright field microscope, magnification ×400, provided with an ocular grid. The total tumor area and the proportion of the total tumor area displaying high, intermediate, or low hAR expression were determined using an ocular grid (lower margin set at 4/100 ocular grid, ie, 0.01 mm^2). Computing the hAR score of a tumor by taking the average scores of hAR-positive and hAR-negative tumor areas would overestimate the weight of low hAR scores, because in most tumors only a minor part of the total tumor area lacked hAR expression. Therefore, the separate hAR scores had to be weighted by the percentage of the tumor area displaying that hAR score to reach the hAR score of a tumor.

The percentage of Ki-67-positive tumor cells was calculated after counting the number of immunopositive tumor cells on a total of 300 cells. For counting of Ki-67-positive cells, areas with the highest proportions of labeled cells were selected visually.[34] PSA immunoreactivity and coexpression of PSA and hAR were assessed semiquantitatively.

Single-Strand Conformation Polymorphism (SSCP) and CAG Repeat Analysis of the hAR Gene

Genomic DNA was isolated from five consecutive 5 µm cryostat sections. Flanking sections were used for immunohistochemical hAR staining and histological examination to be certain that the sample analyzed contained 50% or more tumor cells. hAR exons 2 to 8 were amplified essentially as described.[35] Primers used for exons 5 to 8 were identical to those described.[35] Primers for exons 2 to 4: exon 2, 5′-CATTTATGCCTGCAGGTT and 5′-ATGGCTCTATTTCTGAGATG; exon 3, 5′-GTTTGGTGCCATA-

CTCTGTCCAC and 5′-CTGATGGCCACGTTGCCTATGAA; exon 4, 5′-AAGTCTCTCTTCCTTC and 5′-TGCAAAGGAGTTGGGCTGGTTG; and 5′-CAGAAGCTTACAGTGTCACACA and 5′-GCGTTCACTAAATATGATCC. For exon 4, two partially overlapping fragments were generated.

Standard polymerase chain reaction conditions were: 25 cycles of 1 minute 94 C, 2 minutes 55 or 60 C, and 2 minutes 72 C in a Perkin Elmer Thermal Cycler in the presence of 1 μCi ^{32}P-dATP in a 15 μl reaction mix. The 1 μl of the reaction product was added to 9 μl of a solution containing 98% formamide, 10 mM EDTA (pH 8), and xylenecyanol and bromophenol as dye markers. After denaturation (5 minutes, 100 C), the solution was chilled on ice and 1.5 μl was loaded onto a 6% nondenaturing polyacrylamide gel in 0.5× Tris, boric acid, EDTA (TBE), 10% glycerol. Electrophoresis was at constant power (7 W) overnight at room temperature. Subsequently, the gel was fixed in acetic acid (10%) then dried and exposed to x-ray film for 4 to 16 hours at −70 C using intensifying screens. The sensitivity of the SSCP conditions used was estimated 80 to 90%, as judged from the analysis of known point mutations in the hAR gene in androgen insensitivity. Nontumor DNA served as control.

For analysis of the size of the CAG repeat in exon 1, the procedure and primers as described by Sleddens et al[36] were applied.

R1881 Binding Assay of Prostate Cancer Cytosol

Preparing the Cytosol

Frozen prostate tumor tissue (25 to 150 mg) was pulverized under liquid nitrogen. The resulting powder was suspended with an Eppendorf micropestle in 2 volumes cytosol buffer (40 mM Tris, pH 7.4, 1 mM EDTA, 10% glycerol, 10 mM dithiotreitol, 10 mM sodium molybdate, 600 μM phenylmethane sulfonyl fluoride, 500 μM bacitracin, and 500 μM leupeptin) and cytosol buffer was added to a total volume of four or six times tissue volume (depending on the original amount of tumor tissue). The suspension was centrifugated 10 minutes at 800 × *g*. The resulting supernatant was centrifugated 10 minutes at 100,000 rpm (390,000 × *g*). The supernatant was designated as cytosol. The cytosol was stored at −80 C until use.

AR Binding Assay

Cytosol was incubated with 10 nM [^{3}H]R1881 (Du Pont de Nemours, Den Bosch, The Netherlands) to determine the amount of receptor molecules present. The nonspecific binding was determined by coincubation with a 200-fold molar excess of nonradioactive steroid (2 μM R1881). To avoid interference of other R1881 binding receptors, 5 μM triamcinolone acetonide was added simultaneously. Bound and free steroid were separated using protamine-hydrochloride precipitation, essentially as described[37] for protamine-sulfate precipitation.

Statistical Analysis

The Spearman rank correlation test was used to compare the hAR expression in different Gleason grades in both localized and locally progressive, hormone refractory (HR) human prostate cancer. The Mann-Whitney *U* test was applied to ascertain the correlation between hAR expression and Gleason scores. The Mann-Whitney *U* test was also performed to corroborate differences in proliferative activity between hAR-expressing human prostate cancers and carcinomas lacking F39.4 immunoreactivity.

Results

Immunohistochemical Analysis of the hAR in Prostate Cancer

Most cancer areas in prostatectomy specimens displayed moderate to high hAR expression, as demonstrated with monoclonal antibody F39.4. However, heterogeneity in staining was observed both in the epithelial and stromal component of the cancers. In this study we focused on hAR immunostaining in the malignant epithelial component of the resected specimens. Two patterns of heterogeneous staining could be distinguished, ie, intraregional and interregional heterogeneity (Figure 1). The former pattern is characterized by a substantial admixture of F39.4 immunopositive and immunonegative cells in a single tumor area, the latter by alternation of completely immunopositive and immunonegative areas of prostate cancer (Figure 1). Interregional heterogeneity was observed in 21 of 49 prostatectomy specimens and in 11 of 30 TUR tissue samples.

The carcinomas were subdivided according to histological growth patterns adopted by Gleason. In 49 prostatectomy specimens, 81 separate tumor areas could be identified with different histological patterns representing four of the five histological growth patterns of the Gleason grading system (Table 1, Figure 2A). No significant correlation was found between hAR scores (in prostatectomy material) and Gleason growth pattern (Spearman rank correlation −0.12; $P =$

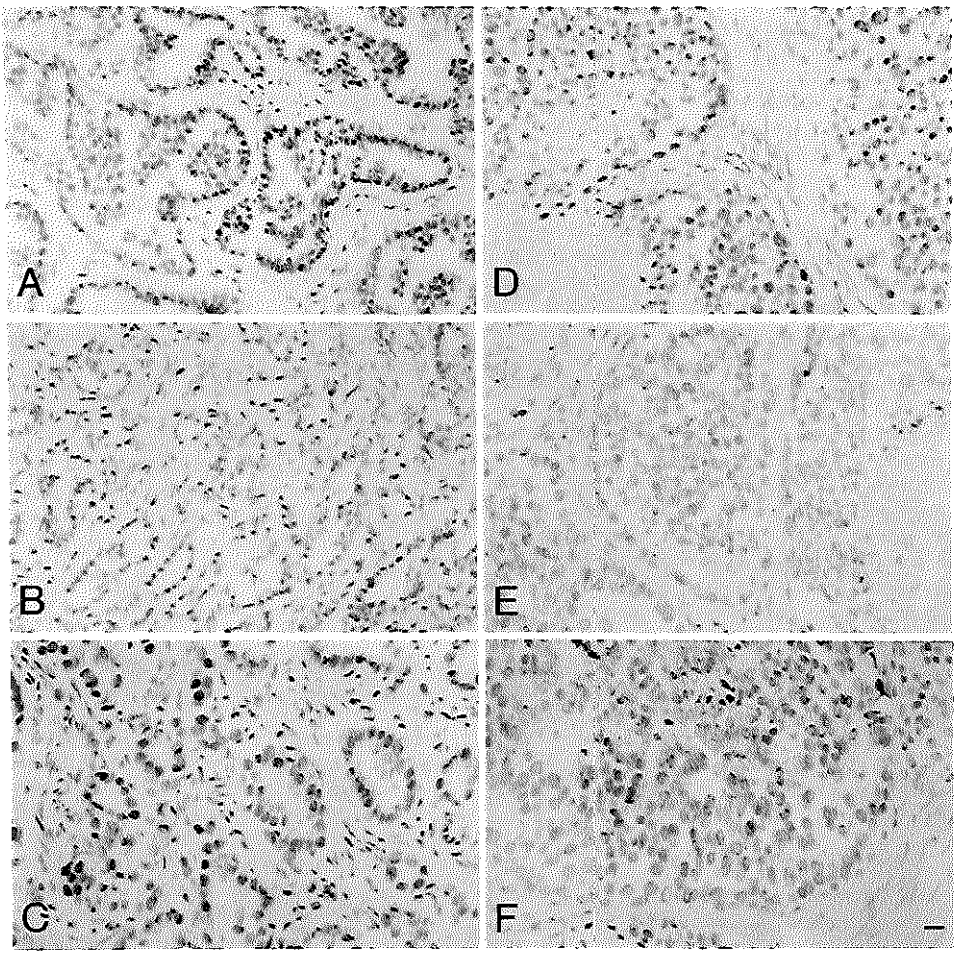

Figure 1. *Heterogeneous F39.4 immunoreactivity of acinar* (A-C) *and solid* (D-F) *prostatic adenocarcinoma visualized with the indirect PAP technique.* A, B, D, E: *Interregional heterogeneity demonstrated by alternation of prostate cancer areas expressing hAR* (A, D) *or lacking F39.4 immunostaining* (B, E). C, F: *Substantial admixture of F39.4-positive and immunonegative tumor cells in a single tumor area characterizing intraregional heterogeneity. Nuclear counterstaining with Mayer's hematoxylin. Original magnification ×320. Bar = 20 μm.*

0.228) (Figure 2A). hAR-negative tumor areas were equally represented by each Gleason grade.

Because of the putative importance of hAR-negative tumor areas in the development of androgen-independent prostate cancer, we measured the relative size of hAR-negative tumor area in the tumor tissue specimens. In only 7 of 81 histological patterns studied (prostatectomy specimens), >25% of the tumor largely lacked immunostaining with F39.4 (Table 2). Most Gleason grades were represented in the seven tumor areas with partial or no hAR expression (Table 2, Figure 2A).

Aware of the heterogeneity in hAR expression in prostate cancer, we were able to calculate weighted average hAR scores, taking into account the percentage of F39.4 immunostained cells and the proportion of the total tumor area displaying that amount of immunoreactivity hAR scores were subsequently correlated with tumor Gleason scores (Table 3). The percentages of F39.4 immunostained cancer cells of moderately (Gleason score 5, 6, and 7) and poorly differentiated (Gleason score 8, 9, and 10) localized prostate cancers did not differ to a great extent. Less differentiated, localized prostatic carcinomas tended

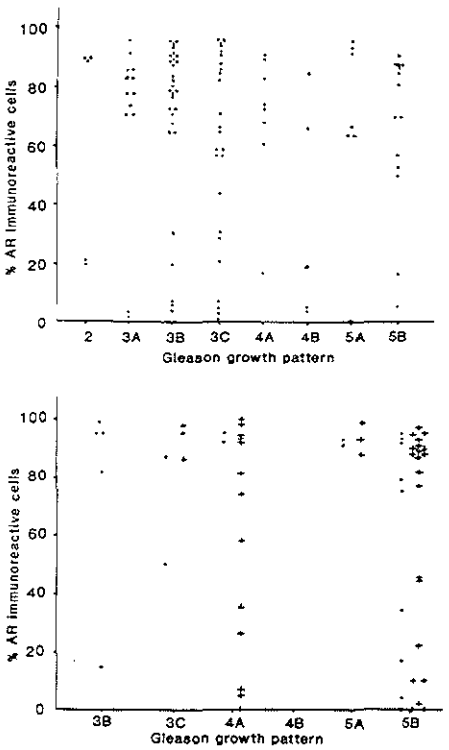

Figure 2. *Top: Percentages of hAR-expressing cancer cells in localized prostate cancer subdivided by histological pattern adopted by Gleason. No correlation was found between hAR scores and Gleason grade (Spearman rank correlation −0.12, P = 0.228). hAR-negative tumor areas are equally represented by each Gleason grade. Bottom: Percentages of hAR-expressing tumor cells in TUR cancer tissue subdivided by histological pattern according to Gleason. (.), Representing TUR specimens of patients without prior endocrine treatment; (+), indicating TUR material from hormonally treated patients. The putative lack of hAR expression in high grade HR prostate cancer was not confirmed (Spearman rank correlation −0.22, P = 0.085).*

toward diminished hAR expression (Mann-Whitney *U* test, $P = 0.054$). Surprisingly, the hAR expression pattern in TUR specimens of untreated prostate cancer patients with locally progressive tumor growth did not vary a great deal from that in prostatectomy specimens (Figure 2B). In this group only one tumor completely lacked hAR expression (Table 2).

As in radical prostatectomy samples, no significant correlation was found between hAR expression and Gleason grade in locally progressive human prostate cancer (Spearman rank correlation −0.22, $P = 0.085$). The percentages of F39.4 immunostained tumor cells in TUR specimens of hormonally treated prostate cancers equalled those found in prostatectomy and TUR specimens of patients without prior endocrine therapy (Mann-Whitney *U* test, $P = 0.217$) (Figures 1, 2, and 5, Table 3). However, three TUR specimens largely lacked hAR expression. These tumors seem to belong to a subset of prostatic carcinomas that behaves biologically different: note the poor differentiation grade and the high proliferation indices. The proliferative activity was assessed immunohistochemically by staining with monoclonal antibody Ki-67. The three tumors largely lacking hAR expression display significantly higher proliferation indices (Ki-67 index: 45 ± 3 [± SD], $n = 3$) compared with hAR-expressing cancers (Ki-67 index: 14 ± 13 [± SD], $n = 25$) (Mann-Whitney *U* test, $P = 0.004$).

Summarizing, our data do not indicate that increasing Gleason score or tumor progression to HR locally progressive disease is associated with a larger proportion of hAR-negative tumor area.

hAR Gene Structure and Hormone Binding Capacity of the hAR Protein in Locally Progressive HR Prostate Cancer

Structural Analysis of the hAR Gene

Because hARs are expressed in hormone-dependent prostate cancers and in the large majority of hormone-independent prostate cancers, we studied the integrity of the hAR gene. We concentrated on those parts of the gene that are known to be frequently involved in pathological situations (androgen insensitivity syndrome [exons 2 to 8] and Kennedy's disease [CAG repeat]).[24,38] Exons 2 to 8 of the hAR gene were analyzed for the presence of mutations by SSCP (Figure 3). The size of the highly polymorphic CAG repeat in exon 1 of the hAR was determined on a denaturing polyacrylamide gel[36,39] (Figure 4). In none of the DNA samples analyzed (18 DNAs from TUR material of patients with HR progressive cancer and 9 DNAs from TUR specimen of patients without prior hormonal therapy) could a mutation in the hAR gene be detected. This strongly indicates that mutations in the hAR gene do not (substantially) contribute to the hormone-dependent or -independent progressive growth of prostate tumors.

R1881 Binding Assay in Cytosols of Prostate Cancer

Because hormone binding is another prerequisite for a structurally intact functional receptor, we engaged on a ligand binding assay in cytosol of HR prostate cancer. Tissue specimens used for ligand binding were selected by their immunohistochemical

Table 2. *Percentage of AR-Negative Tumor Areas in Prostatic Carcinomas Related to Tumor Progression and Therapy*

AR-Negative Tumor Area (%)	Radical Prostatectomy		TUR, No Prior Treatment		TUR, After Hormonal Therapy*	
0–25	74†	(42)	15	(9)	26	(16)
25–50	3	(3)	0	(0)	1	(1)
50–75	2	(2)	0	(0)	0	(0)
75–100	2	(2)	1	(1)	3	(3)

* Orchiectomy, goserelin acetate, cyproterone acetate, or flutamide.
† Number of separate tumor areas with a different Gleason grade, number of patients in parentheses.

Table 3. *AR Expression in Prostate Cancer Related to Tumor Progression and Therapy*

Gleason Score	Radical Prostatectomy	TUR, No Prior Hormonal Therapy	TUR, After Hormonal Therapy*
	AR%†	AR%	AR%
5	77 ± 21 (3)		
6	75 ± 21 (23)	83 ± 5 (2)	98 (1)
7	74 ± 17 (7)	98 (1)	77 (1)
8	65 ± 26 (9)	83 (1)	90 ± 13 (2)
9	56 ± 45 (2)	92 ± 8 (2)	76 ± 12 (6)
10	73 ± 18 (5)	41 ± 36 (4)	65 ± 36 (10)

* Orchiectomy, goserelin acetate, cyproterone acetate, or flutamide.
† Weighted average hAR score ± SD; number of patients in parentheses.

hAR expression pattern. Only those samples displaying epithelial F39.4 immunoreactivity but lacking stromal hAR expression were analyzed to avoid contamination with hAR-positive stromal cells in the cytosol sample. To exclude the possibility of false negative immunostaining with F39.4 in case the reactive epitope is masked due to conformational change of the hAR molecule, we assessed two F39.4 immunonegative prostatic carcinomas for ligand binding properties. All selected F39.4 immunopositive HR prostatic carcinomas (Figure 3) were able to bind R1881 (Table 4). Extracts of two carcinomas lacking hAR immunoreactivity contained a very low level of radiolabeled receptor (Figure 5, Table 4), comparable with the values obtained with thymus cytosol (negative control).

hAR and PSA Coexpression in Prostate Cancer

An immunoenzymatic double labeling was used to visualize the coexpression of PSA and hAR (Table 5). Considering data on PSA/AR coexpression in radical prostatectomy specimens, all but one of the hAR-expressing carcinomas demonstrated at least partial PSA immunoreactivity (48/49). Heterogeneous hAR expression was observed in 48 of 49 cancers. In 41 of 49 carcinomas, tumor areas lacking hAR expression revealed ER-PR1 immunostaining (Figure 5). The TUR samples displayed increased heterogeneity in PSA/hAR coexpression. An increased proportion of tumor areas completely lacking both ER-PR1 immunostaining and F39.4 immunoreactivity was noted in the TUR samples compared with prostatectomy specimens. Interestingly, two locally progressive carcinomas, regardless of hormonal status, completely lacked both hAR and PSA expression. The assumption that PSA expression in the carcinomas reflected the presence of an intact androgen-regulated hAR could not be confirmed, because a proportion of the ER-PR1 immunostained cells lacked detectable F39.4 immunoreactivity.

PSA Content of Glandular Hyperplasia Compared with Prostate Cancer

Prostate specimens with glandular hyperplasia and/or cancer were stained for PSA expression with decreasing concentrations of the monoclonal anti-PSA antibody ER-PR1. The applied maximum primary antibody concentration led to a homogeneous staining of the secretory epithelium of the glandular hyperplastic prostate without background staining. Multiple twofold dilutions of this maximum primary antibody concentration were used in this study and the end point of antibody dilution was determined by the concentration at which no PSA expression was detectable. Comparing the results of the ER-PR1 dilution experiments of nine prostatectomy specimens with both glandular hyperplasia and cancer tissue expressing the hAR, no differences were observed in PSA content, as judged from the end points of antibody dilution. Only one F39.4 immunoreactive cancer sample (Gleason grade 5B) demonstrated diminished PSA expression compared with glandular hyperplasia: the end point of ER-PR1 dilution was reached at a twofold higher primary antibody concentration in the tumor tissue than in the glandular hyperplastic tissue. Examining hAR-positive and hAR-negative regions in the same tumor area, 12 of 16 carcinomas showed the same level of PSA staining

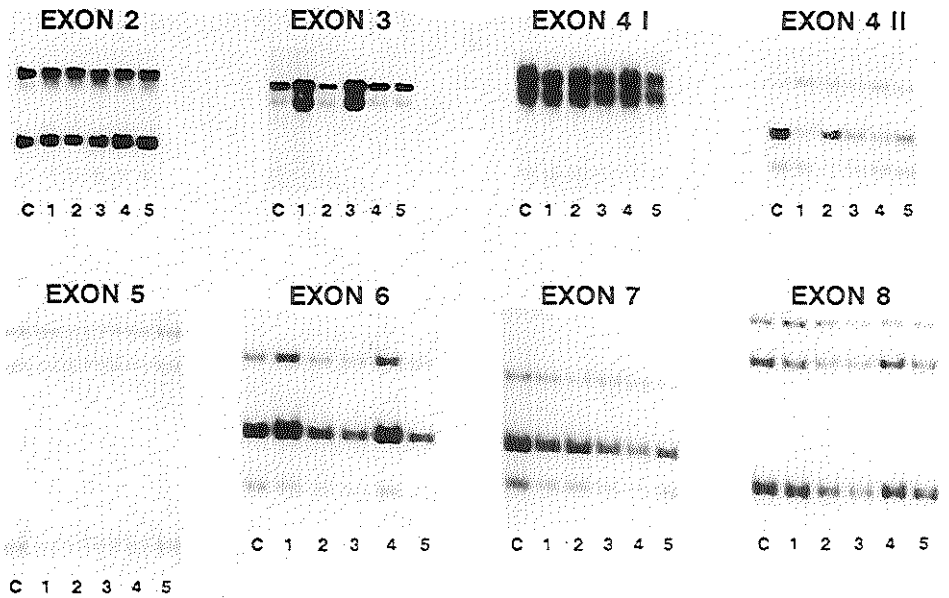

Figure 3. *Polymerase chain reaction-SSCP analysis of the hAR gene from five representative human prostate cancer-derived DNAs (lanes 1–5). The exons 2 to 8 of the hAR gene were amplified and analyzed as described in the Materials and Method section. The control (C) consisted of nontumor DNA*

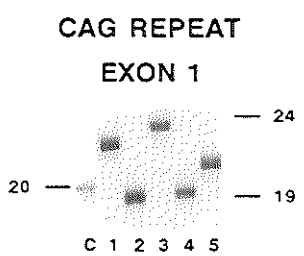

Figure 4. *Length analysis of the CAG repeat in the hAR gene from human prostate cancer-derived DNAs. Of 27 prostate cancer DNA samples the polymorphic CAG repeat in exon 1 was amplified and analyzed as described in the Materials and Method section. Five representative autoradiographs are shown (lanes 1–5). C, control hAR cDNA.*

in either area. In only four cases the hAR-negative tumor area had a twofold lower PSA content than its hAR-positive counterpart.

Discussion

Most prostatic carcinomas in this study reveal heterogeneous hAR expression. Our data are supported by immunohistochemical studies published until now[15–19] except one, stating a consistent qualitative and quantitative hAR immunostaining of epithelial nuclei throughout the different areas studied for each patient.[20] Because most prostatic carcinomas that no longer respond to androgen withdrawal are high grade tumors, it is assumed that hAR content and tumor grade of these tumors are negatively correlated. However, in our material high grade tumors (Gleason score 8 to 10) did not show a significant increase in hAR-negative tumor areas. A tendency to a lower percentage of F39.4 immunoreactive tumor

Table 4. *[^{3}H]R1881 Binding Capacity in Cytosol of Hormone-Resistant Prostatic Carcinoma*

Sample	Therapy	% AR-Positive Nuclei	AR Protein (fmol/mg)
T5–5	Orchiectomy	80	121
T1–14	Orchiectomy	80	328
T1–9	Orchiectomy	80	262*
T2–10	Orchiectomy	90	363*
T1–2	Orchiectomy	90	190
T1–7	Orchiectomy	0	4
T1–8	LH-RH agonist	0	1
Thymus	None	0	3*

* Average of two different tissue specimens.

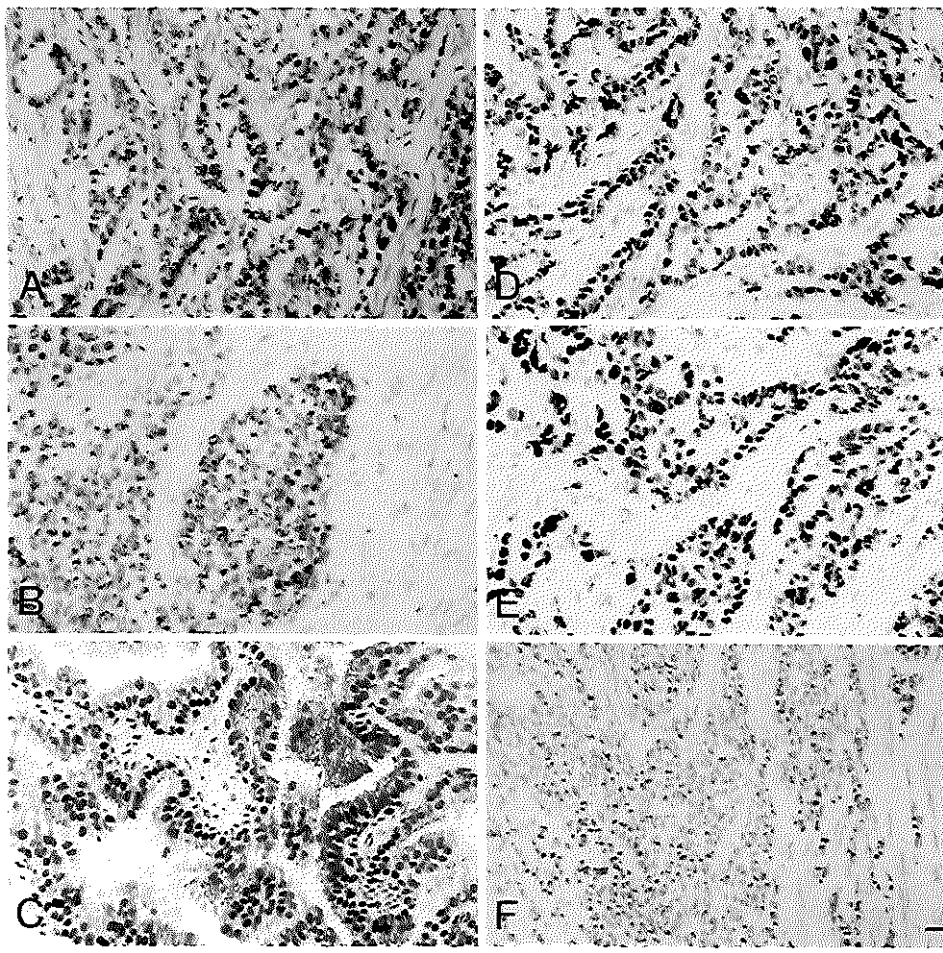

Figure 5A-C: *Coexpression of hAR and PSA visualized by a sequential double immunoenzymatic staining with horseradish peroxidase and alkaline phosphatase as respective enzymatic label.* A, B: *Acinar prostatic carcinoma area* (A) *and solid tumor area* (B) *lacking F39.4 immunoreactivity but expressing PSA.* C: *Prostatic glandular hyperplasia with secretory epithelial cells expressing both hAR and PSA.* D-F: *F39.4 immunoreactivity in HR metastasized high grade prostate cancer. Most metastasized hormonally treated carcinomas demonstrate uniform and intense hAR expression, regardless of duration of endocrine treatment* D, *orchiectomy for 24 months:* E, *orchiectomy for 50 months.* F: *Some HR prostatic carcinomas completely lack hAR expression. Nuclear counterstaining with Mayer's hematoxylin. Original magnification ×320. Bar = 20 µm.*

cells in high grade malignant tumors was noted. Similar observations were reported by several authors, including our group.[15,16,19,20]

Because the androgen unresponsive Dunning tumor sublines that lack AR expression were demonstrated to originate from a heterogeneous tumor consisting of hormone responsive and hormone unresponsive tumor cells, we analyzed prostate cancer in various stages of development for a similar heterogeneity with regard to hAR expression. The presence of F39.4-negative tumor areas in a large proportion of prostatic carcinomas before initiation of therapy would be in line with observations in the Dunning model, suggesting the development of androgen-independent tumors subsequent to endocrine therapy. However, most tumors with local progression, despite endocrine therapy, display generalized high level F39.4 immunoreactivity. This was demonstrated in this study and in a previous paper of our laboratory on a separate group of patients.[17]

Table 5. *AR and PSA Coexpression in Prostate Cancer*

Staining Pattern PSA/AR	Proportion of Carcinomas with each Staining Pattern		
	Radical Prostatectomy	TUR*	TUR†
PSA+/AR+	48/49‡ (97%)	7/10 (70%)	16/20 (80%)
PSA+/AR−	41/49 (83%)	5/10 (50%)	15/20 (75%)
PSA−/AR+	15/49 (30%)	5/10 (50%)	12/20 (60%)
PSA−/AR−	14/49 (28%)	6/10 (60%)	13/20 (65%)

* No prior endocrine therapy.
† Endocrine therapy: orchiectomy, goserelin acetate, cyproterone acetate, or flutamide.
‡ Number of patients; percentage in parentheses.

Thus, the development of androgen unresponsiveness *in vivo* seems to be more complex than suggested by data from animal studies.

It is important to note, however, that not all hormonally treated prostatic tumors express the hAR. In our material two small cell carcinomas completely lacked hAR expression and one high grade carcinoma showed only partial F39.4 immunoreactivity. At this moment we do not know whether the lack of F39.4 immunostaining and the high proliferative activity of these tumors are interdependent events or both merely the consequence of genetic alterations in tumor progression.

Structural alterations of the hAR protein in prostatic cancer may lead to tumor growth even at low levels of androgen. A truncated hAR may constitutively activate transcription, as has been described for AR carboxy deletion mutants transfected in CV1 cells.[40] Mutations in the hormone binding domain could affect the hormone responsiveness of prostatic tumors by altered steroid hormone specificity, as described for the human prostate cancer cell line LNCaP.[25] The hAR gene mutation reported on in human prostatic cancer tissue[26] provided an additional motive to study the structural integrity of the hAR gene. Notwithstanding the attractiveness of the concept of a mutated transcriptionally active hAR to explain androgen unresponsiveness in the presence of an immunohistochemically detectable hAR, no gene mutations were found in the TUR specimens of patients with HR prostate cancer. Data from literature indicate that only 1 of 41 prostate cancers screened for hAR gene mutations revealed a mutation.[26,27]

Prostatic cancer immunoreactivity with F39.4 and structural integrity of the hAR gene do not imply specific hormone binding by the receptor. We showed by R1881 binding that the endocrine therapy-resistant tumors contained hARs with specific hormone binding, a further prerequisite for a structural intact receptor to function.

Because we considered PSA expression an indirect parameter of the functional activity of the hAR,[28,29] we studied coexpression of hAR and PSA using high concentration anti-PSA antibody. The high percentage of PSA-expressing prostate cancers, as demonstrated by ER-PR1 immunostaining, and the complete lack of ER-PR1 immunoreactivity in some poorly differentiated tumors in our material are in line with published data.[41–43] Remarkably, PSA expression was also noticed in tumor areas without F39.4 immunoreactivity. Thus, PSA expression may be regulated by other factors that do not involve hAR-mediated transcription activation. Interestingly, a non-androgen autocrine factor with PSA-inducing activity has been recently described. This prostate-specific autocrine factor could explain the rebound of serum PSA level in castrated tumor-bearing host animals, as observed in an androgen-independent subline of LNCaP.[44]

Tumor areas displaying or lacking hAR expression had comparable ER-PR1 immunoreactivity, as demonstrated in the ER-PR1 dilution experiments. Furthermore, we observed that most HR human prostate cancers growing at low androgen levels partially express PSA, confirming earlier reports on PSA expression after hormonal therapy.[17,45,46] These data indicate that the hAR is only one of the transcription factors implemented in the regulation of PSA expression and therefore cannot be used as the only parameter for the presence of a functionally active hAR in human prostatic carcinomas.

Thus, most prostate cancers, even when progressed to a state of hormone insensitivity, contain a heterogeneously distributed structurally intact hAR with hormone binding capacity. Strikingly, no major differences were observed in hAR expression if one compares the percentages of F39.4 immunoreactive tumor cells in hormone responsive (±75%) and HR carcinomas (±70%). The proliferative activity of HR prostatic carcinomas may be explained by their hypersensitivity to dihydrotestosterone derived of adrenal (androgen) precursors [47] acting by a structurally and functionally intact hAR. A second option to explain hAR-mediated proliferation in HR prostatic carcinomas may be ligand-independent transcription activation of androgen target genes by a membrane receptor-mediated phosphorylation cascade, similarly as described for the progesterone receptor, that can be activated by dopamine.[48] Alternatively, continuous proliferation may be considered a consequence of the presence of either autostimulatory growth factors or derepression of growth inhibitory factors, bypassing hAR-mediated effects on tumor growth.

Acknowledgments

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Regulation of Androgen Receptor Expression in the Human Heterotransplantable Prostate Carcinoma PC-82

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Regulation of Androgen Receptor Expression in the Human Heterotransplantable Prostate Carcinoma PC-82

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ABSTRACT

In vivo effects of androgen withdrawal and substitution on human androgen receptor (hAR) expression were evaluated in the androgen-dependent human prostatic carcinoma tumor line PC-82.

By application of several antibodies reactive with different epitopes of the hAR molecule, hAR protein expression was studied in tumor transplants by immunohistochemistry and immunoblotting. hAR messenger RNA (mRNA) levels were quantitated in PC-82 tumor tissue with a S_1-nuclease protection assay.

Most PC-82 tumor cells (>97%) from testosterone-supplemented mice displayed nuclear hAR protein expression immunohistochemically. The almost complete reduction of nuclear hAR immunoreactivity within 5 days after androgen withdrawal (<10%) was restored after androgen substitution within 1 day. The immunochemical data were confirmed by Western blot analysis. In contrast, no significant changes were observed in hAR mRNA content of PC-82 cells after 5 days of androgen withdrawal.

Correlating hAR expression with proliferative activity of PC-82 tumor tissue during endocrine manipulation, a rapid, castration-induced decline of the percentage of bromodeoxyuridine-labeled cells accompanied the loss of hAR. Androgen substitution in castrated male mice restored the proliferative activity. However, this increase of proliferative activity lagged at least 24 h behind the normalization of the hAR protein level.

In contrast to the steroid receptor down-regulation by homologous ligands observed in other experimental models, our data support the concept of hAR up-regulation by androgen. Since the hAR mRNA content of PC-82 tumor tissue was hardly affected by castration, expression of the hAR in PC-82 is thought to be modulated by translational and/or posttranslational mechanisms. (*Endocrinology* **131**: 3045–3050, 1992)

ANDROGENS mediate their growth-promoting effects on human prostatic cancer cells through their interaction with the human androgen receptor (hAR) (1, 2). In most biopsies from patients with either primary or metastasized prostate cancer the presence of hARs was demonstrated by ligand-binding assays, autoradiography, and immunohistochemistry (3–7).

The hAR, like the other steroid hormone receptors, is a ligand-responsive transcription factor (8). The genomic organization, primary structure, and mechanistic and regulatory aspects of the steroid hormone receptors, and of the hAR in particular, have been reviewed recently (9–11). hAR activation modulates gene expression, resulting in the synthesis of proteins involved in the regulation of cell proliferation and differentiation (11, 12).

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

To study autoregulation of hAR expression *in vivo* we used the well characterized human heterotransplantable prostatic carcinoma PC-82, derived from a primary human prostatic adenocarcinoma (18). PC-82 shares its histological features, its androgen-dependent growth, and the secretion of prostate-specific glycoproteins with clinical prostatic carcinoma (19, 20). The strictly androgen-dependent PC-82 tumor, maintained by serial transplantation in athymic nude mice, slowly regresses in the absence of androgen. Previous studies from our laboratory have shown that PC-82 tissue contains hAR mRNA and protein (17, 21).

This model allows the simple manipulation of serum and tissue androgen levels by sc implantation and removal of androgen-containing (Silastic) implants. PC-82 tumor specimens derived from androgen-deprived and resubstituted mice were analyzed for hAR protein content and hAR mRNA. Androgen-dependent proliferation was evaluated simultaneously.

Materials and Methods

Hormonal manipulation of PC-82

Small fragments of PC-82 tumor tissue (50 mg) were sc transplanted into athymic nude mice. Male mice were supplemented with testosterone (T) to obtain an optimal tumor take (80–85%) and tumor growth. Hormonal substitution was achieved by implanting silastic tubings (Talas, Zwolle, The Netherlands) filled with crystalline steroid (6 mg/tubing; Steraloids, Pawling, NY) (22). Mild ether anesthesia was used

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for tumor and steroid implantation. Mice with exponentially growing tumors were deprived of androgens for 5 days by castration and removal of the T implants approximately 80 days after tumor inoculation. Castration was carried out via the scrotal route under total anesthesia with tribromoethanol (Aldrich, Beerse, Belgium). Mice that were deprived of androgens for 5 days were subsequently resubstituted with T by reimplantation of T containing devices for 1 and 3 days. One hour before killing, the mice were injected ip with bromodeoxyuridine (BrdU) (10 mg/kg). After killing, plasma and tumor tissue were collected. Plasma samples were analyzed for T according to Verjans *et al.* (23).

Antibodies

Monoclonal and polyclonal anti-hAR antibodies were prepared and characterized as described previously (24, 25). Conjugates of synthetic peptides corresponding to the amino acid residues 301–322 (Sp061) of the N-terminal domain, amino acid residues 593–612 (Sp063) of the DNA-binding domain, and amino acid residues 892–910 (Sp066) of the steroid-binding domain were used for immunization of BALB/c mice (Sp061 and Sp063) or rabbits (Sp061 and Sp066) (T.N.O. Medical Biological Laboratory, Ryswyk, The Netherlands).

The specificity of the monoclonal antibody raised against an epitope in the N-terminal domain, designated F39.4 (Sp061), was demonstrated in sucrose gradient density sedimentation analysis, in immunoprecipitation assays, and in Western blots. Moreover, F39.4 is an appropriate antibody for immunohistochemical applications (25, 26).

Monoclonal antibody F52.24.4 (Sp063) recognizes the hAR, glucocorticoid receptor, and to a much lesser extent the estrogen and progesterone receptor (Veldscholte, J., unpublished results). Despite observed cross-reactivity, this monoclonal antibody appeared to be a suitable first step reagent for immunoprecipitation of the hAR.

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

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

Immunohistochemical techniques

Immunohistochemistry for detection of the hAR was performed on cryostat sections as described previously (26). Immunoreactivity was visualized with the peroxidase antiperoxidase complex procedure, followed by incubation with 3,3′-diaminobenzidine (Sigma, St. Louis, MO).

The monoclonal anti-BRdU antibody was applied on frozen sections after a preincubation in 2 N HCl for 30 min at 37 C and two wash steps in 0.1 M borax buffer (pH 8.5) and PBS, respectively. Immunostaining for Ki-67 and BRdU incorporation was visualized in cryostat sections with a peroxidase-conjugated rabbit antimouse immunoglobulin as a secondary reagent and 3,3′-diaminobenzidine (28, 29).

hAR immunoreactivity, as revealed by monoclonal antibody F39.4, was quantitated by counting three random clusters of 100 cells using a Zeiss (Oberkochen, Germany) bright field microscope, magnification × 400, provided with an ocular grid. The percentage of BRdU- and Ki-67-positive PC-82 tumor cells was determined by counting 500 cells.

Preparation of cytosol and nuclear extract

One hundred milligrams of tumor tissue (pooled from two mice) were homogenized in 300 μl buffer A (40 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10% (vol/vol) gycerol, 10 mM dithiotreitol, 0.6 mM phenylmethylsulfonyl fluoride, 0.5 mM bacitracin, and 0.5 mM leupeptin) at 4 C. The homogenate was centrifuged for 10 min at 12,000 × *g*. The supernatant (cytosol) was stored at −80 C until use. The pellet was washed with buffer A containing 0.2% (vol/vol) Triton X-100 and then with buffer A without additions. Subsequently, the pellet was extracted with buffer A (pH 8.5) containing 0.5 M NaCl for 1 h at 4 C. The extract was centrifuged at 105,000 × *g* for 30 min. The supernatant (nuclear extract) was stored at −80 C until use.

Immunoprecipitation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Western blotting, and protein detection with chemiluminescence

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

RNA isolation and S_1 nuclease protection

Total cellular RNA was isolated from four separate PC-82 tumor samples (see Fig. 3) by the guanidinium thiocyanate method (31). For S_1 nuclease protection experiments, 50 μg PC-82 RNA were used in combination with the hAR probe; 10 μg PC-82 RNA were used with the human elongation factor 1α (hEF) control probe. Previous S_1 nuclease assays on LNCaP cells cultured in the presence and absence of androgens demonstrated that hEF mRNA levels did not change after androgen manipulation. The hAR and hEF probes are schematically depicted in Fig. 1. The hAR probe consists of a fragment containing two transcription initiation sites of the hAR gene. It was generated by amplification using the primer combination G-I (32). To obtain the hEF probe, a *SphI-SphI* fragment (469–1161) from clone hEF 1 was subcloned in a pTZ 19 vector (Pharmacia, Uppsala, Sweden) (33). An antisense primer corresponding to positions 650–669 (5′-CACTTGGCTCCAGCATGTTG-3′) was then used in combination with the sequencing primer (15 mer) (NEB, Beverly, MA) to generate the hEF probe. Probes were end-labeled using [γ-^{32}P]dATP and T4 polynucleotide kinase (Gibco BRL, Grand Island, NY) and separated from unincorporated nucleotides using a Sephadex G-50 column. Approximately 1×10^5 cpm probe was annealed to the RNA in 80% formamide, 40 mM piperazine-*N,N′*-bis(2-ethanesulfonic acid) (pH 6.5), 0.4 M NaCl, and 1 mM EDTA overnight at 50 C (hAR) or 42 C (hEF) in a total vol of 30 μl. S_1 Nuclease (Boehringer, Mannheim, Mannheim, Germany) digestions (400 U) were carried out for 1 h at 37 C, and the resulting protected fragments were analyzed on a 6% polyacrylamide/ureum gel (34). Bands were visualized by exposure of the dried gel to Kodak X-AR films (Eastman Kodak Co., Rochester, NY) for 48 h (hAR) or 16 h (hEF).

Results

Effects of hormonal manipulation on hAR immunostaining

To study the effect of androgen manipulation on hAR expression in the PC-82 human prostate tumor, this tumor was propagated on nude mice under various different hor-

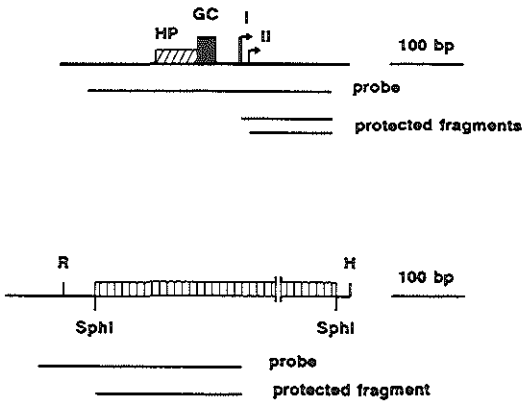

FIG. 1. Probes used in S_1-nuclease protection assays. *Top*, Shown is the hAR gene promoter region. Indicated are the two transcription initiation sites (ARTIS I and II), the position of the homopurine stretch (HP), and the GC box (GC) and underneath the S_1 probe used and the two expected protected fragments. *Bottom*, Shown is the hEF-1 *SphI-SphI* fragment (*striped bar*) cloned in pTZ 19 (*flanking line*) and underneath the S_1 probe used and the expected protected fragment. H, *Hin*dIII; R, *Eco*R1.

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

Hormonal state of male mice	n	pT (nM)	AR (%)	Ki-67 (%)	BRdU (%)
Intact	4	ND	97.2 ± 2.1	11.5 ± 1.7	5.5 ± 1.2
Intact + T implant	6	5.8 ± 1.5[a]	97.5 ± 1.4	14.2 ± 6.1	4.2 ± 1.4
Castrated	8	0.4 ± 0.1	6.1 ± 4.0	0.4 ± 0.3	0.5 ± 0.4
Castrated + T implant[b]	5	18.4 ± 4.6	98.4 ± 1.2	0.5 ± 0.2	0.7 ± 0.4
Castrated + T implant[c]	5	14.9 ± 1.5	97.4 ± 1.6	18.0 ± 10.3	5.4 ± 3.1

pT, Plasma testosterone; ND, not done.
[a] SD.
[b] Implant for 1 day.
[c] Implant for 3 days.

monal conditions. The PC-82 tumor, grown in male mice with a T implant, showed a uniform nuclear immunoreactivity of moderate intensity with monoclonal antibody F39.4 (Table 1 and Fig. 2A). No cytoplasmic staining was observed. A small percentage of the PC-82 cells, particularly mitotic cells, did not express the hAR. Nonspecific immunoreactivity was observed in the stroma-aligned epithelial cells. The hAR expression in PC-82 tissues of intact male recipients was comparable with immunostaining in T-implanted recipients (Table 1).

Androgen withdrawal from PC-82-bearing mice induced a decline in hAR staining of the tumor tissue; percentages of F39.4-reactive cells dropped from approximately 97% in T-implanted male animals to 6% in mice deprived of androgens for 5 days (Table 1 and Fig. 2, A and B).

Repletion of castrated male mice with androgen via a T implant resulted in a restoration of nuclear hAR immunostaining to precastration levels within 1 day after androgen resubstitution (Table 1 and Fig. 2C). The number of immunoreactive PC-82 cells at 1 or 3 days after androgen resubstitution did not differ (Fig. 2, C and D).

Similar experiments were performed on PC-82 tumor tissue grown in female mice with a T implant. In female hosts, like in male mice, PC-82 tissue displayed a rapid decrease of hAR immunostaining after androgen withdrawal (results not shown).

hAR levels in cytosol and nuclear extract

In order to confirm that the observed differences in hAR expression under various hormonal conditions, as observed by immunostaining, resulted from variation in hAR protein levels, hAR expression in PC-82 cells was also studied by a second experimental approach using different antibodies. To this end the hAR was immunoprecipitated with the monoclonal antibody F52.24.4. The isolated receptor complexes were subjected to SDS-PAGE and subsequently to Western immunoblotting with a polyclonal antibody preparation specific for the C-terminal end of the hAR (Sp066).

The results of these experiments are shown in Fig. 3. Lane 5 shows the hAR from LNCaP control cells. In the cytosol samples (lanes 1 and 3) a 110-kDa receptor protein was evident, although a considerable degradation to a 50-kDa receptor fragment was observed, despite the presence of several protease inhibitors in the homogenization buffer. The relative amount of the 110-kDa protein and the 50-kDa degradation product in the cytosol of male mice at 5 days after castration (lane 1) was low as compared to that in PC-82 tumor tissue from castrated (5 days) animals supplemented with T for 1 day (lane 3). The 110-kDa protein (and the 50-kDa band) was absent in the nuclear extracts of castrated animals (lane 2). However, the nuclear fraction of PC-82 carcinoma tissue, grown in T-implanted mice, revealed a considerable amount of the hAR protein (lane 4). The protein band at 45 kDa is nonspecific staining, caused by the chemiluminescence technique used.

Both immunohistochemistry with F39.4 and immunoblotting with the antibodies F52.24.4 and Sp066 indicate a T-induced up-regulation of the hAR protein, as shown by the restoration of the nuclear hAR content after androgen resubstitution.

hAR mRNA detection by S_1-protection assay

A S_1-nuclease protection assay was used to study the effect of hormone withdrawal on the level of hAR mRNA expression. The results of the S_1-nuclease assay are displayed in Fig. 4. Lane 5 depicts the two protected fragments found when control LNCaP mRNA was analyzed. The hAR mRNA content of PC-82 tumor of nude mice at 5 days after castration (lanes 3 and 4) did not differ significantly from that found in the tissue of T-implanted control animals (Fig. 4, lanes 1 and 2). The presence of two different-sized radiolabeled fragments in each of the PC-82 samples analyzed for hAR mRNA is due to the probe used. The probe G-BSSH II is partially complementary to sequences of the first exon of the hAR gene (Fig. 1). Because this hAR fragment contains

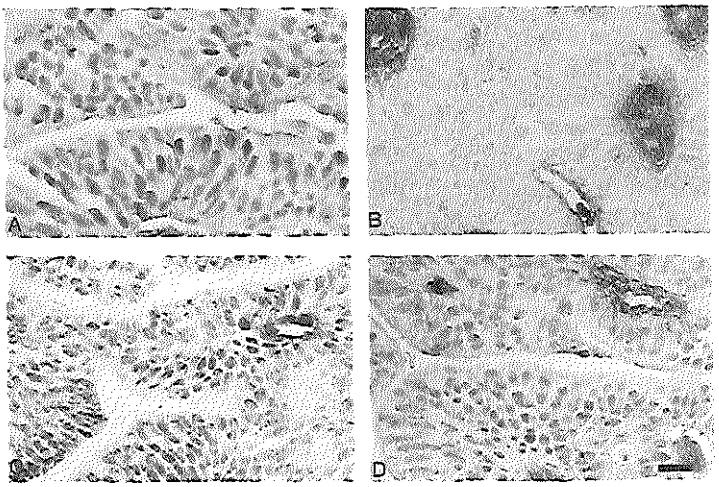

FIG. 2. Immunohistochemistry of PC-82 tumor tissue for AR (×375; no nuclear counterstain). The photographs represent immunostained PC-82 tumor tissue of intact T-implanted male animals (A), of mice depleted of androgens for 5 days (B), and of androgen-resubstituted mice for 1 day (C) and 5 days (D). *Bar* = 20 μm.

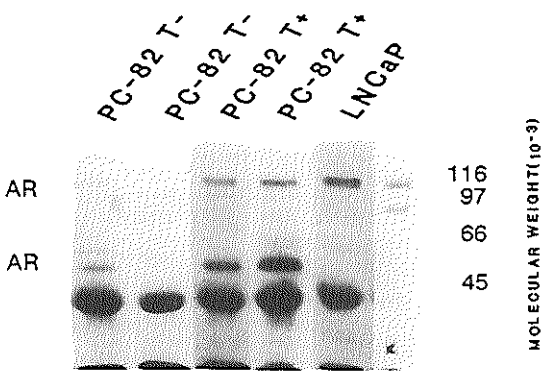

FIG. 3. Western blot analysis of cytosol (lanes 1 and 3) and nuclear fractions (lanes 2 and 4) of PC-82 tumor tissue of male mice at 5 days after castration (lanes 1 and 2) and castrated mice (5 days) resupplemented with T for 1 day (lanes 3 and 4). Lane 5 represents the nuclear extract of LNCaP cells which served as a positive control.

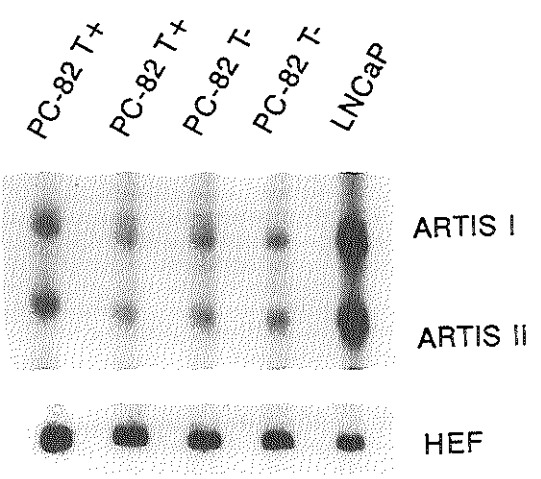

FIG. 4. S_1-Nuclease protection analysis of PC-82 tumor tissue RNA derived from intact T-implanted mice (lanes 1 and 2) and from mice 5 days after androgen depletion (lanes 3 and 4). Lane 5 is control LNCaP RNA.

the hAR transcription initiation sites, ARTIS 1 and ARTIS 2, two different-length hAR mRNA fragments were detected by S_1-nuclease protection assay (Figs. 1 and 4).

The androgen-independent hEF mRNA levels did not differ in various samples of PC-82, indicating that equal amounts of PC-82 tumor tissue RNA were analyzed (Fig. 4).

Androgen dependency of proliferation of PC-82

To confirm the presence of a functional hAR in the examined PC-82 tissues, the effect of androgen manipulation on the proliferative activity of the tumor tissue was analyzed by determination of the percentages of Ki-67 staining and the S-phase fraction by BRdU incorporation. The Ki-67 score, reflecting the number of cycling cells, decreased dramatically within 5 days after androgen withdrawal from approximately 14% in males with a T implant to 0.4% in T-deprived male mice (Table 1). Interestingly, at the moment hAR expression was completely restored, the percentage of cycling cells was still at castration level (Table 1). Three days after implantation of T, the mean percentage of Ki-67-positive nuclei increased to the precastration level (18%) (Table 1).

The BRdU-determined proliferation fraction displayed the same time course as the percent of Ki-67 staining. Almost equal S-phase fractions were observed in precastration and T-resupplemented animals (Table 1).

Discussion

The availability of hAR-specific DNA probes and antibodies against the hAR (24–26, 32; Veldscholte, J., unpublished

results) has permitted comparative analysis of hAR mRNA and receptor protein expression in a well characterized, strictly androgen-dependent human prostatic carcinoma.

In our study, the immunohistochemical staining results of PC-82 tumor tissue with monoclonal antibody F39.4 were in line with immunoprecipitation and Western blot data obtained by application of monoclonal antibody F52.24.4 and polyclonal antibody Sp066. With both methods and different antibodies a decrease in nuclear hAR expression after 5 days of androgen withdrawal and a rapid restoration of hAR immunostaining after androgen resubstitution were observed. Comparing intact (non-T-implanted) male mice and T-implanted male mice, no significant differences in nuclear immunoreactivity between both groups were revealed by immunohistochemical staining with F39.4.

Our immunohistochemical results are in agreement with a report on estrogen and progesterone receptors, stating that steroid receptors reside mainly in the nuclear compartment (35). The hAR protein in immunoblots of the PC-82 cytosol fraction therefore seems to be due to an extraction artefact and most likely represents a less tightly nuclear-bound receptor population.

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

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

hAR mRNA content of PC-82 was determined in our study by a S_1-nuclease protection assay in order to quantitate hAR mRNA of PC-82 tumor cells. The results indicated that in the PC-82 tumor hAR mRNA levels were not substantially influenced within 5 days after androgen withdrawal. This constant hAR mRNA expression in PC-82 is in contrast to previously reported results obtained in rodent prostate and in the LNCaP cell line. Northern blot analysis of prostatic and seminal vesicle tissue of castrated rats revealed an increase in AR mRNA content (14). Furthermore, Quarmby *et al.* (16) reported in another study on the rat ventral prostate a rapid 4-fold decrease of AR mRNA within 8 h after a single injection of testosterone proprionate. Autologous down-regulation of AR mRNA was also observed in LNCaP (16, 17). Using *in situ* hybridization analysis Takeda *et al.* (15) reported a 25% reduction of AR mRNA levels in mouse ventral prostate after castration. Like the hAR mRNA expression of the PC-82 tumor line, the hAR mRNA levels of the genital skin fibroblast strain 704 and the osteosarcoma cell line SA-OS did not change in response to androgens (13).

Altogether our studies suggest that the hormone-dependent hAR expression in PC-82 tumor tissue is regulated by increased translation rate or by stabilization of the receptor protein. Recent experiments performed by Kemppainen *et al.* (39) in COS cells transfected with an AR expression plasmid demonstrated the androgen dependence of hAR turnover (39). In their study, hAR degradation half-life values were 6 h and 1 h in presence and absence of androgen, respectively. However, so far no evidence is available on hAR stabilization by hormone under physiological conditions. It is unlikely that phosphorylation plays a role in androgen-induced receptor stabilization because glucocorticoid and progesterone receptor down-regulation have been demonstrated to be independent of phosphorylation (40, 41). Further studies on the kinetics of hAR expression after androgen withdrawal and resubstitution are needed to unravel the precise mechanism of hAR autoregulation in physiological systems.

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Chapter 9

Discussion

1. Androgen receptor expression in prostate tumors/ cell lines

Immunohistochemical studies on hAR expression in prostate cancer performed until today, allow some general statements to be made [Chapters 4,6 and 7] (1-6). The studies performed thus far used either the polyclonal anti-AR antibodies AR52, AR32 (7,8) and Sp61 [Chapter 10] (9) or the monoclonal anti-AR antibodies AN1-15 (10,11) or F39.4. [Chapter 11] (12). Immunohistochemistry demonstrates that the AR seems to reside mainly in the nucleus. Prostate cancer specimens demonstrate (almost) invariably heterogeneous hAR expression both in the malignant and in the non-malignant tissue [Chapters 4,6 and 7] (1-6). In glandular hyperplastic prostate tissue, high anti-hAR immunoreactivity was observed in the secretory epithelium and weak or absent immunostaining in the basal cell layer. A high percentage of stromal cells was stained as intensely as the secretory epithelium. Data on the correlation between hAR expression and tumor grade in prostate cancer diverge. Most studies reported a decreased hAR staining in high grade carcinomas [Chapters 4 and 7] (1,2,5,6). Yet lack of correlation between hAR immunostaining and grade was also reported (4). Unfortunately, the percentage of hAR expressing tumor cells seems not to be useful in predicting the clinical response of metastatic prostate cancer to androgen withdrawal therapy, since no significant correlation was found between the percentage of hAR-positive malignant cells and time to progression (4).

The link between androgen responsiveness and AR expression, as suggested by [^{3}H]-ligand-binding data on tumor cell lines, has recently been confirmed by determinations of AR mRNA and AR protein content of androgen-dependent and androgen-independent tumors and/or cell lines (13-15). In contrast, hAR expression in most locally progressive, androgen-unresponsive prostatic carcinomas demonstrated predominantly a homogeneous distribution [Chapter 6,7] (3,6). Functional activity of the immunohistochemically detected hAR in the absence of androgen might be explained by either a constitutively activated receptor or by changed ligand affinity. However, neither in localized prostate cancer (16,17) nor in advanced prostatic carcinomas (6,18) have deletions of the hAR gene been found that could result in a constitutively activated receptor. Only 1 of 34 advanced prostatic carcinomas analyzed so far contained a point mutation in exon 4 of the hAR gene (18). Alternatively spliced hAR mRNA could also result in an activated or non-functional protein, but no such data are available for human prostate cancer. Interestingly, the hAR in locally progressive, hormone-refractory prostate cancer demonstrated retained androgen-binding capacity [Chapter 7] (6). Thus, unlike most androgen-independent prostatic carcinoma cell lines and tumor models, the majority of locally progressive prostatic carcinomas contain a hAR with retained androgen-binding capacity. It is yet unknown whether the hAR in these androgen-independent tumors mediates tumor cell proliferation.

2. AR expression and the stem cell concept

The differentiation and maturation from actual prostatic glandular stem cell to mature functioning cells requires qualitative and quantitative changes in the cellular phenotype, leading to functional competence. Recently, proliferation and differentiation markers were used to ascertain differentiation pathways in normal and abnormal prostatic growth.

The application of an immunohistochemical double-staining technique for basal cell keratins and a proliferation marker (Ki-67) indicated that 70 % of the proliferating epithelial cells both in normal and hyperplasic prostates are localized in the basal cell layer (19). Neuroendo-crine cells were demonstrated to be in the G_0 phase of the cell cycle. Remarkably, Ki-67-labeled cells were preferentially situated in the proximity of neuroendocrine, that is chromogranin-A positive, cells (20), suggesting a paracrine effect of neurohormones on basal cell proliferation in the human prostate gland. Secondly, the existence of a transitional population of proliferating cells situated between the stem cells and the mature functional compartment (transit cells) in prostatic epithelium was made plausible by sequential immunohistochemistry of basal cell keratins and PSA. The bidirectional differentiation potential of basal cells was exemplified by the presence of cells with amphicrine characteristics, expressing both PSA and chromogranin-A (21-23). The existence of amphicrine cells had already been reported in neuroendocrine cancers of the human prostate (24,25).

As to AR expression in the different prostate tissue compartments, the secretory epithelium expresses the hAR rather homogeneously. The basal cell layer contains a variable proportion of hAR positive cells (26). The issue of hAR immunoreactivity of neuroendocrine cells in the prostate has not been settled yet. Although one group of investigators claimed that most prostatic neuroendocrine cells express hAR (27), we and others were unable to confirm these observations (23,28,29).

Based on the proliferation data of androgen manipulation experiments in rat ventral prostate and on the expression of differentiation markers in the human prostate, an attractive stem cell concept was proposed for the differentiation of the prostatic epithelium (19) characterized by: 1. three basic cell types linked in a precursor-progeny relationship, that is secretory luminal, basal and endocrine/paracrine (EP) cells, differing in morphology, function and hormonal regulation; 2. a proliferative compartment largely represented by the basal cell layer; 3. EP-cells, which do not proliferate and lack hAR expression; and 4. a subset of potentially androgen-responsive basal cells expressing the hAR.

3. Interpretation of the current findings on hAR expression in hormone refractory human prostate cancer

A. Androgen-mediated growth of advanced prostate cancer

Since locally progressive, hormone-refractory prostate cancer contains an androgen-binding hAR, receptor expression may implicate androgen-mediated tumor growth. A direct explanation for the pronounced hAR expression in hormone-refractory prostate cancer is that low levels of androgen, remaining in prostatic tissue after castration, are able to stimulate the growth of the AR expressing transit cell population of tumor cells. The transit population may expand owing to the androgen-regulated production of growth factors with auto- and paracrine effects on cellular growth. Such factors, for instance EGF, TGFα and bFGF, may originate in the epithelium, as has been described for the Shionogi mammary cancer cell line and the cultured prostate cancer cell lines LNCaP and DU-145 (30-35). In addition, growth factors produced by the stroma are able to stimulate cell division in the epithelial compartment (36-38). Members of the heparin-binding fibroblast growth factor (FGF) family are direct-acting growth regulators of isolated prostate cells. In contrast to the expression of FGF-1 and FGF-2 in prostatic cells, keratinocyte growth factor (KGF/FGF-7) expression is androgen-sensitive. KGF is a specific mitogen for epithelial but not for stromal cells (39). The development of tumor cell clones that are hypersensitive to androgen, as observed in the Shionogi tumor model system (40), may equally well explain increasing tumor volume at low androgen concentrations. Yet androgen concentrations at castration level are generally not believed to stimulate cell division. Changed binding specificity of the hAR for the respective ligand can result in hAR activation by different steroids and/or anti-steroids, as observed in the prostate carcinoma cell line LNCaP (41). However, the hAR gene of only 1 of 75 human prostatic carcinomas analyzed until now displayed a point mutation in exon 4 of the hormone-binding domain associated with altered ligand specificity. This mutated hAR displayed increased transcription activation by adrenal androgens and progesterone (18).

B. Advanced prostate cancer bypassing androgen-mediated growth

Alternatively, locally progressive, hormone refractory prostatic carcinomas may bypass the androgen-mediated pathway of tumor growth regulation. The pronounced hAR expression of the majority of prostatic cancer cells in a microenvironment devoid of androgen may be explained by different androgen requirements for proliferation of tumor cells, maintenance of androgen sensitivity and androgen-regulated transcription of genes not directly involved in proliferation, as suggested from experimental data on cultured Shionogi SC-115 cells (42).

Low androgen levels in hormone-refractory, progressive prostate cancer might likewise maintain hAR regulated gene expression without affecting hAR mediated proliferation. In fact, AR expression itself is regulated by androgen. Autoregulation of AR expression has been studied both at the mRNA level, at the protein level and by ligand-binding techniques in rat ventral prostate (7,8,43-47), rat testis (44), genital skin fibroblasts (48) and in the prostatic tumor lines LNCaP (13,15,49) and PC-82 [Chapter 8] (50,51). Most studies demonstrated a decrease in AR mRNA after T supplementation. Similarly, in most tissues castration induced an increase in AR mRNA expression. However, in rat testis and in the PC-82 prostate carcinoma model, androgen withdrawal did not influence AR mRNA expression levels (44,51). Using *in situ* hybridization Takeda *et al.* observed that AR mRNA expression in mouse ventral prostate was decreased after castration (47). Data from the above mentioned studies on AR autoregulation at the protein level using ligand binding techniques, Western blot and/or AR immunohistochemistry demonstrated that androgens generally up-regulate AR expression.

When the androgen-mediated pathway of tumor cell proliferation is bypassed, the expansion of prostate cancer might be a consequence of stimulation of proliferative activity of both stem cells and transit cells by other factors than androgen or androgen-dependent stimuli. Progression is generally associated with increasing karyotypic variability and chromosomal translocations (52). In human prostatic malignancy the frequency of DNA aneuploidy increases with advancing stage. Altered levels of oncogene expression may accompany the genetic instability of tumors. Tumor suppressor genes, for instance the retinoblastoma gene (53) and the p53 gene (54), have been demonstrated to be mutated in a limited number of primary and lymphogenic metastasized prostate cancers (55-57). In contrast, a mutant p53 gene is a more frequent observation in bone metastases of androgen-independent prostate cancer (58). In our series, three locally progressive, hormone-refractory prostatic tumors lacked both hAR and PSA expression [Chapter 7] (6). Two of these carcinomas contained a mutant p53 protein. The third tumor was entirely composed of neuroendocrine cells. The occurrence of a prostatic neuroendocrine small cell carcinoma might be explained by changes of stem cells or by asymmetric stem cell differentiation (that is, an increased ratio of neuroendocrine to secretory cell differentiation) and subsequent selection (59).

Tumor cells with EP differentiation are often found in prostatic carcinomas, dispersed among non-neuroendocrine cells. A potential role for these EP-cells in stimulating cell division of transit cells may be considered. Serotonin, present in prostatic EP-cells, has a stimulatory effect on cell proliferation (60). Concerning the mechanism of action of neurohormones, steroid hormone receptors can be activated in a ligand-independent manner by the neurotransmitter dopamine. Activation of the dopamine membrane receptor (D1 receptor) may result in phosphorylation or inhibition of dephosphorylation of amino acid

residues of the steroid receptor involved, eventually leading to receptor activation. It can be speculated that the AR in prostatic cells might be similarly activated by a membrane receptor-mediated phosphorylation pathway (61). Whatever the mechanism, the putative role of the prostatic EP-cell in cell proliferation is strengthened by the observation that Ki-67-labeled cells were preferentially situated in the proximity of chromogranin-A positive cells (20).

The involution of the normal prostate after androgen withdrawal is a result of programmed cell death, occurring in the interphase of the cell cycle, a process initiated in the glandular epithelial cells. This event is characterized by the expression of testosterone-repressed prostatic message 2 (TRPM-2), increased intracellular Ca^{2+}, Ca^{2+} and Mg^{2+} dependent endonuclease-induced fragmentation of DNA, and the development of apoptotic bodies (62). The same sequence of events occurred in the strictly androgen-dependent PC-82 human prostatic cancer (63). Androgen-independent tumor lines, for instance the Dunning AT-3 tumor, cannot be induced to the activation of programmed death by androgen withdrawal (62).

Interestingly, glandular epithelia such as in the prostate, in which hormones and growth factors regulate proliferation and involution, demonstrate bcl-2 expression (64). Bcl-2 is a proto-oncogene, encoding an inner mitochondrial membrane protein blocking programmed cell death, first described in a human follicular B-cell lymphoma (65). In the human prostate, the bcl-2 protein is expressed in the cells of the basal compartment in both normal and hyperplastic prostates (64). However, in a recent study a proportion of the localized prostate cancers (14/37) and all hormone-refractory prostatic carcinomas (21/21) were demonstrated to express the bcl-2 gene (66). Although inhibition of programmed cell death in prostate cancer has also to be taken into account as a potential factor in tumor expansion, it does not explain the increased proliferative activity of locally progressive as compared to localized human prostatic carcinomas [Chapter 7] (6,67).

4 Concluding remarks

Despite a heterogeneous hAR expression in localized human prostate cancer, most hormone-refractory carcinomas express the hAR rather homogeneously. Apparently, androgen ablation does not cause a selection for hAR-negative prostatic tumor cells. In contrast, the majority of androgen-independent cancers in animal tumor models lack AR expression. In hormone-independent prostate cancer, AR expression, possibly maintained by low concentrations of intraprostatic androgen, may still mediate androgen-regulated gene expression of both prostatic fibroblasts and epithelial cells. Unraveling proliferation induction, mediated by either epithelium- and/or stroma-derived growth factors or endocrine/paracrine effects of EP-cells, will be a challenge for the time to come.

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Characterization of Polyclonal Antibodies against the N-terminal Domain of the Human Androgen Receptor

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Characterization of polyclonal antibodies against the N-terminal domain of the human androgen receptor

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Summary

Antibodies against the N-terminal domain of the human androgen receptor (hAR) were prepared by two different approaches. Firstly, rabbits were immunized with a β-galactosidase-hAR (amino acids (aa) 174–353) fusion protein. Secondly, two synthetic peptides corresponding to potentially antigenic sites located within this fragment (aa 201–222 and 301–320) were used as immunogens. The obtained antisera contained high titer anti-hAR antibodies as was established with several independent methods (e.g. sucrose gradient centrifugation, immunoprecipitation, Western blotting). The two anti-peptide antisera specifically stained nuclei of glandular epithelial cells in frozen sections of human prostate tissue. Progesterone, estradiol and glucocorticoid receptors were not immunoprecipitated with these antisera.

The specific hAR antibodies provide new tools for the characterization of this steroid receptor as well as for diagnostic purposes in pathology of the human prostate and androgen resistance.

Introduction

Like all steroid hormone receptors, the androgen receptor (AR) is a ligand-responsive transcriptional regulator consisting of a putative DNA-binding domain, a steroid-binding domain at the C-terminal end and a large N-terminally located domain that might be involved in the regulation of gene transcription (Weinberger et al., 1986; Petrovich et al., 1987).

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Antibodies against purified preparations of the oestrogen (ER), progesterone (PR) and glucocorticoid receptors (GR) have been used as tools in elucidating the function of these regulatory proteins (Okret et al., 1984; Logeat et al., 1985; Westphal et al., 1984). In addition, monoclonal antibodies directed against PR and ER are used for the quantification of these receptors in breast tumors, which is an important criterium for endocrine therapy. Similarly, antibodies against the human androgen receptor (hAR) might be useful for studies concerning the functional properties, quantification and localization of this protein. The antibodies could also be of high clinical importance for the characterization of defective receptor forms in individuals with the (partial) androgen

insensitivity syndrome as well as for the quantification of AR in prostate cancer specimens.

Until recently antibodies against the hAR were not available because the low concentration of hAR in target tissues as well as the lability and susceptibility to proteolytic breakdown have hampered large scale purification of the receptor protein. However, autoimmune antibodies (Liao et al., 1985) as well as antibodies produced by immortalized human lymphocytes have been reported (Young et al., 1988). With the recent cloning of cDNA encoding the hAR and the subsequent elucidation of the hAR primary structure (Chang et al., 1988a; Lubahn et al., 1988a; Trapman et al., 1988), tools were provided for the preparation of large amounts of hAR protein fragments that can be used as antigen in the production of antibodies.

In this paper we report the preparation and characterization of a polyclonal anti-hAR antiserum, raised in rabbits against a fusion protein product containing a part of the hAR N-terminal domain. In addition we describe the properties of antibodies against synthetic peptides corresponding to possible predicted antigenic areas in the N-terminal domain of the hAR. The antibodies are shown to be valuable tools in various immunobiochemical techniques: precipitation, blotting and in situ enzyme-immunohistochemistry.

Materials and methods

[17α-*methyl*-^{3}H]Methyltrienolone ([^{3}H]R1881), spec. act. 87 Ci/mmol and [17α-*methyl*-^{3}H]promegestone ([^{3}H]R5020), spec. act. 72.4 Ci/mmol were purchased from NEN-Dupont, Dreieich, F.R.G. [2,4,6,7-^{3}H]Oestradiol, spec. act. 94 Ci/mmol and [1,2,4,6,7-^{3}H]dexamethasone, spec. act. 94 Ci/mmol, were obtained from Amersham, Cardiff, U.K.

Preparation of hAR fusion protein and generation of antibodies

The 542 bp *Pvu*II-*Pvu*II hAR cDNA fragment (derived from clone T4.1. A1 and corresponding to amino acids (aa) 174–353, see Faber et al. (1989), Figs. 1 and 2) was ligated in the *Sma*I site in the polylinker of the bacterial expression vector pEX2 (Stanley and Luzio, 1984) using standard recombinant DNA technology (Maniatis et al., 1982). The orientation of the integrated fragment was established by digestion of the plasmid (pEX-β-gal-hAR) with *Pst*I. β-Galactosidase-hAR fusion protein was produced in *Escherichia coli* POP 2136 during a 2 h induction period at 42°C. Protein was isolated from the bacterial pellet by extraction in a 60 mM Tris buffer (pH 6.8) containing 3% sodium dodecyl sulphate (SDS), 6 M urea, 5% β-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation (10 min, 12000 × *g*) the supernatant was dialyzed overnight at 4°C against phosphate-buffered saline (PBS) containing 1 mM PMSF. The protein extract was once again centrifuged and subsequently used for SDS-polyacrylamide gel electrophoresis (PAGE) analysis or immunization. A New Zealand white rabbit was immunized subcutaneously 2 times at 4-week intervals with approximately 250 μg of protein. The first injection was in complete Freund's adjuvant, the second in incomplete adjuvant and subsequent boosters in PBS. Two weeks after each immunization serum was analyzed for reactivity with the fusion protein by Western blotting.

Selection of putative antigenic sequences

For the selection of putative antigenic sequences predictive algorithms were used: hydrophilicity (Hopp and Woods, 1981; Kyte and Doolittle, 1982), α-helix and β-turns (Chou and Fassman, 1978; Garnier et al., 1978), surface probability (Emini, 1985), flexibility (Karplus and Schulz, 1985), culminating in an antigenicity index according to Jameson and Wolf (1988). Two peptides, SpO60 (amino acids 201–222) and SpO61 (amino acids 301–320), were synthesized.

Synthesis of peptides and immunization protocol

Solid-phase synthesis on Rapid-Amide resin beads was performed essentially according to Merrifield (1963) using Fmoc-protected amino acids (Dupont, Wilmington, U.S.A.) in the procedure as described for the RaMPS System (Dupont, Medical Products, Biotechnology Systems, U.S.A.). High-performance liquid chromatography (HPLC)-grade reagents were used throughout the peptide production procedure. For deprotection piperidine was used. Elongation was controlled at

each step (Kaiser et al., 1970), in case of a proline coupling elongation was checked according to a modification (Pritchard and Aufret, 1986) of the method originally described by Kaiser et al. (1980). Final deprotection and cleavage were performed using a mixture of trifluoroacetic acid, phenol and ethanedithiol followed by precipitation and filtration from diethyl ether. The peptides contained cysteins with a tertiary butyl protection group which was removed using mercuric (II) acetate. The peptides were purified as described elsewhere (Van Denderen et al., 1989). Briefly, the peptides were reduced using β-mercaptoethanol and subsequently purified over G-15 Sephadex (Pharmacia, Uppsala, Sweden) in 5% acetic acid. Fractions showing a single peak in reversed-phase chromatography in a gradient of acetonitrile with 0.1% trifluoroacetic acid, monitored at 214 nm, were pooled and freeze dried. The amino acid composition was confirmed using amino acid analysis of the peptides. The selected AR sequences were prolonged with a cystein to allow coupling to a carrier protein, keyhole limpet hemocyanin with maleimidobenzoyl-*N*-hydroxysuccinimide ester as described elsewhere (Boersma et al., 1988). Control conjugates of the peptides with bovine serum albumin (BSA), based on coupling of the peptides with a carbodiimide (Boersma et al., 1988), were used in enzyme-linked immunosorbent assay (ELISA) for analyses of the anti-peptide responses. Two rabbits (Flemisch Giant random bred, MBL-TNO, Rijswijk, The Netherlands) were immunized with each peptide conjugate; 3 times at 4-week intervals with 250 μg of the conjugate with Freund's complete adjuvant the first time and with incomplete Freund's adjuvant for the other immunizations. Two weeks after each immunization serum was analyzed for reactivity with the peptides and conjugates in an ELISA (Boersma et al., 1988). Pre-immune sera served as controls.

Cell culture

The cell lines LNCaP, NHIK, MCF-7 and T47D were cultured as described previously (Mulder et al., 1978; Keydar et al., 1979; Berns et al., 1984; Van Laar et al., 1989).

Preparation of nuclear extract

LNCaP, MCF-7 and NHIK cells were incubated with 10 nM [^{3}H]R1881, [^{3}H]oestradiol and [^{3}H]dexamethasone respectively for 1 h at 37°C. T47D cells were incubated with 40 nM [^{3}H]R5020 for 1 h at 37°C. After incubation, the cells were collected in TEDGP buffer, pH 7.4 (40 mM Tris, 1 mM EDTA, 10% glycerol (w/v), 10 mM dithiothreitol, 0.6 mM PMSF, 0.5 mM bacitracin) at 0°C and homogenized by using a glass/Teflon homogenizer. The homogenate was centrifuged at 800 × *g* for 10 min at 4°C. The pellet was washed with TEDGP buffer pH 7.4 containing 0.2% Triton X-100 and with TEDGP buffer pH 7.4 without additions.

The nuclear pellet was resuspended in TEDGP buffer, pH 8.5, in the presence of 0.5 M NaCl and 0.25 mM leupeptin and extracted for 1 h at 0°C. The sample was centrifuged at 100 000 × *g* for 30 min.

Measurement of steroid binding

Protamine sulphate (Organon, Oss, The Netherlands) solution (500 μl of 0.5 mg/ml) was mixed with 50 μl nuclear extract and precipitated for 5–10 min on ice in glass tubes which were precoated with 0.1% BSA for 30 min at 30°C. The precipitate was centrifuged for 15 min at 2000 × *g* and washed 4 times with 0.5 ml ice-cold TEDGP pH 7.4 buffer. The precipitate was dissolved in 0.5 ml Soluene-350 (Packard, Downers Grove, IL, U.S.A.) for 15 min at 60°C. Precipitated radioactivity was counted in 5 ml Instagel (Packard). Nonspecific binding (less than 10%) was determined in nuclear extracts of cells which were incubated with radiolabeled ligand in the presence of a 100-fold molar excess of unlabeled ligand.

Sucrose gradient centrifugation

150 μl of [^{3}H]R1881-labeled nuclear extract (0.15 M NaCl), which was incubated for 6 h at 4°C with anti- or pre-immune serum was layered on 10–30% sucrose gradients in the presence of 0.3 M NaCl and centrifuged for 20 h at 370 000 × *g* at 4°C (De Boer et al., 1986).

Double immunoprecipitation

20 μl of [^{3}H]R1881-labeled LNCaP nuclear extract, diluted until the NaCl concentration was 0.15 M, was incubated with 20 μl antiserum or pre-immune serum in various dilutions for 18 h at

4° C. 50 µl goat anti-rabbit serum and 50 µl 1% normal rabbit serum were added and the incubation was continued for 2 h at 4° C. 1 ml of polyethylene glycol (molecular weight (MW) 6000–7000) was then added and the mixture was incubated for an additional 2 h at 4° C before centrifugation at 2400 × *g* for 30 min. The precipitate was washed once with 1 ml PBS containing 1% BSA, centrifuged for 10 min at 2400 × *g* and counted for radioactivity in 10 ml Instagel (Packard).

Photoaffinity labeling of nuclear extracts

Photoaffinity labeling of the androgen receptor with [^{3}H]R1881 in nuclear extracts was performed via irradiation of the sample with an Osram HBO 100 W/W-2 high pressure mercury lamp as described previously (Brinkmann et al., 1985).

Methanol precipitation

One volume of photolabeled nuclear extract from LNCaP cells was added to 5 volumes of methanol, precipitated for 18 h at −80° C and centrifuged at 10000 × *g* for 30 min. The precipitate was prepared for SDS-PAGE.

SDS-PAGE

SDS-PAGE was carried out according to Laemmli (1970) using 8% polyacrylamide gels. After electrophoresis, the slab gel was either subjected to Western blotting or counted for radioactivity as described (Van Laar et al., 1989).

Western blotting

The slab gels were positioned on nitrocellulose paper (Schleicher and Schuell, 0.45 µm) and placed in a Bio-Rad (Richmond, CA, U.S.A.) Trans-Blot cell, filled with 16.5 mM Tris/150 mM glycine/20% methanol (pH 8.3). The transfer was performed at 4° C using 65 V for 17 h. The paper was incubated with the antiserum diluted 1:50 (Tp4) or 1:200 (SpO60 and SpO61) in PBS/0.05% Tween-20 (PBS-Tween) for 1 h at room temperature, washed 3 times for 20 min each with PBS-Tween, and incubated for another hour at room temperature with alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO, U.S.A.), diluted 1:2000 in PBS-Tween. After washing of the nitrocellulose paper, the antibody complexes were stained with a solution of 0.3% (w/v) 4-aminodiphenylamine diazonium sulphate (Sigma), 0.1% (w/v) naphthol phosphate (disodium salt) (Sigma) and 10 mM $MgCl_2$ in 0.2 M Tris-HCl, pH 9.1.

Immunohistochemistry

6 µm-thick cryostat sections of freshly frozen prostatic tissues with nodular glandular hyperplasia were fixed in 4% buffered formalin and dehydrated in chilled methanol and acetone. Incubation with the oligopeptide-specific rabbit antisera (SpO60 and SpO61) diluted 1:500 in PBS containing 0.5% BSA and 0.1% sodium azide (pH 7.8) was done overnight at 4° C. After rinsing in PBS (pH 7.4) an indirect conjugated peroxidase method was applied using a swine anti-rabbit immunoglobulin conjugate (Dakopatts, Glostrup, Denmark) as second step reagent. Reactivity was visualized using hydrogen peroxide and diaminobenzidine (Sigma) as substrate. For distinction of nuclei counterstaining with Mayer's haematoxylin was sometimes applied. Pre-immune sera were used as negative controls. Freshly frozen lymphoma tissue processed similarly as the prostatic tissue served as a specificity control.

Results

Generation of the antibodies

A *Pvu*II-*Pvu*II cDNA restriction fragment (Tp4) encoding part of the hAR N-terminal domain (aa 174–353, see Faber et al., 1989) was ligated to the *lacZ* gene and expressed in *E. coli* (Fig. 1). The lysate, containing large amounts of the fusion protein (about 25%), was used for immunization of rabbits. Antisera which were purified over an affinity column prepared from lysates

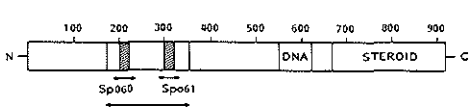

Fig. 1. Schematic diagram of the hAR, illustrating the location of the functional domains and the location of the peptides (SpO60 and SpO61) and the *Pvu*II-*Pvu*II fragment (Tp4) that were used for immunizations. Numbers indicate the amino acid residues.

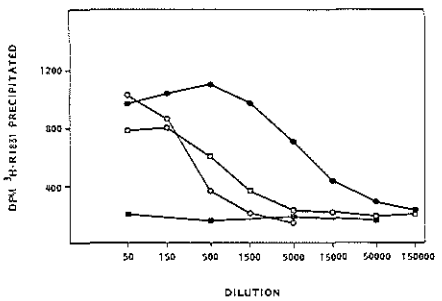

Fig. 2. Immunoprecipitation of [^{3}H]R1881-labeled hAR in nuclear extracts with the antisera Tp4 (○), SpO60 (□), SpO61 (●) or SpO61 pre-immune serum (■). A constant amount of nuclear extract containing 2480 dpm [^{3}H]R1881-labeled hAR was incubated with serial dilutions of the sera. Goat anti-rabbit antiserum was used as a second antibody.

of β-galactosidase producing bacteria were reactive with the β-gal-hAR fusion protein in a Western blot (not shown), indicating that antibodies against the hAR part of the fusion protein were indeed generated. With prediction programme analyzing immunogenicity (Chou and Fassman, 1978; Garnier et al., 1978; Hopp and Woods, 1981; Kyte and Doolittle, 1982), two possible antigenic areas were selected within the same hAR fragment which was used for the preparation of the fusion protein. Two peptides, SpO60 (aa 201–222) and SpO61 (aa 301–320, see Fig. 1 and Faber et al., 1989) were used to raise anti-hAR antibodies in rabbits. In an ELISA assay the anti-peptide antisera were reactive with the material used for immunization. In addition, the antisera recognized the β-gal-hAR (aa 174–353) fusion protein in a Western blot (not shown).

Double immunoprecipitation

The interaction of the three antisera with the native hAR was determined by double immunoprecipitation. A constant amount of [^{3}H]-R1881-labeled nuclear extract, prepared from

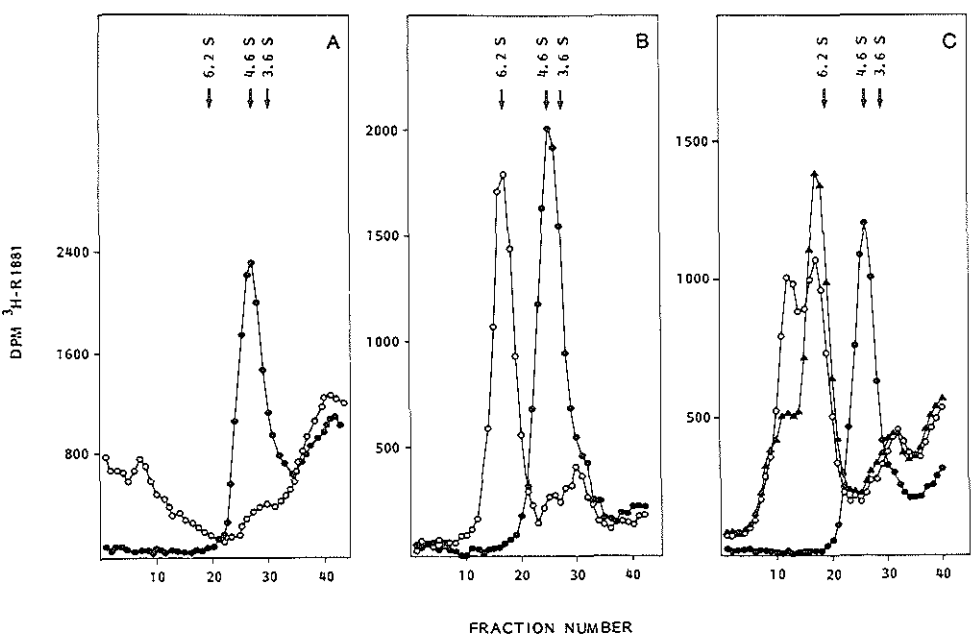

Fig. 3. Sedimentation profiles in 10–30% sucrose gradients of 137 μl [^{3}H]R1881-labeled hAR in LNCaP nuclear extracts (0.15 M NaCl in TEDGP buffer) incubated with (*A*) 13 μl Tp4 (○) or 13 μl pre-immune serum (●); (*B*) 13 μl SpO60 antiserum (○) or pre-immune serum (●); (*C*) 4 μl (○) or 0.8 μl (▲) SpO61 antiserum or 4 μl pre-immune serum (●).

LNCaP cells, was incubated with serial dilutions of the antisera. Immune complexes were precipitated with a second antibody. As shown in Fig. 2, use of serial dilutions of the antisera proportionally decreased the amount of precipitated [^{3}H]-R1881-AR complexes, indicating the presence of specific antibodies against the hAR.

The titres of anti-hAR antibodies in both anti-peptide sera (SpO60 and SpO61) were higher than in the serum raised against the fusion protein (Tp4). Approximately 50% of the [^{3}H]R1881 binding activity could be precipitated with a 1:300 dilution of the Tp4 antiserum. Dilutions of 1:7000 and 1:900 of the antisera SpO61 and SpO60 respectively were able to precipitate 50% of [^{3}H]R1881 binding activity.

Sucrose gradient analysis

In Fig. 3 sucrose density sedimentation analysis is shown of [^{3}H]R1881-labeled hAR in nuclear extracts of LNCaP cells in the presence of the antisera. Incubation of labeled nuclear extracts with each of the antisera resulted in faster sedimentation rates of the 4.6S AR, indicating the presence of specific antibodies in the three antisera that recognize the native hAR. No shifts were observed with pre-immune sera. In the presence of the SpO60 antiserum a 6.7S peak was formed (Fig. 3*B*). In contrast, incubation with the SpO61 antiserum resulted in the generation of two labeled protein peaks of 6.7S and 8.1S respectively (Fig. 3*C*). The ratio between both labeled protein peaks was dependent on the antiserum concentration. The 6.7S complex shifted completely to the 8.1S form in the presence of more antiserum. This suggests that the SpO61 antiserum is directed against two different epitopes on the receptor molecule, whereas one epitope is recognized by the SpO60 antiserum. The Tp4 antiserum shifted the steroid receptor complex to the bottom of the tube (Fig. 3*A*), indicating the formation of large immune complexes.

Specificity of the antibodies

In order to exclude any crossreactivity of the three different antisera with other steroid receptors, we tested receptor specificity with a double-immunoprecipitation assay. For this purpose nuclear extracts containing hPR, hER, hGR and hAR were prepared from T47D, MCF-7, NHIK and LNCaP cells respectively. The results of the immunoprecipitation studies with the three different antisera and the particular receptor preparation are shown in Table 1. Only with the AR preparation a significant radioactive immunoprecipitate was obtained, indicating that none of the other steroid hormone receptors was recognized by the antisera.

TABLE 1

STEROID RECEPTOR SPECIFICITY OF THE ANTISERA

Radiolabeled hAR (2480 dpm), GR (1050 dpm), ER (840 dpm) and PR (2000 dpm) in nuclear extracts of LNCaP, NHIK, MCF-7 and T47D cells, respectively, were incubated with anti- or pre-immune serum in a 1:50 dilution. Goat anti-rabbit antiserum was used as a second antibody. Data are expressed as dpm ^{3}H precipitated.

	hAR	hGR	hER	hPR
Tp4	1018	40	94	45
SpO60	787	43	88	47
SpO61	967	38	94	38
Pre-immune	204	37	100	30

Immunoprecipitation of covalently labeled hAR

To verify that the antisera recognize the intact hAR, receptors that had been covalently labeled with [^{3}H]R1881 were precipitated from LNCaP nuclear extracts with a double-immunoprecipitation assay. The immune precipitate was analyzed on SDS-PAGE. Fig. 4*A* shows the presence of one labeled protein at a position of 110 kDa after methanol precipitation of proteins from photo-labeled LNCaP nuclear extracts. This protein peak represents the intact hAR (Van Laar et al., 1989). The SpO61 and Tp4 antisera precipitated a radioactive labeled protein with exactly the same molecular mass as was precipitated with methanol (Fig. 4*B* and *C*). Similar results were obtained with the SpO60 antiserum.

Western blotting

To examine the interaction of the antisera with the denatured hAR, photolabeled LNCaP cell nuclear extract was fractionated by SDS-PAGE. After transfer to nitrocellulose membranes, blots were incubated with each of the sera. Fig. 5 shows

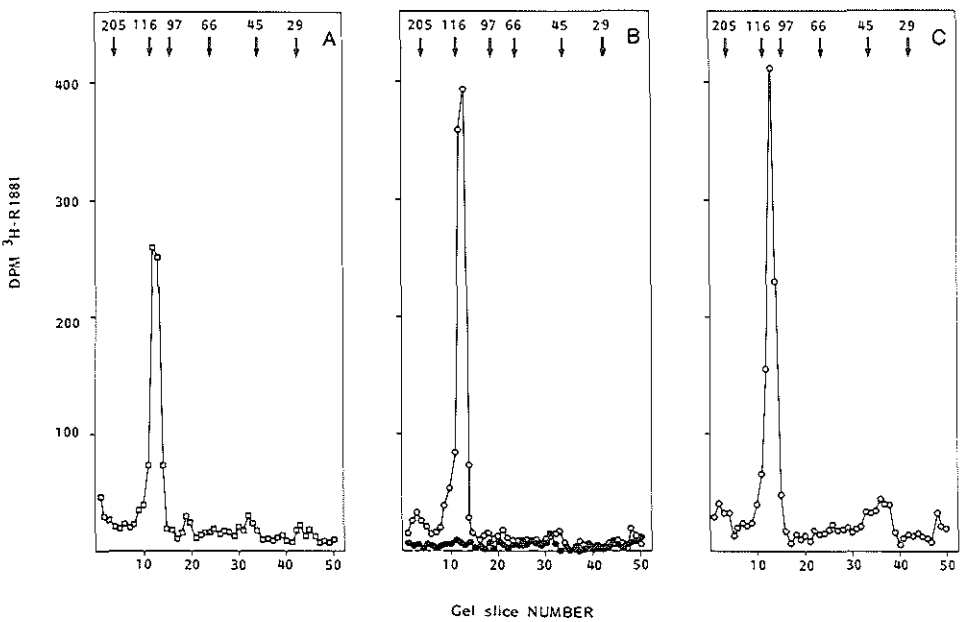

Fig. 4. SDS-PAGE patterns of covalently labeled hAR, precipitated from 800 μl LNCaP nuclear extracts (in 0.15 M NaCl in TEDGP buffer) with (*A*) methanol; (*B*) 4 μl SpO61 antiserum (○) or pre-immune serum (●); (*C*) 16 μl Tp4 antiserum. Immune complexes were isolated with a second antibody.

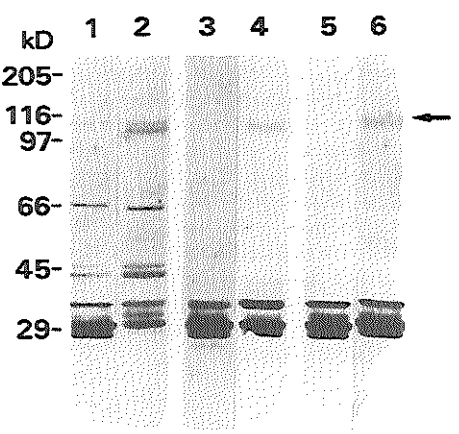

Fig. 5. Immunoblotting of hAR. After SDS-PAGE of nuclear extracts protein was transferred to nitrocellulose and blots were incubated with Tp4, 1:50 (lane 2), SpO60, 1:2000 (lane 4) and SpO61, 1:2000 (lane 6) or the corresponding pre-immune sera in the same dilutions (lanes 1, 3, and 5 respectively).

that a protein with an apparent molecular mass of 110 kDa was identified by the antisera (lanes 2, 4, and 6). A similar protein band was not detectable with the pre-immune sera (lanes 1, 3, and 5). Radioactivity comigrates with the specifically stained protein band.

Immunohistochemistry

Immunostaining with both antiserum SpO60 and SpO61 resulted in a selective nuclear staining of the secretory epithelial cells lining the prostatic glands, whereas no reactivity with the basal cell layer was observed. Reactivity with SpO61 was more intense as compared to the SpO60 antiserum. A rather weak staining reaction with varying proportions of nuclei of stromal cells with antiserum SpO61 was consistently present. Neither antisera yielded reactivity with lymphoma tissues (Fig. 6). In addition, pre-immune sera were negative for nuclear staining of prostate tissue.

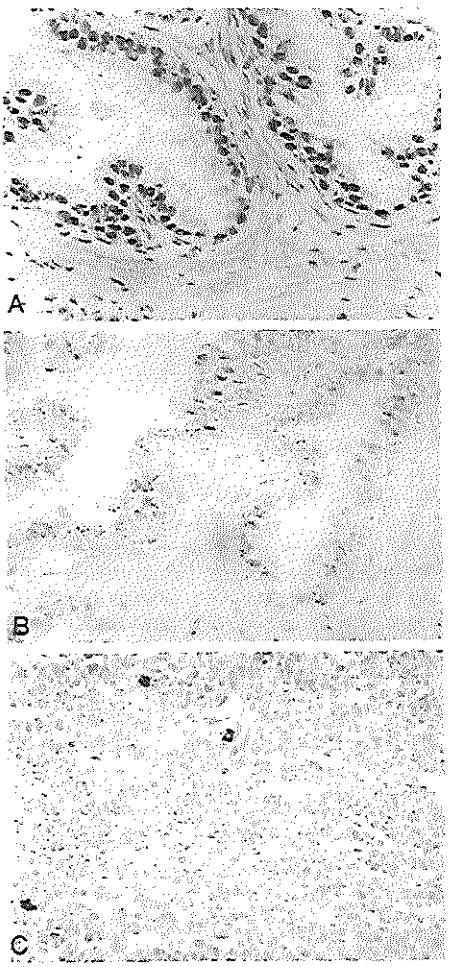

Fig. 6. *A*: Nuclear staining of the secretory epithelial cells of prostatic glands after immunohistochemistry with anti-AR antiserum SpO61. *B*: Prostatic tissue incubated with the pre-immune serum. *C*: Lymphoma tissue incubated with anti-AR antibody SpO61. Magnification 375×, no nuclear counterstaining.

Discussion

This paper describes two different approaches for the preparation of anti-hAR antisera. Both approaches resulted in the generation of high-titre antisera that recognize the native hAR as well as the hAR protein in Western blots. In addition, the peptide-antisera recognize the hAR in nuclei of epithelial cells of benign hyperplastic prostate tissue. The antibodies generated were highly specific for the hAR because crossreactivity with hGR, hPR and hER was absent. In addition, it appeared that AR from other species (e.g. calf) was also recognized (unpublished results). The species specificity, however, has to be investigated further.

In the first approach a bacterially expressed fusion protein product (Tp4), containing a fragment of 180 amino acid residues of the hAR was used for immunization. For two reasons a fragment localized in the N-terminal domain of the receptor protein had been chosen. Firstly, it was known that antibodies against purified preparations of the GR (Carlstedt-Duke et al., 1982; Westphal et al., 1982) and PR (Lorenzo et al., 1988) were predominantly directed against sites in the N-terminal domain, suggesting that this domain is the most immunogenic part of steroid hormone receptors. Secondly, since the steroid- and DNA-binding domains of the hAR share a high homology with similar domains in the hPR, hGR and mineralocorticoid receptor (Chang et al., 1988b; Lubahn et al., 1988b; Trapman et al., 1988) antibodies generated against regions in these domains might crossreact with other steroid hormone receptors.

In the second approach two synthetic peptides (SpO60 and SpO61), corresponding to potentially antigenic regions in the receptor molecule, were selected as antigen. Both amino acid stretches are present within the hAR fragment of the fusion protein product. A similar approach has also been successfully used for the generation of antibodies against a peptide homologous to a hAR fragment flanking the N-terminal zinc finger motif (Lubahn et al., 1988b).

Sucrose gradient analysis of [^{3}H]R1881-labeled hAR in the presence of the Tp4 antiserum resulted in the formation of large immune complexes sedimenting to the bottom of the tube. It is likely that a mixed population of antibody molecules had been generated, recognizing different epitopes on the hAR molecule. This could be expected because a relatively large fragment was used for immuniza-

tion. This result is different from the discrete shifts induced by the anti-peptide sera which are indicative for single-epitope recognition on the labeled hAR. In the presence of the SpO60 antiserum the sedimentation coefficient of the 4.6S [^{3}H]R1881-labeled hAR was shifted to 6.7S suggesting the formation of 1 : 1 complexes of hAR and antibodies. In the presence of the SpO61 antiserum antigen-antibody complexes with sedimentation coefficients of 6.7S and 8.1S were formed. Furthermore, the 6.7S form was converted to 8.1S with increasing antiserum concentrations. This sedimentation profile might be explained by the fact that the antiserum recognizes probably two different epitopes on the receptor molecule.

Both the pre-immune sera and antisera recognize protein bands in Western blots of LNCaP nuclear extracts. One protein band at a position of 110 kDa was stained exclusively with the hAR antisera. There is strong evidence that this band represents the hAR. Firstly, the hAR migrates as a 110 kDa protein on SDS-PAGE (Van Laar et al., 1989). Secondly, although the antisera were raised against different epitopes on the receptor molecule, they all recognized a protein at a similar position in the gel. Thirdly, [^{3}H]R1881 radioactivity comigrated with the stained protein band.

For the characterization of the antisera we exclusively used ligand-bound nuclear hAR. Whether the untransformed hAR is also recognized by the antisera remains to be investigated. Since transformation of steroid receptors might imply dissociation of the receptor from a macromolecular complex including a 90 kDa heat shock protein (Schuh et al., 1985; Sanchez et al., 1987) and/or a conformational change in the receptor molecule, it cannot be excluded that the epitopes, recognized by the antisera are masked in the untransformed receptor form.

The peptide antisera recognize specifically antigens in nuclei of the secretory epithelial cells lining the prostatic glands in frozen sections of prostatic tissue. Some nuclei of stromal cells showed a weak reactivity. A similar AR localization has been observed in rat and human prostatic tissue with different antisera (Lubahn et al., 1988b; Tan et al., 1988) and with radiolabeled R1881 as a ligand (Peters and Barrack, 1987).

The hAR-directed antibodies provide important tools which can be used for studying many aspects of receptor structure and function. It might be possible now to detect the AR without the use of radioactive ligands. Furthermore, AR mutants which lack completely or partially the steroid-binding domain can be detected now. The development of detection methods for aberrant hARs is highly relevant for studies concerning the androgen insensitivity syndrome and for investigations on androgen independency of prostate cancer. Other potential applications of specific AR antibodies are their use for receptor determination with enzyme-linked or radioimmunoassays, and for purification purposes on immunoadsorbent columns.

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Epitope Prediction and Confirmation for the Human Androgen Receptor: Generation of Monoclonal Antibodies for Multi-assay Performance following the Synthetic Peptide Strategy

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Epitope prediction and confirmation for the human androgen receptor: Generation of monoclonal antibodies for multi-assay performance following the synthetic peptide strategy

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The human androgen receptor (hAR) is an important regulatory protein particularly in male sexual differentiation. The investigation of hAR functionality has been hampered by the lack of AR specific monoclonal antibodies recognizing the functional domains of the receptor. Therefore production of high affinity mono-specific polyclonal (PAbs) and monoclonal antibodies (MAbs) directed to the hAR was initiated following the synthetic peptide (SP) strategy. Five hAR specific peptides were selected on the basis of their predicted antigenic properties avoiding homology with other steroid hormone receptors. Peptide specific polyclonal antisera were obtained following selected immunization protocols. Mono-specific polyclonal antibody responses were elicited to all peptides in mice and rabbits. Crossreactivity of the peptide specific antisera with the native hAR in various biochemical assays was observed with two out of five peptides. Peptide SP61 (hAR residues 301–320) was used for the generation of site-directed MAbs specific for the hAR. Specificity for the hAR was established by immunoprecipitation, immune-complex density gradient centrifugation and immunohistochemistry on human prostate tissue sections. The multi-assay performance of the selected high affinity antibodies proved the usefulness of the straight forward peptide approach and opens a wide field of possible biochemical and physiological investigations into questions related to androgen action.

Introduction

Androgens play a major role in male sexual differentiation and they are essential for prostate gland development. They also play a role in tumour genesis in the human prostate. Most human prostatic tumours are at least initially androgen dependent. Androgens exert their action via binding to the intracellular androgen receptor. The activated androgen receptor (AR) subsequently modulates specific gene transcription by interaction with cis-acting hormone responsive elements present in target genes. Steroid hormone receptors consist of three domains: the N-terminal part, which might be involved in the regulation of gene transcription, a DNA binding domain, and a steroid-binding domain at the C-terminus [1,2]. Recently the cDNA sequence of the human androgen receptor (hAR) was elucidated [3,4]. This information provided a basis for the production of new tools to study the hAR system. Since a considerable homology with other steroid receptors (progesterone, oestrogen and glucocorticoid receptors) exists and in addition the purification of a sufficient amount of the protein was not obtained, we decided to use the synthetic peptide (SP) approach to generate site specific, hAR specific, polyclonal and monoclonal antibodies (MAbs). Especially the availability of hAR specific antibodies directed to the different parts of the molecule: the N-terminal regulating part and the DNA and steroid binding domains could be very helpful for immuno-af-

Abbreviations: SP, synthetic peptide(s); hAR, human androgen receptor; ELISA, enzyme-linked immunosorbent assay; KLH, keyhole limpet hemocyanin; BSA, bovine serum albumin; GA, glutaraldehyde; MAb, monoclonal antibody; PAb, polyclonal antibody; R1881, [17α-*methyl*-^{3}H]methyltrienolone.

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finity purification of the receptor. Furthermore, site specific antibodies would facilitate detailed investigation of the AR structure, distribution and its physicochemical properties. Specific antibodies open new possibilities for histochemical localization of the receptor in tissue sections, for diagnostic purposes and to monitor, e.g., AR expression during endocrine therapy for prostatic cancer.

In previous studies, it was shown that when a protein is not available for immunization, for instance for reasons of purification, but the DNA sequence has been determined, synthetic peptides (SPs) become very important tools to generate specific antibodies [5,6]. The use of SPs allowed us to circumvent the difficulty in generating specific antibodies when there exists a strong homology in a family of proteins [7]. SPs are frequently applied in model studies of antigenicity [8–14]. Furthermore, SPs are used to assign the importance of certain amino acid residues (in replacement studies) with respect to the function of a protein domain [15]. SPs may become important as specific subunits in the production of vaccines [16,17].

As the antigenic determinants of the hAR were not known, putative continuous epitopes were selected on the basis of theoretical considerations. In this report, we describe the selection and synthesis of the peptides and the immunization and screening procedures to evoke hAR specific monoclonal and polyclonal specific antibodies. We show that the MAbs are widely applicable in various biochemical and histochemical techniques and that they do not crossreact with other steroid receptors. These properties make them useful diagnostic tools.

Materials and Methods

Peptide synthesis, purification and characterization

Peptides (SP59, SP60, SP61, SP65 and SP66) were synthesized on RapidAmide resin beads using Fmoc protected amino acids (Dupont, U.S.A.) following the procedure as described for the RAMPS System (Dupont, Medical Prod., Biotechnology Syst., U.S.A.). Piperidine was used for deprotection. Elongation was checked each step [18]; the method described by Kaiser et al. [19] was used in case of a proline. Final deprotection and cleavage was performed using a mixture of trifluoroacetic acid, phenol, and ethanedithiol followed by precipitation and filtration from diethyl ether. Mercuric (II)-acetate was used to deprotect the peptides containing cysteine with a tertiary butyl protection group. Cysteine containing peptides were reduced with β-mercaptoethanol for 1 h at pH 8. This procedure also allows to restore the N to O migration in case of serine or threonine. Peptides were purified using liquid chromatography on G-15 Sephadex (Pharmacia) in 5% acetic acid. Fractions were analyzed on a Beckman Ultrosphere 5 μm reversed-phase C18 column using a gradient of acetonitrile with 0.1% trifluoroacetic acid. Fractions with the same major compound and with a high purity were pooled and lyophilized twice. Amino acid analyses were performed to confirm the peptide composition of the hydrolysed peptide using pre-column derivatization of the amino acids [20]. The amount of free thiol groups was determined using DTNB (5′,5′-dithiobis(2-nitrobenzoic acid)) [21].

Coupling methods

All peptides were coupled to immunogenic carrier proteins to enhance antigen presentation (in vivo) or to be used in screening (antigen presentation in assay) [12].

Glutaraldehyde. Essentially the method described by Zegers et al. [14] was applied. The (carrier) protein was dialysed at a concentration of 10 mg · ml^{-1} against 200 ml 0.2% glutaraldehyde (GA) ('Baker' grade 25% in H_2O, J.T. Baker Chemicals, Deventer, Holland) in 0.01 M phosphate-buffered saline, pH 7.2 (PBS) for 16 h at 4°C. The activated (carrier) protein was then dialysed against PBS (three times in 500 ml for several hours) to remove excess GA and transferred to a reaction vessel. The peptide (10 mg · ml^{-1} in distilled water) was added to the GA activated carrier in a molar ratio of 100 molar equivalents. The mixture was stirred for 16 h at 4°C. Remaining active GA groups were blocked by 0.1 ml lysine-HCl (0.2 M) during a 2 h incubation. The excess peptide and lysine molecules were removed by dialysis.

MBS. m-Maleimidobenzoyl-*N*-hydroxysuccinim ide (MBS) ester (Pierce 22310, Rockford, IL, U.S.A.) coupling, conjugating -NH_2 and -SH groups, was performed as described earlier [12]. The MBS was dissolved in dimethylformamide at a concentration of 20 mg · ml^{-1}. To the (carrier) protein (10 mg · ml^{-1} in PBS) MBS was added in a molar ratio of 200 : 1, in three equal portions at 5 min intervals. After incubation for another 20 min at 4°C, under stirring, the mixture was centrifuged to remove any insoluble salts. The supernatant was purified over a PD-10 gel filtration column (Pharmacia, Sweden) to remove excess MBS. To the MBS-activated protein the peptide (10 mg · ml^{-1}) was added in 100-times molar excess and incubated for 1 h at room temperature. The conjugate was then purified by dialysis against PBS.

EDC. For 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC; Sigma E-7750, St Louis, MO, U.S.A.), conjugating -COOH and -NH_2 groups, the modified method as described earlier [13] was used. Briefly, the carrier protein (10 mg · ml^{-1}), EDC (200 mg · ml^{-1}) and the peptide (10 mg · ml^{-1}) were dissolved in 0.5 M *N*-methylimidazole pH 6.0 (Aldrich, M5, 083–4, Brussels, Belgium). The peptide was added to the carrier in a 100 molar excess. After addition of EDC (molar ratio protein/EDC is 1 : 100) the mixture was stirred for 30

min at room temperature followed by dialysis against PBS.

Conjugates of peptides and proteins for screening were made with bovine serum albumin (BSA) and for immunization purposes with keyhole limpet hemocyanin (KLH, Calbiochem, 374811, San Diego, CA, U.S.A.). The conjugates were produced with the aid of various coupling agents (MBS, GA or EDC) and were designated as, e.g., KLH-MBS-SPxx or BSA-EDC-SPxx.

Immunization

Rabbits (Flemish Giant random bred, MBL-TNO, Rijswijk, The Netherlands) were injected subcutaneously three times at 4 week intervals, with 250 μg of the conjugate. First immunization and booster conjugates were emulsified in Freund's complete adjuvant. For the third immunization the conjugate was emulsified in incomplete Freund's adjuvant. Serum was analyzed 14 days after each immunization in ELISA or immunoprecipitation reaction followed by sucrose density gradient centrifugation [22]. Pre-immune sera of the same rabbits served as (negative) controls.

Conjugates of the peptide and KLH were emulsified in Specol [23], and 25 μg was injected intraperitoneally into groups of four (12-week-old female BALB/c) mice. The mice were boosted after 5 weeks with the same dose of conjugates and adjuvant. Seven days after each immunization a blood sample was taken from the tail vein. The serum was assayed in a direct ELISA (in which the antigen was coated directly to the plate by passive adsorption) or in an immunoprecipitation assay.

ELISA

PVC-microtiter plates (Titertek, 77-172-05 highly activated, Flow Laboratories, Irvine, U.K.) were coated overnight at 4°C with 50 μl PBS containing 5 $\mu g \cdot ml^{-1}$ protein, peptide or alternatively the peptide conjugated to BSA (not used for immunization) with a reagent not used in the immunogen. Plates were blocked with 0.5% gelatin in PBS during a 30 minute incubation at room temperature. Sera were diluted in 0.1% gelatin and 0.05% tween-20 in PBS and incubated for 1 h. Alkaline phosphatase conjugated to goat-anti-mouse IgG or swine-anti-rabbit Ig (Kirkegaard and Perry Laboratories, MD, U.S.A.) were used. *p*-nitrophenyl phosphate (1 $mg \cdot ml^{-1}$) in 10 mM diethanolamine + 1 mM $MgCl_2$ at pH 9.8 was used as substrate. After 30 min the absorbance was read at 405 nm in a Titertek Multiskan reader (Flow Laboratories, Irvine, U.K.) against normal mouse serum or pre-immune rabbit serum responses as a blank. The titre in the ELISA was chosen arbitrarily as that dilution which gave an absorbance of 1.0 after 30 min of incubation. A direct ELISA was used to determine the isotype of the MAbs with specific rabbit-anti-mouse-immunoglobulin-subclass anti-sera (Miles Laboratories, Kankakee, IL, U.S.A.).

Immunoprecipitation

The response to the native androgen receptor was analyzed in an immunoprecipitation assay. Sera were incubated at different dilutions for 2 h at 4°C in roller tubes with anti-mouse-IgG conjugated to agarose (Sigma). After centrifugation and washing of the pellet with PBS, 25 μl of a nuclear extract from LNCaP cells (a human cell line of a lymph node carcinoma of the prostate) [24] in which the receptor was labelled with [17α-*methyl*-^{3}H]methyltrienolone ([^{3}H]R1881, a synthetic androgen, NEN-Dupont, Dreieich, F.R.G.), was added. Subsequently 400 μl PBS was added and the mixture was incubated for 2 h at 4°C while rotating [22]. After centrifugation and washing of the pellet, the sample was assayed for the amount of radioactivity. Values are given corrected for background.

Sucrose gradient centrifugation

Essentially the method described by De Boer et al. [25] was followed. Briefly, 50 ml of [^{3}H]R1881-labelled nuclear extract (0.15 M NaCl) from LNCaP cells was incubated for 6 h at 4°C with antiserum or RPMI 1640 culture medium (Flow, Irvine, U.K.) supplemented with 7.5% FCS and 1% NMS as a control and subsequently layered on 10-30% sucrose gradients in the presence of 0.15 M NaCl and centrifugated for 20 h at 370 000 $\times$ *g* at 4°C.

Monoclonal antibodies

Cell fusion was performed essentially as described by Haaijman et al. [26]. Briefly, a spleen cell suspension was prepared 4 days after the second boost. Spleen cells and SP2/0 cells in logarithmic growth were fused at a ratio of 5 : 1 in 40% poly(ethylene glycol) 4000 (Merck, Darmstadt, F.R.G.) + 5% DMSO in PBS. The fused cells (10^5/well in 0.2 ml) were cultured in RPMI 1640 selection medium, containing 1 $\mu g \cdot ml^{-1}$ azaserine and 0.1 mM hypoxanthine, 15% FCS, 2 mM glutamine, 0.1 $mg \cdot ml^{-1}$ streptomycin, 100 $E \cdot ml^{-1}$ penicillin, 1 mM sodium pyruvate, and $5 \cdot 10^{-5}$ M β-mercaptoethanol. After 1 week of culture the azaserine was discontinued and the FCS concentration was lowered to 10%. Selected cell cultures were subcloned by limiting dilution at a density of 0.5 cells/well.

BALB/c mice were injected intraperitoneally with 0.5 ml pristane (2,6,10,14-tetramethylpentadecane 96%, Ega-chemie, Steinheim, F.R.G.). Seven days later the mice were injected with 10^6 monoclonal hybridoma cells in 0.25 ml PBS. Ascites fluid was collected under anaesthesia.

Immunohistochemistry

Samples of prostatic tissue with glandular hyperplasia were removed from prostatectomy specimens immediately after surgery, snap-frozen by immersion in cooled isopentane (-150°C) and stored in liquid

nitrogen. Immunohistochemistry was performed on cryostat sections of 5 μm thickness using the method described by Ruizeveld de Winter et al. [27]. After fixation and rehydration, the slides were incubated overnight with appropriate dilutions of MAbs in PBS (pH 7.8) at 4°C. After several washing steps the reactivity was visualized using horseradish peroxidase conjugated to rabbit-anti-mouse immunoglobulin (DAKO, Denmark) diluted 1 : 100 in PBS containing 5% non-immune human serum and 5% non-immune rabbit serum. Diaminobenzidine and hydrogen peroxide were used as substrate. Control slides were incubated with pre-immune sera or PBS.

Results

Peptide selection

The amino acid sequence of the human androgen receptor [3,4] was used to select stretches of about 20 amino acids, with a high antigenicity index [28,29] with the aid of a computer prediction program (Genetics Computer Group, Wisconsin; [30]). This selection was based on analysis of primary and secondary structure parameters such as hydrophilic character, protein surface probability and expected flexibility [31–36]. Preferable sequences including putative "turn" sequences were selected based on earlier experiences. The cut-off places in sequences were chosen in such a way that amino- and carboxy-termini had a relatively high hydrophilicity index. Because of a strong homology between the hAR and other steroid hormone receptors, peptides were chosen such that amino acid sequences similar to those in other steroid hormone receptors, like progesterone-, oestrogen- and glucocorticoid receptors, were avoided [37–41].

TABLE I

Selected amino acid sequences of the human androgen receptor

SP	Residues	Sequence
SP59	(AA194–213)	CQQQEAVSEGSSSGRAREASG
SP60	(AA201–222)	EGSSSGRAREASGAPTSSKDNYC
SP61	(AA301–320)	EDTAEYSPFKGGYTKGLEGEC
SP65	(AA834–853)	KELDRIIACKRKNPTSCSRRC
SP66	(AA899–917)	CVQVPKILSGKVKPIYFHTQ

Underlined Cysteine was not a part of the hAR sequence, but was added at one of the termini of the peptides for coupling purposes.

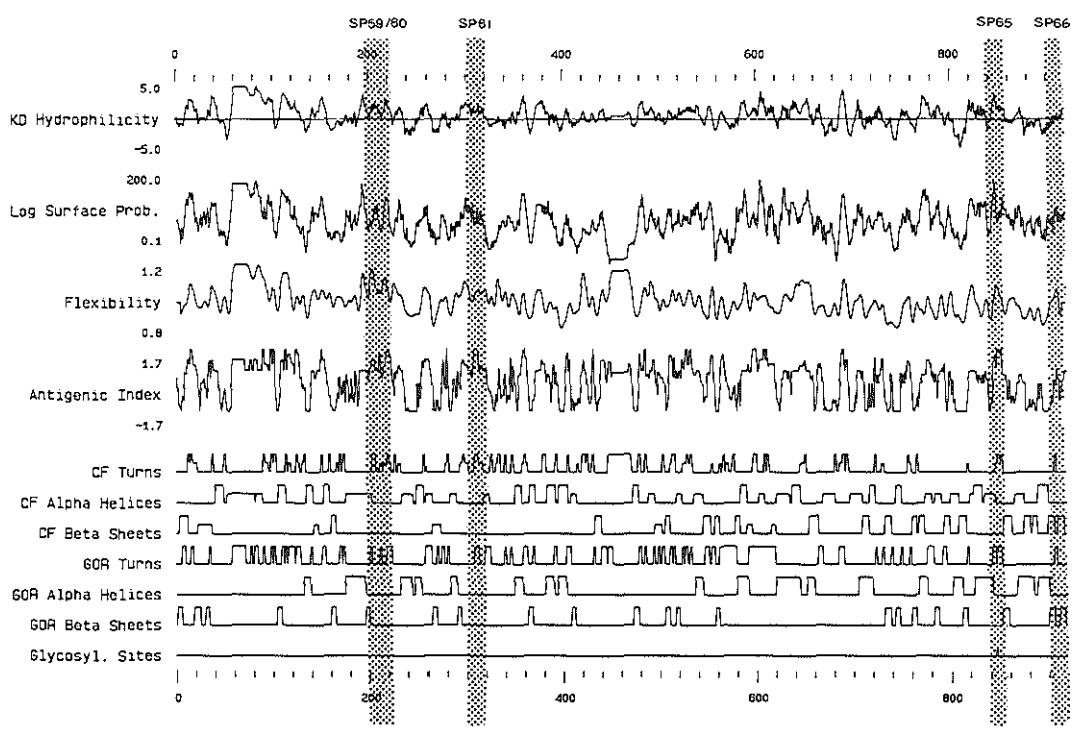

Fig. 1. Physical parameters of the androgen receptor protein predicted on the basis of the amino acid sequence deduced from the DNA sequence. In this plot hydrophilicity data are calculated according to Kyte and Doolittle [34]. CF: Chou and Fassman (1978), GOR: Garnier, Osguthorpe and Robson (1978). See Materials and Methods section.

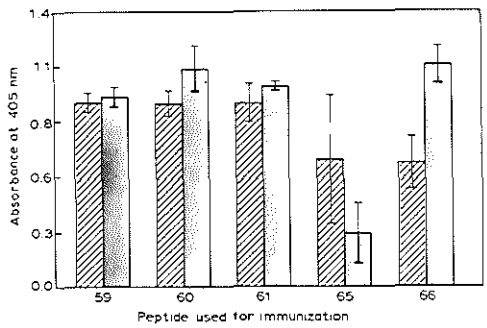

Fig. 2. Response to peptide and conjugate. Reactivity of antibodies in rabbit sera with free peptide or to the peptide conjugated to BSA with EDC determined in ELISA. Rabbits were immunized twice with KLH-MBS-SP conjugates. The serum dilution was 1:500. Results are shown as the mean value with standard bars for two rabbits. Coatings are: peptide, striped bars; BSA-peptide, solid bars.

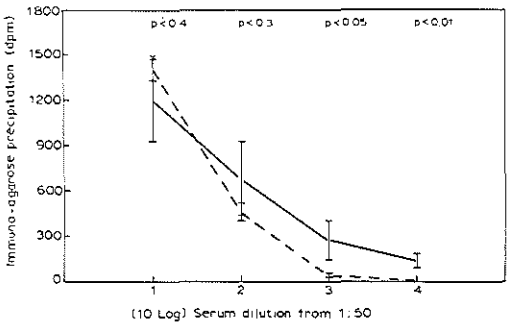

Fig. 4. Reactivity with androgen receptor. The reactivity of antibodies in mouse sera with hAR-steroid complex using immuno-agarose precipitation. Mice were immunized twice with KLH-MBS-SP61 or KLH-GA-SP61. Results are shown for ^{10}log serial dilutions of the sera with an initial serum dilution of 1:50. The mean value with standard deviation bars for three (MBS) or four (GA) mice are given. Solid line represents glutaraldehyde and the dotted line represents MBS.

The selected peptide sequences were matched with protein sequences in the protein sequence database PIR (Protein Identification Resource, National Biomedical Research Foundation, Washington, D.C., U.S.A.) to determine whether the selected sequences showed homology with proteins stored in the database. Even when a mismatch of ten residues (50% homology) in the peptide sequence was allowed, we did not find similar peptide sequences from putative crossreactive (human) proteins except the hAR fragment itself.

The position of the five selected peptides is shaded in Fig. 1. An extra cysteine (at the N-terminus for SP59 and SP66, at the C-terminus for the other peptides) was added for coupling purposes. SP59, SP60 and SP61 are situated in the N-terminal domain and SP65 and SP66 in the steroid binding domain of the protein (Table I).

Generation of monospecific polyclonal antibodies

Each peptide conjugated to KLH with MBS was injected in two rabbits. SP59 was also coupled to KLH with glutaraldehyde. Therefore one rabbit was immunized with KLH-MBS-SP59 and one rabbit with KLH-GA-SP59.

After the second boost, sera from all rabbits as tested in ELISA gave a positive response to the coating of free peptide as well as to the same peptide in a BSA conjugate (Fig. 2). The response towards SP65 was low compared to the response to other peptides. Pre-immune sera of rabbits did not show any response to the peptides. SPs immunized in mice gave essentially similar polyclonal responses as observed in rabbits. All responses were read against non-immune serum as a blank.

Effect of conjugation on antibody responses in mice. At higher serum dilutions, the reactivity of antibodies to SP61 was much lower in sera of mice immunized with conjugates of SP61 prepared with glutaraldehyde (KLH-GA-SP61) than was determined after KLH-MBS-SP61 immunization (Fig. 3, right). The responses

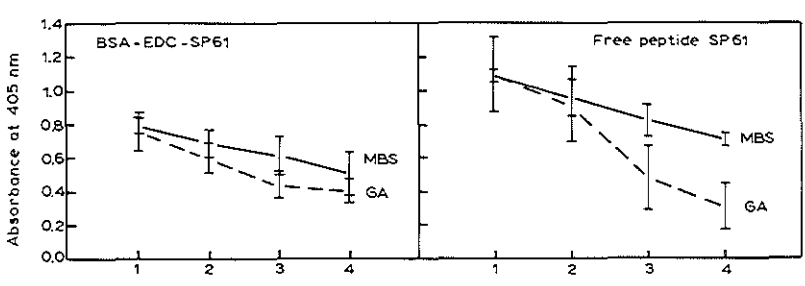

Fig. 3. Reactivity of antibodies in mouse sera with free SP61 (right) and BSA-EDC-SP61 (left) in ELISA. Mice were immunized twice with KLH-MBS-SP61 or with KLH-GA-SP61. The initial serum dilution was 1:100. Results are shown as the mean value with standard deviation bars for three (MBS) or four (GA) mice. The response to the free peptide differs significantly for the two last dilutions ($P < 0.05$ and $P < 0.01$ respectively for a final dilution 1:400 and 1:800).

towards SP61 conjugated with EDC to BSA were similar for both groups (Fig. 3, left). Sera from these mice did not react with other peptides or with BSA. In Fig. 4, the responses towards the human androgen receptor of the sera elicited with the GA and the MBS based KLH-conjugates of SP61, as measured in an immuno precipitation assay, are shown. At higher serum dilutions (higher then 1:5000), the sera from the mice immunized with KLH-GA-SP61 still gave a native protein specific response, while for the sera elicited with MBS conjugate the signal has disappeared.

Recognition of the native protein. The hAR specific antibody response in the rabbit and mouse anti-sera was demonstrated for the peptide SP61 and for SP60 with rabbit sera only, in several independent techniques: sucrose gradient density centrifugation, immunoprecipitation, Western blotting and immunohistochemistry (data not shown).

Generation of monoclonal antibodies

Groups of three or four mice were immunized with KLH-MBS-SP61 and KLH-GA-SP61. Two different conjugates were used in order to present the peptide in various orientations to the immune system [12,42]. Spleen cells of mice immunized with KLH-GA-SP61 were used for cell fusion. The first fusion was highly efficient as all the wells (768) contained proliferating hybridomas. SP61 specific antibody producing clones were identified in a primary selection in ELISA. Anti-SP61 responses were found in 107 out of 768 wells. A second selection was performed in immunoprecipitation and gave 15 positive anti hAR clones. Seven wells containing clones with the highest reactivity were selected for subcloning. Three out of seven lost their activity during subcloning. Four monoclonal cell lines, F39.3.1, F39.4.1, F39.5.1 and F39.6.2, derived from different wells in the original 96-well fusion plates, were selected for ascites production.

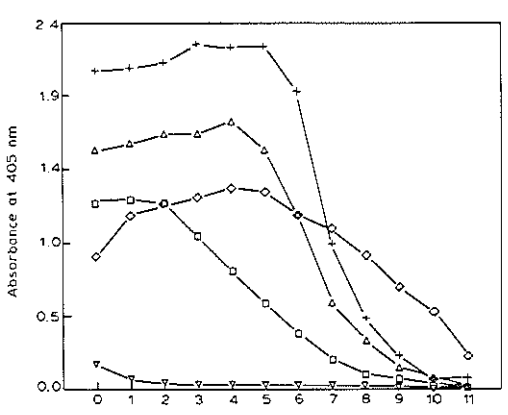

Fig. 5. Titration curve of MAbs to SP61 in a direct ELISA. The titre was expressed as the dilution at which the absorbance is 1.0 after 30 min of incubation. The initial ascites dilution was 1:100 (point 0). □, F39.3.1; +, F39.4.1; ◇, F39.5.1; △, F39.6.2; and ▽, background.

TABLE II

Properties of hAR specific monoclonal antibodies

MAb	Isotype	hAR titre [a]	SP61 titre [b]	Sedimentation rate constant [c]	Immunohistochemistry [d]
F39.3.1	IgG2b κ	<1:100	1:918	4.5S	–
F39.4.1	IgG1 κ	1:13000	1:12800	6.5S	+
F39.5.1	IgG1 κ	1:1300	1:18100	6.5S	–
F39.6.2	IgG2b κ	1:1600	1:7900	6.5S	–

[a] The titre is expressed as the dilution at which the antibody binding to the hAR-steroid complex is 50% of maximum, determined in immuno agarose precipitation.

[b] The titre is expressed as the dilution at which the absorbance is 1.0 after 30 min of incubation with substrate at 25°C determined by ELISA.

[c] Complex formation of antibody binding to R1881-hAR induces a shift in sedimentation constant.

[d] Only F39.4.1. gave strong immunostaining (+) in sections of hyperplastic prostatic tissue. The other MAbs were negative (–). Detailed information in Fig. 7 and in the text.

Characterization of hAR specific monoclonal antibodies

Immunoprecipitation. Specificity for the human androgen receptor was determined by assaying the crossreactive response to other hormone receptors (progesterone, oestrogen and glucocorticoid receptors) in immuno precipitation assays. The four MAbs showed a positive response with the hAR only. The selected MAbs crossreacted with rat AR and calf AR in immunoprecipitation and Western blots. In a comparison of the reactivity of the four anti-AR MAbs, in the agarose precipitation assay, the highest titre was obtained with F39.4.1 ascites (Fig. 5; Table II). Ascites fluid F39.4.1 could be diluted ten times more than both ascites F39.5.1 and F39.6.2. The affinity of MAb F39.3.1 for hAR is very low, the titre being lower than 1:100. Anti-hAR MAb F39.4.1 in this assay crossreacted with the rat-AR (not shown) but the affinity as judged from titration curves might be a factor ten lower than observed for the hAR.

To precipitate 25 fmol hAR, 0.13 μg MAb F39.4.1 was needed. The concentration of IgG was established by measurement of the total protein concentration and from scanning agarose gels to assign the proportion of total protein of the IgG band.

ELISA. The curves of MAbs F39.4.1 and F39.6.2 (Fig. 5) with SP61 at comparable protein concentrations showed a steep slope in a direct ELISA. The slope of MAb F39.5.1 decreased more slowly and showed a

prozone effect at lower dilutions. This might indicate that the affinity of F39.5.1 for SP61 is lower than the affinity of F39.4.1 and F39.6.2. The affinity of F39.3.1 for SP61 is very low as can be derived from both the slope and the titre (Table II).

The isotype of the MAbs (Table II) was determined in a direct ELISA. Both F39.4.1 and F39.5.1 were IgG1 κ and the MAbs F39.3.1 and F39.6.2 were IgG2b κ.

Antigen-antibody complex formation. Formation of antibody-receptor complexes was analyzed by sucrose density gradient centrifugation. In Fig. 6 the precipitation of the androgen receptor in presence or absence of the MAbs is shown. For MAbs F39.4.1, F39.5.1, and F39.6.2 the buoyant density of the steroid-hAR complex in a sucrose gradient shifted approx. from 4.6S to 6.5S due to the binding of the antibody to the complex. For MAb F39.3.1 the shift was less pronounced, only broadening of the 4.6S peak was seen (Fig. 6A).

Immunohistochemistry. Supernatants from 15 cell cultures (tested positive in immunoprecipitation) of anti-SP61 specific MAbs were applied for the indirect immuno-enzymatic staining of human prostate tissue sections. Only MAb F39.4.1 gave strong staining in immuno-histochemistry. Fig. 7 shows hyperplastic prostatic glands surrounded by stromal cells. A strong and specific immunostaining of the nuclei of secretory epithelial cells and some of the stromal cells is obtained using the anti-SP61 MAbs.

Discussion

In this study we demonstrate, that the synthetic peptide strategy leads to the generation of highly specific and desirable reagents that could not be produced following classical approaches. Human androgen receptor antibodies were produced with: known determinant specificity, high affinity and multi-assay performance. The properties of the high affinity hAR specific poly- and monoclonal antibodies are summarized in Table II. Calculation and prediction of putative antigenic sites of the AR to which the MAbs are directed was based on the amino acid sequence derived from the cDNA se-

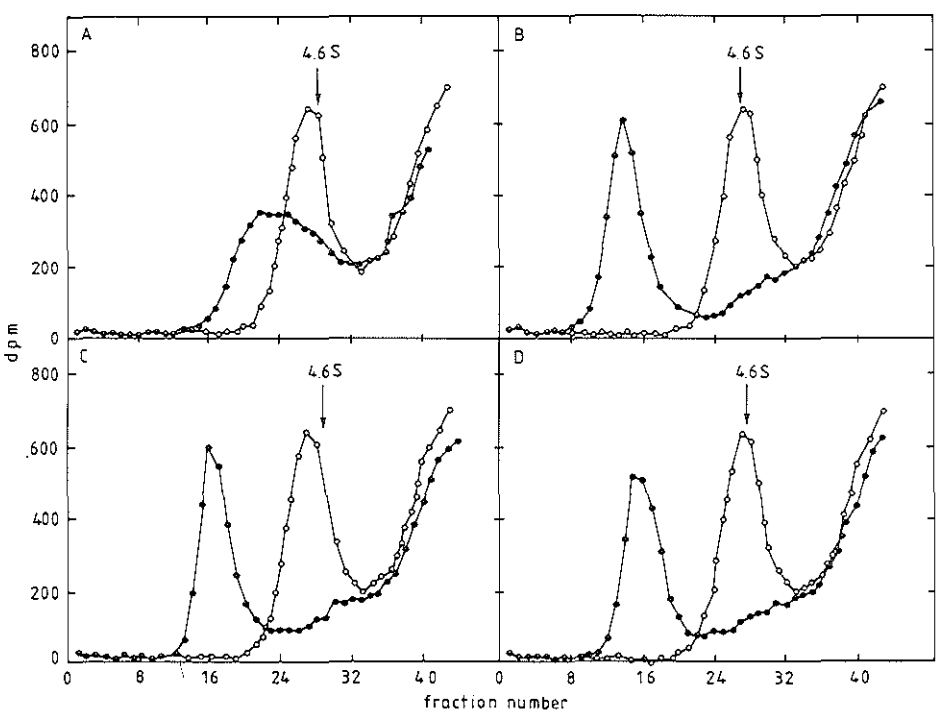

Fig. 6. Complex formation of selected MAbs with the hAR. Complexes were demonstrated by a shift in the sedimentation profile of the [^{3}H]R1881-labelled hAR. Appropriate antibody dilutions were established in separated titration experiments. Sedimentation profiles in 10–30% sucrose gradients of [^{3}H]R1881-labelled hAR in LNCaP nuclear extracts incubated with (A) MAb F39.3.1 (●); (B) MAb F39.4.1 (●); (C) MAb F39.5.1 (●); (D) MAb F39.6.2 (●); or without antiserum (o). The total volume was 100 μl in which the ascites dilution was 1:100. MAbs F39.4.1, F39.5.1 and F39.6.2 show a characteristic sedimentation shift. With MAb F39.3.1 there is an interference of the MAb with the sedimentation properties of the receptor not characteristic for immune-complex formation.

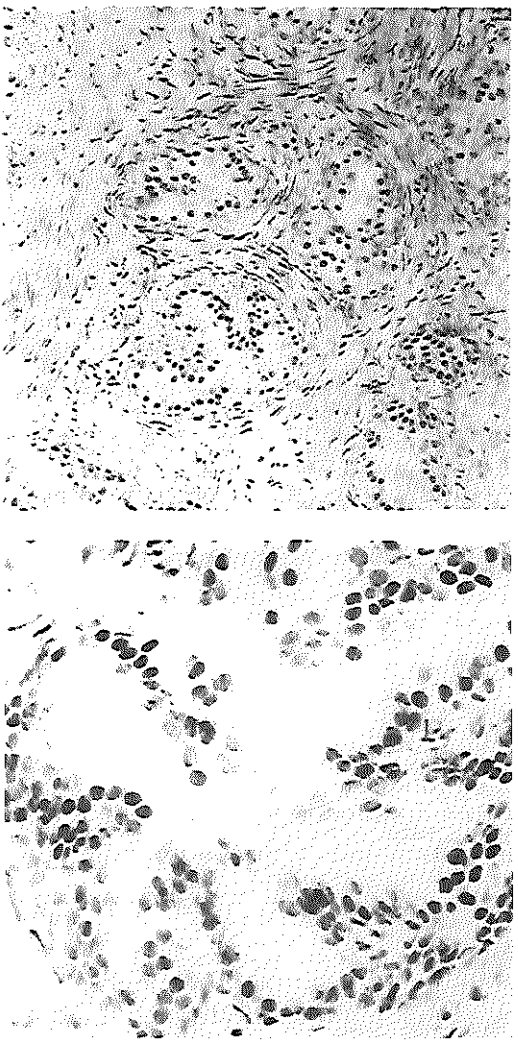

Fig. 7. Top panel: low-power magnification of hyperplastic prostatic tissue immunostained with F39.4.1. Prominent nuclear staining of prostatic glands and a large proportion of stromal cells. No nuclear counterstaining (×100). Lower panel: hyperplastic prostatic gland immunostained with F39.4.1. Intense nuclear staining of secretory epithelial cells lining the glands. Variable staining of stromal nuclei. The basal cells do not show reactivity with F39.4.1. No nuclear counterstaining (×250).

quence. Peptides were selected by exclusion of sequences with a large homology with other steroid receptors (oestrogen, progesterone, glucocorticoid) and selection of putative immunogenic peptides, based on the prediction of the antigenicity index. This approach is limited to prediction of continuous linear epitopes, against which antibodies can be evoked with synthetic peptides.

We elicited a peptide specific response to all peptides selected with a computer algorithm. In two out of five cases antibodies crossreacted with the intact hAR. These two peptides, SP60 and SP61 are both located in the same N-terminal domain of the protein. MAbs were only developed with the peptide that generated the highest response. This N-terminal domain of the hAR showed a relatively low degree of homology (approx. 80%) with N-terminal sequence of the rat AR as compared to the homology observed in the DNA binding domain and the steroid-binding domain [46]. Homology of hAR with the rabbit AR is not known, but the DNA sequence as present in the hAR may be conserved in rabbits as well. For SP61 17 out of 20 amino acids are homologous in human and rat androgen receptors. A putative high degree of homology between hAR and rabbit AR did not prevent the generation of specific hAR antibodies. This can be explained by the fact, that the receptor in general is not or only to a limited degree exposed to the immune system.

Peptides SP65 and SP66 are hydrophilic stretches derived from the C-terminal steroid binding domain. Anti-SP65 and anti-SP66 antibodies were not cross-reactive with the hAR as shown with immuno-precipitation and in immuno-histochemistry. Apart from exposed sites, hinges and protein edge strands, the hydrophilic stretches in a protein may have a function in protein-protein, protein DNA-RNA or protein-steroid interactions. The reason for anti-SP65 and SP66 antibodies being not crossreactive with the hAR might originate in either a difference in 3D structure of the peptide or in the assay systems applied. The sera were screened for the presence of hAR specific antibodies with agarose immuno precipitation. In this assay the hAR is complexed first with a labelled steroid analogue ([^{3}H]R1881) and subsequently the antisera are added. It cannot be excluded that the putative antigenic sites of the hAR are shielded and/or drastically changed by the steroid binding, such that the antibodies cannot recognize the altered hAR. It is also possible that only denatured hAR may be recognized by anti SP65 or anti SP66 antibodies. However, this is unlikely since the immuno-precipitation method and the sucrose gradient centrifugation method applied make use of soluble hAR extracted with 0.15 M. NaCl without any detergents and only mild fixation was used for immuno-chemistry.

Four MAbs with different isotype (IgG1 κ (2×) and IgG2b κ (2×)) and with different affinity for the hAR (varying from moderate to high) were isolated in one fusion after immunization with SP61. The MAbs obtained are site specific for AA301–320 situated in the N-terminal part of the hAR. Anti-SP61 MAbs show a broad applicability as was shown in our performance testing namely in immuno-histochemistry, in immuno-

blotting assays and in immuno-precipitation assays. MAb F39.4.1 has the highest affinity for the hAR (0.0052 μg/fmol hAR) (Table II) but not the highest affinity for SP61. MAb F39.5.1 has the highest affinity for SP61 but hAR binding is moderate (Table II; Fig. 5). Differences in isotype are welcome since this enables double staining studies in immunohistochemistry with different second step (anti-isotype) conjugates. MAbs with low affinity can also be used, e.g., in competition studies.

PAbs against a synthetic peptide of 15 amino acids at the N-terminal end of the DNA binding region of the rat AR, that do recognize the ratAR in immunohistochemistry have been described [43,44]. Chang et al. [45] produced PAbs and MAbs obtained after immunization with β-galactosidase fusion proteins of fragments of the hAR (A: AA331–572 and B: AA 544–822). One of the MAbs raised with the fragment A fusion protein has the highest affinity, and precipitates an amount of 25 fmol AR with 4 μg monoclonal antibody. In contrast, only 0.13 μg was needed for MAb F39.4.1 (this paper) for precipitation of a similar amount of AR. Although non-identical assay conditions could have affected these data it suggests, however, that MAb F39.4.1 has a very high affinity for hAR.

In the present study we have shown, that the strategy using synthetic peptides for MAb production may lead to qualitative and quantitative results, that otherwise only could be obtained at the expense of considerably greater effort, e.g., production of purified fusion proteins. Especially the properties of the hAR: instability, large, not available in purified form made it a good candidate for this type of approach.

Excellent site specific performance of the monospecific polyclonal antibodies as well as for the monoclonal antibodies was demonstrated in different biochemical assays: immunoprecipitation, sucrose density gradient centrifugation and immuno-histochemistry. The antibodies probably recognize the native hAR in immuno-precipitation since the extraction method applied only uses 0.15 M NaCl without use of detergents and only mild fixation was used for tissue sections in immuno-histochemistry. The MAbs have high affinity for hAR (ascites F39.4.1 still effective at 1 : 13000 dilution) and they crossreact with the homologous rat AR and bovine AR.

The hAR-specific antibodies produced are reactive with the N-terminal part of the receptor and are supposed not to interfere with DNA- and steroid-binding and allowing, therefore, biochemical and cell-biological investigations at the molecular level. Furthermore the immuno-histo-cytochemistry at light microscopic and electron-microscopic level should enable now the generation of data on expression of the receptor during growth and development as well as in malignant processes.

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Summary & Samenvatting

Summary

In 1991 prostate cancer was the second leading cause of cancer mortality in the Netherlands. Some 40% of the patients, those with metastatic disease, are treated with hormonal therapy. Eventually all patients will demonstrate hormone-refractory (HR) cancer, the median time to progression being 12-18 months.

The transient character of the remission induced by endocrine therapy can be explained by two mechanisms: 1. the prostate cancers already contained an androgen-independent (human androgen receptor [hAR]-negative) cellular component at the start of therapy and 2. the androgen independence of the initially androgen dependent prostatic carcinomas will develop during endocrine treatment. The premise of this study was the relationship between androgen responsiveness, hAR expression and the likelihood of castration-induced selection of androgen-independent, hAR-negative cancer cells.

The molecular cloning of the human androgen receptor (hAR) cDNA provided the opportunity to generate specific anti-hAR antibodies, to study hAR expression in prostatic carcinomas at the cellular level and to address the issue of developing androgen independence in human prostate cancer.

Chapters 5, 9, and 10 describe the generation and characterization of poly- and monoclonal anti-hAR antibodies. Rabbits and mice were immunized with Sp061, a synthetic peptide corresponding to a potential antigenic site in the N-terminal domain of the hAR (amino acids 301-320). Specific anti-peptide titers in repeatedly immunized rabbits and in mice were determined in ELISA. Subsequently positive sera (or supernatants of hybridomas) were tested with a variety of techniques (immunoprecipitation, sucrose gradient centrifugation, Western blotting and immunohistochemistry) to analyze the properties, including specificity, of the antibodies. The polyclonal anti-hAR antibody Sp61 and the monoclonal anti-hAR antibody F39.4 recognize the hAR in various assays and proved to be a valuable marker in immunohistochemistry. The immunohistochemical characterization of F39.4 is presented in Chapter 5. Using this antibody all male and part of the female reproductive organs demonstrated a predominantly nuclear immunostaining in both the epithelial and stromal compartment. Virtually no immunostaining was seen in lymphatic tissues.

Polyclonal antibody Sp61 was used to determine whether localized prostate cancer already contained an hAR-negative component, which could be responsible for the development of androgen independence. hAR expression was heterogeneously distributed in this group of patients. Poorly differentiated adenocarcinomas contained lower percentages of hAR-positive cells as compared to well and moderately differentiated prostate cancers (Chapter 4). Using monoclonal antibody F39.4 these data were confirmed in an extended series of prostate cancer patients (Chapter 7).

The transition of androgen dependence to androgen independence of prostate cancer

might be associated with modulation of the hAR system (change in hAR expression level). Alternatively, tumor cells in androgen-independent prostatic carcinomas may have bypassed the hAR system. The question of whether androgen-independent prostate cancer contains an altered level of hAR expression was addressed in two series of patients with locally progressive HR prostate cancer (Chapters 6 and 7). The majority of the HR carcinomas were found to display a rather uniform hAR expression. This stands in contrast with the view that hormonal therapy (androgen ablation) selectively induces expansion of receptor-negative tumor cells. Only part of the specimens analyzed (4/48) (almost) completely lacked hAR expression. A subset of HR prostate cancers was evaluated for mutations in the hAR gene, but no such changes were found (Chapter 7). Prostatic carcinomas displaying F39.4 immunoreactivity and containing a structural intact hAR gene were also assessed for their hormone-binding capacity, a further prerequisite for a structural intact receptor to function. The hormone-binding data were in line with the immunohistochemical observations (Chapter 7). Immunoenzymatic double staining for hAR and prostate-specific antigen (PSA) expression was performed to determine hAR functional activity, since PSA expression is known to be an androgen-regulated proces. Even in hAR negative tumor areas PSA immunostaining was observed, indicating that PSA immunohistochemistry is of no use in determining hAR functional activity in human prostatic carcinomas. It is yet unknown whether the hAR expressed in androgen-independent carcinomas — in an environment largely devoid of androgen — actively participates in gene transcription.

The effect of androgen manipulation (withdrawal and/or resubstitution) on hAR messenger ribonucleic acid (mRNA) and protein was assessed in the strictly androgen-dependent human prostatic tumor line PC-82 (Chapter 8). The almost complete reduction of nuclear hAR immunoreactivity within 5 days after castration was restored by androgen resubstitution. These data support the concept of hAR up-regulation by androgen in the human heterotransplantable prostate carcinoma PC-82.

Samenvatting

In 1991 was prostaatkanker na longkanker de belangrijkste oorzaak van kankersterfte in Nederland. Patienten met gemetastaseerd prostaatcarcinoom - ongeveer 40% van het totale aantal patienten met deze maligniteit- worden behandeld met hormonale therapie. De therapie geïnduceerde remissie is slechts van tijdelijke aard. De mediane tijdsduur tot het optreden van hormoon onafhankelijkheid, zich uitend in tumor progressie, bedraagt 12 - 18 maanden. Het slechts tijdelijke effect van de hormonale therapie zou kunnen worden verklaard door hetzij de aanwezigheid van een androgeen onafhankelijke, humane androgeen receptor (hAR) negatieve component in de tumor bij de start van de therapie of door de ontwikkeling van een androgeen onafhankelijke component tijdens de therapie. Uitgangspunt bij de studie was de relatie tussen androgeen gevoeligheid, hAR expressie en het optreden van een selectie van androgeen onafhankelijke, hAR negatieve tumorcellen door androgeen onttrekking.
Het cloneren van het hAR cDNA en de opheldering van de eiwit structuur van de hAR schiep de mogelijkheid specifieke anti-hAR antilichamen te ontwikkelen en de hAR expressie in prostaatcarcinomen op cellulair niveau te bestuderen. Tevens kon de aandacht worden gericht op het probleem van de ontwikkeling van androgeen ongevoeligheid in prostaattumoren.
In de hoofdstukken 5, 9 en 10 worden de ontwikkeling en de karakteristieken van de poly- en monoclonale anti-hAR antilichamen beschreven. Konijnen en muizen werden geïmmuniseerd met Sp061, een synthetisch peptide overeenkomend met een potentieel antigene sequentie in het N-terminale domein van de hAR (aminozuren 301-320). Specifieke anti-peptide titers werden m.b.v. ELISA bepaald in de sera van de meerdere malen geïmmuniseerde konijnen en muizen. Positief geteste sera (of supernatanten van hybridomen) werden op meerder wijzen gekarakteriseerd (immunoprecipitatie, sucrose gradient centrifugatie, Western blot en immunohistochemie). Het polyclonale anti-hAR antilichaam Sp61 en het monoclonale anti-hAR antilichaam F39.4 herkennen de hAR in verschillende tests en zijn waardevolle immunohistochemische merkers gebleken. De immunohistochemische specificiteit van F39.4 wordt beschreven in hoofdstuk 5. Gebruikmakend van dit antilichaam werd een overwegend in de kern gelocalizeerde kleuring gezien van zowel epitheliale- als stromale cellen van de mannelijk- en vrouwelijk genitale organen. Lymphatische organen toonden nauwelijks immunoreactiviteit.
De hAR status van tumorcellen in het gelocalizeerde prostaatcarcinoom werd onderzocht met het polyclonale antilichaam Sp61. De hAR expressie bleek heterogeen te zijn verdeeld in deze tumoren. Weinig gedifferentieerde adenocarcinomen bevatten minder cellen, die de hAR tot expressie brengen dan goed- en matig gedifferentieerde prostaatcarcinomen (hoofdstuk 4). Gebruikmakend van het monoclonale antilichaam F39.4 werden deze gegevens bevestigd (hoofdstuk 7).
De overgang van androgeen afhankelijkheid naar androgeen onafhankelijkheid zou verklaard

kunnen worden door modulatie van de hAR gereguleerde transcriptie (veranderingen in het hAR expressie niveau). Anderzijds zou het kunnen zijn, dat het hAR systeem niet betrokken is bij de progressie van het prostaatcarcinoom naar androgeen onafhankelijkheid. Het hAR expressie niveau van het androgeen onafhankelijk groeiende prostaatcarcinoom werd onderzocht in patienten met locale tumor progressie tijdens hormonale therapie (hoofdstukken 6 en 7). De meerderheid van de hormoon refractoire carcinomen toonden hAR expressie. Deze bevinding is niet in overeenstemming met het concept, dat hormonale therapie (androgeen onttrekking) selectief de groei bevordert van al in een eerder stadium aanwezige tumorcellen, die geen hAR bevatten. Slechts een minderheid van de onderzochte tumoren (4/48) toonde vrijwel geen hAR expressie.
Een deel van de hormoon refractoire prostaatcarcinomen werd onderzocht op het voorkomen van mutaties in het hAR gen. Structurele afwijkingen van het hAR gen werden echter niet gevonden (hoofdstuk 7). Aangezien de mogelijkheid tot het binden van androgenen een voorwaarde is voor het functioneren van een structureel intacte receptor, werd de hormoonbindingscapaciteit van de hAR in een deel van de hormoon refractoire tumoren onderzocht. De carcinomen, die immunohistochemisch hAR expressie toonden, bleken in staat androgeen te binden (hoofdstuk 7).
Aangezien androgenen van belang zijn voor de expressie van prostaat-specifiek antigeen (PSA) werd de coëxpressie van hAR en PSA bestudeerd met een immunohistochemische dubbelkleuringstechniek teneinde een indruk te krijgen van de functionele activiteit van de hAR. De PSA expressie van humane prostaatcarcinomen bleek geen waarde te hebben voor het bepalen van de functionele activiteit van de hAR. Zelfs in groepen tumorcellen zonder waarneembare hAR werd PSA expressie aangetoond. Op dit moment is het nog onbekend of de hAR in androgeen onafhankelijke prostaatcarcinomen — in een omgeving zonder androgenen — actief betrokken is bij de gen transcriptie.
Het effect van hormonale manipulatie (androgeen onttrekking en resubstitutie) op hAR mRNA en hAR eiwit expressie werd bestudeerd in de androgeen afhankelijke prostaat tumorlijn PC-82 (hoofdstuk 8). Androgeen onttrekking (castratie) gedurende 5 dagen leidde tot de afwezigheid van hAR expressie. De hAR expressie niveaux voor castratie en na androgeen resubstitutie waren vergelijkbaar. Deze gegevens ondersteunen het concept van hAR-upregulatie door androgenen in de humane, transplanteerbare prostaat tumorlijn PC-82.

Nawoord

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Curriculum vitae

Jacobus Anne Ruizeveld de Winter werd 1 maart 1959 geboren te Rotterdam. Na het behalen van het Gymnasium diploma en colloquium doctum werd in 1980 aangevangen met de studie geneeskunde aan de Erasmus Universiteit Rotterdam. Na het artsexamen, dat in 1987 cum laude werd afgelegd, was hij gedurende 8 maanden verbonden aan de Afdeling Immunologie van de Erasmus Universiteit, geleid door Prof. Dr. R. Benner. Vanaf december 1988 tot januari 1994 werd hij opgeleid tot patholoog aan de Afdeling Klinische Pathologie van Academisch Ziekenhuis Rotterdam (opleiders achtereenvolgens Prof. Dr. R.O. van der Heul en Prof. Dr. F.T. Bosman) en aan de Afdeling Pathologie van de Stichting Samenwerkende Delftse Ziekenhuizen (opleider Dr. C.A. Seldenrijk). Het onderzoek, zoals beschreven in dit proefschrift, werd in dezelfde periode verricht aan de Afdeling Klinische Pathologie van het Academisch Ziekenhuis Rotterdam onder begeleiding van Dr. Th.H. van der Kwast. Sedert 1 januari 1994 is hij werkzaam als patholoog bij de Stichting Laboratorium Pathologie en Cytologie, PATHAN te Rotterdam.