THE ROLE OF GROWTH FACTORS, STEROID HORMONES AND THEIR RECEPTORS IN THE PROLIFERATION OF HUMAN PROSTATE TUMOR CELLS, LNCaP

DE ROL VAN GROEIFAKTOREN, STEROIDHORMONEN EN HUN RECEPTOREN IN DE HUMANE PROSTAATTUMOR-CELLIJN LNCaP

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CONTENTS

LIST OF ABBREVIATIONS

aFGF	acid fibroblast growth factor
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
DHT	dihydrotestosterone
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
HRE	hormone responsive element
IGF_1	insuline-like growth factor 1
Kd	dissociation constant
LNCaP	lymph node carcinoma of the prostate
M	moles per liter
(m)RNA	(messenger) ribonucleic acid
n	number of determinations
PAP	prostate acid phosphatase
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
SHBG	sex hormone binding globulin
TGFα	transforming growth factor α
TGFβ	transforming growth factor β
v/v	volume/volume

CHAPTER 1

SCOPE OF THIS THESIS

The prostate gland is dependent on androgens for the maintenance of its normal growth and functional integrity. The secretion of testicular testosterone, the major androgen in blood, is dependent on a chain of endocrine events. In the hypothalamus luteinizing hormone releasing hormone is secreted and stimulates the pituitary to release luteinizing hormone. The latter hormone is transported by the blood to the testes which are then stimulated to synthesize testosterone. This hormone reaches the prostate via the blood. In the prostate testosterone is converted into dihydrotestosterone, which specifically binds to cellular androgen receptors. In the cell nuclei the hormone-receptor complex interacts with DNA thereby inducing the transcription of specific genes.

Carcinoma of the prostate is after lung carcinoma, the second most common cancer in men. Little is known about the etiology of prostate cancer, but androgens play a critical role in the growth regulation of neoplastic prostate tissue.

Cancer cells escape growth control to which their normal counterparts are subjected. The unrestrained growth of cancer cells is the result of enhanced metabolic activity involving growth factors, their receptors or intracellular signals generated by binding of growth factors to their receptors. Growth manifests itself in two ways: hypertrophy (increase in cell size) and hyperplasia (increase in cell number). Until recently it was generally considered that the main mechanism that regulates cell growth in multicellular organisms involves some type of either paracrine or endocrine control of growth. These mechanisms imply that the molecules that regulate the growth of a cell are produced by other cells, placing an individual cell under external growth control. The development of the concept of autocrine secretion provided a new framework to account for the rather autonomous nature of the cancer cell. The autocrine hypothesis suggests that a cell can become independent of external growth control by synthesis of its own growth factors for which it possesses functional receptors. Cancer cells may

also fail to synthesize, express or respond to specific negative growth factors which they normally secrete to control their own growth. Alternatively, it has been considered that the number of receptors for growth factors by the cancer cell have changed, resulting in a decreased or increased sensitivity to a certain growth factor (chapter 2).

This thesis deals with a tumor cell line, named LNCaP, which has been derived from a lymph node carcinoma of the human prostate. LNCaP cells show androgen responsive growth. The first aim was to study the possible involvement of growth factors and their receptors in the proliferation of the androgen responsive cell line (chapter 3 to 5).

As described before, the prostate is a target organ for steroid hormones such as testosterone. As most of prostate cancers are androgen responsive, endocrine therapy, directed at androgen deprivation, may influence their growth. Androgen deprivation can be assessed by inhibiting the release of luteinizing hormone releasing hormone and/or luteinizing hormone which results in a decrease in testosterone serum levels. One of the ways this can be achieved is by estrogen therapy. Estrogens mimic testosterone in the feedback mechanism thus blocking secretion of luteinizing hormone releasing hormone and luteinizing hormone and thereby the production of testicular testosterone. Antiandrogens are substances that prevent androgens from expressing their activity (e.g. growth induction) at target tissue by blocking androgen-receptor interaction. The second aim of the thesis was to compare the effects of androgens with other steroids and antiandrogens concerning growth regulation and other physiological processes in the androgen responsive prostate tumor cell LNCaP (chapter 6 and 7).

Finally, we have considered possible effects on LNCaP cells of suramin, a polyanionic compound which has been described to counteract growth factor activity. The effect of suramin on androgen responsive and growth factor responsive growth of LNCaP cells was studied (chapter 8).

CHAPTER 2

GENERAL INTRODUCTION

2.1. INTRODUCTION

Growth and function of the normal prostate is primarily dependent on androgens (Sandberg, 1980; Coffey and Isaacs, 1981). Upon castration (withdrawal of the main source of the androgen, testosterone) the prostate becomes atrophic while secretory activity ceases and all characteristic components, such as acid phosphatase and zinc disappear. Cancer of the prostate is a common disease in elderly man and a relatively high proportion of prostate cancers is androgen dependent. Already in 1941 Huggins and Hodges achieved dramatic success in the treatment of patients with prostate cancer through castration or estrogen administration. However, while most patients with metastatic prostate cancer respond to androgen ablation, the degree and duration of response to this treatment is variable (Blackard et al., 1973) and their prostate cancer relapses to an androgen independent state (Coffey and Isaacs, 1981). To get a better insight into these processes, an understanding of hormonal and nonhormonal requirements for proliferation of prostate tumor cells is essential. Besides androgens, growth factors may play an important role in regulation of proliferation of neoplastic prostate cells, similar to what has been observed for estrogen dependent breast cancer (Lippman et al., 1986, 1987).
The first part of this chapter deals with the mechanism of action of steroid hormones, antihormones, growth factors and oncogenes. Since this thesis describes many experiments concerning the epidermal growth factor receptor in the human prostate tumor cell line LNCaP, this growth factor receptor is discussed in particular . Finally, some models which may explain the relationship between steroid hormones and growth factors in the control of tumor cell proliferation are described.

2.2.1. STEROID HORMONES

Steroid hormones are released into the blood by the steroid

producing endocrine organs (in the male: the testes and adrenals) and are subsequently bound to plasma proteins including serum albumin, corticosteroid binding globulin, and sex hormone binding globulin (SHBG) (Burton and Westphall, 1972; Westphall, 1971). Since this thesis deals with prostate cancer and androgens play an important role, only androgens are discussed. Testosterone is the major circulating androgen in normal males and originates almost completely (95%) from the testes (Lipset, 1970). Approximately 57% of circulatory testosterone is strongly bound to SHBG and 40% is loosely bound to serum albumin. Less than 2% of total testosterone in human plasma is free or unbound and available for diffusion into target organs such as the prostate. The active form of the androgen which influences cell and tissue growth of the prostate is a testosterone metabolite, 5α-dihydrotestosterone (DHT) (Bruchovsky and Wilson, 1968; Anderson and Liao, 1968). DHT has a 2- to 3-fold higher affinity for the androgen receptor when compared to testosterone in most bioassays. The conversion of testosterone to DHT can take place directly in the prostate catalyzed by the enzyme 5α-reductase which is located in the nuclear membrane (Houston et al., 1985). In addition DHT may enter the prostate from the plasma but its low plasma concentration (10% of that of testosterone) and tight binding to SHBG renders it less important as a circulating androgen affecting the prostate (Sandberg, 1980).

2.2.2. MECHANISM OF STEROID HORMONE ACTION IN TARGET CELLS

Following entry of the steroid hormone into the target cell a cascade of events leads to a hormonal effect which ultimately results in expression of cell function or cell proliferation These events (figure 1) include:

- -binding of the steroid hormone (or steroid metabolite) to a specific receptor
- -activation of the receptor and binding to specific DNA sites
- -induction or repression of specific genes.

Steroid hormone receptors have a high affinity for the steroid (Kd = 0.1 to 1 nM) and target cells contain only a limited number of receptor molecules (Baxter and Funder, 1979).

For many years after it was first proposed by Jensen, Gorski and

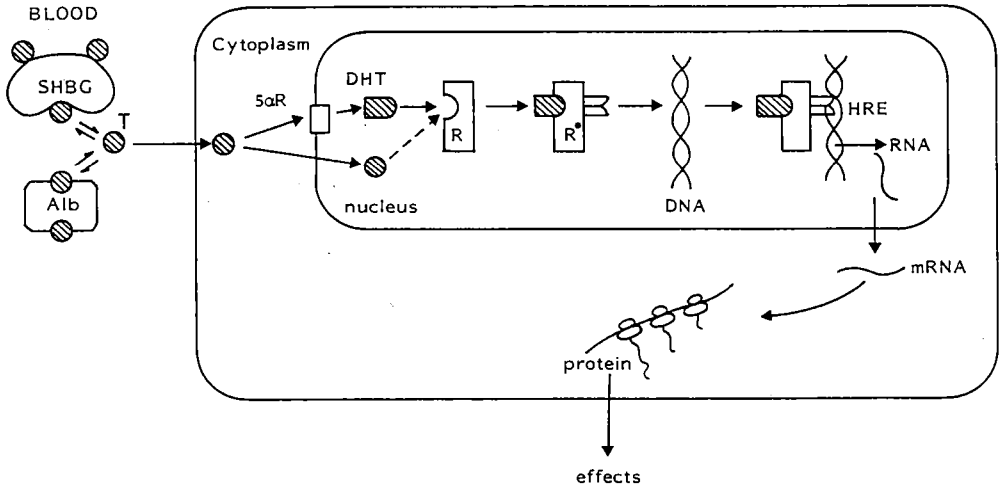

Fig.1. Concept of the mechanism of action of androgens with the assumption that the androgen receptor is located in the nucleus. SHBG, sex hormone binding globulin; Alb, serum albumin; T, testosterone; DHT, dihydrotestosterone; 5αR, 5α-reductase; R, androgen receptor; R*, activated receptor; HRE, hormone responsive element.

their colleagues (1968), the "two step" model had been widely accepted as describing the early events involved in the interaction of steroid hormones with their receptors. The concept of this model implicates that the unoccupied receptors are located in the cytosol. Upon binding of the steroid , the hormone-receptor complex is transformed to the DNA binding state ("activation") and translocated to the nucleus where it elicits the hormonal response essential for gene transcription. The development of monoclonal antibodies against steroid receptors has changed the general acceptance of this "two step" model. Immunocytochemical and biochemical evidence indicates that the unliganded estrogen receptor and progesterone receptor are located predominantly within the nucleus (King and Green, 1984; Welshon et al., 1984; Perrat-Applanat et al., 1985). In contrast, the glucocorticoid receptor has been repeatedly observed in the cytoplasm with an increase in nuclear receptor after glucocorticoid addition (Antakly and Eisen, 1984; Fuxe et al., 1985). No such data are currently available for the androgen receptor but the finding that the enzyme 5α-reductase is located in the nuclear membrane (Houston et al., 1985) may suggest

nuclear localization of free androgen receptor (Figure 1). Studies with monoclonal antibodies against the androgen receptor, should give more information concerning the localization of the androgen receptor.

Complete nucleotide sequences have now been elucidated for the human glucocorticoid receptor (Hollenberg et al., 1985), progesterone receptor (Greene et al., 1986) and estrogen receptor (Green et al., 1986a). More recently, the primary structure of the androgen receptor has been described (Chang et al., 1988; Lubahn et al., 1988; Trapman et al., 1988a). Comparison of these structures reveals that steroid hormone receptors are members of a family of ligand activated regulatory proteins (Green and Chambon, 1988; Evans, 1988). All steroid hormone receptors which have been studied appear to be composed of several functional domains including a ligand binding domain at the C-terminal end, a hinge region, a DNA binding domain, and a N-terminal domain involved in transcriptional response. In general, steroid binding to the ligand binding domain results in an increased affinity of the receptor for DNA. Steroid hormone regulated genes have short cis-acting enhancer sequences termed hormone responsive elements, in or near their transcription units (Jantzen et al., 1987; Klein Hitpass et al., 1987; Strahle et al., 1987). The DNA binding domain of steroid receptors contain highly conserved cysteine residues which may tetrahedrally coordinate zinc to form a "zinc finger" (Green et al., 1986b; Weinberger et al., 1986). These zinc fingers are believed to become unmasked upon hormone binding and to interact with the hormone responsive elements ultimately leading to the transcription of the gene.

2.2.3. ANTIHORMONE ACTION

Antihormones antagonize hormones at target sites to express their activity. Several mechanisms of action have been suggested. It is possible that antihormones bind to the receptor and trap the receptor into a nonactivated (non DNA binding) form. This may occur through interaction with a heat shock protein in the case of glucocorticoid receptors (Groyer et al., 1987). Another possibility is that the antihormone-receptor complexes can bind to HRE's but fail to induce transcription (Webster et al., 1988;

Giuochon-Mantel et al., 1988).

2.3.1. GROWTH FACTORS, GROWTH FACTOR RECEPTORS AND ONCOGENES

The search for substances that regulate growth of eukaryotic cells, woundhealing and organ regeneration has led to the discovery of growth factors. Growth factors are hormone-like polypeptides, either circulating in the blood or locally released, which are recognized by target cells through specific high affinity plasmamembrane receptors. Growth factors and growth factor receptors have been identified as products from proto-oncogenes (c-oncs). The effect of oncogenes in transformed cells might involve constitutive expression of a protein with function identical to that of a factor active transiently during normal cell growth. Proto-oncogenes also might be activated to become transforming genes by genetic changes that alter protein function.

2.3.1.1. EPIDERMAL GROWTH FACTOR AND TRANSFORMING GROWTH FACTOR α

Epidermal growth factor (EGF) stimulates the growth of a wide variety of transformed and nontransformed cells (Carpenter and Cohen, 1979), including prostate cells of canine, murine and human origin (Eaton et al., 1988; Mckeehan et al., 1987; Peehl and Stameyet, 1986). In mice, EGF is synthesized in salivary glands and androgens strongly induce EGF synthesis (Carpenter and Cohen, 1979). Interestingly, EGF or EGF-like activity is also present in the human prostate gland (Elson et al., 1984) and in the secretory fluid of the prostate that forms the seminal plasma (Kishi et al., 1988).
The human EGF protein consists of 53 amino acids translated from a mRNA of 4750 base pairs (Gray et al., 1983; Scott et al., 1983). This mRNA encodes the EGF precursor protein. The structure of the EGF precursor protein resembles a transmembrane receptor of which the extracellular domain contains seven amino acid sequences with high degree of homology to EGF.

Transforming growth factor α (TGFα) is a 50 amino acid polypeptide that is structurally related to EGF (Marquard et al.,

1983; 1984; Derynck et al., 1984; Lee et al., 1985). Expression of the TGFα gene has been demonstrated in a variety of tumors (Derynck et al., 1984). Like EGF, TGFα is encoded within a larger precursor protein (Derynck et al., 1984; Lee et al., 1985). Mature TGFα can be released from this precursor by the action of elastase like enzymes (Ignotz et al., 1986). Secreted TGFα often exists as a multiple species of 5 to 20 kdalton. This heterogeneity can be explained by differential cleavage of the external precursor domain and heterogeneous glycosylation of TGFα (Texido and Massaguè, 1988).
TGFα and EGF which both act via the EGF receptor, are about equivalent in their ability to stimulate DNA synthesis in various cell lines (Schreiber et al., 1986). They induce anchorage independent growth of immortalized fibroblasts in the presence of transforming growth factor β (Anzano et al., 1983) and induce eyelid opening in new born mice (Smith et al., 1985). However, TGFα is more potent in inducing neovascularization (Schreiber et al., 1986) and woundhealing (Schultz et al., 1986).

2.3.1.2. EGF RECEPTOR

The EGF receptor is involved in actions of both EGF and TGFα (Downward et al., 1984). This 170 kdalton glycoprotein consists of an extracellular ligand binding domain, a hydrophobic transmembrane region and an intracellular catalytic domain. Ligand binding to the extracellular domain of the receptor stimulates the tyrosine kinase activity of the intracellular domain. This leads to increased self-phosphorylation as well as phosphorylation of exogenous substrates (Gill et al., 1987; Martin, 1986; Carpenter,1987; Thomson and Gill, 1985). While several proteins are known to be phosphorylated in vitro or in vivo, the identity of some and functional significance of all are at present unknown.
Schlessinger et al. (1988) have postulated an allosteric oligomerization model for receptor activation in which EGF induced oligomerization leads to activation of the catalytic properties of the kinase domain by interaction of neighbouring cytoplasmic domains. In vitro site directed mutagenesis was used to generate various EGF receptor mutants. NIH-3T3 cells lacking

endogenous EGF receptors were transfected with different constructs and shown to generate various EGF receptor mutants. Using this approach it was shown that the kinase activity of the EGF receptor is essential for signal transduction (Schlessinger et al., 1988; Honegger et al., 1987) while autophosphorylation was not essential for receptor signalling (Honegger et al., 1988). The precise composition of the transmembrane domain is not essential for receptor activity and this favours the oligomerization model for receptor activation (Kashles et al., 1988).

Binding of EGF to the cell membrane also activates biochemical processes such as alteration of intracellular free calcium, pH, and rapid increase of transcription of c-fos followed by activation of c- myc (Saywer and Cohen, 1981; Moolenaar et al., 1982; Muller et al., 1984; Greenberg and Ziff, 1984).

Another important consequence of occupancy of the EGF receptor is ligand induced receptor down-regulation by internalisation of the receptor-ligand complex. EGF receptors which are diffusely distributed on the cell surface (Haigler et al., 1979) undergo rapid ligand induced lateral mobility (Schlessinger et al., 1978), clustering in coated pits (Haigler et al., 1978), endocytosis (Hertel and Perkins, 1987), and extensive degradation (Stoscheck and Carpenter, 1984). The EGF receptor dissociates from its receptor in the acid environment of the endosome but both ligand and receptor are degraded after fusion of the endosomes with the lysosomes (Carpenter, 1987). Subsequent to EGF treatment a compensatory increase in EGF receptor synthesis has been observed in several epithelial cell lines resulting from an elevation in EGF receptor mRNA levels (Clark et al., 1985; Earp et al., 1986; Kudlow et al., 1986).

Parameters for receptor activity (e.g.: dissociation constant and receptor concentration) are frequently derived from data of [^{125}I]EGF binding to intact cells (Carpenter, 1985). In some cell types EGF receptors are described as a homogeneous population exhibiting a single affinity for the growth factor (Carpenter and Cohen, 1976; Vlodavsky et al., 1978). However, both high affinity (Kd= ± 0.1 nM) and low affinity (Kd = ± 1 nM) receptors exhibited on the same cell type has also been reported (King and Cuatrecasas, 1982; Kawamoto et al., 1983). Since there appears to

be a single EGF receptor gene, these receptor classes could be the result of post-transcriptional modifications (e.g.: phosphorylation) or receptor-receptor interactions.
Most cells display average EGF receptor numbers within the range of 20,000 to 200,000 per cell, with the exception of the A431 cell line (Haigler et al., 1978) and several other epithelial cell lines (Filmus et al., 1985; Kamata et al., 1986) that have approximately 2,000,000 receptors per cell.

There is evidence that the EGF receptor is in some way related to oncogenic transformation both by mutation and overexpression. The v-erb B transforming gene of avian erythroblastosis virus can be derived, by retroviral transduction, from the c-erb B gene which codes for the avian EGF receptor (Yamamoto et al., 1983; Downward et al., 1984; Lin et al., 1984; Ullrich et al., 1984; Xu et al., 1984). The transforming capacity of the v-erb B appears to result from truncation of the ligand binding domain of the receptor, causing a constitutively "on" configuration and persistent protein kinase activity of the catalytic domain of the receptor.
The high concentration of EGF receptors in A431 cells results from gene amplification (Gill et al., 1987). Variants of A431 cells have differing extents of gene amplification and EGF receptor expression. When these variant cell lines are implanted in nude mice or cultured in the presence of EGF, the concentration of EGF receptors is directly correlated with growth of these cells. A high concentration of EGF receptors appears to facilitate growth of tumors in vivo and of tumor cells in vitro (Santon et al., 1986).
Oncogenic transformation frequently results from enhanced expression of TGFα (Todaro et al., 1980). In vitro studies indicate that different human tumor lines release TGFα into their culture medium thus stimulating these cells to grow. This observation has led to the hypothesis of uncontrolled secretion of autocrine growth factors that results in malignant transformation (Sporn and Todaro, 1980).
There are, however, no indications yet, that oncogenic transformation is associated with overexpression of EGF.

2.3.2. TRANSFORMING GROWTH FACTOR β

Type β transforming growth factors (TGFβ) are ubiquitous proteins synthesized by normal and transformed cells (Derynck et al., 1985; 1987) and found at high levels in blood platelets (Assoian et al., 1983) indicating their role in woundhealing. A wide range of cell types respond to TGFβ and many different cellular responses are elicited by these factors. For example, TGFβ blocks adipogenesis, myogenesis, and hematoisis while it promotes chondrogenesis and epithelial cell differentiation in vitro (Massaguè et al., 1987; Massaguè et al.,1986; Ellingsworth et al., 1986). TGFβ is known primarily as a growth inhibitor of in vitro proliferation of most cell types tested including tumor derived epithelial cells (Tucker et al., 1984; Knabbe et al., 1987). Growth stimulatory effects of TGFβ have been observed so far only in mesenchymal cells. There is evidence that this stimulatory effect of TGFβ on fibroblastic cells is indirect through induction of c-sis and autocrine stimulation of platelet derived growth factor (PDGF)-like activity (Leof et al., 1986a; 1986b).

Little information is available on a possible role of TGFβ in the regulation of normal and neoplastic prostate growth. The hormone independent human prostate tumor cell PC3 contains high levels of mRNA for TGFβ (Derynck et al., 1987) and TGFβ protein has been purified from medium conditioned by tamoxifen stimulated PC3 cells (Ikeda et al., 1987). TGFβ transcripts have been found in other androgen independent cell lines but not in androgen dependent prostate tumor cells (Trapman et al., 1988b). TGFβ receptors are detected in rat ventral prostate (Kypraianou and Isaacs, 1988). So far there are no data on effects of TGFβ on prostate derived cells.

TGFβ activity is associated with a family of molecules that are made up as disulfide- linked dimers of at least three types of homologous polypeptides (Derynck et al., 1985; Cheifetz et al 1987; Martin et al., 1987; Ten Dyke et al., 1988; Derynck et al., 1988). TGFβ monomers are encoded within larger precursor proteins that consist of a N-terminal hydrophobic leader sequence (signal peptide), a glycosylated middle region and a C-terminal mature TGFβ sequence. These larger inactive secretory products are

cleaved to form mature disulfide-bonded dimers (Massaguè et al., 1987). Acidification of cell secretory products is often required to detect TGFβ activity (Lawrence et al., 1984; Coffey et al., 1986). For TGFβ1 it has been shown that this homodimer is noncovalently associated with a disulfide-bonded complex of one dimeric remnant of the precursor and a single molecule of the so called TGFβ1 binding protein (Miyazono et al., 1988; Wakefield et al., 1988). In vitro studies show that enzymatic removal of the carbohydrate structures in the remnant TGFβ precursor produces biologically active TGFβ1 from this latent complex (Miyazono and Heldin, 1989).

The homodimers TGFβ1 and TGFβ2 are widely distributed but the heterodimer TGFβ1,2 has been found in porcine platelets only (Cheifetz et al., 1987). The homodimer TGFβ3 is mainly expressed in cell lines from mesenchymal origin.

Three types of cell surface receptors have been identified as 65 kDa (Type I), 85-95 kDa (Type II) and 250-350 kDa (Type III) affinity labeled receptor complexes on electrophoresis gels (Massaguè et al., 1987). They coexist in many cell types, have a high affinity for TGFβ1 and varying degrees of affinity for TGFβ2 (Cheifetz et al., 1988). Recent studies suggest that the Type I receptors are the main mediators of TGFβ inhibition of epithelial cell proliferation (Boyd and Masague', 1989). TGFβ receptors are apparently quite different from other growth factor receptors since no kinase activity or other enzymatic activity has been detected thus far.

One possible mechanism by which TGFβ may affect cell proliferation is by modulating the number of high affinity EGF receptors expressed at the cell surface. In the presence of EGF, TGFβ stimulates the proliferation of normal rat kidney cells which strongly correlates with an increase in high affinity EGF receptors (Assoian et al., 1984; Takehara et al., 1987). High affinity EGF receptors are down-regulated by TGFβ in rat heart endothelial cells which are growth inhibited by TGFβ (Takehara et al., 1987).

It has been suggested that TGFβ could be oncogenic through decrease in its expression or activity (Sporn and Roberts, 1985), normal cells may produce TGFβ to control their own growth. The

loss of this negative growth control may be one of the mechanisms that contributes to unrestrained growth of tumor cells. Human lung carcinoma cells A459 secrete large amounts of TGFβ in the inactive, latent form. Acid activation of the conditioned medium of these cells or addition of exogenous TGFβ results in potent inhibition of cell proliferation (Sporn et al., 1986) suggesting that the uncontrolled proliferation of the cells is due to their inability to activate the secreted TGFβ.

2.3.3. FIBROBLAST GROWTH FACTORS

Fibroblast growth factors (FGF), characterized as either acidic (aFGF) or basic (bFGF), trigger the proliferation and differentiation of a wide variety of mesoderm and neuroectoderm derived cells (Gospodarowicz et al., 1986; 1987). FGF's are potent angiogenic factors both in normal and tumor tissue and play a critical role in woundhealing.
Concerning the possible role of FGF's in regulation of proliferation of prostate cells, several reports are of interest. Prostatotropin, a growth factor now known to be homologous to aFGF (Crabb et al., 1986), stimulates the growth of normal and neoplastic prostate epithelial cells (McKeehan et al., 1987; Crabb et al., 1986). Basic FGF stimulates proliferation of both stromal and epithelial cells of the prostate (Gospodarowicz et al., 1987) and mRNA for bFGF is present in normal prostate tissue, benign hyperplastic prostate tissue as well as carcinoma of the prostate (Mydlo et al.,1988).
Studies on the structure of bFGF have shown that the molecule is a single chain polypeptide composed of 146 amino acids, it also exists as an amino terminal truncated form, missing the first 15 amino acids (Esch et al., 1985a). This truncated form of bFGF is as potent as native FGF (Neufeld and Gospodarowicz, 1985; Gospodarowicz et al., 1985). Acid FGF is a single chain polypeptide of 146 amino acids that also exists in a truncated form lacking 6 amino terminal residues (Esch et al., 1985b). The mechanism by which aFGF and bFGF are secreted is unknown. Since they lack an amino terminal hydrophobic leader sequence which facilitates secretion, it has been suggested that they are released by cell leakage or cell lysis.

Although there is a 60% homology between aFGF and bFGF there are several significant differences. Basic FGF has a greater affinity to heparin, is 30 to 100 times more potent as a mitogen to fibroblasts, and has a greater angiogenic potential (Gospodarowicz et al., 1987; Thomas, 1987) than aFGF. These different biological potencies may be explained by the differences in affinity of the growth factors to their receptors. Both aFGF and bFGF can interact with the same two 145 kDa and 125 kDa receptor species (Neufeld and Gospodarowicz, 1986). Basic FGF appears to display a higher affinity than aFGF for the 145 kDa receptor species whereas aFGF displays a higher affinity than bFGF for the 125 kDa species.
In contrast to the receptor for EGF (Cohen et al., 1980), PDGF (Frackleton et al., 1984) and IGF1 (Jacobs et al., 1983), FGF receptors are not autophosphorylated on tyrosine residues upon ligand binding (Gospodarowicz et al.,1986). However, phosphorylation of tyrosine residues of non-receptor proteins by aFGF and bFGF has been demonstrated both in vivo and in vitro (Coughlin et al., 1988).

There are three additional members (int-2, hst/KS3 and FGF5) of the FGF family which show considerable homology of the amino acid sequence when compared to aFGF and bFGF, ranging from 35% to 55% identity in commonly aligned regions (Thomas, 1988). The expression of the normal mouse int-2 gene is amplified by the integration of the viral enhancer sequence of mouse mammary tumor virus (Smith et al., 1988). This is an example of insertional mutagenesis since the provirus does not code for int-2 but integrates adjacent to it so that the expression of this normal mouse gene is amplified by the viral enhancer sequence. Two virtually identical oncogenes were isolated as transforming genes from Kaposi's Sarcoma (KS) and human stomach tumor (hst) (Yoshida et al., 1987; Zahn et al., 1988). Another gene coding for the FGF5 protein was found in human bladder carcinoma DNA (Zahn et al., 1988). In contrast to aFGF and bFGF, these three members of the FGF family contain a hydrophobic leader sequence which should facilitate their secretion and therefore permit access of these FGF's to FGF receptors. In this way growth stimulation of tumor cells can take place in an autocrine fashion.

2.4. RELATIONSHIP BETWEEN STEROID HORMONES, GROWTH FACTORS AND CELL PROLIFERATION

Androgens are required for the normal development of the prostate, but their function in prostate cancer seems to be permissive. The failure of endocrine therapy in prostate cancer through interference with androgen production results from the development of androgen independent cells. More successful approaches to cure prostate cancer could come from investigations directed towards signals resulting from the androgen stimulus. In prostate cancer it could be that factors that mediate androgen control of growth in normal cells have changed or - in the case of androgen independent tumors - are no longer under the regulatory influence of androgen. These factors may include growth factors and their receptors.

In addition to a direct action of androgen to trigger cell proliferation (Figure 2a), several models for the mechanism of androgen action can be postulated comparable to estrogen action in breast cancer (Lippman et al., 1986; 1987; Vignon et al., 1984). Androgens may affect the production and/or secretion of growth factors that leads to autocrine growth stimulation (Figure 2b). This mechanism of action has been well documented for estrogens in breast cancer. For example, human breast cancer cells MCF7 secrete growth factors into their culture medium among which are TGFα, IGF1, TGFβ and PDGF (Westley and Rochefort, 1980; Salomon et al., 1984; Dickson et al., 1986a; Bates et al., 1986; Huff et al., 1986). Some of these are estrogen regulated in hormone dependent cells and are constitutively expressed in cells that have acquired hormone independence either spontaneously or by v-ras transfection (Kasid et al., 1985; Lippman et al., 1986, 1987). Furthermore, these secreted growth factors are capable of stimulating proliferation of estrogen deprived MCF7 cells in culture and promote tumor formation by MCF7 cells in nude mice (Dickson et al., 1986b). Information on a similar action of androgen is scarce. However, studies on androgen responsive (SC3) and androgen independent (SC4) cell lines have been derived from a Shionogi 115 mouse mammary tumor (Nonomura et al., 1988) show that SC3 cells secrete growth factors upon androgen stimulation. These growth factors stimulate the proliferation of both androgen

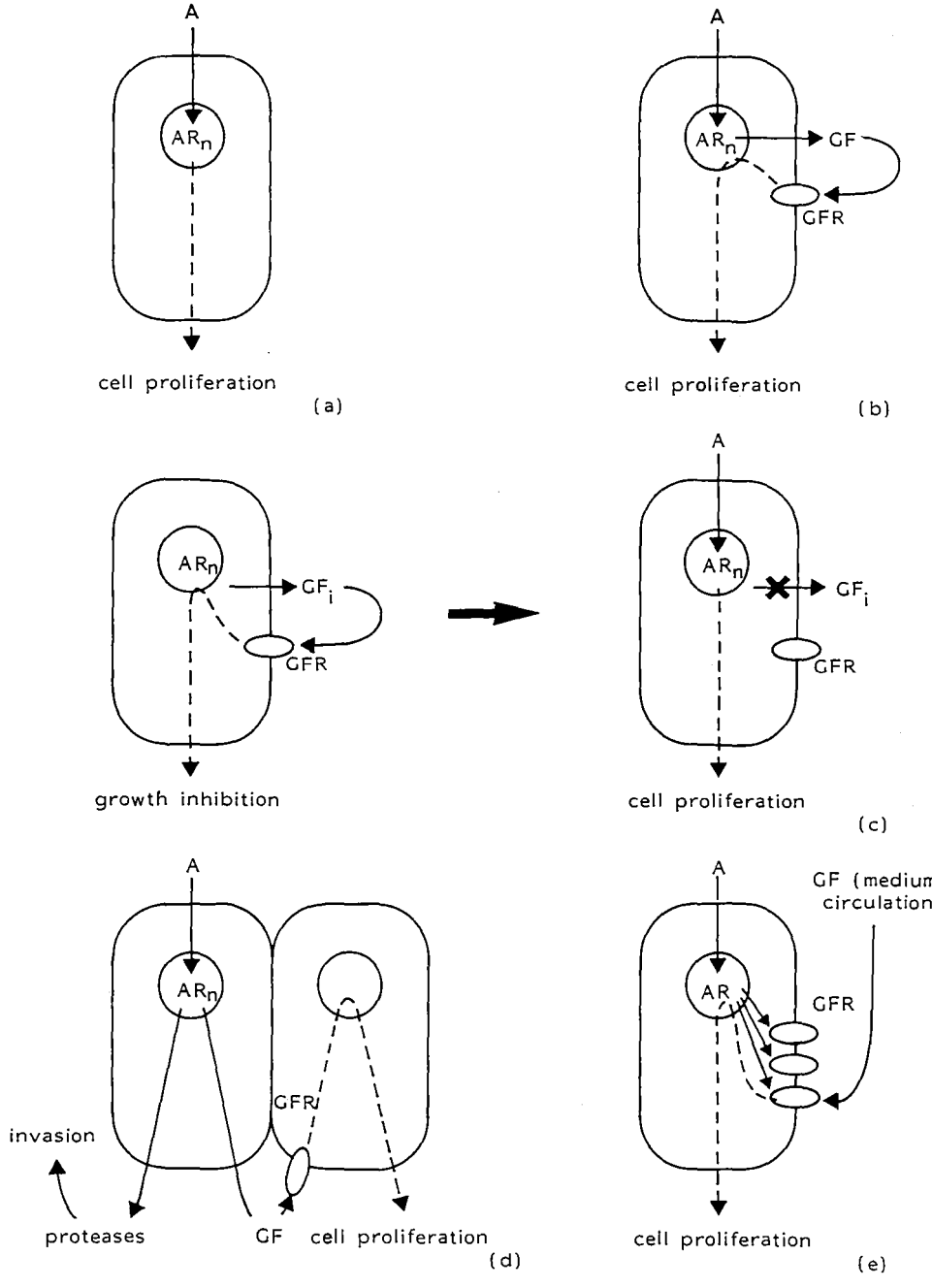

Fig.2. Models of androgen action. A, androgen; AR_n, nuclear androgen receptor; GF, growth factor; GFR, growth factor receptor; GF_i, inhibitory growth factor

deprived SC3 cells and SC4 cells, indicating an autocrine and paracrine (Figure 2d) mode of androgen action in vitro. Since growth regulation seems to be a balance between growth stimulating activity and growth inhibitory activity, transformation might also be correlated with the failure of tumor cells to synthesize, express or respond to specific negative growth factors which they normally release to control their own growth (Sporn and Roberts, 1985). Thus steroid hormones could suppress the secretion of inhibitory growth factors by tumor cells (Figure 2c). Estrogens have been shown to decrease the secretion of TGFβ by MCF7 cells therefore contributing to increased growth of these cells upon estrogen stimulation (Knabbe et al., 1987). Antiestrogens appeared to inhibit growth of MCF7 cells via at least two mechanisms: increase of secretion of total TGFβ and activation of the latent form of TGFβ into a biologically active form (Knabbe et al., 1987). Furthermore, estrogen independent breast tumor cells are growth inhibited by conditioned medium of antiestrogen treated hormone dependent MCF7 cells. This indicates that both autocrine and paracrine mechanisms may be involved in the action of antiestrogens, mediated by TGFβ.

In addition to autocrine growth factors, proteases (plasminogen activator, collagenase and lysosomal cathepsins) may play an important role in tumor growth and invasion (Figure 2d). The protease cathepsin D is secreted by estrogen dependent human breast cancer cells under estrogen control and it is constitutively produced by estrogen independent tumor cells (Rochefort et al., 1987). This protease also displays autocrine mitogenic activity (Vignon et al., 1986; Morisset et al., 1986). This mitogenic activity of proteases has been suggested to arise from the release of growth factors from their precursors or binding proteins via enzymatic activity (Rochefort et al., 1987). Another potential indirect effect of androgens could be to increase the sensitivity to endocrine or autocrine factors by increasing the number of receptors for these factors (Figure 2e). Prostate cells contain EGF receptors (Maddy et al., 1987) and androgen treatment results in a decrease of the number of EGF receptors in normal (rat) prostate (Traish and Wotiz, 1988; St-Arnaud et al., 1988). The effect of androgens on EGF receptor

levels in tumor cells has not been described but other classes of steroid hormones have been shown to increase EGF binding to tumor derived cells in culture (Fanger et al., 1984; Murphy et al., 1986).

2.5. THE LNCaP CELL LINE

Several model systems have been developed for the study of human prostate cancer. Among these model systems, PC-82 (Steenbrugge et al., 1987), PC-EW (Hoehn et al., 1984) and HONDA (Ito et al., 1985) are examples of transplantable human prostate tumors in athymic nude mice which show androgen dependent growth. These tumors lack the ability of continuous growth in vitro.
Only a limited number of human prostate tumor cell lines show continuous growth in vitro (Kaign et al., 1979; Webber, 1980; Claas and Steenbrugge, 1983). This thesis deals with the LNCaP (Lymph Node Carcinoma of the Prostate) cell line which was established by Horoszewicz et al (1983) from a metastatic supraclavicular lymph node from a 50 year old patient with prostate adenocarcinoma. LNCaP cells contain androgen receptors and, until now, it is the only cell line that shows androgen responsive growth in vitro. The cells express prostate specific antigen and prostate acid phosphatase (PAP). In addition, androgens regulate the production and secretion of PAP (Horoszewicz et al., 1983; Schulz et al., 1985) and the release of a 40 kDa protein (Berns et al., 1986) by LNCaP cells. This cell line therefore seems to be a suitable model to study androgen responsive growth and has thus gained much interest during the last two years (Hierowsky et al., 1987; Trapman et al., 1988; Wilding et al., 1989a, 1989b; Konig et al., 1989; Sonnenschein et al., 1989).
From the parental LNCaP-FGC (fast growing colony) line several sublines have been derived which differ in their growth response to androgen. The LNCaP-r (resistant) line developed by Hasenson et al (1985) is an example of an androgen independent subline. The different sublines established in various institutes have recently been reviewed by Steenbrugge et al (1989). This thesis describes experiments with the androgen responsive subline, LNCaP-GJ.

2.6. REFERENCES

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CHAPTER 3

ANDROGENS STIMULATE BOTH GROWTH-RATE AND EPIDERMAL GROWTH FACTOR RECEPTOR ACTIVITY OF THE HUMAN PROSTATE TUMOR CELL LNCaP.

(published in: The Prostate 12: 55-63, 1988)

3.1. ABSTRACT

LNCaP cells (derived from a lymph node carcinoma of the human prostate) contain androgen receptors and show androgen responsive growth in vitro. Maximal effects on growth were seen at 0.1 nM of the synthetic androgen R1881 or 0.2 nM of epidermal growth factor (EGF); both compounds independently increased the growth rate 2-3 times.

EGF-receptors were measured after 6 days culture in the presence or absence of 0.1 nM R1881. A 2.3 fold increase in receptor number/cell was found when binding was measured at 0°C (from 12,500 to 28,900 sites/cell in stimulated cells). The Kd value (0.45 nM) was not affected by androgen treatment. The increase of EGF-receptor activity was first observed between 6 and 12 h after exposure to androgen.

It is concluded that LNCaP cells are sensitive to low concentrations of EGF (or EGF-like compounds) and that one of the mechanisms involved in androgen action on these cells is an increase of EGF-receptor expression at the cell surface.

3.2. INTRODUCTION

An understanding of hormonal and non-hormonal requirements for prostate tumor cell growth is essential for the study of the mechanisms involved in the proliferation of prostate tumors and the design of new therapies against prostate cancer. Many prostate and mammary carcinoma respond with an increased growth rate upon androgen and estrogen stimuli respectively. The mechanism by which steroid hormones act upon these tissues is not fully understood. Several mechanisms of action have been proposed. Firstly, the observation that malignant cells in culture require less exogenous growth factors than their normal counterparts led to the proposal of an autocrine mechanism of

growth control, e.g.: transformed cells produce and respond to their own growth factors (Sporn and Todaro, 1980). Autocrine growth stimulation seems to play an important role in the mammary carcinoma cell MCF7. These cells secrete a collection of growth factors some of which are estrogen regulated in hormone dependent cells (Lippman et al., 1986; Dickson et al., 1986; Westley and Rochefort, 1980). The hypothesis of an autocrine mechanism has been extended to include the possibility that transformation is correlated to the failure of cells to synthesize, express or respond to specific negative growth factors (Knabbe et al., 1985). Another potential indirect effect of steroid hormones could be up-regulation of growth factor receptors. For example, the number of epidermal growth factor receptors in T47D, MCF7 and HeLa S_3 cells is increased after hormonal treatment of these cells (Fanger et al., 1984; Murphy et al., 1986).

Growth of androgen responsive prostate tumor cells might be controlled by androgens in a way comparable to the regulation of growth of mammary carcinoma cells by estrogens. The LNCaP cell line (Lymph Node Carcinoma of the human Prostate) has been used as a model for the study of growth regulation by androgens in prostate tumors. LNCaP cells show continuous growth in vitro, produce prostate specific acid phosphatase and contain androgen receptors. These cells respond to androgens with an increased growth rate and secretion of androgen dependent proteins (Horoszewicz et al., 1983; Berns et al., 1986).

It has been shown that epidermal growth factor (EGF) can stimulate the growth of normal and tumor epithelial cells obtained from the human and rat prostate (Peehl and Stamey, 1986; McKeehan et al., 1987). EGF, a 6 kD single chain polypeptide (Taylor et al., 1972), has been shown to enhance cell proliferation of transformed and nontransformed cells including MCF7 cells (Carpenter and Cohen, 1979; Osborn et al., 1980). The receptor for EGF is an integral 170 kD membrane protein with an extracellular binding domain that serves to bind the ligand (EGF or transforming growth factor alfa), a transmembrane region and an intracellular domain exhibiting tyrosine kinase activity in response to ligand binding. This tyrosine kinase activity is presumed to be involved in the activation of cell growth (reviewed in: Goustin et al., 1986; Martin, 1986; Gill et al.,

1987).
In the present study we show stimulating effects of androgens and EGF on growth of LNCaP cells and provide evidence for an androgen dependent increase in EGF-receptor number per cell.

3.3. MATERIALS AND METHODS

3.3.1. REAGENTS

R1881 (17β-hydroxy-17α-methyl-estra-4,9,11-trien-3-one) a synthetic androgen was obtained from New England Nuclear (Boston, MA). Unlabeled epidermal growth factor (EGF) from mouse submaxillary gland was obtained from Sigma (receptor grade). Mouse ^{125}I-EGF (Amersham, s.a.: 140 Ci/g) was used immediately after Sephadex G25 gel filtration.

3.3.2. CELL CULTURE

The LNCaP cell line (derived from a fast growing colony of a lymph node carcinoma of the prostate) was a gift from Dr. Horoszewicz (Buffalo, NY). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were maintained in RPMI 1640 medium (GIBCO) with added penicillin and streptomycin, supplemented with 7.5% (v/v) heat inactivated (30 min, 56°C) fetal calf serum (GIBCO). Cells were trypsinized and plated out once a week with a medium change after 3-4 days. LNCaP cells between passages 65 and 70 were used for the experiments in this report.

3.3.3. GROWTH STUDIES

Cells were trypsinized and plated out in 24 well dishes (Falcon) at a cell density of $2x10^4$ cells/ cm^2 in 5% charcoal stripped fetal calf serum (obtained by two times treatment with dextran coated charcoal - 1% charcoal, 0.1% dextran - in order to remove steroid hormones). During three days cells were allowed to attach and to start growth, then medium was changed and the indicated hormones were added (steroid hormones in ethanol, final ethanol percentage less than 0.1%). For 6 day cultures medium

with hormones was renewed after 3 days. Cells were counted using a Bürker-chamber. The cells were washed twice with phosphate buffered saline (GIBCO) and dissolved in 1 M NaOH for measurement of protein and DNA content.
Similar growth studies were performed in growth factor inactivated 5% charcoal stripped fetal calf serum, which was a gift of Dr. E.J.J. van Zoelen (Hubrecht Laboratorium, Netherlands Institute for Developmental Biology, Utrecht). Growth factor activity in serum was destroyed by treatment with dithiothreitol (the method for preparing is fully described in (Van Zoelen et al., 1985). When such serum is supplemented to culture media instead of regular serum, normal rat kidney cells become quiescent unless defined polypeptide growth factors like insulin and EGF are added (Van Zoelen et al., 1985).
In some experiments serum free culture conditions were used. LNCaP cells do not attach to plastic culture flasks in the absence of serum. Therefore, cells were first plated in the presence of 5% charcoal stripped serum and after 3 days medium was removed and cells were washed twice with phosphate buffered saline. Thereafter cells were grown for 6 days in medium without serum in the presence or absence of hormones (medium was changed after 3 days).

3.3.4. EGF BINDING ASSAYS

EGF binding assays were performed essentially as described by Carpenter (1985). Cells were plated into 24 well dishes in medium (1.0 ml) with 5% charcoal treated serum. After attachment for 3 days, the cells were grown for 6 days in the presence or absence of 0.10 nM R1881 in medium with 5% charcoal stripped serum (medium was changed after 3 days). For the binding assay cells were washed twice with phosphate buffered saline and preincubated for 1h at 37°C with 1.5 ml buffer A (RPMI 1640 with penicillin and streptomycin, and supplemented with 0.1% bovine serum albumin), medium was removed and the preincubation repeated. For quantitative binding analysis the cells were incubated in 0.2 ml medium with ^{125}I-EGF plus unlabeled EGF (total EGF concentrations per well 0.10, 0.20, 0.30, 0.45, 1.30, 3.30 and 30 nM). Four wells were used for each EGF concentration and

incubation was performed for 4h at 0°C. After incubation the cells were suspended in 1 ml ice-cold buffer A and centrifuged for 5 min at 2200 g. The supernatant was removed and the radioactivity of the cell pellets was measured. Scatchard analysis of binding data was performed after correction for non-specific binding (non-specific binding contributed for 10% or less to the total binding). The amount of EGF-receptors was expressed per microgram of cellular DNA. The amount of EGF-receptors per cell was calculated from the correlation between cell number and DNA content.

3.3.5. OTHER PROCEDURES

Protein content of cell cultures was estimated according to Bradford (1976) and DNA content according to Hinegardner (1971).

3.4. RESULTS

3.4.1. EFFECTS OF ANDROGENS AND EGF ON GROWTH OF LNCaP CELLS IN DIFFERENT MEDIA

LNCaP cells cultured in medium, freed of endogenous steroids by charcoal treatment, showed an increased growth rate in the presence of androgens. The synthetic androgen R1881 stimulated growth maximally at a concentration of 0.1 nM (Berns et al., 1986). This steroid was also used in the present study. Although the specificity of R1881 is limited - it binds to androgen and progestagen receptors with equal affinity - the effects in LNCaP cells were entirely mediated through androgen receptors, because progestagen receptors were not detectable in LNCaP cells (Berns et al., 1986). The effects of EGF and R1881 on the growth of LNCaP cells were compared (fig.1). Addition of 0.2 ng EGF/ml to medium with 5% charcoal stripped fetal calf serum approximately doubled DNA content versus control cultures. Under optimal conditions EGF (1 ng/ml, 165 pM) decreased doubling time from approximately 140 to 70 hours. With 0.1 nM R1881 a comparable increase in growth rate was found. EGF had no additive effects on R1881 stimulated growth, because no difference was found in DNA content (or cell numbers) between cells stimulated with R1881

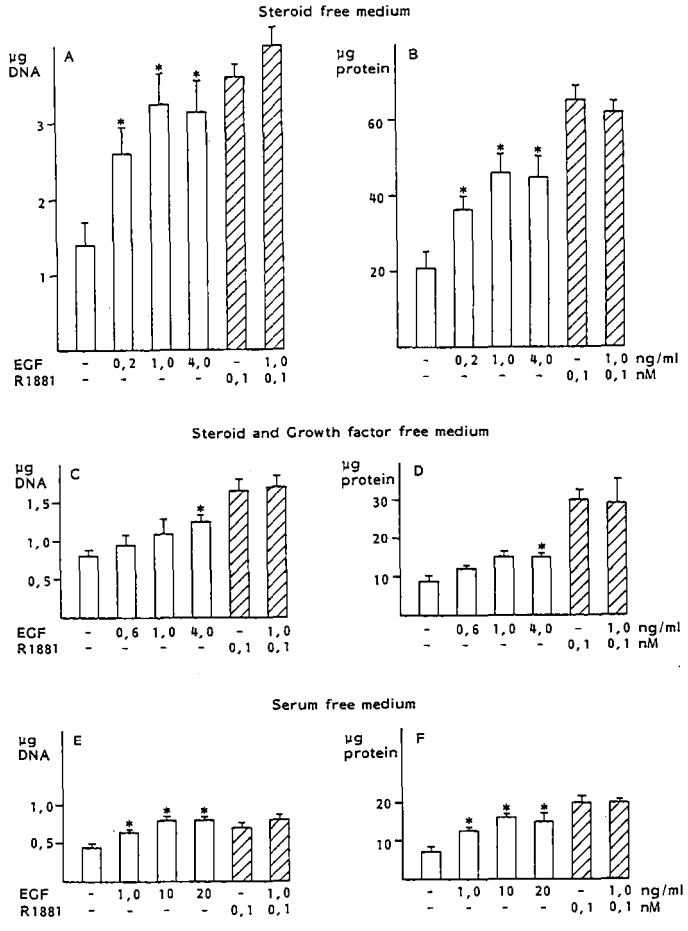

Fig.1: Effects of androgen (R1881) and epidermal growth factor (EGF) on growth of LNCaP cells in different media.
Equal amounts of cells were plated out and cultured for 6 days in the presence or absence of EGF and R1881 as indicated (EGF concentration is expressed as ng/ml; 1 ng EGF/ml is equal to 1.67×10^{-10}M EGF). Steroid free medium: charcoal stripped 5% serum; steroid and growth factor free medium: charcoal stripped 5% serum with an additional treatment to inactivate growth factors; serum free medium: culture in absence of serum as described in the method section. Mean value and standard deviation of three experiments are shown. For EGF stimulated cultures significant differences with controls ($p < 0.05$) are indicated (*).

alone, or with EGF and R1881 together (fig. 1A). The protein content of R1881 stimulated cells - grown in serum containing media-was higher than of EGF stimulated cells (fig. 1B and D); this difference is also expressed in different morphology of the cells (larger, more rounded up cells after R1881 stimulation). Fetal calf serum might contain small amounts of growth factors, especially EGF, therefore the growth studies were repeated with growth factor inactivated medium and with serum free medium. Both basal growth rate and growth rate after stimulation was smaller under these conditions, although the qualitative patterns were not changed (fig 1C-F). These results show that serum factors are necessary for optimal growth of LNCaP cells, however, they do not mask the stimulation of cell growth by EGF or androgens when medium with 5% charcoal treated fetal calf serum is used.

3.4.2. TIME AND TEMPERATURE DEPENDENCE OF ^{125}I-EGF BINDING

The time course of ^{125}I-EGF binding at 0°C and 20°C to LNCaP cells grown in the presence or absence of R1881 is illustrated in fig. 2. Specific binding of ^{125}I-EGF was time and temperature dependent; equilibrium was reached between 2 and 4 hours for R1881 treated as well as untreated cells at both temperatures. Specific binding was approximately two fold higher at 20°C than at 0°C. At both temperatures specific EGF binding to R1881 treated cells was increased versus control cells. Non-reversible steps may have contributed to the apparently higher equilibrium binding obtained at 20°C. At 0°C internalization and degradation would make a negligible contribution to the uptake of radioactivity, so the binding reaction can be considered reversible (Carpenter, 1985). In subsequent studies the cells were incubated with EGF for 4 h at 0°C.

3.4.3. ANDROGEN INDUCED ENHANCEMENT OF EGF BINDING

Scatchard plots of binding data were obtained by incubation of LNCaP cells with increasing concentrations of labeled and non-labeled EGF. This type of analysis permitted an accurate estimation of the time dependent enhancement of EGF binding under the influence of an optimal concentration of androgen (0.1 nM

R1881). The binding data obtained for the LNCaP cells were well-fitted by a one-site binding model resulting in linear Scatchard plots.

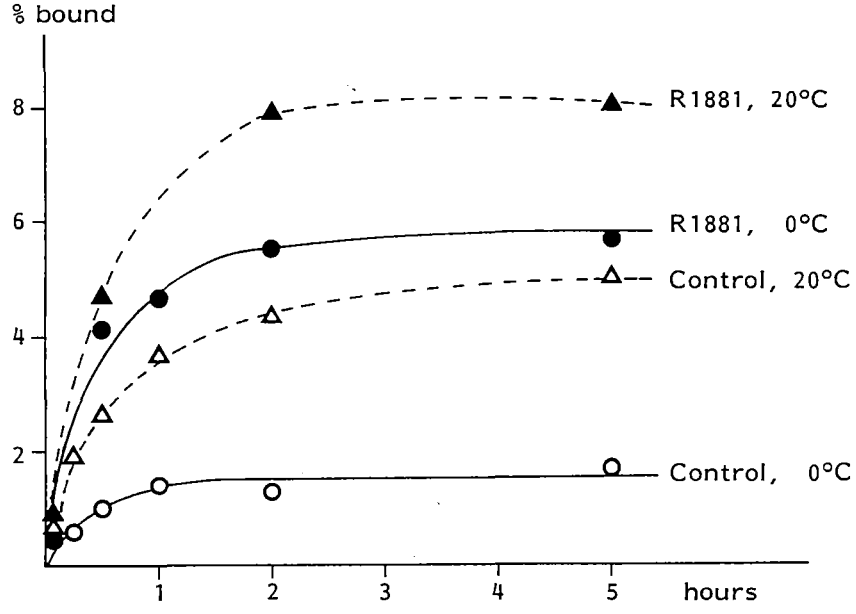

Fig.2: Time and temperature dependence of ^{125}I-EGF binding to LNCaP cells. Cells (1-2x10^5 cells in 2 cm^2 dishes in buffer A) were incubated with a non-saturating amount (0.1 nM) of ^{125}I-EGF at 0°C or at 20°C for different time periods. Solid symbols represent cell cultures stimulated for 6 days with 0.1 nM R1881. Data represent average values of duplicate measurements and were corrected for non-specific binding. For comparison of curves the binding data were recalculated for 8x10^4 cells both for androgen stimulated and control cultures.

Representative results obtained after culturing the cells for 1 or 6 days in the presence or absence of androgens are shown in fig. 3; the results were corrected for the difference in number of cells between androgen treated and control cultures. When EGF binding was studied at 0°C a 2.3 fold increase in EGF-receptors was observed in the androgen treated versus the control cells. There was no difference in Kd (0.45 ± 0.11 nM, n=8) of the EGF-receptors between treated and control cells suggesting that enhancement of EGF binding was due to increased receptor numbers. The time course of the increase in receptor numbers per cell is shown in table I. Between 6 and 12 h after addition of androgens the EGF-receptor level starts to increase and reaches a plateau after 3 days.

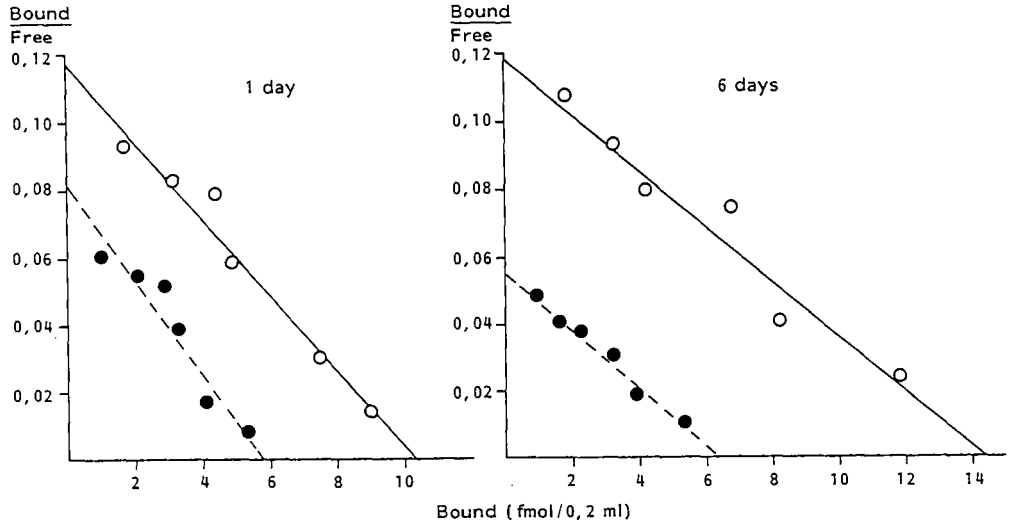

Fig. 3: Scatchard plots of EGF binding to LNCaP cells. Scatchard plots were obtained after incubation with ^{125}I-EGF at 0°C as described in the method section. The concentration of bound ligand is expressed per well with 0.2 ml medium. Representative results of cells cultured for 1 or 6 days in the presence (open symbols) or absence (solid symbols) of 0.1 nM R1881 are shown. Wells contained variable amounts of cells (2-5x10^5 cells). For comparison of Scatchard plots the concentration of bound ligand and the bound/-free ratio were recalculated for 3x10^5 cells.

Table I: Time course of androgen effect on EGF-receptor activity of LNCaP cells.

Period	Sites/cell control (A)	Sites/cell R1881 (B)	Stimulation factor (B/A)
3 h	11,250	12,010	1.07
6 h	11,250	13,030	1.16
12 h	15,680	19,930	1.27
24 h	11,800	20,730	1.76
3 days	10,540	21,890	2.08
6 days	12,560	28,940	2.30

Cells were cultured in medium with 5% charcoal stripped serum in the presence or absence of 0.1nM R1881 for the period indicated and EGF-receptors were measured thereafter at 0°C as described in the method section. The number of EGF-receptors was calculated from Scatchard plots of binding data as shown in figure 3.

3.5. DISCUSSION

EGF is a potent mitogen for a wide variety of cells (Carpenter and Cohen, 1979). In the present investigation we have showed that EGF approximately doubled both protein and DNA synthesis in human prostate tumor cells when added to medium with 5% charcoal treated fetal calf serum. Maximal increase was observed after addition of 1 ng EGF/ml (165 pM). When serum free medium was used 10 ng EGF/ml stimulated cell growth maximally. Similar results were found by Osborne et al. (Osborne et al., 1980) with the human breast cancer cell MCF7. The growth rate of the MCF7 cells was increased maximally at 10 ng EGF/ml serum free medium. EGF has also been shown to stimulate the growth in vitro of normal and tumor epithelial cells from the human and rat prostate (Peehl and Stamey, 1986; McKeehan et al., 1987). In contrast to these studies, where androgens did not effect growth rate, the hormone sensitive LNCaP cells were stimulated by low doses of the synthetic androgen R1881 to the same extent as by EGF. Androgens but not EGF also raised the total protein content per cell compared to the controls.

EGF-receptors have been convincingly demonstrated in human prostate tissue (Maddy et al., 1987) and it was therefore of interest to study the effect of androgens on the EGF-receptor level in LNCaP cells. EGF binding data obtained in this study, revealed an increase of EGF binding to LNCaP cells treated with androgen versus control cells. When assayed at 0°C, a temperature at which internalization of the complex of EGF and receptor plays no significant role (Carpenter, 1985; Goldstein et al., 1985), EGF binding to androgen treated cells was increased more than 2 fold. A raise in EGF-receptor levels was first observed between 6 and 12 hours after addition of androgens, in line with the comparatively late effects of androgens on induction of new protein synthesis. Since there was no difference in Kd-value of the receptor for EGF between androgen treated and control cells, the data suggested that androgens induced up-regulation of available EGF-receptor molecules at the cell surface. Up-regulation of EGF-receptor expression by steroid hormones has been demonstrated in various cell systems. Estrogens stimulate uterine growth and the uterine content of EGF-receptors increased after estrogen treatment in vivo (Mukku and

Stancel, 1985). Glucocorticoids were able to regulate both the number of EGF-receptors and their distribution into high and low affinity components in HeLA S_3 cells (Fanger et al., 1984). Murphy et al. (1986) demonstrated up-regulation by progestagens of the EGF-receptor expression in progesterone receptor positive human breast cancer cells (MCF7 and T47D). In these studies the sensitivity among the cell lines investigated was related to the presence and concentration of cellular progesterone receptors.

EGF-receptor up-regulation by androgens might be one important mechanism of action of this steroid hormone to increase the growth rate in LNCaP cells. Since androgens stimulate LNCaP cell growth in serum free or growth factor depleted media (which contain no EGF-like activity nor other growth factors), induction of growth factor production and/or secretion by androgens might also play a significant role in androgen action. Derynck et al. (1987) screened human tumors and tumor cell lines for the presence of mRNA coding for transforming growth factor α (TGFα) and the EGF-receptor. Many tumor cells derived from solid tumors synthesize TGFα and all of these cells also synthesize EGF-receptor mRNA. This suggests that TGFα could act in an autocrine fashion in these solid tumors since TGFα has been shown to act through the EGF-receptor (Todaro et al., 1980; Massague, 1983; Carpenter et al., 1983).
In this context it is of interest that the TGFα messenger has been demonstrated in LNCaP cells (J Trapman, personal communication).

In conclusion, we demonstrated that EGF stimulates the growth of LNCaP cells and that androgen responsive growth of these cells might in part be caused by an increase of EGF-receptor expression by androgens. The LNCaP cells used in the present study were carefully selected for androgen sensitivity. Preliminary results have shown that androgen sensitivity is lost in some cultures, while other subcultures are entirely androgen dependent. It will be of interest to compare the degree of androgen sensitivity with expression of EGF-receptors and the possible secretion of EGF-like activity and/or other growth factor activities by these different LNCaP mutants. These studies could help in the elucidation of the mechanisms involved in the development of hormone independence in prostate cancer.

3.6. ACKNOWLEDGMENTS

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CHAPTER 4

ANDROGENS AND TGFβ MODULATE THE GROWTH RESPONSE TO EGF IN HUMAN PROSTATE TUMOR CELLS (LNCAP)

(published in: Molecular and Cellular Endocrinology 60: 101-104, 1988)

4.1. ABSTRACT

Treatment of LNCaP human prostatic cancer cells with 0.1 nM of the synthetic androgen, R1881, resulted in a 3-fold stimulation of growth in 6 days. Of several growth factors tested (PDGF, IGF_1, insulin and EGF) only EGF (1 ng/ml) stimulated cell growth (2-fold). This stimulatory effect of EGF was inhibited for approximately 70% by 0.02 ng TGFβ/ml. EGF (1 ng/ml) acted synergistically with R1881 (0.1 nM) on LNCaP cells to induce cell proliferation (7-fold increase in cell growth). This synergistic effect was inhibited by TGFβ (0.05 ng/ml). In conclusion: human prostatic LNCaP cells are sensitive to EGF. Androgen increases and TGFβ decreases the growth response to EGF. This effect of TGFβ on an androgen responsive system has not been observed before.

4.2. INTRODUCTION

It has been suggested that growth factors and their receptors may play an important role in growth regulation of steroid hormone-dependent tumor tissues (Lippman et al., 1987). Hormone-dependent human breast cancer cells (MCF) secrete specific growth factors upon estrogen treatment. Progestins up-regulate the number of EGF receptors in the human breast cancer cell T47D (Murphy et al 1988). The possible role of growth factors and their receptors in prostatic cancer is less well documented but it is very likely that androgens regulate growth of these tumors similarly to estrogen regulated breast cancer (Wilson et al., 1987). Previous studies demonstrated that the human prostatic cell LNCaP shows androgen responsive growth (Horoszewicz et al., 1983). Furthermore, androgens increase the number of EGF receptors expressed at the cell surface 2- 3-fold

(Schuurmans et al., 1988). We now present evidence that this androgen induced increase in EGF receptor number is correlated with an increase in growth response to EGF. In addition we show that TGFβ partly inhibits the stimulatory effect of EGF.

4.3. MATERIALS AND METHODS

4.3.1. REAGENTS

R1881 (17β hydroxy-17α-methyl-estra-4,9,11 trien-3-one), a synthetic androgen was obtained from New England Nuclear. EGF from mouse submaxillary gland was purchased from Sigma (receptor grade). TGFβ, highly purified from human platelets was obtained from Sanbio. IGF_1 and insulin were from Amersham and PDGF from Calbiochem.

4.3.2. CELL CULTURE

The LNCaP cell line (derived from a fast-growing colony of a lymph node carcinoma of the human prostate) was a gift from Dr. Horoszewicz (Buffalo, NY). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were maintained in RPMI 1640 medium (Gibco) with added penicillin and streptomycin, supplemented with 7.5% (v/v) heat-inactivated (30 min, 56°C) fetal calf serum (Gibco). Cells were trypsinized and plated out once a week with a medium change after 3-4 days. LNCaP cells between passages 65 and 70 were used for the experiments in this report.

4.3.3. GROWTH STUDIES

Cells were trypsinized and plated out in 24 well dishes (Falcon) at a cell density of $2x10^4$ cells/cm^2 in 5% charcoal stripped fetal calf serum (obtained by two times treatment with dextran-coated charcoal - 1% charcoal, 0.1% dextran - in order to remove steroid hormones). During 3 days cells were allowed to attach and to start growth, then the medium was changed and the indicated growth factors and/or steroid hormones were added (steroid hormones in ethanol, final ethanol percentage less than

0.1%). For 6 day cultures, medium with hormones and/or growth factors was renewed after 3 days unless indicated otherwise. The cells were washed four times with phosphate-buffered saline (Gibco) and dissolved in 1 M NaOH for measurement of DNA content according to Hinegardner (1971).

4.4. RESULTS

Cells were cultured for 6 days in the absence or presence of various concentrations of different growth factors (medium change after 3 days). Figure 1 shows that IGF_1 (0.01ng - 10ng/ml) insulin (0.01ng - 1ug/ml), PDGF (0.3ng/ml - 30ng/ml) or TGFβ (0.01ng - 5ng/ml) had no effect on cell proliferation. However, EGF stimulated growth in a dose-dependent manner (figure 1).

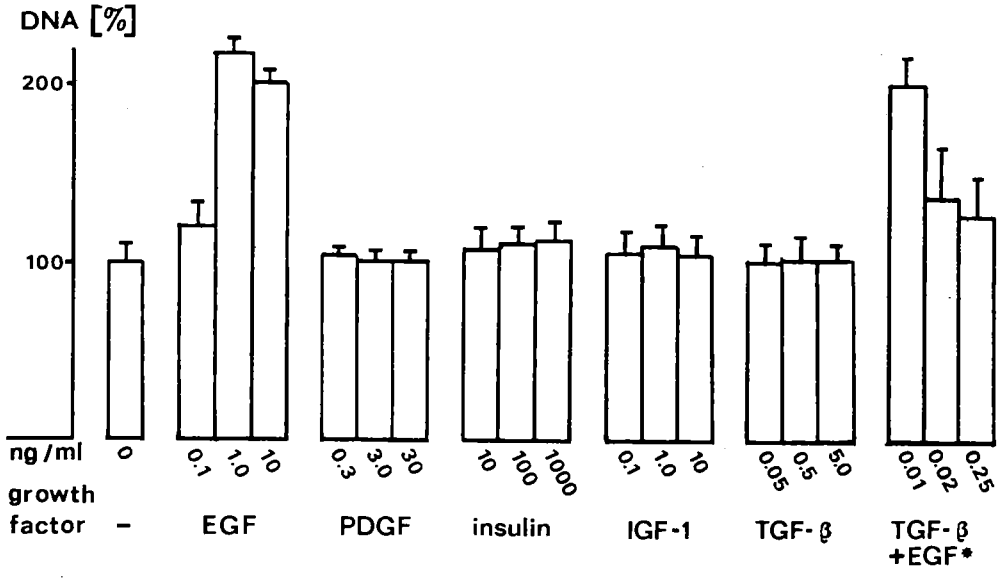

Fig. 1. Effect of different growth factors at various concentrations on growth of LNCaP cells. DNA content per culture was measured after 6 days treatment with or without (-) indicated growth factors. DNA content per culture was expressed relative to DNA content of control cultures (controls were set at 100%). Mean values and standard deviations of triplicate cultures are shown. *, the last three bars show the effect of three concentrations of TGFβ in the presence of 1 ng EGF/ml.

Maximal growth was observed at 1 ng EGF/ml. When 1 ng EGF/ml was added in combination with IGF_1, insulin or PDGF, growth

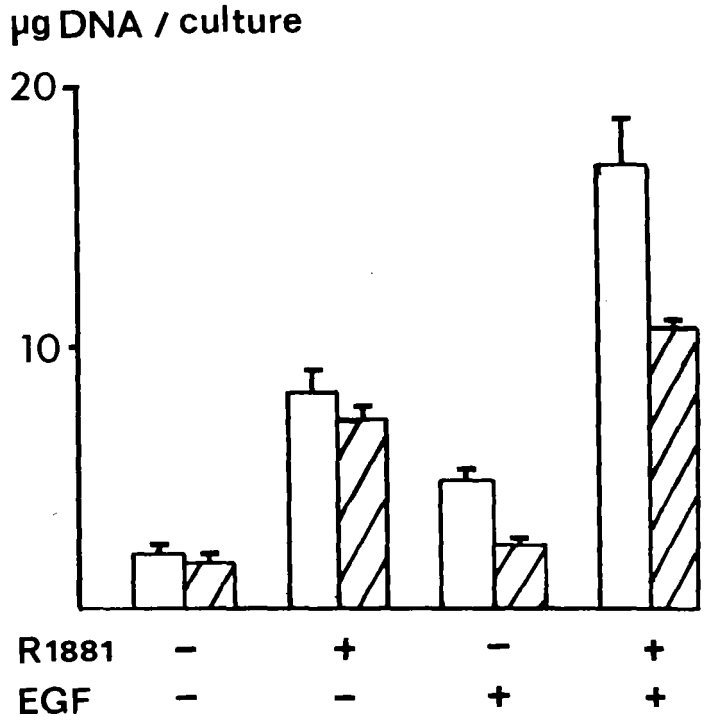

Fig. 2. Effect of transforming growth factor β. LNCaP cells were grown with androgen (0.1 nM R1881) and/or epidermal growth factor (1 ng EGF/ml) in the absence (open bars) or presence (shaded bars) of transforming growth factor β (0.05 ng/ml). Culture media were changed daily. DNA content per culture was measured after 6 days treatment with indicated hormone and growth factors. Mean and standard deviation of triplicate cultures of one series of experiments are shown.

stimulation was similar to adding EGF only (data not shown). Interestingly, the stimulatory effect of EGF on LNCaP cell proliferation was partly inhibited by TGFβ. Already at a concentration of 0.02 ng TGFβ/ml, the stimulatory effect of 1 ng EGF/ml was inhibited for 70% (figure 1).

Previous studies showed that androgens increase the number of EGF receptors per cell in LNCaP (Schuurmans et al., 1988). We therefore next examined whether up-regulation of EGF receptors by androgens might increase the growth response of LNCaP cells to EGF. The synthetic androgen R1881 was used in the present study. Although the specificity of R1881 is limited- it binds the androgen and progestagen receptor with equal affinity- the effects in LNCaP cells are entirely mediated through androgen

receptors because progestagen receptors were not detectable in LNCaP cells (Berns et al., 1986). Cells were cultured in the absence or presence of 0.1 nM R1881 and/or 1 ng EGF/ml for 6 days. Figure 2 shows that EGF acted synergistically with R1881 on LNCaP to induce cell growth (5- to 7-fold increase). This effect was observed only when the culture medium with hormone was changed daily. Figure 2 also shows that TGFβ counteracts the stimulatory effect of EGF both in the presence and absence of the synthetic androgen. TGFβ has only a limited (not significant) effect on R1881-stimulated cell growth.

4.5. DISCUSSION

The LNCaP cell line (derived from a lymph mode carcinoma of the human prostate) has been used as a model for the study of growth regulation by androgens in prostatic cancer (Horoszewicz et al., 1983; Berns et al., 1986). LNCaP cells contain androgen receptors and respond to androgen with increased growth rate and secretion of prostatic acid phosphatase. Recently, we reported that EGF also stimulated LNCaP cell proliferation (Schuurmans et al., 1988). The present paper describes the effects of several other growth factors on cell proliferation. PDGF, insulin and IGF_1, had no effect. TGFβ which inhibits the growth of cells derived from various sources (Knabbe et al., 1987; Takehara et al., 1987) did not affect basal growth rate of LNCaP cells but it did counteracts the EGF-induced growth stimulation.
Prostate cells contain EGF receptors (Maddy et al., 1987). It has been shown that androgens down-regulate EGF receptor levels in normal (rat) prostate (Traish and Wotiz, 1987). In contrast androgens up-regulate EGF receptor levels in LNCaP tumor cells (Schuurmans et al., 1988). Fig.2 provides evidence that these androgen-induced EGF receptors are functional: EGF acts synergistically with androgens to induce cell proliferation. To observe this effect, the growth factor had to be added daily to LNCaP cell cultures. An explanation for the need of daily addition of EGF could be that upon binding to its receptor, EGF is rapidly internalized and metabolized (Schlessinger, 1988). The synergistic effect of EGF with androgen was counteracted by TGFβ. This result suggests that androgens increase the number of EGF

receptors and TGFβ decreases the effectiveness of EGF to stimulate growth via this increased number of receptors.
Hormone-dependent breast cancer cells, MCF_7, secrete TGFβ (Knabbe et al., 1987). Estrogens which stimulate MCF_7 cell proliferation, decrease the secretion of TGFβ by these cells and increase secretion of an EGF-like activity (transforming growth factor α). Antiestrogens which inhibit MCF_7 cell proliferation increase the secretion of TGFβ by these tumor cells. Little information is available on a possible role of TGFβ in the regulation of prostate tumor growth. The hormone-independent prostate tumor cell from the PC3 cell line contains high levels of mRNA for TGFβ (Derynck et al., 1987) but so far no effects of TGFβ on prostate tumor proliferation have been described. In the present study we showed that in androgen-responsive LNCaP cell TGFβ partly inhibited the stimulatory effect of EGF on proliferation both in the presence or absence of androgen. However, TGFβ did not directly affect androgen-responsive growth. These results make it less likely that androgen-responsive growth is mediated by regulation of secretion of an EGF-like activity which in turn acts in an autocrine manner to stimulate growth. If the latter mechanism played a role one would expect TGFβ to inhibit the growth response to this autocrine EGF-like activity in a comparable way as observed for exogenously added EGF. Studies are in progress to characterize further the relationship between androgens, antiandrogens and transforming growth factors and their receptors in the LNCaP prostate tumor cell line.

4.6. ACKNOWLEDGEMENT

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CHAPTER 5

THE INVOLVEMENT OF GROWTH FACTORS IN THE CONTROL OF LNCaP CELL PROLIFERATION

5.1. INTRODUCTION

There is considerable interest in the possible role of growth factors with respect to the control of cancer cell proliferation. De Larco and Todaro (1978) and Sporn and Todaro (1980) proposed the concept of autocrine growth factors: cancer cells produce and respond to their own growth factors. Application of this concept to hormone dependent growth suggests that steroid hormones might induce target cells to secrete locally acting mitogens. On the other hand, steroid hormones can also act by inhibiting the secretion of growth inhibitory factors. Indeed, estrogens regulate the production and secretion of different growth factors in several estrogen dependent human breast cancer cell lines (Lippman et al., 1986, 1987). Estrogens increase the secretion of growth stimulatory factors such as transforming growth factor α (TGFα), while they decrease the secretion of growth inhibitory factors such as transforming growth factor β (TGFβ) (Knabbe et al., 1987).

In prostate cancer, androgens might act by mechanisms comparable to that for estrogens in breast cancer. This chapter deals with experiments which we have performed with the androgen responsive human prostate tumor cell line LNCaP-GJ. The main purpose of these experiments was to resolve the question: do androgens regulate LNCaP cell proliferation by inducing the release of growth factors that act in an autocrine fashion?

There is good evidence to support the suggestion that the growth stimulatory effect of androgen may be mediated by growth factors in an autocrine mechanism. Nonomura et al. (1988) have reported that mouse mammary carcinoma cells, SC115, show androgen responsive growth in vivo and in vitro. After removal of androgen autonomous cancer cells develop from androgen responsive SC115 cells. An androgen responsive cloned cell line (SC4) and an androgen unresponsive cloned cell line (SC115) have been developed from a SC115 tumor (Nonomura et al., 1988). SC4 cells

produced growth factor-like activity only upon stimulation by androgens while SC3 cells produce growth factor-like activity independent of androgens. Medium conditioned by androgen responsive cells stimulated the proliferation of androgen unresponsive cells and of androgen responsive cells. Furthermore, androgen unresponsive SC3 cells showed continuous growth in vitro only if cells were plated in high density. This may indicate that at higher cell density the concentration of secreted proteins was high enough to maintain autonomous growth.

Table I summarizes our data on some of the growth characteristics of the LNCaP-GJ cell line. Cells showed continuous growth in

Table I. Growth characteristics of LNCaP-GJ cells in culture

CULTURE CONDITION	PLATED CELLS/ CM^2	CONTINUOUS GROWTH	DOUBLING TIME (HOURS)
FCS[1])	2×10^4	yes	40
ccFCS[2])	2×10^4	no	124
ccFCS	2×10^5	yes	124
ccFCS + 1 ng EGF/ml	2×10^4	yes	63
ccFCS + 0.1 nM R1881	2×10^4	yes	48

[1]): fetal calf serum; [2]) charcoal treated fetal calf serum.

vitro when cultured in RPMI 1640 supplemented with fetal calf serum. Cells which were plated at low density in charcoal treated (in order to remove steroid hormones) fetal calf serum proliferated only slowly and after one passage they ceased to grow. Addition of androgens or epidermal growth factor (EGF) to charcoal treated fetal calf serum, increased the growth rate and cells can be passaged continuously. Interestingly, LNCaP cells can be passaged continuously in charcoal treated fetal calf serum in the absence of steroid hormones or growth factors if they are plated at higher cell density. These data indicate that growth factors may be involved in the growth regulation of LNCaP cells.

5.2. GROWTH RESPONSE TO GROWTH FACTORS

In previous studies we have found that LNCaP cell proliferation is stimulated by epidermal growth factor (EGF) but not by insulin, insulin-like growth factor 1 (IGF1) or platelet derived growth factor (PDGF) (see chapter 4). In addition, figure 1 shows that basic fibroblast growth factor (bFGF) and, to a lower extent, acid fibroblast growth factor (aFGF) can stimulate the proliferation of LNCaP cells. Hierowski et al. (1987) partially purified human prostate growth factor (hPGF) from human benign hyperplastic and cancerous prostate tissue. This factor showed strong homology to bovine aFGF and occurred only in very low amounts in normal prostate tissue. Hierowski et al. (1987) showed that partially purified hPGF from benign hyperplastic or cancerous prostate tissue stimulated DNA synthesis of LNCaP cells.

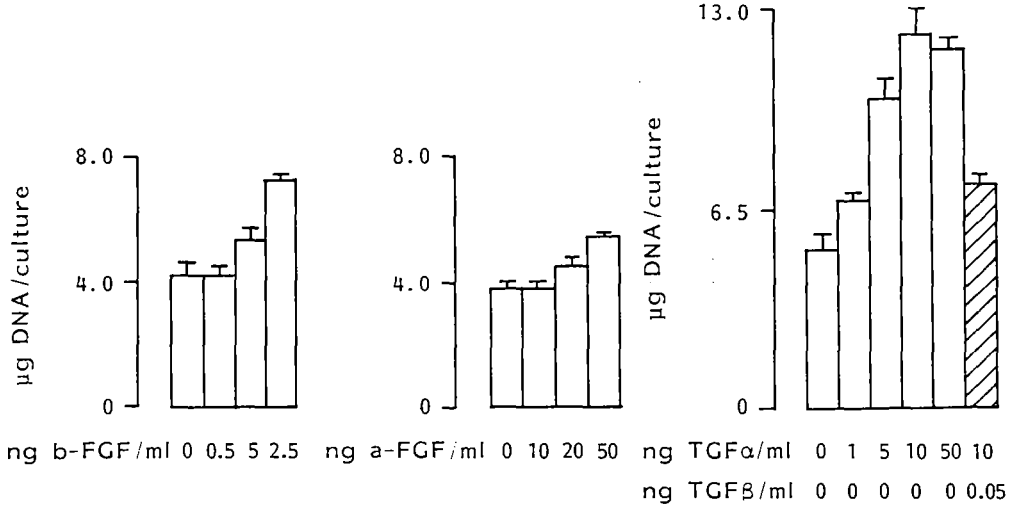

Fig. 1. Growth response of LNCaP cells to TGFα, TGFβ, aFGF or bFGF. LNCaP cells were plated at a density of $2x10^4$ cells/cm^2 in RPMI 1640 medium supplemented with 5% charcoal treated fetal calf serum. After 3 days medium was changed and growth factors were added at indicated concentration. Medium with growth factors was renewed after 3 days. DNA content per culture was measured according to Hinegardner (1971) after 6 days of treatment. Data represent mean DNA content per culture and standard deviations of triplicate cultures.

LNCaP cells are also sensitive to TGFα (figure 1). EGF and TGFα both act via the EGF receptor and have been shown to be

equipotent in stimulating DNA synthesis in a variety of cell lines (Schreiber et al., 1986). However, optimal stimulation of LNCaP cell proliferation has been observed at 0.17 nM (1ng/ml) EGF or 1.80 nM (10ng/ml) TGFα (chapter 4; figure 1). We have no explanation yet for this observation that an approximately tenfold higher concentration of TGFα than EGF was needed for optimal growth response.
TGFβ had no effect on LNCaP cell proliferation when added to the culture medium alone (chapter 4; Wilding et al., 1989). However, in the presence of EGF (chapter 4) or TGFα (figure 1) it counteracted the growth stimulatory effects of EGF and TGFα.
In summary, LNCaP cell proliferation can be stimulated by EGF, TGFα, aFGF and bFGF. TGFβ can counteract the stimulatory effect of EGF and TGFα. LNCaP cell proliferation is not affected by insulin, IGF1, or PDGF.

5.3. RELEASE OF PROTEINS BY LNCaP CELLS

It has been shown that LNCaP cells release prostate specific acid phosphatase (chapter 7; Horoszewicz et al., 1983; Schulz et al., 1985). The production and secretion of this protein is increased by dihydrotestosterone or the synthetic androgen R1881. Furthermore, Berns et al. (1986) have studied the release of proteins in the culture medium of LNCaP cells by incubating the cells with [35S]methionine. Among the labelled secreted proteins, a 40 kDa species appeared. The release of this protein was increased by treatment with androgens. The function of this protein however, is unknown.

5.4.1. PREPARATION AND EFFECTS OF CONDITIONED MEDIUM OF LNCaP CELLS

We have attemped to study also whether proteins secreted by LNCaP cells show autocrine growth factor-like activity using conditioned medium of LNCaP cells cultured in the absence (CCM) or presence (RCM) of 0.1 nM R1881 (a synthetic androgen). We have investigated the effect of the CCM and RCM on proliferation of steroid deprived LNCaP cells.

5.4.2. PREPARATION AND CONCENTRATION OF CONDITIONED MEDIUM

LNCaP cells were plated at 50% confluency in RPMI 1640 medium supplemented with 5% charcoal treated fetal calf serum. Cells were allowed to attach for 3 days. Subsequently, half of the cultures received 0.1 nM R1881 while the other half had no hormone treatment. After 24 hours the medium was discarded and cell monolayers were washed with phosphate buffered saline. Medium was replaced with serum free RPMI 1640 medium plus (RCM) or minus (CCM) 0.1 nM R1881. This conditioned medium was collected after 24 hours and clarified by centrifugation. Cells were exposed to fresh serum free medium with (RCM) or without (CCM) 0.1 nM R1881 for another 24 hours. Conditioned medium collected during the first and second 24 hours were pooled, lyophilized and dissolved in a small volume of aquabidest. The composition of the solvent was changed by sephadex G25 qel filtration in RPMI 1640 supplemented with 5% charcoal treated fetal calf serum. Finally this medium was passed through a 0.2 µm filter. Following this procedure secreted proteins were dissolved in the original volume.

We also collected CCM and RCM as described above and concentrated the secreted proteins before addition to steroid deprived LNCaP cells. In brief CCM and RCM were concentrated by amicon filtration (filter cut off: 1 kDa). Half of this CCM or RCM was directly lyophilized while the other half of the medium was dialyzed (dialysis tube cut off: 3 kDa) against 1 M acetic acid in order to activate latently present growth factors (Lawrence et al., 1984; Coffey et al.,1986). After dialysis the samples were lyophilized. Lyophilized samples were dissolved in aquabidest. Composition of the solvent was changed by sephadex G25 gel filtration in RPMI supplemented with 5% charcoal treated fetal calf serum. Finally the medium was passed through a 0.2 µm filter. In this way the original volume of CCM and RCM was reduced 50-fold.

5.4.3. EFFECT OF CCM OR RCM ON GROWTH OF STEROID DEPRIVED LNCaP CELLS

Unconcentrated CCM or RCM was added to steroid deprived cultures of LNCaP cells for 6 days (one medium change after 3 days). No growth promoting activity of either CCM or RCM was observed. Since androgens might also act through inhibition of secretion of growth inhibitory factors, the effect of CCM and RCM in the presence of 1 ng EGF/ml or 0.1 nM R1881 was also studied. For example, if LNCaP cells secrete TGFβ in the absence of androgen, we would expect CCM to counteract the growth stimulatory effect of EGF. However, neither CCM nor RCM affected the proliferation induced by the growth factor or the steroid hormone.
Similar growth experiments were performed as described above with concentrated acidified CCM and RCM. We observed no mitogenic effects of CCM or RCM on steroid deprived LNCaP cells.

5.4.4. EGF-LIKE ACTIVITY IN CONDITIONED MEDIUM

All media (CCM and RCM) were analyzed also for the presence of EGF-like activity. EGF-like activity was assayed by competitive binding of CCM and RCM samples with [^{125}I]EGF using placenta membranes as a source of EGF receptors. No EGF-like activity was detected in any CCM or RCM sample (detection limit: 0.2 ng EGF/ml).

5.4.5. CONCLUSION

The results as presented do not demonstrate the presence of autocrine growth factor activity in medium conditioned by LNCaP cells, either in the presence or in the absence of androgen. Furthermore, EGF-like activity was not detected by a radioreceptor assay.

5.5.1. LNCaP CELLS DO NOT PRODUCE TGFβ

Exogenously added TGFβ counteracts the growth stimulatory effect of EGF and TGFα (chapter 3; figure 1). In our experiments neither CCM nor RCM of LNCaP cells affected the growth stimulatory effect

of EGF on steroid deprived LNCaP cells. These observations suggest that LNCaP cells themselves do not secrete TGFβ. This is in agreement with recent findings of Wilding et al. (1989). They analyzed conditioned medium by immunoprecipitation and radioreceptor assay and observed a secretion of TGFβ into the media by androgen independent human prostate tumor cells but not by androgen responsive LNCaP cells. Furthermore, mRNA encoding for TGFβ was not detectable in LNCaP cells (Trapman et al, 1988; Wilding et al., 1989). Interestingly, comparable to LNCaP cells, androgen responsive human prostate tumors transplantable in nude mice contained no TGFβ mRNA (Trapman et al., 1988). In contrast, TGFβ mRNA was detectable in androgen independent human prostate tumor cell lines.

5.5.2. LNCaP CELLS PRODUCE TGFα PROTEIN

Although we could not detect EGF (TGFα) activity in the radioreceptor assay, Trapman et al. (1988) demonstrated that mRNA encoding TGFα is present in LNCaP cells. Our failure to detect TGFα in CM might result from receptor mediated uptake of secreted material at a rate sufficient to remove most of it. It has been demonstrated that TGFα can be rapidly taken up by human breast cancer cells (Björge et al., 1989). TGFα was detectable in culture medium of these cells only when its uptake was blocked by anti-EGF receptor monoclonal antibodies. Immunoreactive TGFα could not be detected in the medium of cells with unblocked EGF receptors.

Another possibility may be that TGFα protein is produced, but not secreted into the medium. The precursor of TGFα (pro-TGFα) occurs as a membrane protein (Texido and Massague, 1988). Release of the mature TGFα from the outer domain of pro-TGFα occurs through proteolytic action of elastase-like enzymes. A variety of transformed cells, including human tumor cells also secrete larger forms of TGFα in varying proportions (Todaro et al., 1980; Dart et al., 1985), suggesting that the elastase-like cleavage is incomplete. Furthermore, pro-TGFα expressed on the cell surface has been shown to bind to EGF receptors on adjacent cells, leading to signal transduction (Wong et al., 1989; Branchmann et al., 1989). This observation has led to the

hypothesis of juxtacrine growth stimulation.

We have used an anti-TGFα antibody (MF9) in experiments to detect TGFα by an immunohistochemical approach. MF9 was produced from hybridomas from mice immunized with a 17-residue synthetic peptide corresponding to the carboxyl terminal sequence of rat TGFα. This monoclonal antibody recognizes human TGFα but not EGF

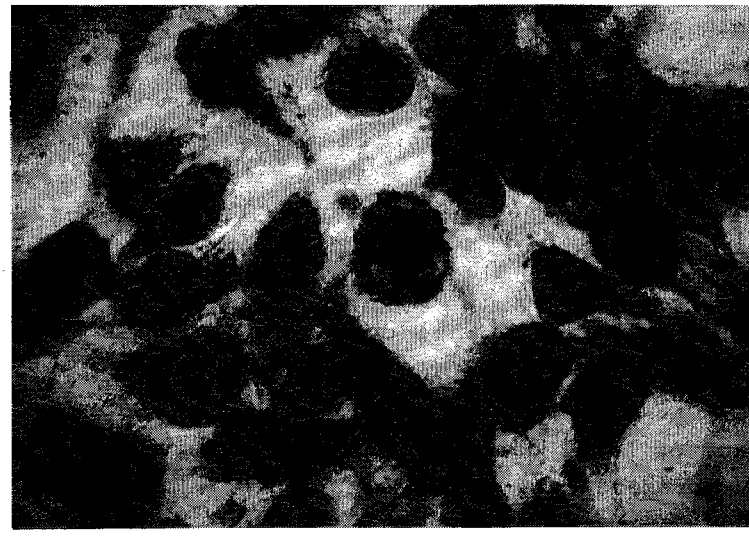

Fig. 2. Immunohistochemical staining of LNCaP cells for the presence of immunoreactive TGFα. LNCaP cells were grown on culture slides in RPMI 1640 medium supplemented with fetal calf serum (which thus contains steroid hormones). Culture slides were fixed with ice-cold methanol, and were stored in 70% ethanol. Endogenous peroxidase activity was blocked by incubating the slides with 1% hydrogen peroxide in methanol for 30 min. Slides were rinsed in tap water and rehydrated for 2 min in 0.01 M PBS, pH 7.4. Monospecific antibody binding was blocked by incubating slides in 10% normal rabbit serum in PBS for 30 min. 100 µl of the MF9 monoclonal antibody (100 µg/ml) was added to each monolayer culture and incubated overnight at 4°C. Slides were brought to room temperature washed in PBS and incubated with a 1:20 dilution of peroxidase-conjugated rabbit antimouse immunoglobulin G. Slides were washed in PBS and the bound antibody was visualized in 0.6 mg/ml 3,3'-diaminobenzidine tetrahydrochloride in 0.05 M Tris, 0.03% H_2O_2, pH 7.6 for 5 min (brown staining). Slides were washed in tap water and counterstained in hematoxylin (blue staining). Brown staining, which indicates the presence of immunoreactive TGFα, was not observed when incubation with MF9 was omitted.

(Kobrin et al., 1986). Figure 2 shows that immunoreactive TGFα is present indeed in LNCaP cells. Whether TGFα is present in the membrane cannot be concluded from this experiment. Electron-microscopy after immuno-gold labeling with the MF9 antibody of LNCaP cell sections might give conclusive evidence about the precise localization of TGFα.

It has been shown that expression of mRNA encoding TGFα in LNCaP cells is not regulated by androgens (Trapman et al., 1988). It would be interesting to examine whether androgens regulate the TGFα protein or whether the protein is constitutively expressed even in the absence of androgen.

We have previously demonstrated that EGF receptors are present on LNCaP cells (chapter 3). Thus both the growth factor and its receptor are produced by LNCaP cells. Interestingly when plated out at high cell density, LNCaP cell cultures can be passaged continuously in the absence of androgen (Table I, this chapter). One could speculate that with such high cell densities pro-TGFα (if present on the membrane) could activate EGF receptors of adjacent cells leading to a growth response.

Androgens increase the number of EGF receptors expressed at the cell surface of LNCaP cells (chapter 3). It could be that these receptors represent only a portion of the EGF receptors that are induced by androgens in LNCaP cells. The other portion of androgen induced EGF receptors might not be expressed at the cell surface and could be activated intracellularly by TGFα. This kind of mechanism of growth factor receptor activation has been shown to play an important role in v-sis transformed normal rat kidney cells (Keating and Williams, 1988). Activation of PDGF receptors in these cells occurs by v-sis encoded proteins in intracellular compartments.

Further studies are needed to demonstrate a possible involvement of these juxtacrine and intracellular mechanisms of growth factor action in LNCaP cells.

5.6. ACKNOWLEDGEMENTS

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CHAPTER 6

REGULATION OF GROWTH AND EPIDERMAL GROWTH FACTOR RECEPTOR LEVELS OF LNCaP PROSTATE TUMOR CELLS BY DIFFERENT STEROIDS

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6.1. SUMMARY

The growth of LNCaP cells, derived from a lymph node carcinoma of the human prostate, was stimulated by different hormones. Optimal growth (3 to 4 fold increase in DNA content per culture versus controls) was observed at 0.1 nM R1881 (a synthetic androgen), 1 nM progesterone or 10 nM estradiol. Triamcinolone acetonide had no effect. Dihydrotestosterone maximally stimulated cell growth at 10 nM. When the culture medium was changed four times in 6 days instead of twice, optimal growth was observed at 1 nM dihydrotestosterone. This indicates that a rapid metabolism of dihydrotestosterone influenced growth response. LNCaP cells contained considerable amounts of androgen receptors (920 fmol/mg cytosol protein) while progestagen, estrogen and glucocorticoid receptors were absent. The affinity of steroids for the androgen receptor decreased in the order of: R1881 (relative binding affinity: 100.0) > dihydrotestosterone (67.7) > progesterone (29.4) > testosterone (23.8) > estradiol (4.3) > triamcinolone acetonide (< 0.1). Effects on cell growth of these steroids paralleled their affinity for the androgen receptor. The number of EGF receptors per cell increased in a dose dependent manner upon treatment with various hormones. Again the amount of steroid needed for maximal effects reflected the affinity of the steroid for the androgen receptor. An approximately two fold increase in EGF receptor number was observed within 24 h and before an increase in growth could be detected. Actinomycin-D and cycloheximide inhibited the hormonally induced increase in EGF receptor numbers.

We conclude that the androgen receptor in LNCaP shows not only considerable affinity for androgens, but also for progesterone and estradiol. This correlates well with hormonal induction of EGF receptors and hormonally induced growth by these steroids.

6.2. INTRODUCTION

It remains a challenge to establish the nature of the relationship between prostatic androgen receptors and the growth response of prostatic cancer to androgens. It has been widely accepted that androgens exert their effect through binding with an androgen receptor, probably as most other steroid hormone receptors located in the nucleus (King and Green, 1984; Welshons et al., 1984), followed by association of the steroid receptor complex with specific DNA sites. In normal rat prostate the androgen-receptor complexes can either induce (Zhang and Parker, 1985; Kandala et al., 1985) or repress (Montpetit et al., 1986) the transcription of specific genes. A difficulty in studying androgen regulated tumor growth has been the lack of in vitro androgen dependent systems. Some progress has been made in the last few years in developing in vitro models for prostatic carcinomas. From the human cell lines available at present (Webber, 1980; Kaign et al., 1979; Horoszewicz et al., 1983) only the LNCaP (lymph node carcinoma of the human prostate) line shows androgen responsive growth in vitro (Horoszewicz et al., 1983; Berns et al., 1986). LNCaP cells contain considerable amounts of androgen receptors and secrete prostatic acid phosphatase. Androgen treatment of these cells also increases epidermal growth factor (EGF) receptor numbers expressed at the cell surface (Schuurmans et al., 1988). The EGF receptor mediates the biological signals of EGF (Carpenter and Cohen, 1979), transforming growth factor α (Marquardt et al., 1984) and vaccinia virus growth factor (Stroobant et al., 1985). The predominant biological response of target cells to these three ligands is enhancement of cell growth (Carpenter, 1987). EGF also stimulated the growth of LNCaP cells (Schuurmans et al., 1988). Interestingly, EGF- like activity has been shown to be present in male reproductive tissues and fluids including the prostate (Elson et al., 1984).

The present investigation describes hormonally induced growth of LNCaP cells by different steroids. The correlation between androgen receptor affinity for various hormones, hormonally induced EGF receptors and hormonal stimulation of cellular growth has been studied. A positive correlation was found between growth

response and affinity of different steroids for the androgen receptor and both progestagens and estrogens considerably stimulated the growth of this prostate tumor cell considerably.

6.3. MATERIALS AND METHODS

6.3.1. MATERIALS

[^{3}H] 17β-Hydroxy-17α-methyl-estra-4,9,11-trien-3-one ([^{3}H]R1881, s.a.: 3.2 TBq/mmol) and radioinert R1881 were purchased from New England Nuclear Corp. (Boston, USA). [1,2,6,7-^{3}H]testosterone (3.9 TBq/mmol, [2,4,6,7-^{3}H]oestradiol (s.a.: 3.3 TBq/mmol), [1,2,6,7-^{3}H]progesterone (s.a.: 3.1 TBq/mmol) and 5α-dihydro[1,2,4,5,6,7-^{3}H]testosterone (s.a.: 5.5 TBq/mmol) were obtained from Radiochemical Centre Amersham, England. Triamcinolone acetonide was obtained from Sigma (St. Louis, USA). All other steroids were purchased from Steraloids Inc. (Wilton, USA). Reagents for enzyme immunoassay of progestagen receptors were obtained from Abbott (Chicago, USA).
Unlabeled EGF from mouse submaxillary gland was obtained from Sigma (receptor grade). Mouse ^{125}I-EGF (s.a.: 140 Ci/g, Amersham) was used following removal of free iodine by Sephadex G25 gel filtration.

6.3.2. CELL CULTURE

The LNCaP cell line was a gift from Dr. Horoszewicz (Buffalo, USA). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were maintained in RPMI 1640 medium (GIBCO, USA) with penicillin and streptomycin, supplemented with 7.5% (v/v) heat- inactivated (30 min, 56°C) fetal calf serum (GIBCO, USA). Cells were trypsinized and plated out once a week with a medium change after 3-4 days. LNCaP cells between passages 65 and 70 were used for the experiments described in this report.

6.3.3. GROWTH STUDIES AND ASSAY OF [^{3}H]STEROID METABOLISM

Cells were trypsinized and plated out in 24 multi-well dishes (Falcon) at a cell density of $2x10^4$ cells/cm^2 in 5% charcoal

treated fetal calf serum. During 3 days cells were allowed to attach and to start growth, then medium was changed and indicated hormones were added at different concentrations. After 3 days medium with hormones was renewed. At day 6 cells were washed four times with phosphate buffered saline and dissolved in 1M NaOH. DNA content was estimated according to Hinegardner (1971). To study the metabolism of the various hormones during these growth studies cells were cultured as described in medium containing 10 nM steroid hormone for 6 days. At day 3 medium was changed and 0.5 µCi of radiolabeled plus unlabeled steroid was added at an end concentration of 10 nM. After another 3 days the medium was collected and extracted twice with an equal volume of ethylacetate. The solvent was evaporated to dryness, the residue was dissolved in 50 µl ethanol and the samples were applied to silicagel thin layer plates (Merck, Darmstadt, FRG). The plates were developed twice in dichloormethane/ether, 85:15 (v/v). Radioactivity was detected with a Panax thin layer scanner (Redhill, UK).

6.3.4. PREPARATION OF CYTOSOL OF LNCaP CELLS; SCATCHARD PLOT ANALYSIS AND COMPETITION STUDIES OF THE ANDROGEN RECEPTOR

LNCaP cells were grown in T180 flasks (Nunc) in 7.5% fetal calf serum. At confluency, medium was removed and new medium containing 5% charcoal treated fetal calf serum was added. After 3 days cells were washed with phosphate buffered saline and trypsinized; soybean trypsin inhibitor (Sigma) was added and cells were centrifuged for 10 min at 100g. The cell fraction was then homogenized in ice cold TEGD buffer (40 mM tris-HCl, 1 mM EDTA, 10% (w/v) glycerol 10 mM molybdate and 10 mM dithiotreitol, pH 7.4) with 15 strokes of a Potter-Elvehjem homogenizer at 900 rpm. The cytosol was prepared by centrifugation at 105,000g for 1 h at 0°C.
For Scatchard plot analysis cytosol was incubated with increasing concentrations of [^{3}H]R1881 (0.5 - 10 nM) at 4°C for 18 h. Separation of bound and unbound steroid was achieved by adsorption of unbound ligand with 0.3% (w/v) charcoal -0.03% (w/v) dextran for 15 min at 4°C and centrifugation at 2000g for 5 min.

Competition studies were performed by incubating cytosol with 5 nM [^{3}H]R1881 in the presence of 0, 5, 50, 500 or 5000 nM R1881, 5α- dihydrotestosterone , testosterone , progesterone , estradiol or triamcinolone acetonide . Specific binding of [^{3}H]R1881 was measured after separation of bound and unbound steroid as described above. [^{3}H]dihydrotestosterone and [^{3}H]testosterone metabolism by LNCaP cytosol was studied under conditions of the binding assay. Cytosol (1 ml) was incubated with 100 nM [^{3}H]steroid during 18 h at 0°C and subsequently extracted and analyzed as described.

6.3.5. PROGESTAGEN RECEPTOR ASSAY

The presence of progestagen receptors was analyzed by Scatchard plot and enzyme immunoassay. For Scatchard plot analysis cytosol (prepared as described above) was incubated with [^{3}H]ORG2058. Non-specific binding was determined with radioinert ORG2058.
Enzyme immunoassay of progestagen receptor in cytosol of LNCaP was performed as described by Blankenstein et al. (1987).

6.3.6. EGF BINDING STUDIES

Cells were plated out in 24 multi-well dishes in 5% charcoal treated fetal calf serum at a density of 1.6×10^5 cells/cm^2. After attachment for 3 days, cells were grown for 24 h in the presence or absence of indicated hormones at different concentrations. Next EGF binding assays were performed essentially as described previously (Schuurmans et al., 1988). Cells were washed twice with phosphate-buffered saline and preincubated for 1 h at 37°C with 1.5 ml buffer A (RPMI 1640 with penicillin and streptomycin, supplemented with 0.1% bovine serum albumin), the medium was removed, and the preincubation was repeated. Next, cells were incubated in 0.2 ml buffer A with ^{125}I-EGF plus unlabeled EGF (total EGF concentrations per well: 0.10, 0.20, 0.45, 1.30, 3.30 and 30 nM). Two wells were used for each concentration, and incubation was performed for 4 h at 0°C. After incubation the cells were suspended in 1 ml ice-cold buffer A and centrifuged for 5 min at 2,200 g. The supernatant was removed, and the radioactivity of the cell pellets was measured. The amount of

specific EGF binding per cell upon hormonal treatment was expressed relative to specific EGF binding per control cell.
The hormonal induction process of EGF receptors was studied by testing the effects of either cycloheximide or actinomycin-D. Cells were cultured in the presence or absence (control) of 0.1 nM R1881 for 12 h. Part of the control cells and part of the R1881 treated cells received either 80 nM actinomycin-D during the first 6 h or 50 µm cycloheximide during all 12 h. After this 12 h period cells were still viable as confirmed by a propidium-iodide/fenyldiacetate staining test. EGF binding was measured as described (Schuurmans et al.,1988).

6.4. RESULTS

6.4.1. EFFECTS OF STEROID HORMONES ON CELL GROWTH

LNCaP cells were cultured for 6 days in charcoal stripped fetal calf serum at various concentrations of different hormones. At day 6, DNA content per culture was determined (figure 1). Growth of LNCaP cells showed to be hormone concentration dependent. Optimal growth (3 to 4 fold increase in DNA content per culture versus control cultures) was observed at 0.1 nM R1881, 1 nM progesterone or 10 nM estradiol. Higher doses of these steroids were less or not stimulatory.
In contrast to the other steroids tested, dihydrotestosterone was rapidly metabolized by LNCaP cells . After 3 h already 67% of dihydrotestosterone was metabolized under growth assay conditions. After 72 h no dihydrotestosterone could be detected, while more than 50% of other steroids added was still present after this time period. We further examined whether this metabolism of dihydrotestosterone could account for the higher concentrations needed to stimulate cell growth than expected. Cells were cultured for 6 days in the presence or absence (control) of dihydrotestosterone. In half of the control cultures and half of the dihydrotestosterone-treated cultures medium was changed twice (day 0 and 3) while in the other half of the cultures medium was changed four times (days 0, 1, 3 and 4). By increasing the number of medium changes from two to four times optimal growth response shifted from 10 nM to 1 nM

dihydrotestosterone (figure 2).

6.4.2. HORMONE BINDING SPECIFICITY

Previous studies showed that LNCaP cells contain androgen receptors (Berns et al.,1986; Horoszewicz et al., 1983). Cytosol was incubated with increasing concentrations of the

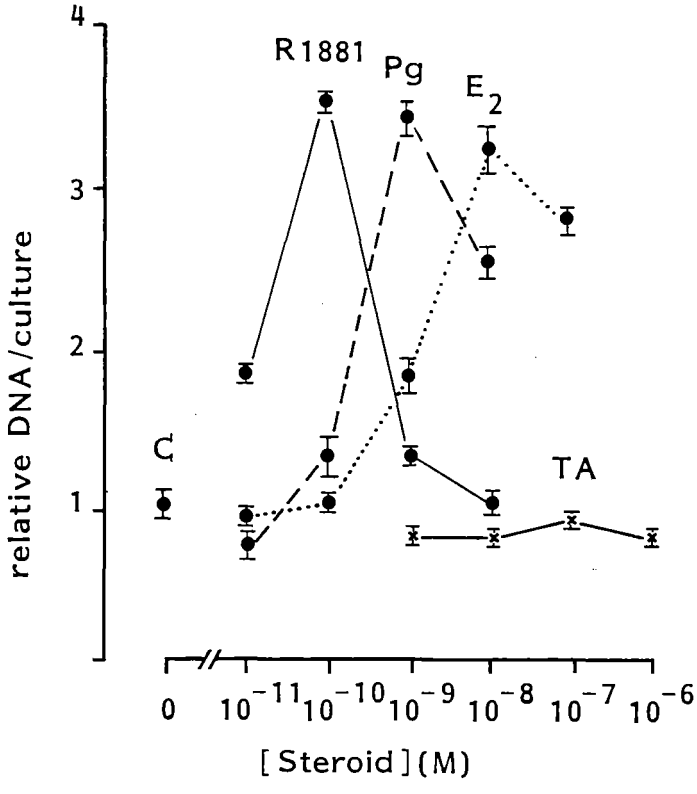

Figure 1 : Effects of various hormones on growth of LNCaP cells after 6 days. Growth is expressed as mean DNA content per culture relative to control cultures (c): R1881 (●——●), progesterone (Pg: ●-●), estradiol (E_2: ●·····●). Bars represent standard deviations of triplicate experiments.

non-metabolizable synthetic androgen [^{3}H]R1881. Scatchard plot analysis revealed a single binding component with high affinity (Kd, 0.4 nM) and a binding capacity of 920 fmol/mg cytosol protein (figure 3).

Steroid binding specificity of this cytosolic androgen receptor was measured by incubating cytosol of LNCaP cells with 5 nM

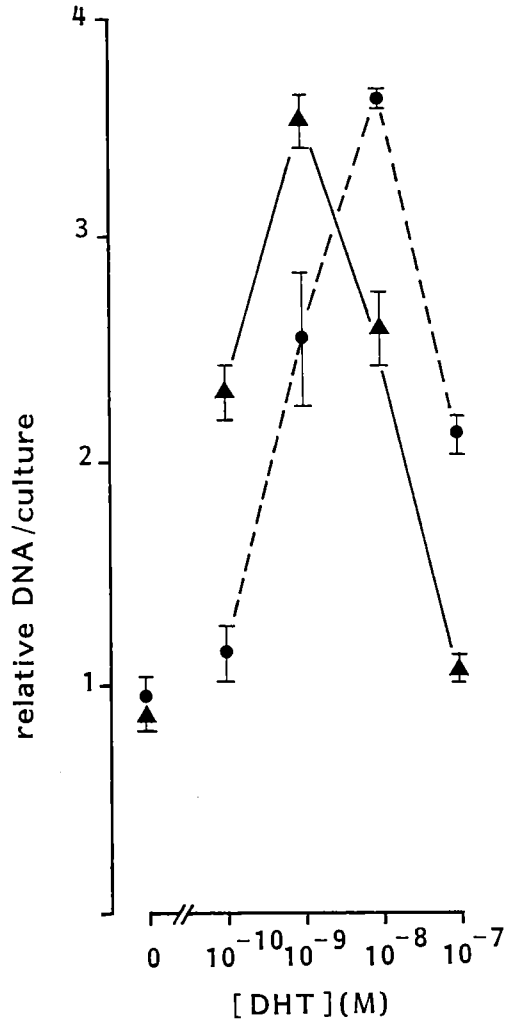

Figure 2 : Effect of dihydrotestosterone on growth of LNCaP cells in a 6 day period. Medium, with or without dihydrotestosterone, was changed at days 0 and 3 (●------●) or at days 0, 1, 3 and 4 (▲——▲). Growth is expressed as mean DNA content per culture relative to control cultures. Bars represent standard deviations calculated from triplicate experiments.

[^{3}H]R1881 in the presence of various concentrations unlabeled R1881, dihydrotestosterone, testosterone, progesterone, estradiol or triamcinolone acetonide. Figure 4 shows the competitive binding curves of different steroids for the androgen receptor. Relative binding affinity (RBA) values were calculated from these data as the ratio of concentration of unlabeled steroid and concentration R1881 required to inhibit [^{3}H]R1881 binding for 50%

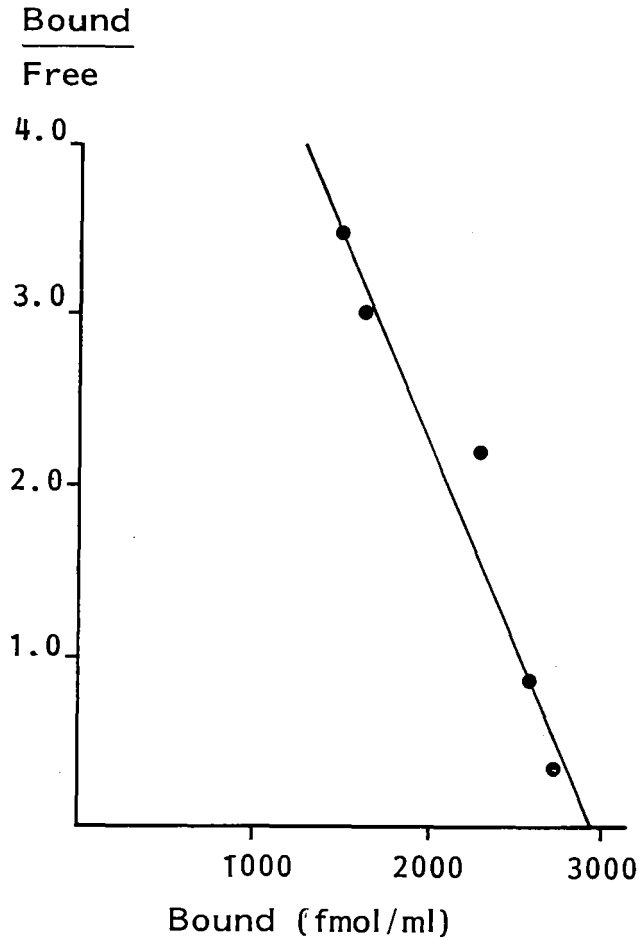

Figure 3 : Scatchard plot analysis of specific [^{3}H]R1881-binding to LNCaP cytosol protein. Cytosol (6 mg protein/ml) was incubated with six concentrations of [^{3}H]R1881 ranging from 0.5 to 10 nM. Parallel tubes contained an additional 1 µM unlabeled R1881 Bound and free R1881 were separated using a dextran-coated charcoal assay.

(table 1). Dihydrotestosterone and testosterone were not metabolized under binding assay conditions as analyzed with thin layer chromatography. The RBA value for R1881 was set at 100. The androgen receptor showed the highest affinity for R1881 and dihydrotestosterone respectively. The affinity for testosterone was approximately equal to that for progesterone. Estradiol also competed significantly with [^{3}H]R1881 for androgen receptor binding. Triamcinolone acetonide did not compete with [^{3}H]R1881

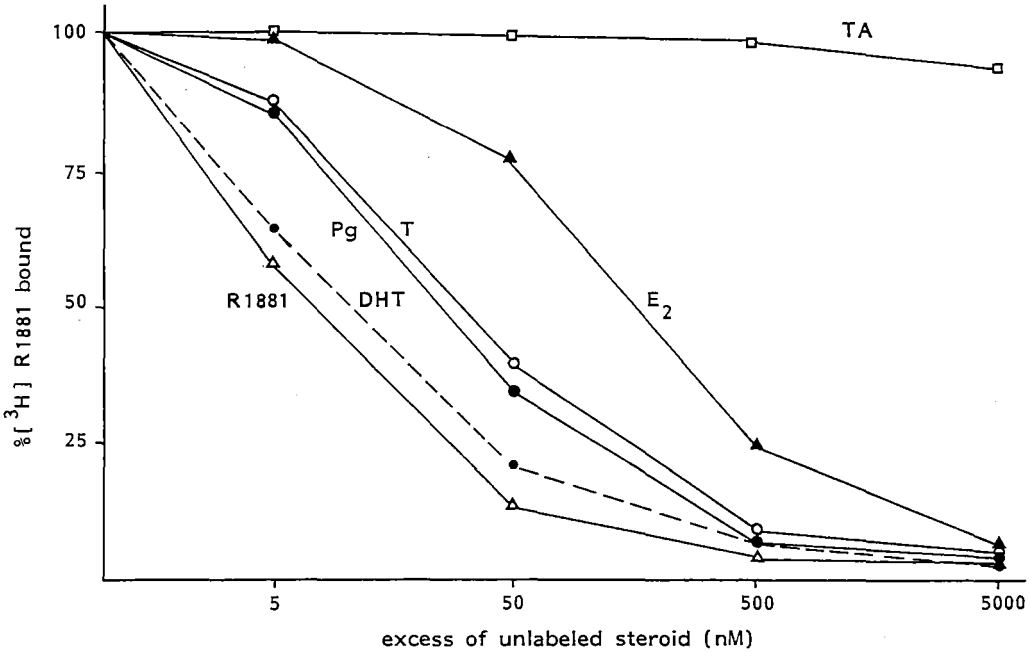

Figure 4 : Competitive binding curves of different steroids for the androgen receptor in LNCaP cells. Cytosol fractions were incubated with 5 nM [^{3}H]R1881 and 0, 5, 50, 500 or 5000 nM of unlabeled R1881 (△—△), dihydrotestosterone (DHT: ●---●), testosterone (T:○—○), progesterone (Pg:●—●), estradiol (E_2:▲—▲) or triamcinolone acetonide (TA:□—□.)

Table I Relative binding affinity (RBA)* of different steroids for the androgen receptor in LNCaP

steroid	RBA value
R1881	100.0
dihydrotestosterone	66.7
testosterone	23.8
progesterone	29.4
estradiol	4.3
triamcinolone acetonide	<0.1

*RBA values were calculated from figure 1 as ratio of unlabeled steroid and R1881 concentrations required to inhibit [^{3}H]R1881 binding by 50% The RBA of R1881 was set at 100

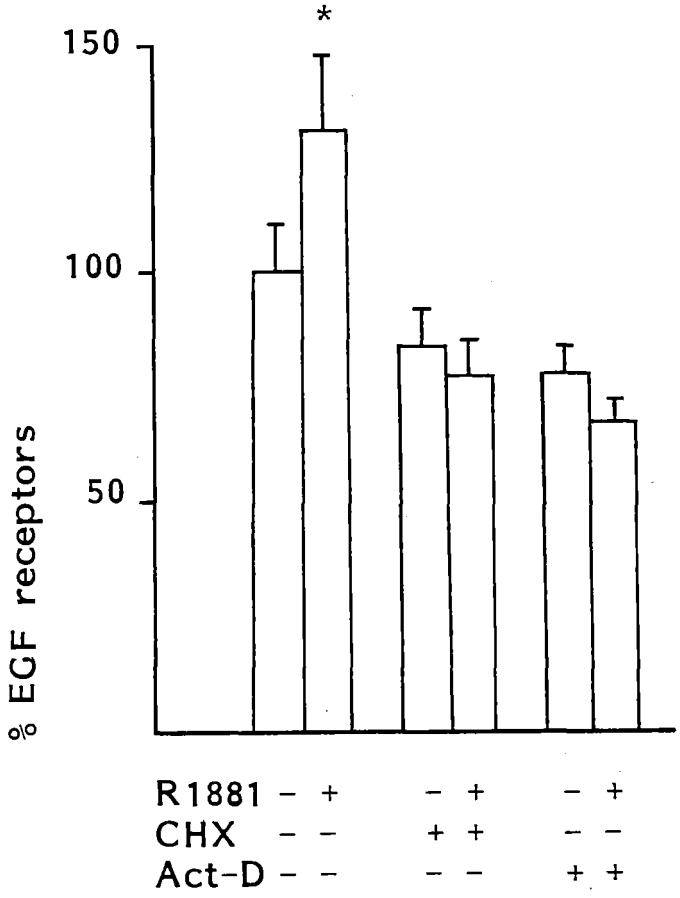

Figure 5 : Effects of cycloheximide (CHX) and actinomycin-D (Act-D) on EGF receptor induction by 01 nM R1881. Cells were grown in the presence (+) or absence (-) of indicated compounds as described in "Materials and Methods". Bars represent standard deviations, calculated from 3-5 experiments (* = P <0.5).

binding up to 1000 fold excess. Since R1881 also shows a high affinity for the progestagen receptor the absence of this steroid receptor was verified by Scatchard plot analysis with labeled progestagen and enzyme immunoassay of progestagen receptors. With both assays the progestagen receptor level was below 10 fmol/mg protein).

6.4.3. HORMONAL REGULATION OF THE EGF RECEPTOR

Previous studies showed that the synthetic androgen R1881 increases both growth rate and EGF receptor activity in LNCaP

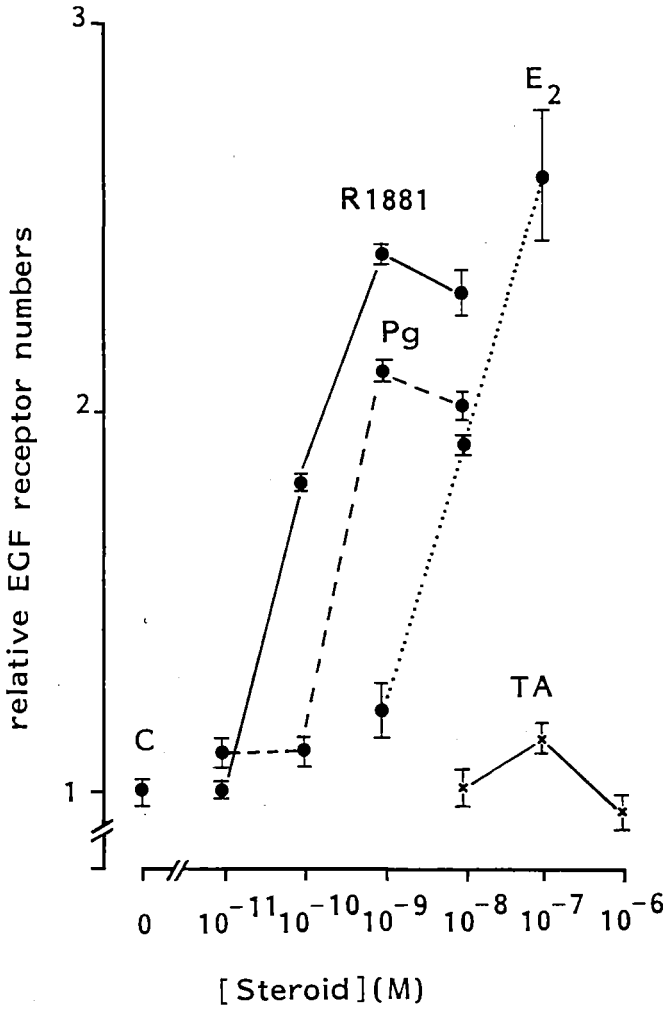

Figure 6 : Effect of various hormones on EGF receptor induction after 24 h treatments. Data are expressed relative to the number of EGF receptors of a control cell (c): R1881 (●——●), progesterone (Pg:●- - -●), estradiol (E_2:●····●) or triamcinolone acetonide (TA:×——×). Bars represent standard deviations

cells (Schuurmans et al., 1988). An increase in EGF receptor number was first observed after 6 to 12 h exposure to 0.1 nM R1881. After 24 h EGF receptor numbers increased from ± 11,000 EGF receptors per control cell to ± 20,000 EGF receptors per steroid-treated cell. To study the mechanism by which androgens increase receptor levels in LNCaP, we tested the effects of the inhibitors cycloheximide and actinomycin-D on the induction process. To limit the exposure time to these toxic agents a short

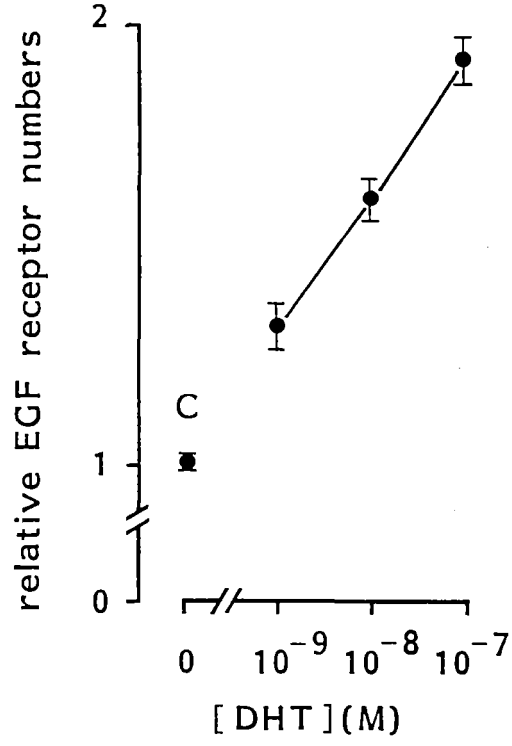

Figure 7 : Effect of various concentrations dihydrotesterone on induction of EGF receptors. EGF binding was measured 24 h after addition of dihydrotestosterone and analyzed by Scatchard plots. Data are expressed relative to the number of EGF receptors of a control cell (c). Bars represent standard deviations.

exposure time was chosen. EGF receptor levels increased significantly after 12 h treatment with 0.1 nM R1881 (figure 5: 30% increase; paired t-test, n=5, $P < 0.01$). To study the effects of the inhibitors, LNCaP cells were grown for 12 h with or without 0.1 nM R1881 and cycloheximide or actinomycin-D as described in "Materials and Methods". Both cycloheximide and actinomycin-D completely abolished the increase in EGF receptor levels following androgen administration (figure 5). The EGF receptor numbers in the presence of the inhibitors were even lower than control levels which might be explained by the EGF receptor half-life in the absence of new synthesis.

6.4.4. HORMONAL INDUCTION OF EGF RECEPTORS

EGF binding to LNCaP cells was measured after 24 h treatment with

various hormones at different concentrations. The number of EGF receptors per cell increased in a dose dependent manner upon treatment with R1881, progesterone, or estradiol while triamcinolone acetonide had no effect (figure 6). Dihydrotestosterone also increased EGF receptor levels in a dose-dependent manner (figure 7). Since dihydrotestosterone is rapidly metabolized during the 24 h culture period, higher concentrations were required to affect EGF receptor levels than expected from steroid binding studies (table 1). R1881 increased EGF receptor levels at lower concentrations (0.1 nM) than progesterone (1 nM) and estradiol (10 nM). Triamcinolone acetonide did not affect EGF receptor levels up to 1 μM. These data positively correlate with androgen receptor affinity for these ligands (figure 4) and induction of cell growth (figure 1).

6.5. DISCUSSION

The LNCaP cell line has been described as a model for prostate cancer (Horoszewicz et al.,1983). In the present study we showed that not only androgens but also progesterone and estradiol stimulated LNCaP cell growth. Therefore the binding properties of cytosolic androgen receptors in LNCaP have been further characterized. We confirmed that it showed a high affinity (Kd, 0.4 nM) and limited capacity (920 fmol/mg cytosol protein) for the synthetic androgen R1881. Furthermore R1881 and DHT were the most potent competitors for [^{3}H]R1881 binding in the competitive binding assay. It was striking that progesterone competed as strongly as testosterone with [^{3}H]R1881 for binding to the androgen receptor. R1881 also binds the progesterone receptor. However, competition of progesterone with [^{3}H]R1881 was not due to the presence of progestagen receptors as checked with Scatchard plot analysis using a labeled progestagen and with monoclonal antibody based enzyme immunoassay of progestagen receptors. Estradiol also competed to a limited extent with [^{3}H]R1881 binding. Berns et al. (1986) demonstrated the absence of estrogen receptors in LNCaP by a ligand binding assay and application of an immunocytochemical assay with monoclonal antibodies directed against the estrogen receptor from MCF7 cells. The glucocorticoid triamcinolone acetonide did not compete

with [^{3}H]R1881 binding. The relative binding affinity values of different steroids (except for dihydrotestosterone) correlated with the effect on cell growth. Although dihydrotestosterone showed to be the most potent competitor for [^{3}H]R1881 binding to the androgen receptor, higher concentrations than expected from this observation were needed to stimulate cell growth. However, the rapid metabolism of dihydrotestosterone could explain this result: growth was stimulated at lower concentrations of dihydrotestosterone when the culture medium was changed more frequently.

The binding data (capacity and affinity) are comparable to that published for HONDA (635 fmol/mg cytosol; kD, 1.2 nM), a serially transplantable human prostatic cancer in nude mice (Ito et al., 1985). The LNCaP cells showed a higher binding capacity for R1881 than PC-82, another serially transplantable human prostatic tumor in nude mice (120 fmol/mg cytosol protein; Brinkmann et al., 1987). The binding capacity was also much higher than in the hormone unresponsive LNCaP-r subline (16 fmol/mg cytosol protein; Hasenson, 1985). The binding specificity of the androgen receptor in LNCaP differs from that in HONDA and PC-82 because it showed high affinity for progesterone and estradiol also competed with [^{3}H]R1881 binding.

The steroid specificity pattern of the androgen receptor positively correlated with the induction of EGF receptors by various hormones e.g.: the higher the affinity for a steroid, the lower concentrations are required to increase EGF receptor levels.

Actinomycin-D and cycloheximide inhibited the hormonally induced increase in EGF receptor numbers. This indicates that the EGF receptor induction process is at least in part regulated at transcriptional level. Increase of transcription of specific genes by steroids has been described for several systems.

Several cell lines have been described to overexpress the EGF receptor of which the most noted is the A431 cell line with 10^6 receptors per cell (Haigler et al., 1978). In a study with variants of the A431 cell line having different levels of EGF receptor it was demonstrated that those variants having a high number of EGF receptors produced larger tumors much more quickly than those variants bearing lower numbers of EGF receptors

(Santon et al., 1986). In the present study we also show that increased expression of EGF receptors correlates with increased growth. These results suggest but do not proof a direct involvement of EGF receptors in growth stimulation mediated by the androgen receptor. A direct proof could come from transfection studies with anti-sense mRNA of the EGF receptor. In such studies, which are beyond the scope of this report, EGF receptor-mRNA is not available for translation and this should lead to a decreased growth activity.

Up-regulation of EGF receptor levels has been described in several cells upon treatment with estrogen (Mukku and Stancil, 1985), progesterone (Murphy et al., 1986) and glucocorticoids (Fanger et al., 1984). In contrast to these and our findings, androgen down-regulation has been demonstrated in normal rat prostate (Traish and Wotiz, 1987). Castration of mature rats resulted in a 3 to 6 fold increase in EGF binding, while treatment of castrated rats with dihydrotestosterone decreased the number of binding sites. Thus in this normal adult rat prostate tissue androgens might prevent growth by down-regulation of EGF receptors while in transformed LNCaP cells androgens up-regulate EGF receptors which correlates with increased growth. It might be of interest to study the effect of androgens on EGF receptor levels in benign prostate hypertrophs (BPH), a condition which also escapes normal growth control.

Tumor cells exhibit uncontrolled proliferation which in part may be explained by an autocrine mechanism of growth control e.g. cells produce and respond to their own growth factors (Sporn and Todaro, 1980; Lippman et al., 1987). Recent findings concerning growth of pancreatic cancer cells which overexpress EGF receptors and also produce TGFα suggest this as an efficient mechanism for cancer cells to obtain a growth advantage (Smith et al., 1987). Studies are in progress to find out whether LNCaP cells might produce TGFα. In our laboratory various LNCaP sublines are cultured which differ in androgen dependency. It is of interest to study whether growth factor secretion and/or growth factor receptor levels may differ among these various sublines.

6.6. ACKNOWLEDGEMENT

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CHAPTER 7

EFFECTS OF ANTIANDROGENS ON LNCaP HUMAN PROSTATE TUMOR CELLS IN VITRO

7.1.SUMMARY

LNCaP cells (derived from a lymph node carcinoma of the human prostate) show androgen responsive growth. Antiandrogens and estradiol competed with androgen for binding to the androgen receptor. The relative binding affinity of various ligands for the androgen receptor decreased in the order of: R1881 (a synthetic androgen; relative binding affinity, RBA: 100.0), cyproterone acetate (a steroidal antiandrogen, RBA:12.5), estradiol (RBA:4.3) RU 23908 (a nonsteroidal antiandrogen, RBA:1.0). Optimal growth (3- to 4-fold increase in DNA content of 6-day cell cultures versus controls) was observed after addition of 0.1 nM R1881. The antiandrogens did not suppress the androgen responsive growth. At a concentration of 100 nM, both cyproterone acetate and RU 23908 even stimulated growth to an extent comparable to that observed after addition of androgen. Cyproterone acetate and RU 23908 also increased the number of epidermal growth factor receptors expressed at the cell surface to a comparable level as did the androgen. Like androgens, cyproterone acetate, RU 23908 or estradiol stimulated the secretion per cell of prostate specific acid phosphatase in the culture fluid. In conclusion, antiandrogens can exert striking stimulatory effects on the proliferation of LNCaP cells, a phenomenon not observed before concerning androgen responsive human cell lines. It is discussed that LNCaP cells may contain a defective androgen receptor system.

7.2. INTRODUCTION

The prostate gland is dependent on androgens for the maintenance of its growth and functional integrity (Sandberg, 1980). Androgens exert their effects on target tissue through binding to the androgen receptor, followed by association of the androgen-receptor complex with specific binding sites on DNA (Rushmere et

al., 1987). This androgen receptor complex can either induce (Zhang et al., 1985) or repress (Montpertit et al., 1986) transcription of specific genes.

Prostate cancer is a common disease in men and androgens play a critical role in the growth regulation of neoplastic prostate tissue. Huggins and Hodges (1941) defined the regulatory role of testicular androgens in prostate cancer. They demonstrated dramatic clinical responses in men with metastatic prostate cancer following castration or treatment with estrogen. The use of estrogens in treatment of prostate cancer is hampered by their side effects (Byar, 1973). The search for other endocrinologically active agents led to the development of antiandrogens, such as cyproterone acetate and its derivates (steroidal antiandrogens) and flutamide and its derivates (nonsteroidal antiandrogens) (Neumann et al., 1970; Raynaud et al., 1979; Neri et al., 1972).

Cyproterone acetate can suppress pituitary gonadotropin secretion and it has antiandrogenic properties as evidenced by competitive inhibition of formation of androgen receptor complexes in androgen target tissues (Fang and Liao, 1971) resulting in decreased nuclear retention of dihydrotestosterone (DHT) and DHT-receptor complexes (Brinkmann et al., 1983; Callaway et al., 1982) and finally in reduced target organ weights (Neumann and Steinbeck, 1974). In vivo studies with RU 23908 (flutamide derivate, anandron) showed that it inhibits the androgen induced increase of rat prostate weight and androgen dependent gonadotropin feedback giving rise to an increase of LH and testosterone (Raynaud et al., 1979). Its mechanism of action on target cell function seems comparable to that of steroid antiandrogens in that it is able to inhibit testosterone binding to cytoplasmic androgen receptors effectively (Raynaud et al., 1979).

The information on antiandrogenic effects on human prostate cell lines is scarce, because of the very limited number of androgen responsive cell systems. Among the six permanent growing human prostate cell lines described up to now (Isaacs, 1987) only the LNCaP (Lymph Node Carcinoma of the Prostate) cell line developed by Horoszewicz et al. (1983) shows androgen dependent growth. LNCaP cells contain considerable amounts of androgen receptors

while estrogen and progestagen receptors are not detectable (Schuurmans et al., 1988a). Androgen treatment of these cells enhances the synthesis and secretion of prostate specific acid phosphatase (PAP) (Schulz et al., 1985). Furthermore androgens increase the number of epidermal growth factor (EGF) receptors in LNCaP cells, whereas EGF acts synergistically with androgens on LNCaP cells to induce cell proliferation (Schuurmans et al., 1988b,c).
The purpose of the present study was to investigate the effects of a steroidal (cyproterone acetate) and nonsteroidal (RU 23908) antiandrogen on LNCaP cell proliferation, secretion of PAP and expression of EGF receptors. In contrast to our expectation both antiandrogens exerted stimulatory effects on growth of LNCaP cells.

7.3. MATERIALS AND METHODS

7.3.1. MATERIALS

[^{3}H]17β-Hydroxy-17α-methyl-estra-4,9,11-trien-3-one ([^{3}H]R1881, spec. act.: 87 Ci/mmol) and radioinert R1881 were purchased from New England Nuclear (Boston,MA).
1,2α-Methylene-6-chloro- 6-17α-hydroxy- progesterone acetate (cyproterone acetate) was a gift from Schering (Berlin, FRG). 5,5Dimethyl-3-[4 nitro-3-(trifluoro-methyl)phenyl]-imidazolidine-2,3 dione (RU 23908, anandron) was a gift from Roussel Uclaf (Paris, France). 9α-Fluoro-11β,21-dihydroxy-16α,17α-isopropylidenedioxy-1,4-pregnadiene-3,20-dione (triamcinolone acetonide) was obtained from Sigma (St. Louis, MO).
Unlabeled EGF from mouse submaxillary gland was obtained from Sigma (receptor grade). Mouse ^{125}I labeled EGF (spec. act.: 140 Ci/g, Amersham) was used following removal of free iodine by Sephadex G25 gelfiltration.

7.3.2. CELL CULTURE

The LNCaP cell line was a gift of Dr. Horoszewicz (Buffalo, NY). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2

in air. LNCaP cells, subline FGC-GJ (Steenbrugge et al., 1989), between passages 65 and 70 were used for our experiments.Cells were maintained in RPMI 1640 medium (Gibco, Grand Island, NY) with penicillin and streptomycin, supplemented with 7.5% (v/v) heat inactivated (30 min, 56°C) fetal calf serum (Gibco). Cells were trypsinized and plated out once a week with a medium change after 3-4 days.

7.3.3. GROWTH STUDIES

Cells were trypsinized and plated out in 24 multi-well dishes (Falcon, Oxnard, CA) at a density of $2x10^4$ cells/cm^2 in 5% (v/v) charcoal-treated (in order to remove steroid hormones) fetal calf serum. For 3 days cells were allowed to attach and to start growth, then the medium was changed and indicated compounds were added at different concentrations. After 3 days medium was replaced with fresh medium containing the same compounds. At day 6 the cells were washed 4 times with phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+} (GIBCO, Grand Island, NY), and dissolved in 1 M NaOH. DNA content was estimated according to Hinegardner (1976).

7.3.4. COMPETITION STUDIES OF THE ANDROGEN RECEPTOR IN CYTOSOL OF LNCAP CELLS

Cytosol of LNCaP cells was prepared as described previously (Schuurmans et al., 1988a). Competition studies were performed by incubating cytosol for 18 h at 0°C with 5 nM [^{3}H]R1881 in the presence of 0, 5, 50, 500 or 5000 nM R1881, cyproterone acetate, RU 23908 or triamcinolone acetonide. Specific binding of [^{3}H]R1881 was measured after separation of bound and unbound steroid. This separation was achieved by adsorption of unbound ligand to 3 g/l charcoal - 0.03 g/l dextran for 15 min at 4°C and centrifugation at 2000 g for 5 min.

7.3.5. EGF BINDING STUDIES

Cells were plated out in 24 multi-well dishes in 5% (v/v) charcoal-treated fetal calf serum at a density of 1.6x105

cells/cm_2. After attachment for 3 days, cells were grown for 24 h in the presence or absence of indicated compounds. Next, EGF binding assays were performed as described (Schuurmans et al., 1988b). Briefly, cells were washed with PBS (without Ca^{2+} and Mg^{2+}), and preincubated for 2 h at 37°C with binding buffer (RPMI 1640 with penicillin and streptomycin, supplemented with 1 mg/ml bovine serum albumin). Subsequently cells were incubated with binding buffer containing ^{125}I-labeled EGF plus unlabeled EGF. After incubation cells were suspended in ice-cold binding buffer and centrifuged for 5 min at 2200 g. The supernatant was removed and the radioactivity of the cell pellets was measured. Scatchard analysis of binding data was performed after correction for nonspecific binding. The amount of EGF binding was expressed as fmol EGF bound per mg DNA.

7.3.6. ASSAY OF PROSTATE SPECIFIC ACID PHOSPHATASE

Cells were plated out in T25 culture flasks (Nunc, Roskilde, Denmark) in 5% (v/v) charcoal treated fetal calf serum at a density of $2x10^4$ cells/cm^2. After attachment for 3 days, cells were grown in the absence or presence of 0.1 nM R1881, 100 nM cyproterone acetate, 100 nM RU 23908, 100 nM estradiol or 100 nM triamcinolone acetonide. At indicated days DNA content per culture and the concentration of prostate specific acid phosphatase in culture fluid was measured.
The concentration of this enzyme in culture fluid was assayed immunologically with a commercially available test kit (Enzygnost PAP) from Behring Diagnostica according to the recommendations of the supplier. The limit of detection was 0.2 µg enzyme/l. The antibodies of this kit do not crossreact with other acid phosphatase isoenzymes.

7.4. RESULTS

7.4.1. EFFECTS OF ANDROGEN AND ANTIANDROGENS ON GROWTH OF LNCaP CELLS

LNCaP cells were cultured for 6 days in medium containing 5% (v/v) charcoal treated fetal calf serum and various

concentrations of different compounds. At day 6, DNA content per culture was determined (Figure 1). The synthetic, non-metabolizable androgen R1881 was used for study of growth stimulatory effects. In LNCaP cells the effects of R1881 are mediated by the androgen receptor because progestagen receptors are absent in these cells (Schuurmans et al., 1988a). R1881 stimulated growth maximally at a concentration of 0.1 nM (3- to 4-fold increase in DNA content per culture versus control

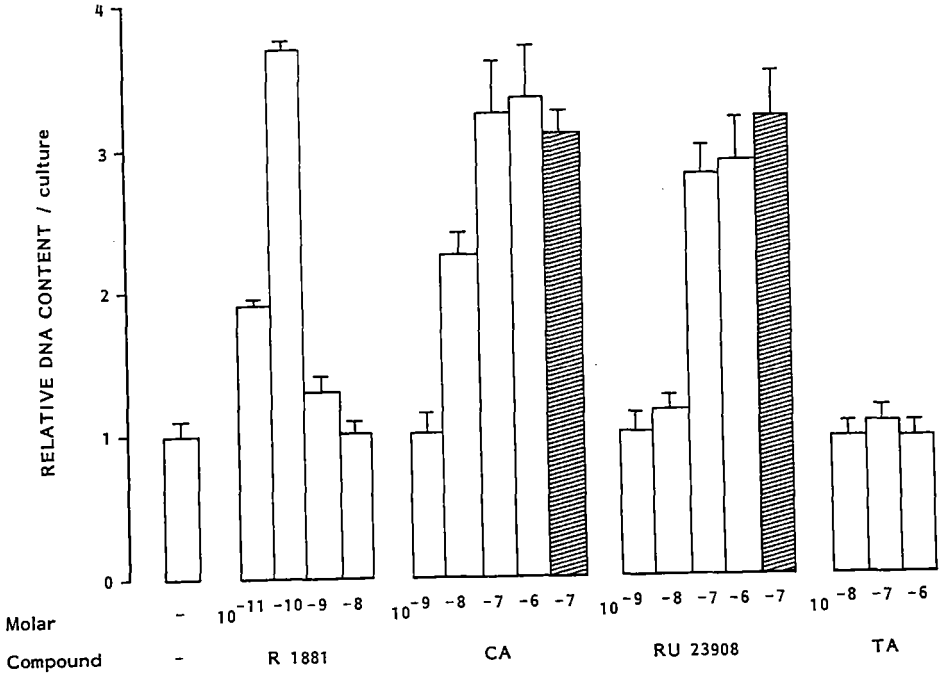

Fig. 1. Effects of various concentrations of R1881, cyproterone acetate (CA), anandron (RU 23908) and triamcinolone acetonide (TA) on growth of LNCaP cells after 6 days treatment with indicated compounds. Growth is expressed as mean DNA content per culture relative to control cultures (-).
Shaded bars show the effect of indicated antiandrogen plus 0.1 nM R1881. Standard deviations of triplicate experiments are shown.

cultures). As observed before (Schuurmans et al., 1988a) stimulation at higher concentration of R1881 is limited. Cyproterone acetate stimulated cell growth twofold at 10 nM while at this concentration RU 23908 had no effect. At a concentration of 100 nM, both cyproterone acetate and RU 23908 stimulated LNCaP cell proliferation to the same extent as 0.1 nM R1881. When 0.1

nM R1881 was added to the cell cultures in combination with 100 nM cyproterone acetate or 100 nM RU 23908, growth stimulation was similar as observed for R1881 or antiandrogens added alone (Figure 1). The synthetic glucocorticoid triamcinolone acetonide did not affect growth at concentrations up to 1 μM.
In a separate series of experiments the effect of long-term cyproterone acetate treatment was studied. LNCaP cells were cultured for 42 days in the presence of 0.1 nM R1881 or 100 nM cyproterone acetate. Cells were replated weekly and the medium with or without the steroids was changed after 3 to 4 days. Continuous stimulation was observed for this period by both R1881 and cyproterone acetate compared to controls (results not shown).

7.4.2. ANDROGEN RECEPTOR BINDING

Previous studies showed that LNCaP cell cytosol contains androgen receptors with high affinity (Kd, 0.4 nM) and a binding capacity of 920 fmol/mg cytosol protein (Schuurmans et al., 1988a). The binding specificity of the androgen receptor was measured by incubating cytosol of LNCaP cells with 5 nM [^{3}H]R1881 in the presence of various concentrations of unlabeled R1881, cyproterone acetate, RU 23908 or triamcinolone acetonide. Figure 2 shows the competitive binding curves for these compounds for the androgen receptor. Relative binding affinity (RBA) values were calculated from these data as the ratio of concentration of unlabeled compound and concentration of R1881 required to inhibit [^{3}H]R1881 binding by 50% (Table I). The RBA value of R1881 was set at 100. The androgen receptor showed the highest affinity for R1881 followed by cyproterone acetate and RU 23908. Triamcinolone acetonide did not compete with [^{3}H]R1881 binding up to 1000-fold excess. The values for estradiol and progesterone obtained in a previous study (Schuurmans et al., 1988a) are also included in Table I.

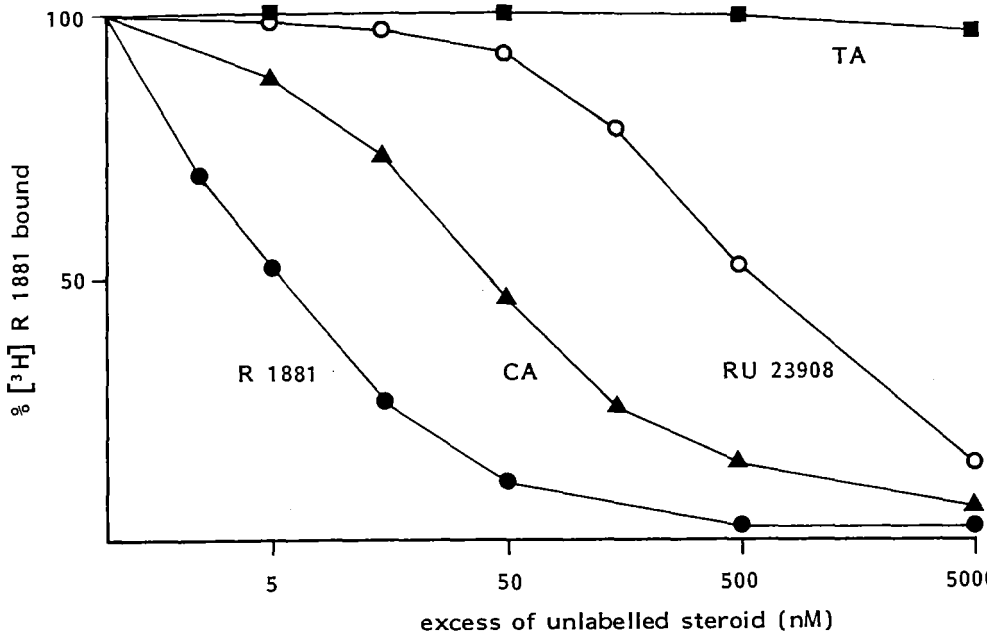

Fig. 2. Competitive binding curves of different compounds for the androgen receptor in LNCaP cells. Cytosol fractions were incubated for 18 h at 0°C with 5 nM [^{3}H]R1881 and 0, 5, 50, 500, or 5000 nM of unlabeled R1881 (●——●), cyproterone acetate (▲——▲), RU 23908 (o——o) or triamcinolone acetonide (■——■). Specific binding of [^{3}H]R1881 was measured after separation of bound and unbound steroid.

Table I. Relative binding affinity (RBA) of different ligands for the androgen receptor in LNCaP.

ligand	RBA value
R1881	100.0
cyproterone acetate	12.5
RU 23908	1.0
triamcinolone acetonide	< 0.1
progesterone[a])	29.4
estradiol[a])	4.3

[a])Data from reference 16.

7.4.3. INDUCTION OF EGF RECEPTORS

Previous studies demonstrated that androgens increase both growth

rate and EGF receptor activity in LNCaP cells (Schuurmans et al., 1988b). EGF binding to LNCaP cell monolayers was measured after 24 h treatment with various compounds (Table II). Both antiandrogens (100 nM) increased EGF binding to LNCaP cells to a level comparable to the binding obtained after addition of R1881 (0.1 nM). Triamcinolone acetonide had no effect on EGF binding to LNCaP cells.

Table II. Effect of different compounds on EGF binding to LNCaP cells.

condition	bound fmol EGF/mgDNA
control	495 ± 20
100 nM triamcinolone acetonide	480 ± 25
0.1 nM R1881	720 ± 25
100 nM cyproterone acetate	745 ± 10
100 nM RU 23908	690 ± 10

7.4.4. SECRETION OF PROSTATE SPECIFIC ACID PHOSPHATASE

LNCaP cells were cultured for various periods of time in the absence or presence of 0.1 nM R1881, 100 nM cyproterone acetate, RU 23908 or triamcinolone acetonide. Growth curves of cells treated with antiandrogens paralleled that of cells treated with R1881 (Figure 3A). Figure 3B shows the time course of secretion of prostate acid phosphatase (PAP) in the culture fluid expressed as µg PAP per 100 µg DNA. R1881 and cyproterone acetate were the most potent stimulators of PAP secretion (3- to 4-fold increase after 6 days versus control cells). The amount of PAP per 100 µg DNA secreted by cells upon treatment with RU 23908 was doubled after 6 days versus control cells. For control cells and cells treated with triamcinolone acetonide the amount of PAP secreted when expressed per 100 µg DNA remained constant after 3 days. We previously reported that 10 nM estradiol also stimulated cell growth (Schuurmans et al., 1988a). Treatment of LNCaP cells with 10 nM estradiol increased PAP secretion to the same extent as obtained after addition of 0.1 nM R1881 (64 µg PAP/100 µg DNA at day 6).

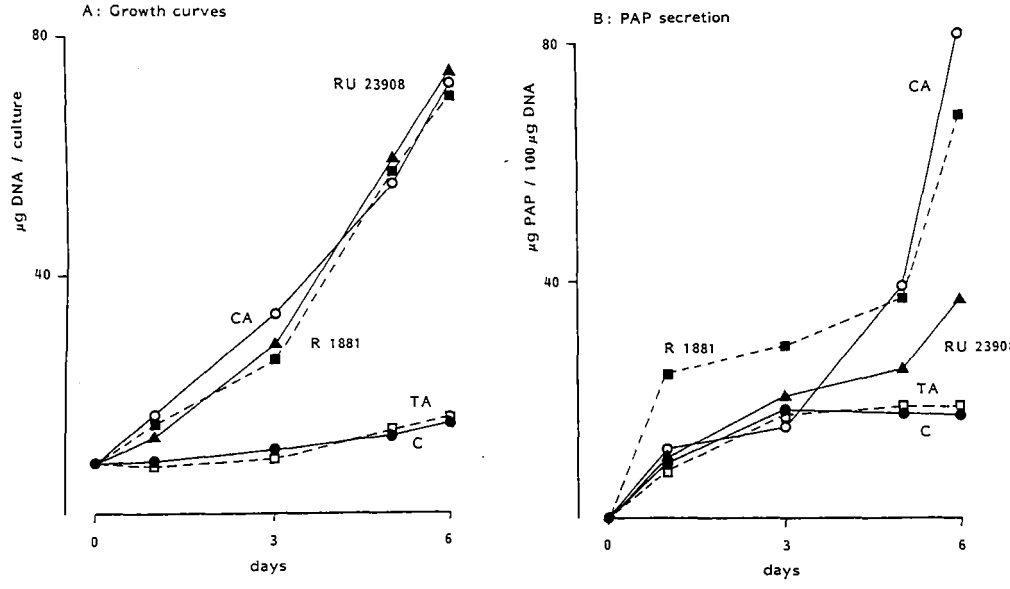

Fig. 3. Growth curves and time course of secretion of prostate specific acid phosphatase by LNCaP cells. Cells were cultured in the absence (●—●) or presence of following compounds: 0.1 nM R1881 (■--■), 100 nM cyproterone acetate (○—○), 100 nM RU 23908 (▲—▲) or 100 nM triamcinolone acetonide (□---□). DNA content per culture (A) and prostate specific acid phosphatase (PAP) secreted in the culture fluid (B) was measured as described in "Materials and Methods".

7.5. DISCUSSION

Horoszewicz et al. (1983) characterized the LNCaP cell line and demonstrated its utility as a model for laboratory studies on human prostate cancer in vitro. LNCaP cells showed androgen dependent growth and we intended to study growth inhibitory activity of antiandrogens in this cell system. The biological effects of a steroidal and a non-steroidal antiandrogen on these human prostate tumor cells were compared with effects of the androgen R1881. In contrast to our expectation both cyproterone acetate and RU 23908 exerted striking stimulatory effects on LNCaP cell proliferation, EGF receptor induction and secretion of PAP.

In vitro studies with tumor cells of nonhuman origin have demonstrated that antiandrogens inhibit androgen dependent growth. The mouse mammary tumor cell line derived from Shionogi carcinoma 115 shows androgen dependent growth which can be

inhibited by flutamide or cyproterone acetate (Yates and King, 1981; Jung-Testas and Baulieu, 1987). RU 23908 antagonizes the effects of testosterone on growth of rat prostate derived cancer cell lines (Shain and Huot, 1986). Since the LNCaP cell line is the only established androgen responsive prostate tumor system of human origin it is not possible to investigate if loss of proper response to antiandrogens by metastatic prostate tumor cells is a general phenomenon for this type of cells in culture. In addition it is important to know if in vivo insensitivity to antiandrogens in advanced metastatic prostate cancer in some cases passes through a stage of stimulation of the tumor by antiandrogens. Growth stimulatory effects by other types of antihormones on hormone responsive cells have been observed before. Long-term therapy with tamoxifen (a nonsteroidal antiestrogen) has been examined in athymic mice bearing human breast MCF7 tumors and development of tamoxifen-dependent growth was shown (Gottardis and Jordan, 1988).

Cyproterone acetate and RU 23908 inhibit androgen binding to LNCaP cytosolic androgen receptors. RU 23908 binds only to androgen receptors (Moguilewsky et al., 1986) while R1881 and cyproterone acetate show considerable affinity for progestin receptors (Raynaud and Ojasoo, 1986). However, the effects of the two latter ligands are entirely mediated via the androgen receptor since progestin receptors are absent in LNCaP cells (Schuurmans et al., 1988a). Cyproterone acetate shows a higher affinity for androgen receptors than RU 23908 (Moguilewsky et al., 1986; Raynaud and Ojasoo, 1986; Ojasoo and Raynaud, 1983), in agreement with the observation that cyproterone acetate stimulates growth of LNCaP cells at a lower concentration than RU 23908.

The antiandrogens and estradiol stimulated PAP secretion into the culture medium of LNCaP cells. Estradiol and cyproterone acetate were more potent than RU 23908; the differences in stimulatory effect reflected the variation in relative binding affinity to the androgen receptor. The concentration of PAP is often elevated in the serum of patients at the metastatic stage of prostate carcinoma (Vihko et al., 1980; Chu et al., 1978). In short-term cultures of human prostatic tissues an enhanced production of PAP can be observed in response to physiological

levels of testosterone and dihydrotestosterone (Hudson, 1981). R1881 is the most effective stimulator of PAP expression in LNCaP cells (Schulz et al., 1985). This may be explained by the facts that R1881 firmly binds to androgen receptors and is only slowly, if at all, degradated to inactive products in prostate tissue, in contrast to testosterone and dihydrotestosterone. Schulz et al. (1985) showed that estrogens but not triamcinolone acetonide can increase PAP secretion in the culture fluid. We confirm these observations in the present study. However, we recently demonstrated that LNCaP cells contain no estrogen receptors but an androgen receptor which shows considerable cross reactivity with estradiol (Schuurmans et al., 1988a). Therefore we believe that estrogens act via the androgen receptor in LNCaP cells and not via estrogen receptors as has been suggested (Schulz et al., 1985).

EGF can stimulate the growth of LNCaP cells and androgens increase the number of EGF receptors expressed at the cell surface (Schuurmans et al., 1988b). EGF acts synergistically with androgen on LNCaP cells to induce cell proliferation, probably due to up-regulation of EGF receptors by androgen (Schuurmans et al., 1988c). The antiandrogens also exerted stimulatory effects with respect to induction of EGF receptors in LNCaP cells. Both cyproterone acetate and RU 23908 increased EGF receptor levels.

One of the mechanisms by which antihormones might antagonize steroid hormones at the molecular level is at the level of transcription. Upon binding of the antihormone to the receptor, this complex binds to hormone responsive elements of DNA but fails to induce the transcription activation function. This transcription activation function is probably located in the steroid hormone binding domain of the receptor (Webster et al., 1988; Guiochon-Mantel et al., 1988). As mentioned before, the androgen receptor in LNCaP significantly crossreacts with progesterone and estradiol (Schuurmans et al., 1988a). The androgen receptor has thus lost its ligand-binding specificity and antiandrogens act as activators alike androgens instead of inhibitors. This raises the interesting hypothesis that the steroid binding domain of the androgen receptor in LNCaP cells differs from a normal androgen receptor. This difference could be caused by changes in the structure of the receptor gene or by

changes in posttranscriptional or postranslational processes. Recently the primary structure of the androgen receptor has been described (Chang et al., 1988; Lubahn et al., 1988; Trapman et al., 1988). This makes it possible to investigate if the deviant behaviour of the androgen receptor in LNCaP cells is due to structural modification.

7.6. ACKNOWLEDGEMENT

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CHAPTER 8

ANTIPROLIFERATIVE EFFECTS OF SURAMIN ON ANDROGEN RESPONSIVE LNCaP CELLS

8.1. ABSTRACT

The effect of the polyanionic drug suramin on androgen responsive human prostate tumor cells (LNCaP) was studied. The proliferation of LNCaP cells was stimulated two- to threefold by the synthetic androgen R1881 (0.1 nM) or EGF (1 ng/ml). Suramin (0.01 to 1.0 mM) inhibited the growth of LNCaP cells in a dose dependent way, both in the presence and absence of androgen or EGF. Growth was arrested in the G0/G1 phase of the cell cycle, but was resumed after removal of suramin. EGF binding to LNCaP cells was measured either in the presence or absence of suramin. Suramin decreased the affinity of EGF binding sites on LNCaP cells with a two- to eightfold increase in Kd at 0.1 and 1.0 mM suramin, respectively. In conclusion: suramin counteracts the growth stimulatory effects of both androgen and growth factor on LNCaP cells.

8.2. INTRODUCTION

Steroid hormones and growth factors are involved in the complex regulation of cell proliferation of hormone sensitive tumors. For example, in human breast cancer cells, estrogens exert their action through coordinated control of production and secretion of a collection of growth factors (Dickson and Lippman, 1987). These growth factors act in an autocrine or paracrine way by binding to their cell surface receptors. In addition, steroid hormones may control cell proliferation by modulating the affinity or level of expression of growth factor receptors (Fanger et al., 1986; Murphy et al., 1986).

Modalities in treatment of cancer patients directed to interference with growth factors or growth factor receptors are scarce. Recently, preliminary data on the application of growth factor antagonists have been reported (Kosano and Takatani, 1986;

Yaish at al., 1988). One of the compounds, suramin, may be of interest for clinical practice. Suramin is a polysulphonated naphthylurea (MW: 1429) that has been used since 1920 for the treatment of african trypanosomiasis and onchocerciasis (Tanon and Jamut, 1924; Olenick, 1975; Hawking, 1978). Suramin has been shown to act as a polypeptide growth factor antagonist for platelet derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF) and transforming growth factor beta (TGFβ) (Coffey et al., 1987).
The purpose of the present investigation was to study the effects of suramin on androgen responsive LNCaP cells. In LNCaP cells androgens, growth factors and their receptors play an important role in the regulation of cell proliferation (Schuurmans et al., 1988 a,b) In this paper we show that suramin can counteract the stimulatory effects of androgens as well as of growth factors on growth of LNCaP cells.

8.3. MATERIALS AND METHODS

8.3.1. CHEMICALS

8,8'-[Carbonylbis [imino-3,1-phenylenecarbonyl imino (4-methyl-3,1-phenylene) carbonyl-imino]]bis-1,3,5-naphtalenetrisulfonic acid hexasodium salt (suramin), a product from Bayer AG, was obtained from Mobay Chemical Corporation (New York, NY).
The synthetic androgen R1881 (17β-hydroxy-17α-methyl-estra-4,9,11-trien-3-one) was purchased from New England Nuclear (Boston, MA). EGF from mouse submaxillary gland was obtained from Sigma Chemical Company (St. Louis, MO, USA). Mouse [^{125}I]EGF (specific activity: 140 Ci/g, Amersham, UK) was used after removal of free iodine by Sephadex G25 gel filtration.

8.3.2. CELL CULTURE

LNCaP cells, a gift from Dr. J. Horoszewicz (Buffalo, NY) were cultured as described previously (Schuurmans et al., 1988a). LNCaP cells between passage 65 and 70 were used for our experiments.

8.3.3. GROWTH STUDIES

Cells were plated in 24 multi-well dishes (Falcon, Oxnard, CA) at a density of $2x10^4$ cells/cm^2 in RPMI 1640 medium supplemented with 5% (v/v) charcoal treated fetal calf serum. The cells were allowed to attach and initiate growth for 3 days. The medium was changed and cells were grown in the presence or absence of different concentrations of suramin (0.01-1mM) with or without 0.1 nM R1881 or 1 ng EGF/ml (one medium change after 3 days). At day 6 cells were used to measure DNA content according to Hinegardner (1971).
To study whether the effect of suramin on LNCaP cell proliferation is reversible, cells were plated in T25 Flasks (Nunc, Roskilde, Denmark) in RPMI 1640 medium containing 5% (v/v) charcoal treated fetal calf serum at a density of $2x10^4$ cells/cm^2 and allowed to attach for 3 days. Medium was changed and the cultures received suramin (0.1 mM) with either 1 ng EGF/ml or 0.1 nM R1881. After 6 days part of the cultures were rinsed four times with PBS and dissolved in 1M NaOH for estimation of DNA content. The remaining cultures were rinsed with PBS and grown in the presence or absence of 1 ng EGF/ml or 0.1 mM R1881 with or without 0.1 mM suramin for another 11 days (medium change every 3 days) and DNA content per culture was measured as described.

8.3.4. FLOW CYTOMETRY

Flow cytometry was performed as described before (Bontenbal et al., 1989). Briefly, bromodeoxyuridine, a marker for DNA synthesis, (BrdUrd, Serva, Heidelberg, BRD) was added to the LNCaP cells 30 minutes before harvesting. Cell nuclei were isolated and incubated both with FITC labeled anti-BrdUrd and propidium iodide (a marker for the amount of DNA). Suspended nuclei were analyzed by dual parameter flow cytometry.
Excitation- and emission wavelengths of FITC and propidium iodide were 494;517 and 540;625 nm respectively. For each sample at least 10^4 events were analyzed.

8.3.5. EGF BINDING ASSAY

EGF binding assays were performed as described previously (Schuurmans et al., 1988a). Dulpicate cell cultures were incubated with ^{125}I-EGF plus unlabelled EGF (final EGF concentration/well ranged from 0.1-30 nM) in the presence or absence of suramin for 4 hr at 4 C. Radioactivity of the pellets was measured and Scatchard analysis of binding data was performed after correction for non-specific binding.

8.4. RESULTS

8.4.1. SHORT TERM EFFECTS OF SURAMIN ON GROWTH

LNCaP cells were cultured for 6 days with various concentrations of suramin, either in the absence or presence of 0.1 nM R1881 or 1 ng EGF/ml (figure 1). R1881 and EGF stimulated LNCaP cell proliferation 3- and 2-fold respectively (growth stimulation expressed as increase of DNA content per culture versus control cultures). Suramin inhibited the growth stimulatory effects of EGF and R1881 in a dose dependent manner (figure 1). Growth of control cultures was also inhibited by suramin. Already at a concentration of 0.01 mM, suramin partially inhibited the growth stimulatory effect of R1881 while the growth stimulatory effect of EGF was only slightly affected. Suramin completely blocked LNCaP cell proliferation at a concentration of 1 mM even in the presence of EGF or R1881.

8.4.2. LONG TERM AND REVERSIBLE EFFECT OF SURAMIN ON GROWTH

The effect of prolonged exposure (over 6 days) of LNCaP cells to 0.1 mM suramin was studied. Table I shows that DNA content per culture decreased, when suramin was added. Interestingly, after 17 days of culture, this decrease in DNA content was more pronounced in cell cultures treated with suramin in the presence of growth stimulatory factors (EGF or R1881) when compared to cell cultures treated with suramin alone. Table I also shows that withdrawal of suramin from the cell cultures after 6 days allows recovery of growth stimulation by EGF or R1881. Furthermore,

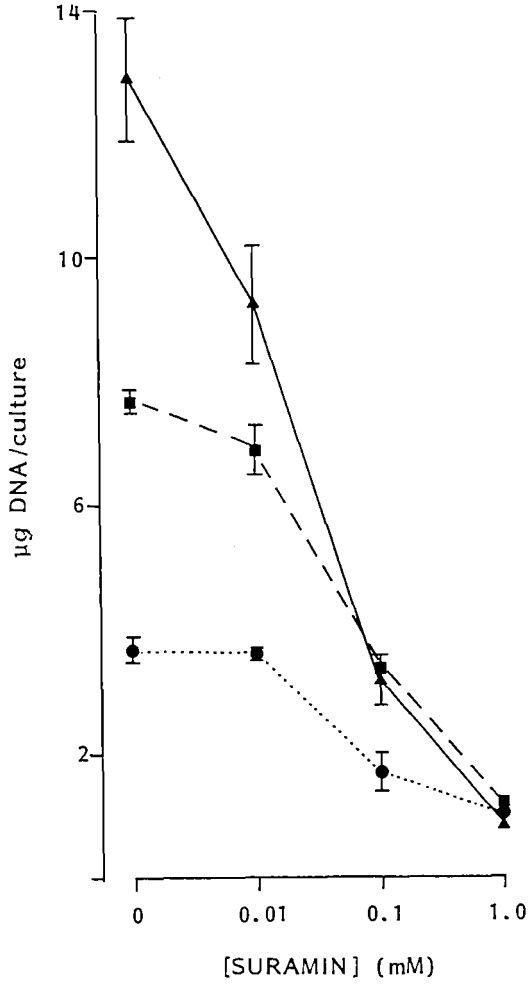

Fig.1. Effects of suramin and growth factors on growth of LNCaP cells. LNCaP cells were cultured for 6 days in the presence of various concentrations of suramin (0 to 1 mM) in RPMI-1640 supplemented with 5% (v/v) charcoal treated fetal calf serum only (●·····●) or with added 1 ng EGF/ml (■- - -■) or 0.1 nM R1881 (▲——▲). Data represent mean DNA content of triplicate cultures with standard deviation. At the start of the culture periods, all cultures contained 1.0 ± 0.1 µg DNA.

after pretreatment with suramin for 6 days, the increase in DNA in the second culture period upon stimulation with 1 ng EGF was approximately equal to stimulation by 0.1 nM R1881. This is in contrast with the observations that androgens are more potent mitogens for LNCaP cells when compared to EGF if cells are cultured for 6 days in the absence of suramin (figure 1).

TABLE I: Long term and reversible effects of suramin on growth of LNCaP cells.

Culture conditions	µg DNA/culture		
	day 6	day 17	
	(a)	(b)	(c)
suramin	+	+	-
control	37.4	32.2	47.2
1 ng EGF/ml	36.6	14.1	82.4
0.1 nM R1881	41.6	6.6	74.2

Cells were cultured for 6 days in the presence of 0.1 mM suramin (control) or with 1 ng EGF/ml or 0.1 nM R1881 (column a). Thereafter growth was continued as indicated in the presence (column b) or absence (column c) of suramin. Data represent the mean of duplicate experiments.

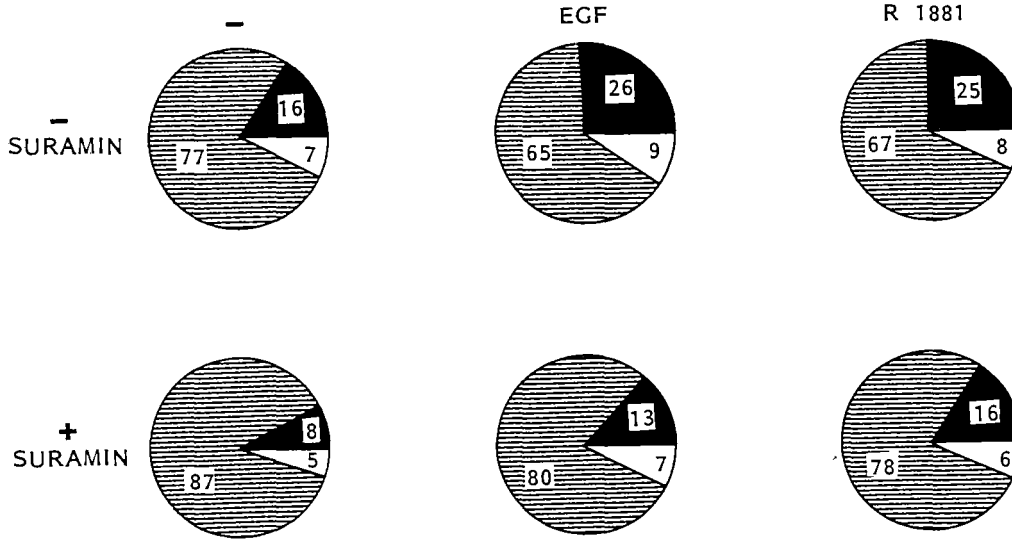

Fig.2. Dual parameter flow cell cytometric analysis of LNCaP cells cultured for 24 hours in the absence (control) or presence of 0.1 nM R1881 or 1 ng EGF / ml with and without 1 mM suramin. Percentages of cells in S phase (black), G1/0 phase (hatched) and G2/M phase (open) are shown (mean of 3 experiments) in a pie-shaped diagram. Standard deviations (not shown) were less than 8% of the mean.

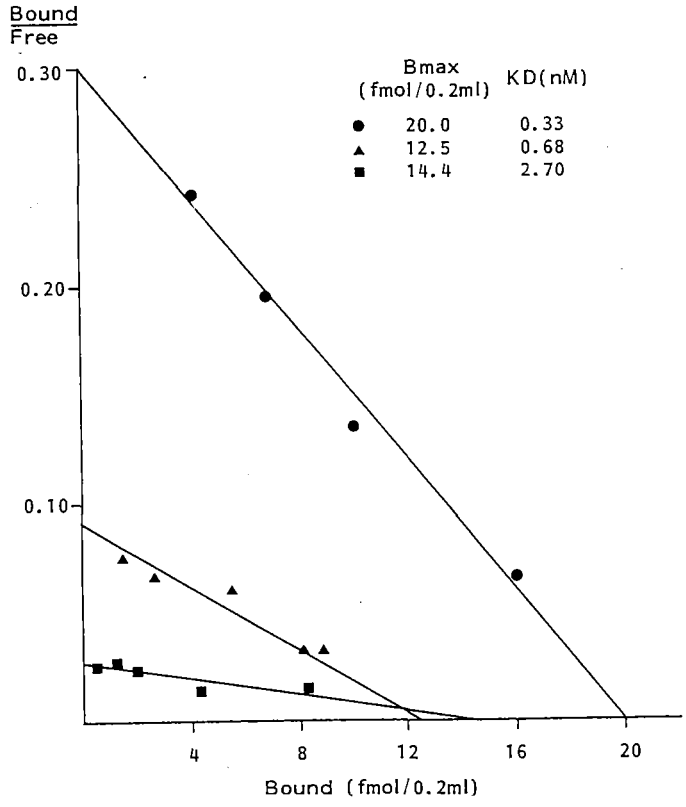

Fig.3. Scatchard plot analysis of EGF binding to LNCaP cells in the presence or absence of suramin. Binding data were obtained after incubation with ^{125}I-EGF in the absence (●) or presence of 0.1 mM suramin (▲) or 1.0 mM suramin (■) at 0°C for 4 hours. The concentration of bound ligand is expressed as fmol per well with 0.2 ml medium. Each well contained 3x10^5 LNCaP cells.

8.4.3. EFFECTS OF SURAMIN ON CELL CYCLE KINETICS OF LNCaP CELLS

The effects on cell cycle kinetics of suramin in the presence and absence of 1 ng EGF/ml or 0.1 nM R1881 were studied. Cells were allowed to attach and initiate growth for 3 days. The percentage of cells in G0/G1 phase and S phase after these 3 days was 74 ± 1 and 17 ± 1 respectively (mean and standard deviation, n=3). Cells were grown for 24 hours in the presence or absence of EGF or R1881 with or without 1.0 mM suramin. After treatment of LNCaP cells with EGF or R1881 the percentage of cells in the S phase increased about two fold whereas the percentage of cells in the G0/G1 phase decreased compared to control cells (figure 2). A

decrease in the percentage of cells in the S phase was observed after treatment of the cells with 1.0 mM suramin either in the presence or absence of EGF or R1881. Upon treatment with suramin the percentage of cells in the G0/G1 phase increased while the percentage of cells in G2/M was only slightly or not affected by suramin.

8.4.4. EFFECTS OF SURAMIN ON EGF BINDING TO LNCaP CELLS

The mechanism by which suramin inhibits the growth response of LNCaP cells to EGF was studied in more detail. EGF binding data were obtained by incubation of LNCaP cells with increasing concentrations of labeled and nonlabeled EGF in the absence or presence of suramin. The binding data were well fitted by a one-site binding model resulting in linear Scatchard plots (figure 3). Incubation of LNCaP cell cultures with EGF in the absence of suramin revealed a high affinity EGF receptor with a Kd of 0.33 nM. When the EGF binding assays were performed in the presence of 0.1 mM or 1 mM suramin, the Kd of the EGF receptor increased two- and eightfold, respectively (figure 3). In addition, the number of measurable EGF binding sites (B max) was decreased.

8.5. DISCUSSION

The results presented in this study demonstrate that in short term cultures (6 days), suramin is a reversible inhibitor of growth of LNCaP cells. Suramin counteracts the stimulatory effect of the growth factor (EGF) and the steroid hormone (androgen). Upon treatment with suramin for 24 hours, cells are predominantly arrested in G0/G1 phase of the cell cycle. Long term (17 days) exposure of LNCaP cells to suramin caused a decrease in DNA content per culture, indicating cell death.
The antiproliferative effect of suramin on growth of other cell types has been demonstrated (Coffey et al., 1987; Betsholtz et al., 1986; Spigelman et al., 1987). The exact mechanism by which suramin acts on growth factor related events is uncertain. Our EGF binding data indicate a decrease in binding affinity of EGF for the EGF receptor and a decrease of measurable EGF binding sites. This indicates that suramin either competes with EGF for

binding to EGF receptors or binds EGF and so prevents interaction of EGF with its receptor. It has been demonstrated that suramin also interfers with binding of other growth factors (FGF, TGFα-PDGF and TGFβ) to their receptors (Coffey et al., 1987; Betsholtz et al., 1986; Coughlin et al., 1988). Particular attention has been paid to the interference of suramin with PDGF. It has been shown that suramin acts by inhibiting binding of PDGF to its receptor and by dissociation of bound PDGF from its cells surface receptor (Sjölund and Thyberg, 1989).

The present results demonstrate that suramin also inhibits steroid (androgen) responsive growth. In chapter 5 we studied whether androgens exert their growth stimulatory effect on LNCaP cells indirectly by inducing LNCaP cells to secrete growth factor like activity in the culture medium. We could not demonstrate the presence of autocrine growth factor activity in medium conditioned by LNCaP cells. However, TGFα and EGF receptor are present on LNCaP cells (chapter 5; Schuurmans et al., 1988a). If suramin acts through prevention of the interaction of growth factors and their receptors, the inhibitory effect of suramin on androgen responsive growth supports the idea that androgens indeed act indirectly by affecting growth factors and their receptors. However, more information on the mechanism of action of suramin is needed to draw such conclusions.

Suramin has already been used in a clinical trial to test its activity as an anti-cancer drug (Stein et al., 1989). From that trial it was concluded that suramin can be an active agent in treatment of metastatic cancer. Studies using in vivo models, like the Dunning or the PC82 prostate tumor model, are necessary to give a better insight on the impact of suramin in treatment of prostate cancer.

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CHAPTER 9

GENERAL DISCUSSION

One of the major problems in hormone dependent tumors concerns the changes in cancer cells that result in alterations of their responsiveness to hormones. While many prostate and mammary tumors are initially dependent on androgens and estrogens respectively, following endocrine therapy they frequently develop into a hormone independent and metastatic phenotype. Hormone dependent tumors appear to become hormone independent and unresponsive after acquisition of an intermediate hormone independent but responsive phenotype (Matsuzawa et al., 1983; Humphries and Isaacs, 1982; Foulds, 1968).

Patients with advanced prostate cancer initially respond to androgen ablation therapy (orchiectomy or estrogen administration). However, the degree and duration of response are variable because of development of androgen independent tumor cells. Endocrine therapy can only increase the time to progression and at present it is impossible to predict on an individual basis which patient will respond to hormonal therapy and for how long. Based on the androgen dependent nature of prostate cancer and the fact that androgens act via androgen receptors, it was proposed (Walsh, 1975) that the androgen receptor content might reflect the degree of responsiveness of the tumor to androgen withdrawal. It was suggested that androgen independent cells might have few or no androgen receptors, while cells that contain androgen receptors were expected to be androgen sensitive. In patients with breast cancer the probability of response to endocrine therapy is related to the presence and concentration of estrogen and progestin receptors (DeSombre et al., 1979; Byar et al. 1979; Alanko et al., 1985). However, the relationship between androgen receptor levels and response to endocrine therapy in prostatic carcinoma has not been demonstrated unequivocally (Trachtenberg and Walsh, 1982; Aubel et al., 1988; Martin et al., 1987). It is not that surprising if one considers that insensitivity to steroids has been shown to occur irrespective of the presence of functional steroid receptors (Dabre and King, 1987). Hence, the presence of steroid

hormone receptors does not necessarily indicate a response to relevant steroid hormones. Furthermore, binding of androgen to its receptor is only the initial step in a complex pathway leading to the physiological effect of the hormone. The effect of androgen on proliferation of cells may not be a direct effect but rather one mediated by other regulators. These latter factors could be growth factors and their receptors.

Human prostate cancer is a complex disease and the aspects of this type of cancer can be investigated only with appropriate animal and in vitro systems. This thesis deals with the LNCaP cell line. Among the permanent human prostatic cell lines described up to now, this cell line is of particular interest because LNCaP cells show androgen responsive growth (Horoszewicz et al., 1980,1983; Berns et al., 1986). In addition the cell line maintains markers which are characteristic for prostate cancer, e.g.: cells express organ specific prostate antigen and secrete prostate specific acid phosphatase (Horoszewicz et al., 1983; Schulz et al., 1985).
This in vitro system may serve as a good tool to study the direct effects of compounds that are of interest in human prostate cancer. Table I summarizes the effects of various compounds on growth of LNCaP cells, as described in the previous chapters.

One of the aims of the experiments described in this thesis was to explore the involvement of growth factors and their receptors in the control of prostate tumor cell growth. LNCaP cells respond to exogenously added growth factors (table I). In the absence of steroid hormones the growth of these cells can be stimulated by EGF or FGF. This may indicate the limitations of growth inhibition by androgen deprivation alone. Cells can escape the inhibitory effect on growth caused by androgen ablation by responding to growth factors.
We have found that LNCaP cells contain EGF receptors (chapter 3). Altough we could not detect autocrine growth factor activity in medium conditioned by LNCaP cells, one of the ligands for the EGF receptor, TGFα, was also detectable in LNCaP cells (chapter 5). The fact that TGFα and EGF receptor are both present in LNCaP cells opens the possibility that cells can be stimulated to grow

Table I: Effect of various compounds on proliferation of LNCaP cells

compound	effect on growth 1)	chapter
steroid hormones:		6
androgen (DHT, T, R1881)	+	
progestagen (Pg, R5020)	+	
estrogen (17b-E2)	+	
glucocorticoid (TA)	0	
antiandrogens:		7
steroidal (CA)	+	
nonsteroidal (R23908)	+	
growth factors:		3-5
EGF/TGFα	+	
aFGF/bFGF	+	
insulin/IGF1	0	
PDGF	0	
TGFβ 2)	0/-	
suramin 3)	-	8

1) += stimulation, -= inhibition, 0= no effect
2) TGFβ has no effect on basal growth (0) but inhibits the growth stimulation by EGF
3) suramin inhibits growth in the absence and presence of androgen or EGF

in the absence of androgen stimuli via this TGFα/EGF receptor system. At high cell density cells show androgen independent growth (chapter 5) and it would be of interest to study whether TGFα and EGF receptor are involved in this androgen independent growth. Figure 1 illustrates the possible routes which could be involved in the androgen independent but TGFα/EGF receptor dependent growth. These autocrine, paracrine, juxtacrine and intracellular mechanisms of TGFα action could be modulated by androgens in androgen responsive tumor cells. Indeed, androgens increase the number of EGF receptors expressed at the cell surface (chapter 3) and thereby increase the sensitivity of LNCaP cells to EGF (chapter 4). Development from androgen responsive to androgen unresponsive growth may be due to loss of androgen control on this growth factor-receptor system. Loss of control might occur at the growth factor or response level; androgen independent cells can obtain the ability to produce growth factors or their receptors which are produced by dependent cells only in the presence of androgen. In vitro studies with Shionogi carcinoma cells suggest that such mechanisms can play a role in

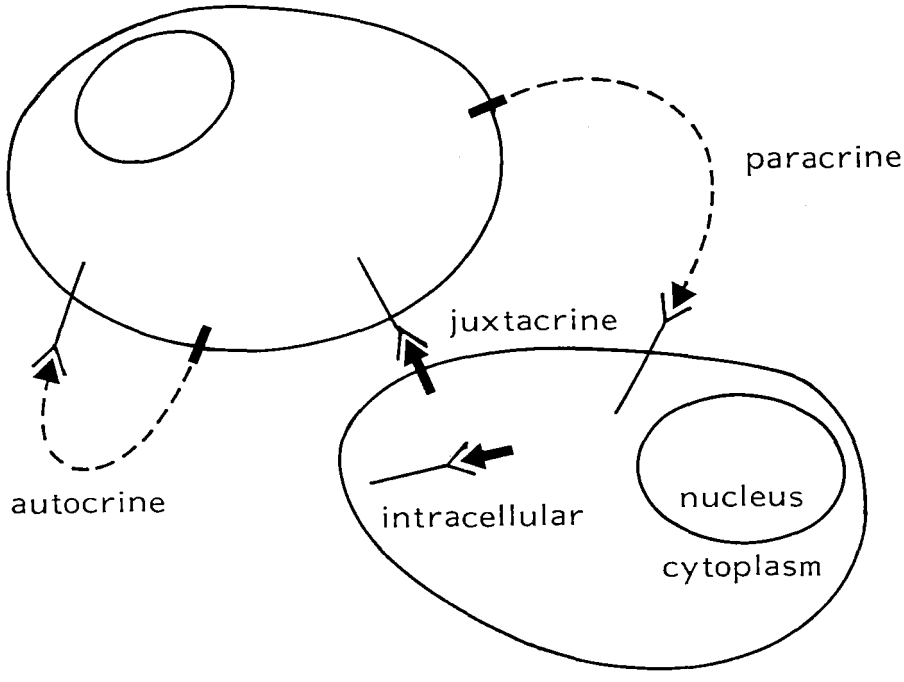

Fig.1: Possible mechanisms of EGF receptor activation by TGFα. The precursor of TGFα (proTGFα = ⟶) occurs as a transmembrane protein. The dotted lines indicate the release of mature TGFα (►) from the outer domain of proTGFα. —≺ = EGF receptor.

progression from androgen dependent cells to androgen independent cancer cells (Noguchi et al., 1989). Androgen independent Shionogi tumor cells derived from androgen responsive cells produce growth stimulatory factors which act in an autocrine and paracrine fashion. Another study with these Shionogi carcinoma cells suggests that progression to androgen independence may also occur by a mechanism by which cells obtain the ability to respond to growth factors (Nonomura et al., 1988) and autonomously produce growth factor receptors.

TGFβ inhibits the stimulatory effect of EGF or TGFα on growth of LNCaP cells (chapter 4 and 5). TGFβ did not affect androgen responsive growth, which makes it unlikely that androgen responsive growth is mediated by TGFα in an autocrine or paracrine way. However, other interactions (juxtacrine or intracellular) between TGFα and the EGF receptor are possible (figure 1) and these interactions may be affected by androgens but not by TGFβ.

If growth factors and their receptors are involved in the process

of tumor progression, interference with growth factor action should be considered also as a possible therapy for prostate cancer. Especially if one considers the observations that other hormones than androgens and even antiandrogens (chapter 7; Wilding et al., 1989) can stimulate proliferation of prostate tumor cells. Suramin, a polyanionic compound, has been demostrated to counteract the effects of growth factors (Coffey et al., 1987). In chapter 8 we demonstrate the inhibitory effect of suramin on growth of LNCaP cells both in the presence of growth factors and androgen.

Several aspects of the study described in this thesis need further investigation:

Although we demonstrated the presence of TGFα and its receptor in LNCaP cells, it remains to be established whether growth can be initiated via this pathway. Growth studies in which the interaction between TGFα and the EGF receptor is blocked by antibodies, directed either against TGFα or the EGF receptor should give more insight in the importance of this growth factor-receptor system in the control of proliferation. A recent study on human breast cancer cells revealed that constitutive expression of TGFα may be essential but as such not sufficient to induce the fully estrogen independent phenotype (Clarke et al.,1989). This may be not that surprising if one considers that estrogens affect not only the expression of TGFα but of a collection of growth factors in hormone dependent tumor cells.

The growth response of LNCaP cells to basic and acid FGF needs further study. Addition of combinations of different growth factors to LNCaP cells may reveal important additive or synergistic effects on growth of the cells. For these studies it is of importance to develop a serum free culture of LNCaP cells. Unknown serum factors may mask the response to factors under study.

The multi-hormonal responsiveness of LNCaP cells and the stimulatory effects on growth of antiandrogens may indicate a deviant androgen receptor system in LNCaP cells. Studies at the molecular level of the androgen receptor are in progress and should give conclusive evidence whether or not the androgen

receptor is mutated. Interestingly, a single point mutation in the steroid binding domain of the androgen receptor of labial skin fibroblasts derived from complete androgen insensitivity syndrome (AIS) males has recently been reported (Lubahn et al., 1989). Cells isolated from these complete AIS males were shown previously to contain a functionally altered androgen receptor protein with reduced binding affinity for dihydrotestosterone and enhanced binding of progesterone (Brown et al., 1982).

Several sublines of LNCaP are available which differ in their response to androgens. The cell line studied in this thesis shows androgen responsive growth and is most closely related to the original LNCaP-FGC line developed by Horoszewicz et al. (see: Steenbrugge et al., 1989). Hasenson et al. (1985) have described an androgen unresponsive subline. Comparison of hormone responsive and unresponsive sublines concerning their response to growth factors may give more insights in the process of tumor progression.

The LNCaP cell line is the only prostate tumor cell line of human origin that shows androgen responsive growth. Because there are no other androgen responsive cell lines of prostate cancer patients, it is impossible to compare the characteristics of the LNCaP cell line with similar cell lines. It appears to be important in this regard to develop additional hormone sensitive human prostate cancer models.

Finally, the effects of compounds on growth have been studied in vitro. One of the advantages of cell cultures above the study of tumors in vivo is the more precise control of factors affecting the cells. A disadvantage of in vitro studies is that the culture conditions may differ from the situation in vivo where the tumor cells are in contact with other cell systems. Therefore the studies using in vivo models (like PC-82 or Dunning tumor model) are necessary in addition to in vitro studies.

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SUMMARY

Carcinoma of the prostate is the second most common cancer among men. A relatively high portion of prostate cancers is androgen dependent.

Prostate cancer can be studied with appropriate animal and in vitro systems. This thesis deals with the LNCaP cell line, isolated from human prostate tumor cells. These LNCaP cells contain androgen receptors and respond to androgens with an increase in cell proliferation. The aims of the present study as described in chapter 1, were:

a. to investigate whether growth factors and their receptors play a role in the proliferation of androgen responsive LNCaP cells
b. to compare the effects of androgens with the effects of other steroid hormones and antiandrogens on growth and other metabolic processes of LNCaP cells
c. to study the effect of the polyanionic compound suramin- which interferes with growth factor action - on the growth of LNCaP cells.

Chapter 2 describes background information on mechanisms of action of steroid hormones, antihormones and growth factors. Some models are presented that describe the possible relationship between steroid hormones and growth factors in the control of cell proliferation.

Chapter 3, 4 and 5 contain the results which were obtained in the study of effects of growth factors on proliferation of LNCaP cells. The proliferation of the cells can be stimulated by EGF, TGFα, aFGF or bFGF. TGFβ can counteract the stimulatory effects of EGF and TGFα. Cell proliferation is not affected by insulin, IGF1 or PDGF.

EGF acts synergistically with androgen on LNCaP cells to induce cell proliferation. This synergistic effect of androgen and EGF is inhibited by TGFβ. Furthermore, treatment of LNCaP cells with androgen increases the number of EGF receptors expressed at the cell surface. These observations have led to the conclusion that LNCaP cells are sensitive to EGF/TGFα and that androgens increase the growth response to EGF which can be explained by upregulation of EGF receptor levels by androgen (chapter 3 and 4).

The main purpose of the experiments described in chapter 5 was to

resolve the question whether androgens affect the cell proliferation of LNCaP cells indirectly by inducing the cells to release growth factors that act in an autocrine manner. The results that were obtained did not support the presence of autocrine growth factor activity in medium conditioned by LNCaP cells, neither in the presence nor in the absence of androgens. However, with a monoclonal antibody directed against human TGFα, the presence of TGFα on and/or in LNCaP cells was demonstrated. Thus TGFα and its receptor appear to be present in LNCaP cells and their possible involvement in LNCaP cell proliferation is discussed.

Chapter 6 describes that progestagen and estrogen receptors are not detectable in LNCaP cells. Interestingly, progesterone as well as estradiol can stimulate the growth of LNCaP cells. These results indicate a sensitivity for non-androgenic steroids. Progesterone or estradiol also increase the number of EGF receptors expressed at the cell surface of LNCaP cells. The glucocorticoid triamcinolone acetonide does not affect growth or EGF receptor numbers. The relative binding affinity of the androgen receptor for the various steroids parallels the effects on cell growth; steroids with a high affinity for the androgen receptor stimulate cell growth at lower concentration than steroids with lower affinity for the androgen receptor. In contrast to expectation, it is shown in chapter 7 that antiandrogens (cyproterone acetate and anandron) do not antagonize the androgen responsive growth of LNCaP cells but have striking stimulatory effects on the growth of the cells. Cyproterone acetate as well as anandron also increase the number of EGF receptors expressed at the cell surface and increase the secretion of prostate specific acid phosphatase in the culture medium by LNCaP cells. It is concluded that LNCaP cells contain a modified androgen receptor system, both with respect to steroid binding specificity (chapter 6) and antiandrogen sensitivity (chapter 7).

Chapter 8 describes the results of studies on the effects of suramin on LNCaP cells. Suramin is a polyanionic compound that can interfere with growth factor action. Addition of suramin appeared to decrease the binding of EGF to LNCaP cells. Furthermore, it counteracts the growth stimulatory effects of EGF

and androgens on growth of LNCaP cells.
Finally in chapter 9 the results of the previous chapters are discussed, especially with respect the involvement of growth factors in the process of tumor progression. Future studies are proposed.

SAMENVATTING

De prostaat of voorstanderklier behoort tot de mannelijke geslachtsorganen en is gelegen rondom de urinebuis op de plaats waar deze uit de urineblaas komt. De klier scheidt een vloeistof uit die het hoofdbestanddeel vormt van de zaadvloeistof welke bij de zaadlozing aan de zaadcellen wordt toegevoegd.
De struktuur en funktie van de normale prostaat is afhankelijk van de aanwezigheid van mannelijke geslachtshormonen (androgenen). Het belangrijkste androgeen is testosteron. Dit hormoon wordt geproduceerd in de zaadballen (testikels). Testosteron bereikt via het bloed de zogenaamde doelwitcellen, zoals bijvoorbeeld prostaatcellen. In de prostaatcel wordt het testosteron grotendeels omgezet in een ander androgeen, dihydrotestosteron. Beide androgenen behoren evenals bijvoorbeeld progesteron en oestradiol (de vrouwelijke geslachtshormonen) tot de klasse van steroidhormonen. Steroidhormonen oefenen hun werking uit op een cel via binding aan een eiwit dat in de cel voorkomt, de steroidhormoonreceptor. Elke klasse van steroidhormonen werkt via een specifieke receptor.
Androgenen werken via de androgeenreceptor. Door binding van het androgeen (testosteron of dihydrotestosteron) aan zijn receptor, ontstaat een complex dat een interaktie aangaat met het erfelijke materiaal, DNA. Dit leidt tot de expressie van een gen, dat wil zeggen, informatie opgeslagen in het DNA wordt "afgelezen" (transcriptie) en dit wordt "vertaald" in een eiwit (translatie). Dit eiwit heeft een bepaalde funktie in of buiten de cel. In het geval van de prostaatcel, bijvoorbeeld, wordt de produktie van het enzym zure fosfatase op deze wijze door androgenen geregeld. Dit enzym is een bestanddeel van de zaadvloeistof.
Afwijkingen van de prostaat doen zich vooral voor op oudere leeftijd. Prostaathypertrofie is een goedaardige vergroting van de prostaat en wordt veroorzaakt door groei van weefsel langs de urinebuis wat leidt tot vernauwing van de urinebuis ("oude mannetjeskwaal"). Prostaatkanker komt minder vaak voor dan prostaathypertrofie, maar is in Nederland en in vele andere Westerse landen na longkanker de meest voorkomende doodsoorzaak door kanker bij mannen. De groei van prostaatkankercellen is in eerste instantie afhankelijk van androgenen. Patienten waarbij de

prostaatkankercellen niet meer beperkt zijn tot de prostaat, maar zijn uitgezaaid (metastase) naar andere plaatsen in het lichaam, krijgen vaak een hormonale behandeling. Deze behandeling is gericht op verminderde produktie van androgenen waardoor de groei van prostaatkankercellen meestal tijdelijk geremd wordt. Zo kan de produktie van testosteron worden beperkt door castratie waarbij de zaadballen worden verwijderd (orchidectomie). Er ontwikkelen zich echter in de loop der tijd ook prostaatkankercellen die in afwezigheid van androgenen kunnen groeien: androgeen-onafhankelijke cellen. Hoe het ontstaan en de groei van deze cellen voorkomen kan worden is nog onduidelijk.

Om prostaatkanker bij de mens te kunnen bestuderen zijn verschillende modelsystemen ontwikkeld. In dit proefschrift wordt een studie beschreven over prostaatkankercellen van menselijke oorsprong. In 1980 is van een patient met uitgezaaide prostaatkankercellen een aantal cellen geïsoleerd. Het is toen gelukt deze cellen te laten groeien buiten het lichaam in kweekflessen. De cellen zijn LNCaP genoemd, naar de Engelse afkorting van Lymph Node Carcinoma of the Prostate. Het voordeel van zo'n kweeksysteem is, dat de cellen onder goed te controleren omstandigheden gemanipuleerd kunnen worden. Er dient enige voorzichtigheid te worden betracht met de interpretatie van resultaten van dit soort in vitro ("in de buis") onderzoek en extrapolatie van de gegevens naar de situatie in het menselijk lichaam. Het kan echter belangrijke gegevens verschaffen over effekten van hormonen en over het werkingsmechanisme van hormonen in prostaatkankercellen.

LNCaP cellen bevatten androgeenreceptoren en androgenen stimuleren de groei van deze cellen. Zoals eerder vermeld binden androgenen aan de androgeen-receptor die zich in de cel bevindt. Voordat echter het uiteindelijke hormonale effekt zichtbaar is, zoals bijvoorbeeld celdeling, voltrekken zich een aantal gebeurte-nissen in de cel. Naar aanleiding van resultaten van experi-menten met borstkankercellen ontstond het idee dat androgenen de celdeling misschien indirekt stimuleren door middel van groeifaktoren. Groeifaktoren zijn hormoonachtige eiwitten die betrokken zijn bij groei, embryonale ontwikkeling, wondheling en bloedvatvorming. Het onderzoek was gebaseerd op de hypothese, dat de prostaatkankercel onder invloed van androgenen groei-

faktoren zouden kunnen produceren en uitscheiden. Deze groeifaktoren zouden na sekretie weer kunnen worden gebonden door receptoren van dezelfde cel welke de groeifaktoren produceert (autocrien mechanisme) of door een andere cel (paracrien mechanisme). De binding van groeifaktoren aan hun receptoren zou uiteindelijk kunnen leiden tot celdeling. Een andere mogelijkheid is, dat onder invloed van androgenen het aantal receptoren voor groeifaktoren stijgt waardoor een cel gevoeliger wordt voor een groeifaktor.

De doelstellingen van de in dit proefschrift beschreven experimenten waren gericht op beantwoording van de volgende vragen:

1. spelen groeifaktoren en hun receptoren een rol bij de groei van LNCaP cellen?
2. wat is het effekt van verschillende steroidhormonen en antihormonen op de groei van LNCaP cellen?
3. wat is het effekt van suramine op de groei van LNCaP cellen?

Allereerst is bestudeerd of toevoeging van verschillende groeifaktoren aan het kweekmedium een effekt heeft op de groei van LNCaP cellen. Uit de resultaten van de hoofdstukken 3 t/m 5 van dit proefschrift blijkt dat de volgende groeifaktoren een stimulerend effekt hebben op de groei van LNCaP cellen: epidermale groeifaktor (EGF), transformerende groeifaktor alpha (TGFα) en fibroblast groeifaktoren. Andere groeifaktoren zoals insuline, insuline-achtige groeifaktor 1 en groeifaktor uit bloedplaatjes, hebben geen effekt op de groei. Groeifaktoren kunnen ook een remmend effekt op de groei hebben. Een interessante waarneming is dat de transformerende groeifaktor beta (TGFβ) het groeistimulerend effekt van EGF en TGFα op LNCaP cellen remt.

EGF en androgenen versterken elkaar (werken synergistisch) met betrekking tot groeistimulatie van LNCaP cellen. Dat wil zeggen dat LNCaP cellen veel sneller groeien wanneer EGF en androgenen beide aanwezig zijn in het kweekmedium vergeleken met de situatie waarin EGF en androgenen afzonderlijk in het kweekmedium aanwezig zijn. Door toevoeging van androgenen aan het kweekmedium van LNCaP cellen neemt het aantal EGF receptoren per LNCaP cel toe. Mogelijk verklaart dit het synergistisch effekt van androgenen en EGF op de groei; androgenen maken de cellen gevoeliger voor EGF

door middel van toename van het aantal EGF receptoren (hoofdstukken 3 en 4).
Uit de resultaten van hoofdstuk 5 blijkt niet dat androgenen ook nog effekten hebben op de sekretie van groeistimulerende of groeiremmende faktoren door LNCaP cellen. Autocriene en paracriene mechanismen lijken geen rol te spelen bij de groeiregulatie van LNCaP cellen door androgenen. Wel is aangetoond dat LNCaP cellen TGFα bevatten. EGF en TGFα zijn twee verschillende groeifaktoren die echter beide werken via interaktie met dezelfde EGF receptor. LNCaP cellen bevatten dus de groeifaktor (TGFα) en de bijbehorende receptor (EGF receptor). Het aangrijpingspunt voor de receptor bevindt zich echter aan de buitenkant van de celmembraan. Wanneer cellen met elkaar in kontakt komen, is het dus mogelijk dat TGFα van de ene cel bindt aan de op de membraan van een andere cel aanwezige EGF receptor. Op die manier kan de groei van LNCaP cellen via een zogenaamd juxtacrien mechanisme worden gestimuleerd. Hierbij wordt het TGFα dus niet door de cel uitgescheiden, zoals bij autocriene en paracriene mechanismen het geval is. Verder onderzoek zal moeten uitmaken of dit juxtacrien mechanisme een rol speelt in de groeiregulatie van LNCaP cellen.
In hoofdstuk 6 worden experimenten beschreven die laten zien dat niet alleen androgenen maar ook andere steroiden een stimulerend effekt hebben op de groei van LNCaP cellen. Zowel progesteron als oestradiol kunnen de groei stimuleren ondanks het feit dat de specifieke receptoren voor deze beide steroidhormonen niet aantoonbaar zijn. Deze resultaten kunnen mogelijk verklaard worden door het feit dat de androgeenreceptoren in LNCaP cellen hun bindingsspecificiteit voor androgenen verloren zijn. Het blijkt dat hoe hoger de affiniteit van de receptor voor een steroid is, des te minder van het betreffende steroid nodig is om de groei van LNCaP cellen te stimuleren.
Antiandrogenen zijn stoffen die de werking van androgenen vaak tegenwerken. Deze stoffen worden soms gebruikt bij de behandeling van prostaatkanker. Twee bekende antiandrogenen zijn cyproteronacetaat en anandron. In hoofdstuk 7 wordt beschreven dat beide antiandrogenen het groeistimulerende effekt van androgenen op LNCaP cellen, niet remmen. In tegendeel, beide antiandrogenen hebben zelfs een stimulerend effekt op de groei

van LNCaP cellen. Tevens neemt het aantal EGF receptoren per LNCaP cel toe wanneer deze antiandrogenen aan het kweekmedium worden toegevoegd. Uit hoofdstuk 6 en 7 kan worden geconcludeerd dat LNCaP cellen mogelijk een afwijkend androgeenreceptor systeem bevatten.

Een alternatief om de groei van LNCaP cellen te remmen, wordt beschreven in hoofdstuk 8. In de literatuur is een stof beschreven, suramine, welke de binding van groeifaktoren aan hun receptoren verhindert. Dus groei welke gereguleerd wordt via groeifaktoren wordt door suramine geremd. Suramine blijkt de groei van LNCaP cellen te remmen, zowel in aanwezigheid van EGF als van androgenen. Suramine is in staat de binding van EGF aan LNCaP cellen te verminderen. Verdere studies zullen uitsluitsel moeten geven over de mogelijke toepasbaarheid van suramine in de behandeling van prostaatkanker.

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NAWOORD

Velen hebben op enigerlei wijze een bijdrage geleverd aan dit proefschrift. Enkelen wil ik hier met name bedanken.
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CURRICULUM VITAE

Alexander Lambertus Gerardus Schuurmans werd geboren op 26 maart 1959 te Gouda. In 1971 begon hij aan zijn middelbare schoolopleiding aan het St. Antoniuscollege te Gouda. In 1977 werd het VWO-B diploma behaald en werd gestart met de biologiestudie aan de Rijksuniversiteit Utrecht. Het doctoraalexamen werd afgelegd op 25 februari 1985 met als hoofdvakken Experimentele Embryologie en Scheikundige Dierfysiologie (Limnologisch Instituut te Nieuwersluis). Tevens werd de bevoegdheid tot het geven van onderwijs in de biologie verkregen.

Vanaf januari 1986 tot oktober 1989 was hij als wetenschappelijk assistent verbonden aan de afdeling Biochemie II van de Faculteit der Geneeskunde van de Erasmus Universiteit Rotterdam, alwaar het in dit proefschrift beschreven werk werd verricht. Het onderzoek dat in deze periode is uitgevoerd, werd ten dele financieel gesteund door de Nederlandse Organisatie voor de Kankerbestrijding, Stichting Koningin Wilhelmina Fonds. Sinds december 1989 is hij als toegevoegd onderzoeker verbonden aan de Vakgroep Kindergeneeskunde, Fakulteit der Geneeskunde, Rijksuniversiteit Utrecht.