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Androgens Inhibit the Proliferation of a Variant of the Human Prostate Cancer Cell Line LNCaP

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The paradoxical androgen response of R2, a subline of the human prostate cancer cell line LNCaP, is described here. Two androgens (DHT and R1881) decreased, in a dose-dependent manner, R2 cell proliferation and [³H]thymidine incorporation. These ligand and cell specific effects were accompanied by an increase in the metabolism of the vital dye MTT and in cell protein content. Both androgens increased the doubling time and the percentage of G_0–G_1 cells. No evidence of androgen-induced apoptosis was found. Cloning allowed the selection of two cell populations on the basis of the response to 10 nM of R1881. Long term culture of uncloned R2 cells with R1881 modified reversibly the pattern of androgen response. R2 was compared to the androgen-stimulated LNCaP-FGC subline to investigate the causes of their different androgen responsiveness. The androgen receptor (number, affinity for hormones and antihormones, sedimentation constant and molecular weight) and androgen receptor genes (exon size and exon 8 sequence) were found to be identical in the two sublines. EGF stimulated LNCaP-FGC but not R2. Both cells were slightly stimulated by basic FGF but were insensitive to IGF-I and TGF β1. In conclusion: (1) androgens inhibit the proliferation of R2 cells possibly by introducing a G_0–G_1 block; (2) this inhibition is incomplete because, at least in part, the R2 cell population is heterogeneous; (3) chronic androgen treatment induces reversible cell adaptation; and (4) there is no evidence that the loss of the classical stimulatory effect of androgen on cell proliferation and the gain of inhibitory effect are due to androgen receptor alteration or to a specific action of one of the four growth factors tested.

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INTRODUCTION

Androgens stimulate the proliferation of prostate epithelial cells and maintain their differentiation. Therefore, the blockade of androgen production or action is used as adjuvant treatment of stage C and D prostate cancer [1]. Castration and administration of estrogens, LHRH analogs and antiandrogens may cause symptomatic and objective remissions in patients. However, the benefit of such treatments is transitory and generally tumors continue to grow. Since prostate cancer is becoming the most common carcinoma in males it is of major interest to identify the mechanisms responsible for such an evolution.

Androgen sensitive cell lines are potentially useful for investigating *in vitro* the molecular mechanisms responsible for the loss of androgen and antiandrogen sensitivity *in vivo*. LNCaP [2] is one of the rare cell lines whose proliferation is controlled by androgens and antiandrogens, thus numerous sublines and clones were selected [3] from it and characterized. Most works have been performed on the fast growing colony

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Abbreviations: AR, androgen receptor; BCA, bicinchoninic acid; CT-FCS, charcoal-treated fetal calf serum; DHT, 5α-dihydrotestosterone; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; FCS, fetal calf serum; FGF, fibroblast growth factor; IGF 1, insulin-like growth factor; MTT, 3-(4,5-dimethylthiazolyl-2yl)-2,5diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; R1881, 17β-OH-17α-methyl estra-4,9,11 triene-3-one; R5020, 17β-ethyl cetone-17α-methyl-estra-4,9 diene-3-one; TGF β1, tumor growth factor.

LNCaP-FGC [4–8] and few studies were done with the LNCaP-R, so designated because it was considered to be androgen resistant [9]. To simplify reading and to use a common designation LNCaP-FGC will be referred to as LNCaP and LNCaP-R as R2. The proliferation of LNCaP cultured in medium supplemented with charcoal-treated FCS is stimulated by androgens in a biphasic dose-dependent manner within a narrow concentration range below 1 nM [4–8]. There is no evidence for any stimulation at higher concentrations and the maximum effect was found with DHT or R1881 $\leq$0.1 nM. Antiandrogens and other compounds known to bind androgen receptors in this experimental model, such as progestins and estradiol [4–8], also stimulated cell proliferation in a dose-dependent manner. The androgen action on R2 proliferation was shown to be clearly different: DHT had no stimulatory effect up to 10 nM and is inhibitory at 100 nM [9]. However, these studies were performed in FCS supplemented with ovine prolactin and the difference between the androgen response patterns in the two types of cells might be due to a different cell environment. To our knowledge, effects of other androgen receptor ligands have not been tested on these cells.

In this work we compare the effects of androgens (and other androgen receptor ligands) on the proliferation of R2 and LNCaP cells cultured in the same conditions. We further characterize the inhibitory effect of androgens and analyse the heterogeneity of the R2 subline. In addition, we report comparative studies on androgen receptor characteristics and growth factor effects in the two cell populations with the aim to explore the mechanisms responsible for the change of androgen response patterns.

MATERIAL AND METHODS

Reagents

The sources of hormones and antihormones were as follows: DHT, 17β-estradiol and progesterone, Sigma (St Louis, MO); R5020 and R1881, New England Nuclear (Boston, MA); cyproterone acetate (6α-chloro-17α-acetoxy-1,2α-methylene-4,6-4-pregnandiene-3,2-dione), Schering A.G. (Berlin, Germany); 4-OH-flutamide (2-methyl-*N*-[4-OH-3-(trifluoromethyl)-phenyl] propanamide), Schering Corp. (Kenilworth, NJ); nilutamide (RU 23908; 5,5-dimethyl-3-[4-nitro-3(trifluoromethyl)-phenyl]2,4-imidazolidinedione), Roussel-UCLAF (Romainville, France); all-trans retinoic acid; Sigma (St Louis, MO); 5α-dihydro [1α,2α,(n)-^{3}H]testosterone, sp. act. 3.7 $\times$ 10^{12} TBq/mmol, Amersham (Buckinghamshire, England) and [17α-methyl-^{3}H]R1881, sp. act. 3.22 TBq/mmol, New England Nuclear (Boston, MA). These compounds were dissolved in ethanol. The sources of growth factors were as follows: human EGF, Genzyme (Boston, MA); porcine TGF β_1, R&D (Minneapolis, MN); recombinant human IGF-I, Kabivitrum-Pharmacia (Saint Quentin en Yvelines, France) was a gift of P. Chatelain and the mouse basic FGF, a gift of J. Saez, was purified as described [10]. These compounds were stored at -70°C, dissolved in acetic acid (0.1 M).

Cells

R2 cells [9] were kindly provided by G. J. van Steenbrugge. They were used between passages 20 and 101. LNCaP cells [2] were from the ATTC (Rockville, MD). They were used between passages 19 and 87. Both cells were propagated routinely in RPMI 1640 medium, TechGen International (Les Ullis, France) supplemented with 7.5% FCS, Gibco (Paisley, Scotland) without evidence of any change of androgen responsiveness.

Cell treatment with hormones, antihormones and growth factors

Cells were trypsinized and plated out in RPMI 1640 medium supplemented with 2.5% CT-FCS obtained after treatment with dextran-coated charcoal [11] because this condition allowed the best androgen stimulation of LNCaP. The serum substitute Ultroser SF, IBF (Villeneuve la Garenne, France) was used in one experiment. Plating was performed in 96-well dishes for proliferation studies, in a T flask (25 cm^2) for flow cytometry and in 24-well dishes coated with extracellular matrix produced by endothelial cells of bovine cornea [11] for measuring [^{3}H]thymidine incorporation. Extracellular matrix allowed strong attachment to support and thus avoided cell loss during the medium changes required for [^{3}H]thymidine incubation and cell wash as described below. For other analyses the medium was not changed to avoid cell loss. Hormones and antihormones were added once immediately after plating. Growth factors were added both immediately after plating and every 2 days.

Control cells were cultured with the same amount of vehicle (ethanol or acetic acid) as the cells treated with the highest concentration of hormones, antihormones or growth factors.

Analyses of hormone antihormone and growth factor effects

Cells were detached with trypsin for counting, viability assay, DNA assay and flow cytometry. Cells were counted with a Coulter counter ZM, Coulter Electronics (Luton, England). Cell viability was estimated with the trypan blue (0.2% v/v) exclusion test. For DNA assay, cells were sonicated in PBS, treated with RNAse (Sigma) for 30 min at 37°C and incubated with Hoechst 33 258 0.1 μg/ml (Polysciences, Warrington, PA). Fluorescence was measured with the DNA fluorimeter TK 0100 Hoefer (San Francisco, CA). Calf thymus DNA was used as a standard. For flow cytometry PBS-washed cells were fixed with ethanol (70%

v/v), stored at $-20^{\circ}C$, centrifuged and suspended in PBS. The analysis was performed on 5×10^4 cells stained with propidium iodide (Cycle test DNA kit, Becton Dickinson). The cytofluorograph FACScan (Becton Dickinson) was calibrated with chicken red blood cells. The percentages of cells in the cell cycle phases were calculated with the Cell Fit program based on the RFIT model.

The following analyses were performed without previous treatment with trypsin: protein assay, [^{3}H]thymidine incorporation and MTT assay. For protein assay PBS-washed cells were lysed in Tris 50 mM pH 7.4, EDTA 10 mM, SDS 0.5% and BCA reagent (Pierce, Rockford, IL) was used according to the manufacturer's instructions. For [^{3}H]thymidine incorporation PBS-washed cells were incubated for 2 h in serum-free medium containing [^{3}H]thymidine (2 μCi/ml, sp. act. 3.17 TBq/mmol) CEA (Saclay, France). Cells were washed and radioactivity counted as described in [12]. The MTT assay was performed as follows: MTT 0.5 mg/ml (Sigma) was added into each well. The incubation was carried out at 37°C for 5 h in 5% CO_2. The formazan crystals formed were dissolved with 100 μl SDS (10%) HCl (0.01 N) overnight at 37°C. The absorbance was measured with a Dynatech microplate reader MR 5000 at 550 nM and corrected for absorbance of medium without cells.

Hormone specific uptake and competition

Cells cultured for 2 days in RPMI 1640 medium supplemented with CT-FCS (2.5%) were trypsinized and washed with PBS. AR was assayed according to Eil *et al.* [13]. Briefly, cells (1×10^6 in 0.8 ml) were incubated for 1 h at 37°C with increasing concentrations of [^{3}H]DHT in the absence or in the presence of the non-radioactive DHT and they were washed twice before counting the radioactivity. The specific binding was taken as the difference between the radioactivity bound to cells in the absence of non-radioactive DHT and that bound in its presence. For competition assays, increasing concentrations of various compounds were added to the medium in the presence of [^{3}H]R1881 (1 nM) for 1 h at 37°C and cells were processed as above.

Western blots

Cells cultured in RPMI 1640 medium supplemented with FCS (7.5%) were harvested, washed and sonicated in Tris–HCl (50 mM, pH 7.4), Na_2MoO_4 (10 mM), KCl (0.4 M), EDTA (1 mM), PMSF (0.1 mM), R1881 (20 nM) and glycerol (20%). Cell extract was centrifuged for 45 min at 180,000 *g* at 2°C. Supernatants (80 and 100 μg proteins) were analysed by SDS–polyacrylamide electrophoresis (polyacrylamide 10% and bis-acrylamide 0.27%). Proteins were transferred by electrophoresis onto nitrocellulose and receptors were detected by a double antibody method. The first antibody, a monoclonal anti-human androgen receptor antibody (F39.4.1, Sanbio, Uden, Netherlands), was diluted at 1:50 in PBS containing Tween 20 (0.2%) and bovine serum albumin (2%) and incubated for 1 h at room temperature and overnight at 4°C. The second antibodies, polyclonal (goat) anti-mouse immunoglobulins labelled with alkaline phosphatase, Immunotech (Luminy, France), were diluted (1:500) in the same buffer and incubated for 1 h at room temperature. The enzyme activity was revealed with Nitro Blue Tetrazolium and 5-bromo 4-chloro 3-indoyl phosphate with gentle agitation for 10 min in the dark.

PCR amplification

Genomic DNA was prepared from R2 and LNCaP cells, genital skin fibroblasts and blood samples by standard methods. Twelve pairs of primers were used to amplify the coding regions of the AR gene. Their sequences and positions are those described by Lubahn *et al.* [14]. They were synthesized using a 391 DNA synthesizer (Applied Biosystem, Foster City, CA). Preparative PCR (100 μl) were carried out with a 480 DNA Thermo Cycler or a 9600 GenAmp PCR system (Perkin Elmer Cetus, Northwalk, CT). Genomic DNA (250 ng) was incubated with primers (25 pmol each), dNTPs (100 μM each), $MgCl_2$ (1.5 mM), Tris–HCl (67 mM, pH 8.8), $(NH_4)_2SO_4$ (16.6 mM), Tween 20 (0.1%). DMSO (10%) was also added to amplify the exon 1. The mixtures were heated (95°C × 10 min) before the addition of Taq Polymerase (2.5 U, Eurobio, Les Ullis, France) at 85°C. Thirty cycles of amplification (95°C × 1 min, 58–60°C × 1 min, 72°C × 1 min) were performed and mineral oil was removed with chloroform. PCR products were precipitated with ammonium acetate–ethanol, dissolved in H_2O and 10% were loaded on 1% agarose gel.

Sequencing

The sequencing of double strand PCR products was carried out with the T7 Sequenase kit (Pharmacia Biotech, Piscataway, NJ) and dATP^{35}S (>1000 Ci/mmol, NEN DuPont de Nemours, France) according to the manufacturer's indications and with purified PCR products (10 μl), primers (5 pmol) and DMSO (10%). Electrophoresis on a sequencing gel (6% acrylamide/bisacrylamide, 8 M urea, 1 × TBE) was run under 1300 V, 55 W, 40 mA. Gel was treated with ethanol and dried under vacuum. Autoradiography was performed for one night at room temperature with an X-OMAT AR film (Kodak, Rochester, NY).

Expression of the results

For each dose–response experiment each assay was performed in triplicate (CV: 7%). The results are shown as means ± SD except for those of Fig. 1. Groups were compared using paired Student's *t*-test. Differences between groups were considered

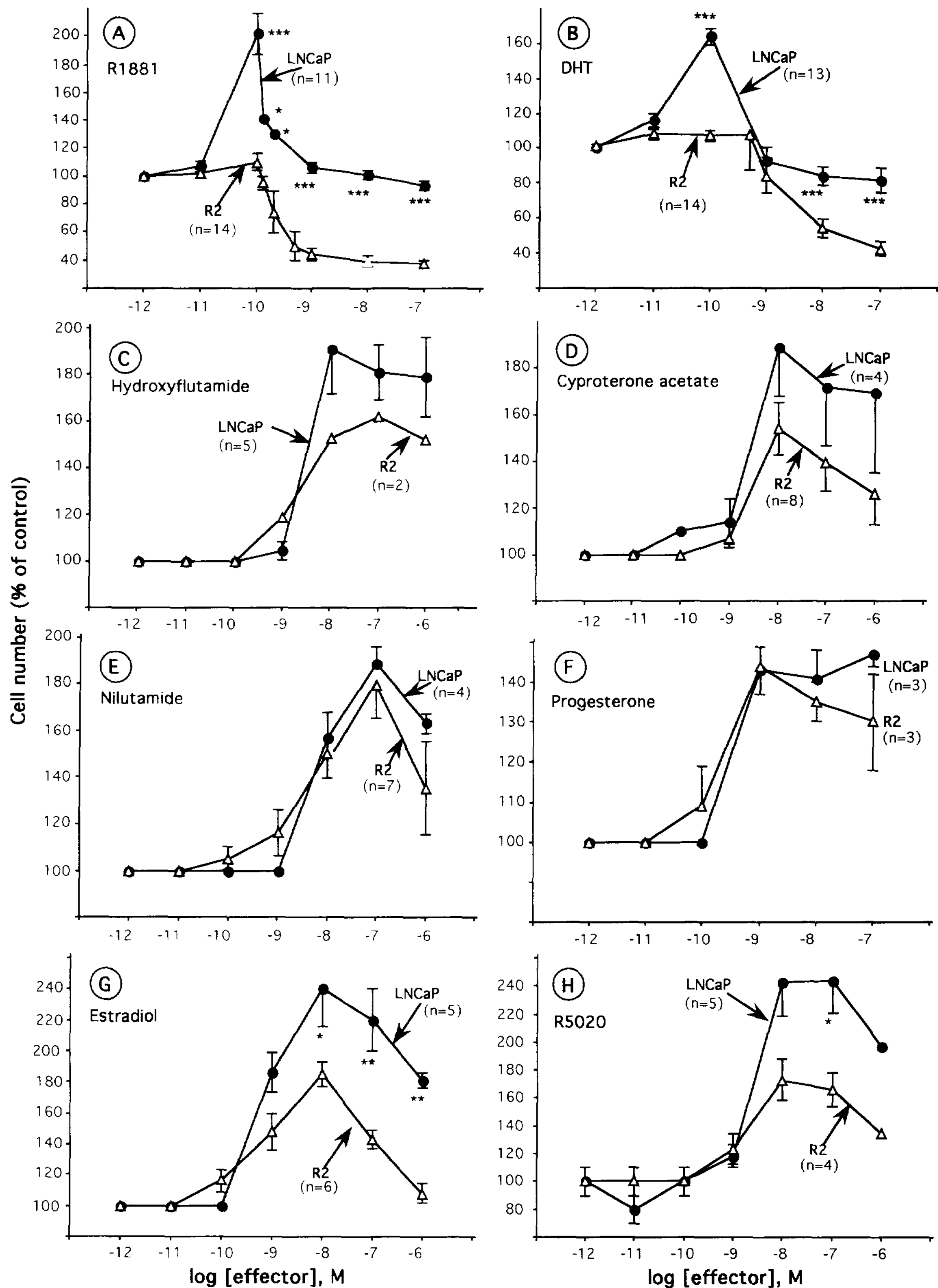

Fig. 1. Cell proliferation. LNCaP (●) and R2 cells (△) were seeded in 96-well plates and cultured in the presence of increasing concentrations of the test compound indicated in the left upper corner of each graph. Cells were counted 7 days later. Results are given in % of control (means ± SEM). *n*, number of experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

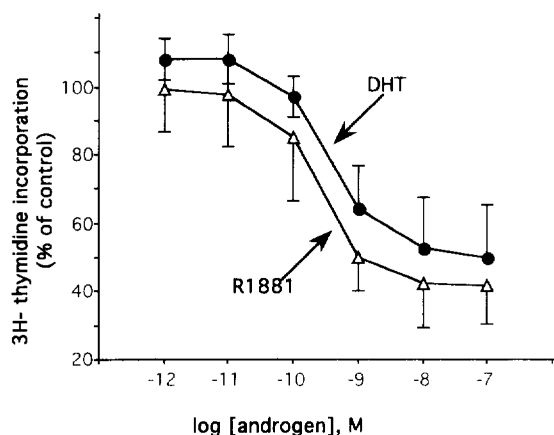

Fig. 2. [^{3}H]Thymidine incorporation. R2 cells in suspension in RPMI 1640 medium supplemented with CT-FCS (2.5%) were seeded in 24-well plates and cultured for 24 h in the presence of increasing concentrations of DHT (●) or R1881 (△). Labeling with [^{3}H]thymidine (2 μCi/ml) was performed for 2 h. Results are given in % of control (means ± SD of 3 experiments).

statistically significant at $P < 0.05$. To allow comparison between two groups of different size the first experiments in a chronological order were selected.

RESULTS

Androgen inhibition of R2 cell proliferation

R1881 and DHT decreased in a dose-dependent manner the cell yield after 7 days of culture in RPMI 1640 medium containing 2.5% CT-FCS [Fig. 1(A, B)]. The difference between the lowest inhibition observed after each androgen treatment was not statistically significant: 37 ± 14% of control (range 18–62%) vs 42 ± 15% (range 19–62%). The EC_{50} of R1881 (~0.5 nM) was lower than that of DHT (~5 nM). This phenotype was found between passage numbers 20 and 101. Sometimes a stimulation was observed at 0.01 or 0.1 nM of either androgen but it was not statistically significant.

The androgen dose–response of R2 is clearly different from that of LNCaP cultured in the same conditions [Fig. 1(A, B)]. Indeed, the most obvious androgen response of LNCaP was, as reported previously [4–8], a biphasic stimulation with a maximum at ≈0.1 nM R1881 (202 ± 47% of control, range 157–312%, $n = 11$) and DHT (164 ± 25% of control, range 146–202, $n = 13$). Sometimes an inhibition was observed at 100 nM of either androgen but it was not statistically significant: the cell yield was 93 ± 10 and 81 ± 18% after R1881 and DHT treatments. Three antiandrogens and other compounds which compete with androgens (see below) stimulated in a dose-dependent fashion both R2 and LNCaP proliferation [Fig. 1(C–H)]. Thus, the androgen inhibition of R2 cells cultured in 2.5% CT-FCS is cell and ligand specific. The same dose-dependent inhibition of R2 cells by androgens was observed when cells were cultured with FCS (7.5%) or serum substitute (2%).

In 2.5% CT-FCS, R1881 (100 nM) increased the doubling time from 42 h up to 70 h (similar results were obtained with DHT). A statistically significant inhibition was observed from 5 days. A 24 h treatment with each androgen decreased in a dose-dependent manner the [^{3}H]thymidine incorporation into DNA (Fig. 2). R1881 (100 nM for 7 days) increased the percentage of G_0–G_1 cells from 76.3 ± 4.5% to 89.8 ± 2.1% ($n = 3$). There was no evidence for a simultaneous increase of dead cells, i.e. cells stained by trypan blue (3–8% of spontaneously floating and trypsinized cells) or containing less DNA than G_0–G_1 cells as measured by flow cytometry. Neither was there found by electrophoresis of nuclear DNA any androgen-induced DNA

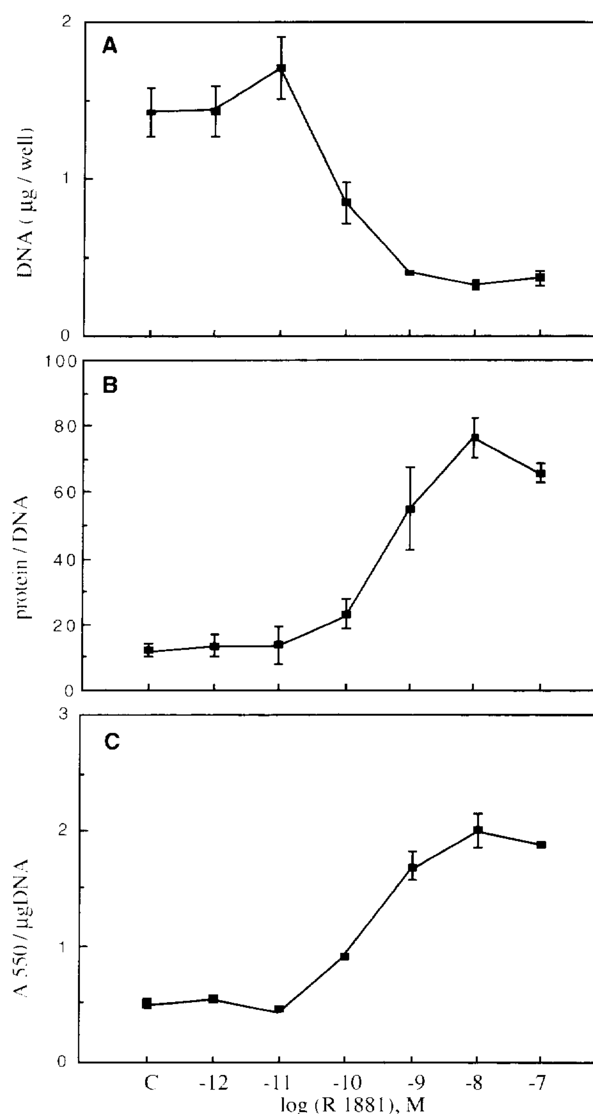

Fig. 3. Cell metabolism. R2 cells were seeded in 96-well plates and cultured in the presence of increasing R1881 concentrations. 7 days later DNA, proteins and the formazan production from MTT were measured in separate wells. Results are means ± SD of triplicates. (A) DNA yield, (B) protein/DNA ratio, (C) absorbance at 550 nm normalized per μg DNA.

Table 1. Evolution of the androgen responsiveness of R2 cells cultured in the presence of R1881 (100nM) and CT-FCS (2.5%) for long periods of time

Experiment number	Long term treatment with R1881 at 100 nM: Passages (start, end)	Time (days)	Responses to R1881 at the end of treatment (% of control): 10^{-12} M	10^{-10} M	10^{-8} M
1	52, 55	56	96	131	167
	52, 65	168	95	184	162
2	49, 60	180	93	147	131
	49, 68	235	122	168	224
	49, 81	349	86	171	200

R2 cells were cultured in medium containing R1881 (100 nM) and CT-FCS (2.5%) between the passage numbers and for the periods of time indicated. Seven days after R1881 withdrawal the responses to 7 days treatment with R1881 were measured.

degradation in either cells (not shown). In addition, R1881 dose-dependent increases of MTT reduction activity and cell protein content were associated with the dose-dependent decrease of DNA yield observed after 7 days treatment (Fig. 3). The increase of protein content was accompanied by cell hypertrophy.

R2 heterogeneity and consequences of chronic androgen treatment on further androgen responsiveness

Five clones were selected from R2 propagated in 7.5% FCS. Three clones exhibited a typical R2-like androgen inhibition while the others were marginally inhibited by or insensitive to 10 nM R1881.

The androgen responsiveness of the R2 subline was dramatically modified by chronic treatment with R1881 (100 nM): the proliferation of these cells was stimulated by androgens both at 0.1 and 10 nM R1881 (Table 1) instead of being inhibited. We looked for the stability of this phenotype by culturing pretreated cells for increasing number of passages in culture medium containing 7.5% FCS but no exogenous androgen. Cells pre-treated for 4 passages (52–55, experiment 1, Table 1) lost the feature to be stimulated at 0.1 and 10 nM R1881 (Fig. 4, experiment 1); after 10 passages cells recovered the typical inhibition at 10 nM. Cells pre-treated for 20 passages with R1881 (49–68, experiment 2, Table 1) displayed the LNCaP phenotype from the passage 4 after androgen withdrawal till passage 19 (Fig. 4, experiment 2). Thus, the stimulatory response both to low and high R1881 concentrations required the continuous presence of this compound and the 10 nM effect changed faster than the response to 0.1 nM.

Taken together these results suggest that R2 cells propagated in FCS 7.5% are heterogeneous and/or that the cell environment (androgen concentration and cloning conditions) might modify their androgen responsiveness.

Search for molecular mechanisms

The mechanisms of the dual effects of androgens were looked for at the level of the androgen receptor and growth factor responsiveness by comparing R2 to LNCaP cells.

No significant difference was found between the following parameters: (1) the ligand binding characteristics (Table 2) including the number of androgen binding sites, the affinity for [^{3}H]DHT and the relative binding affinities for compounds active on cell proliferation; (2) the shape and size of receptor i.e. the sedimentation constant (~8S) at low ionic strength (Fig. 5) and the molecular weight (~110,000) measured by SDS–polyacrylamide electrophoresis (Fig. 6); and (3) the size of exons estimated by 1% agarose electrophoresis (not shown) and the sequence of exon 8 (Fig. 7). Both cells contained the G to A mutation identified as T868A by Veldscholte *et al.* [15] and T876A by Lubahn *et al.* [14] in LNCaP cells. This mutation was not found in foreskin fibroblasts (Fig. 7).

We investigated the effects of EGF known to stimulate the proliferation of LNCaP [16] and growth factors known to stimulate (basic FGF and IGF-I) or inhibit (TGF-β1) the proliferation of some epithelial cells. EGF stimulated LNCaP as previously reported but not R2. Both cells were slightly stimulated by basic FGF (Table 3) and insensitive to IGF-I and TGF β1 (0.1–10 ng/ml).

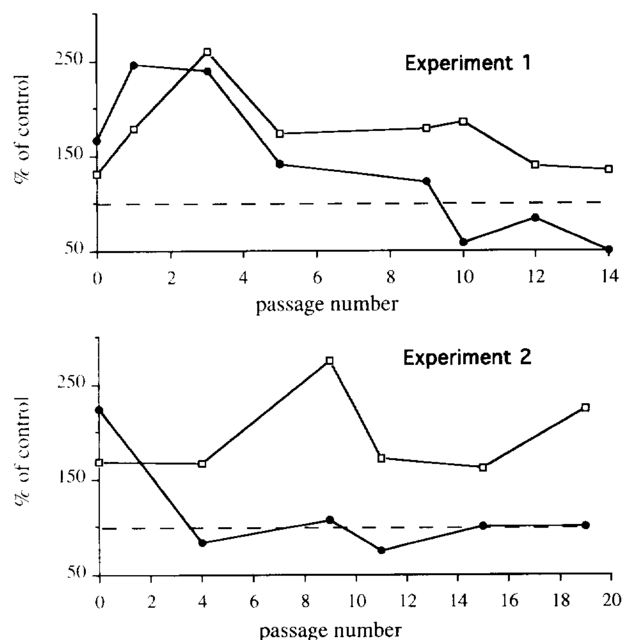

Fig. 4. Evolution of the androgen responsiveness of R2 cells cultured in the presence of 7.5% FCS for increasing number of passages after the arrest of chronic treatment with R1881 (100 nM). Cells were cultured in medium containing R1881 (100 nM) and CT-FCS (2.5%) either for 4 passages (52–55, experiment 1, Table 1) or for 20 passages (49–68, experiment 2, Table 1) before they were cultured in the presence of FCS (7.5%) without R1881. Following the number of passages indicated cells were seeded and cultured in medium containing CT-FCS (2.5%) and the responses to 7 days treatment with 0.1 nM (□) or 1 nM (●) R1881 were measured.

Table 2. Ligand binding characteristics

	R2		LNCaP	
	Whole cells	Cytosol	Whole cells	Cytosol
Binding sites (number per cell)	54,400 ± 13,600*	48,300 ± 7000†	58,800 ± 7900*	43,000 ± 5000†
K_D (nM DHT)	1.6 ± 0.6*	0.7 ± 0.1†	0.8 ± 0.3*	1.2 ± 0.5†
Relative binding affinity†				
R1881	100	nd	100	nd
DHT	55 ± 5	nd	45 ± 9	nd
Progesterone	37 ± 7	nd	22 ± 4	nd
Estradiol	24 ± 9	nd	13 ± 3	nd
Cyproterone acetate	5 ± 1	nd	2 ± 1	nd
R5020	3 ± 1	nd	2 ± 1	nd
Triamicinolone acetate	0	nd	0	nd

Binding sites and K_D were determined from seven-point Scatchard graphs. The relative binding affinities were calculated from competition experiments performed with [^{3}H]R1881 (1 nM) and increasing competitor concentrations. Results are the means ± SD of 5 experiments or 3 experiments.

Further comparison between androgen and LNCaP cells

The two cell lines may be identified by phase contrast microscopy: (i) R2 cells are more tightly attached to the plastic of flasks than LNCaP cells either in the presence of serum or in serum-free medium and they do not form clusters as LNCaP do; and (ii) although cells of similar shape and size are found in both cell lines, R2 contain in addition larger cells. R2 cells grow faster than LNCaP in medium containing CT-FCS (2.5%): the doubling times after seeding at 2×10^4 cells/cm^2 were ~42 h and ~144 h (not shown). R1881 (0.1 nM) shortened the doubling time of LNCaP down to ~84 h. Each cloning by dilution was successful with R2 cells while one clone only was obtained from LNCaP; it responded like the uncloned LNCaP. The difficulty of cloning by dilution is in agreement with the observation according to which the growth rate of LNCaP decreases when the cells are seeded at low density (results not shown and [17]).

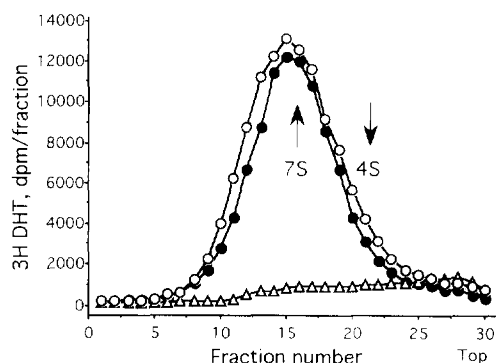

Fig. 5. Ultracentrifugation. Extracts of R2 (○, △) and LNCaP cells (●) were incubated with [^{3}H]DHT (1 nM) for 1 h either in the absence (●, ○) or in the presence (△) of 100-fold unlabeled DHT. Charcoal-treated samples were centrifuged (105 min × 60,000 rpm in VTi 65 rotor) and the radioactivity of each fraction was counted. Arrows: position of ^{14}C-labeled γ globulins (7S) and ovalbumin (4S).

DISCUSSION

This paper extends R2 cell characterization. The first report [9] outlined that these cells were unresponsive to DHT (≃100 nM) and paid less attention to the inhibition of cell proliferation by high DHT concentration (1 μM). Here, we show that androgen inhibition is indeed specific and dose-dependent. The ED_{50}s (R1881, 0.5 nM; DHT: 5 nM) are low enough to suggest that this observation may have biological significance.

Thus, as far as the androgen inhibition of cell proliferation is concerned, R2 cells appeared to us more androgen responsive than previously reported. This change may be due to the difference between the culture conditions: 2.5% CT-FCS instead of 10%

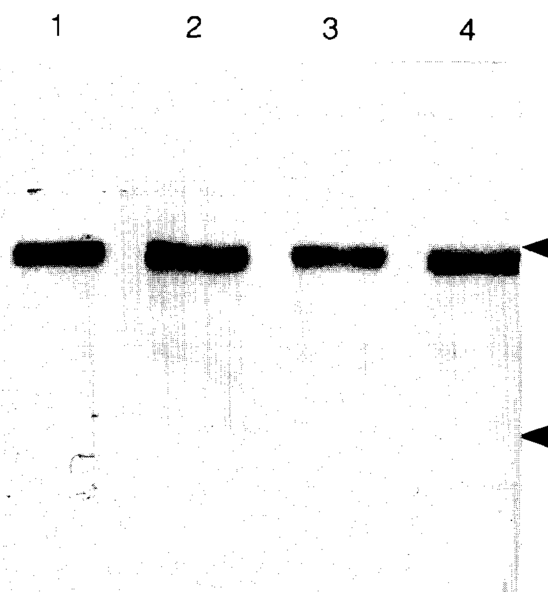

Fig. 6. Western blot. R2 (lanes 1 and 2) and LNCaP (lanes 3 and 4) extracts were submitted to polyacrylamide gel electrophoresis. After electrotransfer androgen receptors were revealed with a two-antibodies method. Lanes 1 and 3: 80 mg proteins. Lanes 2 and 4: 100 mg proteins. Arrows: positions of the standard proteins, *E. coli* β-galactosidase (M_r 116,000) and bovine serum albumin (M_r 68,000).

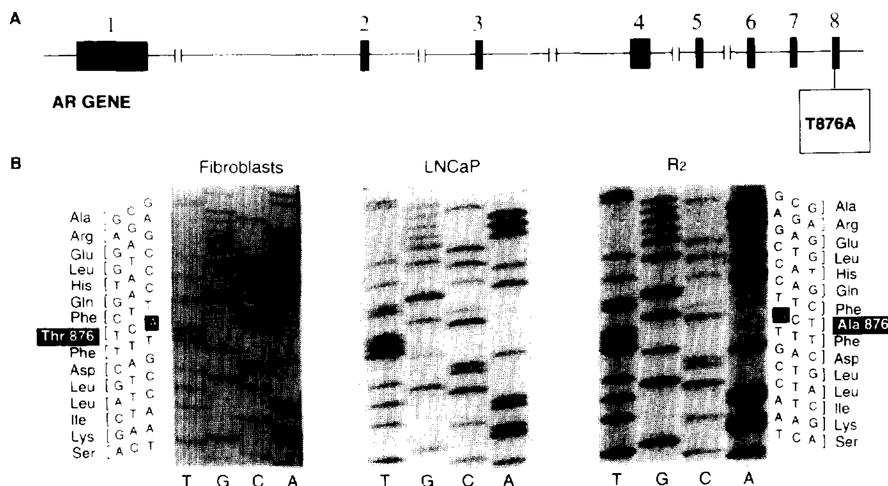

Fig. 7. Exon 8 sequence of the AR gene of normal human fibroblasts, LNCaP and R2 cells. (A) Localization of the mutation T876A according to the numbering of Lubahn *et al.* (B) Sequence of a part of the exon 8. The T876A mutation is the unique alteration found in the 350 bp of this exon.

FCS (not treated by charcoal) plus prolactin (20 μg/l). A time-dependent increase of androgen inhibition may be excluded since a previous work [9] was performed with cells between passage numbers 14–28 and we did find the same response between passage numbers 20 and 101.

Comparative studies (androgen response patterns, growth rate, behavior on plastic, etc.) demonstrate clearly that R2 and LNCaP cells are quite different; nevertheless, finding the same mutation in the exon 8 of the androgen receptor confirms that R2 is an LNCaP variant. It is important to stress that the difference between the androgen response patterns was found in cells treated in the same conditions (2.5% CT-FCS) since culture conditions are known to alter the androgen response to LNCaP [17, 18].

There are two mechanisms by which androgens may decrease the cell yield: blocking cell cycle progression and increasing cell death. We found evidence for the first mechanism but not for the second. Indeed, following androgen treatment, the proportion of G_0–G_1 cells increased and the thymidine incorporation decreased while the proportion of dead cells, i.e. those excluding dye or containing less DNA than G_0–G_1 cells did not increase. In addition, androgens increased the cell protein content and the metabolism of the vital dye MTT, an effect just opposite to that expected for a cytotoxic drug [19, 20]. Therefore, it is proposed that androgens inhibit R2 cell proliferation by blocking cells in the G_0–G_1 phase. Conversely, the androgen stimulation of LNCaP cells is accompanied by an increase in the proportion of cycling cells in the S phase [21]. Thus, check point(s) in the G_1 phase appear to be target(s) for positive and negative control of cell proliferation by androgens and other serum factors. We did not attempt to find a relationship between the putative androgen-increase of dehydrogenase activity revealed by MTT assay [22] and the cell cycle block.

The diversity of androgen responsiveness of the R2 subline according to previous androgen treatment and that of R2 clones may be explained as follows: (1) the partial inhibition of the R2 subline may be due to the presence of two cell populations, only one was inhibited by high concentrations of R1881; (2) cloning by dilution allowed the selection of cells from each population; (3) the stimulation of R2 by R1881 at low and high concentrations after long term culture in the presence of 100 nM R1881 (Table 1) may be due to reversible cell adaptation (Fig. 4).

The difference between the androgen effects in R2 and LNCaP is not accompanied by any detectable change of androgen receptor affinity and binding capacity. The relative binding affinities of the compounds tested on cell proliferation are similar to those previously reported [5, 6, 15]. A size polymorphism of nuclear receptors has been shown in some experimental models since the first description of A and B progesterone receptors [23, 24]. In the present work such a

Table 3. Effects of growth factors on cell proliferation

Growth factor			
Type	Concentration (ng/ml)	R2	LNCaP
EGF (n = 4)	0.1	86 ± 15	138 ± 40
	1	121 ± 11	181 ± 40
	10	113 ± 11	205 ± 67
Basic FGF (n = 2)	0.05	104 ± 4	113 ± 11
	0.1	137 ± 11	116 ± 9
	0.25	124 ± 25	127 ± 12
	0.5	136 ± 18	144 ± 20
	1	143 ± 7	139 ± 18

Cells were seeded and cultured in medium containing CT-FCS (2.5%) and increasing concentrations of growth factors. Growth factors were added every 2 days. n, number of experiments. Results (means ± SD) are given in % of control.

polymorphism was revealed neither by Western blot with the antibodies used nor by ultracentrifugation in sucrose gradient. In addition the sizes of the AR gene exons are the same in R2 and LNCaP. These results, taken with the following observations strongly suggest that the stimulation–inhibition switch is not due to a mutation in the AR gene: LNCaP is stimulated or inhibited by androgens according to environmental conditions [18, 25], chronic androgen treatment induces reversibly an alteration of androgen responsiveness of R2 and finally R2 is inhibited by androgens and stimulated by antiandrogens while both compounds are supposed to bind to the same receptors. It is proposed that R2 and LNCaP cells contain either different "sensor(s)" of the conformation changes induced on AR by androgens and antiandrogens or different transcription auxiliary factors.

From growth factor studies it appears unlikely that the dual effect of androgens is due to a specific action on the production or action of one of the factors tested. However, this conclusion has to be taken with caution since the growth of LNCaP, insensitive to exogenous IGF-I, is inhibited either by antisense oligonucleotides of IGF-I receptor mRNA or IGF-I analogs competing for IGF-I receptor binding sites [26].

In conclusion, this work and those reported by others [27] demonstrate that according to cell type and cell environment, androgens stimulate or inhibit the proliferation of cultured prostate tumor cells. The androgen sensitive genes responsible for this dual effect remain to be found. Although no objective remission of metastatic adenocarcinoma of the prostate was observed in a series of 67 patients [28] who received testosterone it appears to us of interest to test the androgen responsiveness of tumors whose growth is stimulated by antiandrogens [29, 30].

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