

STIMULATORY EFFECTS OF ANTIANDROGENS ON LNCaP HUMAN PROSTATE TUMOR CELL GROWTH, EGF-RECEPTOR LEVEL AND ACID PHOSPHATASE SECRETION

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Summary—LNCaP cells (derived from a lymph node carcinoma of the human prostate) show androgen responsive growth. Progestagens, estradiol and antiandrogens competed with androgens for binding to the androgen receptor in the cells to a higher extent than in other androgen-sensitive systems. Optimal growth (3–4 fold increase in DNA content of 6 day cell cultures vs controls) was observed after addition of the synthetic androgen R1881 (0.1 nM). Both steroidal and non-steroidal antiandrogens did not suppress the androgen responsive growth. At a concentration of 10 nM cyproterone acetate or 100 nM RU 23908, growth was even stimulated 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 probably due to a defective androgen receptor system. It is discussed that comparable changes in the specificity of the androgen receptor in prostate cancer cells may give these cells an advantage in growth rate and may contribute to development of tumors characterized as hormone independent.

INTRODUCTION

Among the six permanent growing human prostate cell lines described up to now [1] only the LNCaP (lymph node carcinoma of the prostate) cell line developed by Horoszewicz *et al.* [2] shows androgen dependent growth [2–5]. This increase in growth rate is not due to neutralization of serum factors as has recently been suggested [6]; an increase in growth rate by androgens is also seen in growth factor inactivated or serum-free medium [7]. LNCaP cells contain considerable amounts of androgen receptors while estrogen and progestagen receptors are not detectable. However, estrogens and progestagens compete for binding to the androgen receptor in LNCaP cells and have stimulatory effects on growth of the cells [5].

In the present study the effects of a steroidal (cyproterone acetate) and non-steroidal (RU 23908) antiandrogen on LNCaP cell prolifer-

ation, secretion of PAP and expression of EGF receptors were investigated. We demonstrate that these androgen-dependent processes were stimulated by antiandrogens.

EXPERIMENTAL

Materials

[³H]R1881 (methyltrienolone, 87 Ci/mmol), radioinert R1881 and R5020 were purchased from New England Nuclear (Boston, MA). Cyproterone acetate was a gift from Schering (Berlin, F.R.G.). RU 23908 (anandron) was a gift from Roussel Uclaf (Paris, France). Triamcinolone acetonide was obtained from Sigma, U.S. Mouse ¹²⁵I labeled EGF (140 Ci/g, Amersham) was used following removal of free iodine by Sephadex G25 gel filtration.

Tumor cells

The LNCaP cell line was a gift of Dr Horoszewicz [2]. Cells were cultured and growth characteristics were analyzed as described previously [5]. The PC-EW human prostate tumor [8], grown in a nude mouse, was kindly provided by Dr van Steenbrugge.

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Steroid and EGF binding studies

Cytosol of LNCaP cells was prepared and competition studies with steroids were performed as described before [5, 9]. EGF binding to LNCaP cell monolayers was studied as reported before [5].

Assay of prostate specific acid phosphatase

The concentration of prostate specific acid phosphatase 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 μ g enzyme/l. The antibodies of this kit do not cross react with other acid phosphatase isoenzymes.

RESULTS

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 (Fig. 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 [5]. R1881 stimulated growth maximally at a concentration of 0.1 nM (3–4 fold increase in DNA content per culture vs control cultures). As observed before [5] 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 (Fig. 1). The synthetic glucocorticoid triamcinolone acetonide did not affect growth at concentrations up to 1 μ M.

In a separate series of experiments the effect of long-term cyproterone acetate treatment was

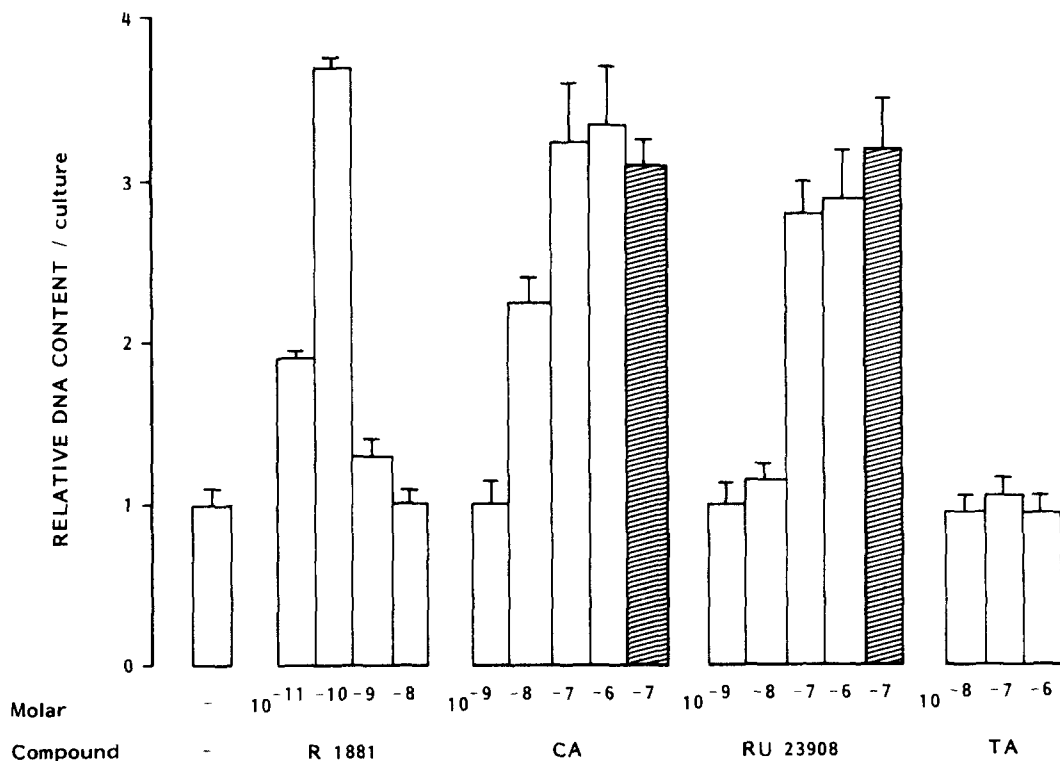


Fig. 1. Effects of various concentrations of R1881, cyproterone acetate (CA), anandron (RU 23908) and triamcinolone acetonide (TA) on growth of LNCaP cells after 6 days treatment with indicated compounds. Growth is expressed as mean DNA content per culture relative to control cultures (—). Shaded bars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

Table 1. Relative binding affinity (RBA) of different ligands for the androgen receptor in LNCaP cells and PC-EW tumor

Ligand	RBA value	
	LNCaP	PC-EW
R1881	100	100
Dihydrotestosterone	88	83
Progesterone	17	0.3
Cyproterone acetate	12.5	—
R5020	8.4	0.3
Estradiol	2.4	0.2
Ru 23908	1.0	—
Triamcinolone acetonide	<0.1	<0.1

—, Not determined.

studied. Continuous stimulation was observed for 42 days by both R1881 and cyproterone acetate compared to controls.

Androgen receptor binding

The binding specificity of the androgen receptor was measured by incubating cytosol of LNCaP cells or PC-EW tumors with 5 nM [³H]R1881 in the presence of various concentrations of steroids and antiandrogens. Relative binding affinity (RBA) values were calculated from competitive binding curves [9] as the ratio of concentration of unlabeled compound and concentration of R1881 required to inhibit [³H]R1881 binding by 50% (Table 1). The RBA value of R1881 was set at 100. In LNCaP cells the affinities of the receptor for both progesterone and R5020 (a synthetic, non-metabolizable

Table 2. Effect of different compounds on EGF binding to LNCaP cells

Condition	Bound (fmol EGF/mg DNA)
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

progesterone), were much higher than in the transplantable prostate tumor PC-EW. In addition, the receptor obtained from the LNCaP cells showed considerable affinity for estradiol, cyproterone acetate and RU 23908.

Induction of EGF receptors

Previous studies demonstrated that androgens increase both growth rate and EGF receptor activity in LNCaP cells [7]. EGF binding to LNCaP cell monolayers was measured after 24 h treatment with various compounds (Table 2). 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.

Secretion of prostate specific acid phosphatase

The time course of secretion of prostate acid phosphatase (PAP) in the culture fluid is shown in Fig. 2. PAP secretion in the culture fluid

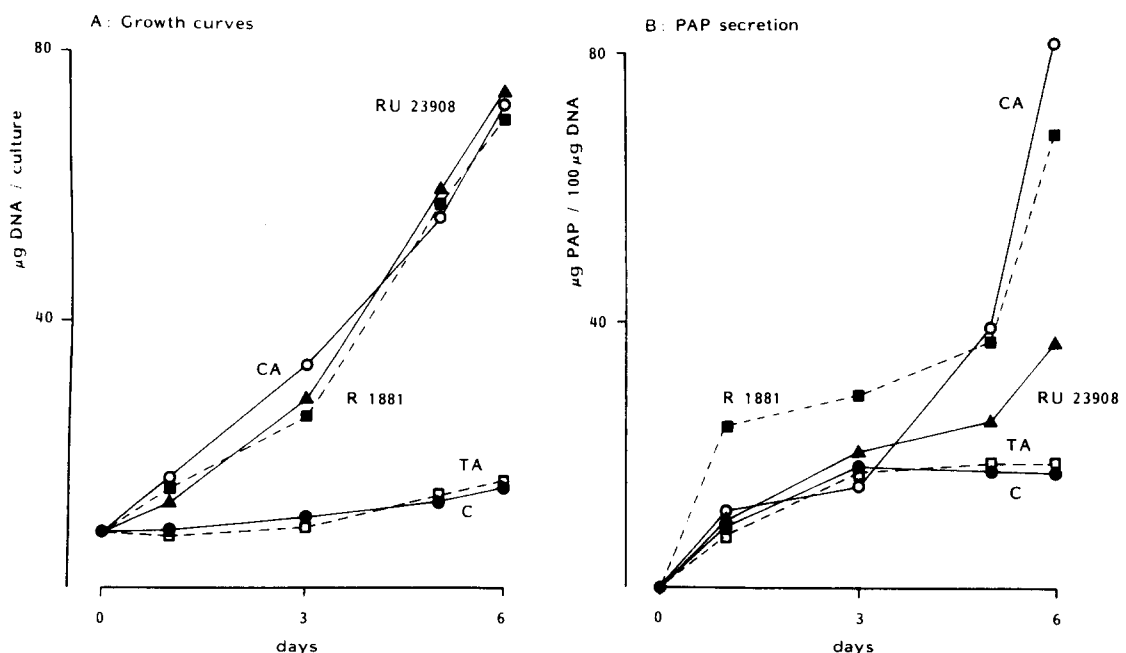


Fig. 2. Growth curves (panel A) and time course of secretion (panel B) of prostate specific acid phosphatase by LNCaP cells. Cells were cultured in the absence (●—●) or presence of following compounds: 0.1 nM R1881 (■—■), 100 nM cyproterone acetate (○—○), 100 nM RU 23908 (▲—▲) or 100 nM triamcinolone acetonide (□—□).

was calculated and expressed per 100 μg DNA in order to correct for differences between cell numbers in control and hormone treated cultures. R1881 and cyproterone acetate were the most potent stimulators of PAP secretion; a 3–4 fold increase after 6 days vs control cells was observed. RU 23908 doubled PAP secretion compared to controls. For control cells and cells treated with triamcinolone acetonide the amount of PAP secreted remained constant after 3 days. We previously reported that 10 nM estradiol also stimulated cell growth [5]. Treatment of LNCaP cells with 10 nM estradiol increased PAP secretion to the same extent as observed after addition of 0.1 nM R1881 (64 μg PAP/100 μg DNA at day 6).

DISCUSSION

The biological effects of a steroidal and a non-steroidal antiandrogen on LNCaP 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. Recently, similar results have been obtained by Wilding *et al.* [10], who showed agonistic effects of hydroxyflutamide, RU 23908 and cyproterone acetate with respect to growth rate and thymidine incorporation by LNCaP cells. In a preliminary study from our laboratory [4] stimulatory effects of cyproterone acetate were not seen when cells were cultured in 15% fetal calf serum. Removal of endogenous steroids from the culture medium (by charcoal stripping) is essential for study of the effect of added steroids on biological characteristics of the cells.

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 [11]. R1881 is the most effective stimulator of PAP expression in LNCaP cells [3]. This may be explained by the facts that R1881 firmly binds to androgen receptors and is only slowly, if at all, decomposed to inactive products in prostate tissue, in contrast to testosterone and dihydrotestosterone. Schulz *et al.* [3] showed that estrogens but not triamcinolone acetonide can increase PAP secretion in the culture fluid. We confirm these observations and showed that also antiandrogens stimulated PAP secretion by LNCaP cells. Estradiol and cyproterone acetate were more potent than RU 23908; the differ-

ences in stimulatory effect reflected the variation in relative binding affinity to the androgen receptor.

EGF can stimulate the growth of LNCaP cells and androgens increase the number of EGF receptors expressed at the cell surface [7]. EGF acts synergistically with androgen on LNCaP cells to induce cell proliferation, probably due to up-regulation of EGF receptors by androgen [12]. 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.

Cytosolic fractions of LNCaP cells and PC-EW transplantable prostate tumor tissue differ with respect to the binding specificity of the androgen receptor. The receptor in LNCaP cells has a broad specificity and binds in addition to androgens especially progestagens with high affinity. Cyproterone acetate and RU 23908 compete with androgens for androgen binding sites in LNCaP cells. RU 23908 binds only to androgen receptors while R1881 and cyproterone acetate show considerable affinity for progesterin receptors [13]. However, the effects of the two latter ligands are entirely mediated via the androgen receptor since progesterone receptors are absent in LNCaP cells [5]. Cyproterone acetate shows a higher affinity for androgen receptors than RU 23908 [13], in agreement with the observation that cyproterone acetate stimulates growth of LNCaP cells at a lower concentration than RU 23908.

Since the LNCaP cell line is the only *in vitro* growing 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. However, it was recently communicated (S. E. Harris *et al.*, J. Trapman *et al.*, unpublished studies) that the androgen receptor in LNCaP cells contains a mutation at aminoacid 877 (thr to ala) in the C-terminal part of the steroid binding domain. The mutation is in an essential conserved region of the steroid binding domain of the receptor. At the similar position in the glucocorticoid receptor a cysteine (cys 754) can be covalently linked to the glucocorticoid triamcinolone acetonide by photo-affinity labeling [14]. 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 [15, 16]. It is tempting to speculate that the mutation in the steroid binding domain of the androgen receptor of LNCaP cells makes it possible for antiandrogens and for different steroids (progestagens, estrogens) to activate this transcription activation function.

The results of the present study imply that further studies on tumor samples obtained from prostate carcinoma are needed to investigate the cause of an unfavorable response to hormonal therapy. *In vivo* in advanced metastatic prostate cancer the tumors may pass through a stage in which they are stimulated by antiandrogens or estrogens.

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