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Inhibition of growth and induction of apoptosis by androgens of a variant of LNCaP cell line

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Abstract

Here are described the effects of androgens, and other molecules known to bind to androgen receptors (AR), on MOP cells established from the human prostate cancer cell line LNCaP. MOP cells contained AR: 100 000 binding sites/cell, K_D for 5α dihydrotestosterone (DHT) 0.5 nM, size 110 kDa. The AR gene has the same repetition polymorphism in exon 1 and the T876A mutation in exon 8 as LNCaP. The proliferation of MOP cells in culture was repressed by the synthetic androgen 17β-hydroxy-17-methyl-estra-4,9,11-trien-3-one (R 1881), the antiandrogen cyproterone acetate (CYPA), estradiol (E2), progesterone and the synthetic progestin promegestone: 17,21 dimethyl-19 nor-4,9 pregnandiene-3,20 dione (R 5020). The number of cells recovered after 7 days decreased to ≈40% of controls. ED_{70}s ranged between 50 pM for R 1881 to 50 nM for E2 and CYPA. Treatment with R 1881 decreased [^{3}H]thymidine incorporation into DNA and increased dramatically the doubling time. R 1881, CYPA and E2 blocked the cell cycle between G1 and S phases and they induced apototosis as demonstrated by the increase of blebs on the plasma membrane, nuclear fragmentation, TdT-mediated dUTP nick end-labeling (TUNEL)-positive cells and internucleosomal DNA breaks. In athymic nude mice, testosterone enanthate prevented the growth of MOP tumors and, when tumors did develop, brought about regression. However, the tumors did not regress completely and finally escaped treatment. In conclusion, a variant of the LNCaP cell line has been established. With these cells it was possible to confirm that androgens paradoxically repress the growth of some prostate cancer cells both in culture and in vivo. In addition it is demonstrated in culture but not in vivo, for the first time to the authors' knowledge, that a synthetic androgen is able to induce apoptosis of cells established from human prostate carcinoma.

Keywords: LNCaP cell line; Androgens; Apoptosis

1. Introduction

Carcinoma of the prostate is one of the most frequent solid tumors in human males [1,2]. Tumors which do not cross the capsule may be cured by surgery or radiotherapy. Those which grow out of the prostatic capsule and/or give rise to metastases cannot be cured, but the majority regress under adjuvant treatment comprising androgen deprivation and/or antiandrogens [1]. Under these conditions tumors regress, mainly because cell death surpasses cell division [3], but generally escape treatment, i.e. they grow although androgens are maintained at a low level and antiandrogens at a high

Abbreviations: AR, androgen receptor; CaP, carcinoma of prostate; CT-FCS, charcoal-treated fetal calf serum; CYPA, cyproterone acetate, 6α-chloro-17α-acetoxy 1,2α-methylene-4,6,-4-pregnandiene-3,2-dione; DHT, 5α dihydrotestosterone; E2, 17β estradiol; EC_{70}, concentration of a compound which allows the recovery of 70% of the number of control cells; R 1881, 17β-hydroxy-17-methyl-estra-4,9,11-trien-3-one; R 5020, promegestone: 17,21 dimethyl-19 nor-4,9 pregnandiene-3,20 dione; SHBG, sex hormone binding globulin; TCA, trichloroacetic acid; TdT, terminal deoxynucleotidyl transferase; TUNEL, TdT-mediated dUTP nick end-labeling.

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level. When tumors escape, cell divisions outdo cell death [3] and several alterations of tumor cells may occur: (a) the proportion of androgen-responsive cells may decrease in the tumor while that of androgen-unresponsive cells such as DU145 [4] and PC3 [5,6] may increase; (b) cells, such as LNCaP [7], whose growth is stimulated by some antiandrogens [8,9] may appear; (c) cells which are hypersensitive to androgens may also arise. They may be activated by the residual androgens present in castrated patients. Such cells have been established from a Shionogi tumor — an androgen-sensitive mouse mammary tumor — which escaped after castration. Amplification of the androgen receptor (AR) gene, which may be associated with an increase of androgen responsiveness, was reported in advanced prostate tumors [10,11]. Finally one provocative hypothesis which has been put forward is that some carcinoma of prostate (CaP) cells may grow faster after androgen deprivation because their growth is slowed by androgens [12–14]. As an example, it was demonstrated that the growth of R2 cells which spontaneously arose from LNCaP cells cultured in serum-containing medium [15] was inhibited by androgens without evidence of apoptosis [12].

The present work was designed to establish cells with a phenotype which may be representative of tumor cells which escape treatment. It was hypothesized that the chronic culturing of cells such as LNCaP (whose growth is known to be stimulated by androgens), in a medium containing androgen-depleted FCS might favor the emergence of a phenotype similar to that of CaP cells after castration. Here the conditions under which a new cell line has been established, some of its features, and its responses to androgens in culture and in nude mice are described.

2. Materials and methods

2.1. Cells and test compounds

LNCaP cells were obtained from the American Type Tissue Collection (Rockville, MD). They were maintained by culturing in RPMI 1640 medium supplemented with 7.5% FCS. The MOP sub-line was established in the laboratory as follows: LNCaP cells at passage 28 were cultured for 1 year in RPMI 1640 medium containing 5% charcoal-treated fetal calf serum (CT-FCS). As the growth rate decreased, a large majority of cells were no longer attached to the plastic and a fast-growing colony of attached cells appeared from which the MOP cell population was derived. This subline has been maintained in RPMI supplemented with 5% CT-FCS. Inhibition of MOP cells by the synthetic androgen 17β-hydroxy-17-methyl-estra-4,9,11-trien-3-one (R 1881) has been shown since passage 32 up to the current passage 118. The effects of various compounds on cell growth were studied as follows. Cells were plated in RPMI 1640 medium containing 2.5% CT-FCS and the test compounds were added 24 h later without changing the culture medium. Cells were counted with a Coulter counter. For [^{3}H]thymidine incorporation assay, cells were cultured for 2 h with 1 μCi/ml [^{3}H]thymidine (CEA, Saclay, France), washed and radioactivity was counted after trichloroacetic acid (TCA) precipitation. For counting mitoses and apoptotic cells, cells were fixed on slides with ethanol-acetic acid (3/1) and colored with Hoechst 33258 (1 μg/ml) for 15 min. Five hundred cells were examined in triplicates for calculating mitotic and apoptotic index: mitotic or apoptotic cells per thousand cells. Sex hormone binding globulin (SHBG) was purified by affinity chromatography [16]. R 1881 and promegestone: 17,21 dimethyl-19 nor-4,9 pregnandiene-3,20 dione (R 5020) were obtained from New England Nuclear (Boston, MA), dihydrotestosterone (DHT), estradiol (E2) and progesterone from Sigma (St. Louis, MO) and cyproterone acetate, 6α-chloro-17α-acetoxy 1,2α-methylene-4,6,-4-pregnandiene-3,2-dione (CYPA) from Schering A.G. (Berlin, Germany).

2.2. Mice and tumors

Swiss nude (nu/nu) female mice (IFFA CREDO), 5–7 weeks old, were housed according to the guidelines of the French Ministère de l'Agriculture, in a non-free environment, six to eight mice per cage. Cells ($2–3 \times 10^6$) in 0.2 ml Matrigel (Collaborative Research, Beadford, MA) diluted 1/2 in RPMI 1640 were injected sc. into the right flanks of the mice. Tumor sizes were measured weekly and tumor volume was calculated using the formula: length × width × height × 1/2. Injections of cells and hormones and collection of blood samples were performed under ether anesthesia. Androtardyl® (testosterone enanthate) was injected every 10 days sc. in the left flanks. Control mice received the same volume of vehicle by the same route. Testosterone was measured by an in-house radioimmunoassay with an intra assay variation of 3% and a coefficient of variation of 9% [17].

2.3. Flow cytometry

For in situ end labeling of DNA strand breaks, cells were processed as described [18]. In short, floating and trypsin-detached cells were fixed first in 1% paraformaldehyde, PBS (15 min, 0°C) then in ethanol (70% v/v, 20 min, −20°C), washed in the terminal deoxynucleotidyl transferase (TdT) buffer, incubated (60 min, 37°C) with TdT (Boehringer, Mannheim, Germany) in TdT buffer containing 0.62 nM biotin-dUTP, 0.34 nM each dCTP, dATP and dGTP, washed twice

with PBS, incubated (30 min, 18°C) with 4 × SSC, 0.1% Triton X100, 5% low-fat powdered milk, 30 μg/ml RNAse and 2.5 μg/ml FITC-avidin, washed with PBS and finally incubated with propidium iodide (10 μg/ml). Due to the numerous steps required, there was a loss of 10–30% cells. To avoid a bias which could result from a preferential loss of cells in a particular phase of the cell cycle or apoptosis, the distribution of cells according to their DNA content was analyzed using a simplified protocol. Floating and trypsin-detached cells were: fixed in ice-cold 70% ethanol, centrifuged, suspended in PBS, incubated with 4 μg/ml DNAse-free RNAse (Boehringer) at 37°C and finally with 50 μg/ml propidium iodide 30 min before cell analysis. Flow cytometry was performed with the FACSscan Becton Dickinson using LYYSIS II and cell-FIT programs.

2.4. Electrophoresis of DNA

Floating cells were pooled with cells detached by the trypsin-EDTA treatment. They were washed with PBS and incubated with 10 mM Tris–HCl (pH 7.4), 5 mM EDTA, 0.5% Triton X100. The samples were centrifuged at 12 000 × *g* for 20 min. The supernatants were incubated at 37°C with 4 μg/ml DNAse-free RNAse (Boehringer) for 30 min and with 0.1 mg/ml Proteinase K for 60 min DNA was extracted with phenol–chloroform, recovered by ethanol precipitation and centrifugation (12 000 × *g* for 20 min), dissolved in water and analyzed by electrophoresis on 2% agarose gel containing ethidium bromide. DNA and protein levels were measured as described previously [12].

2.5. AR assays and AR gene studies

Hormone binding and receptor size were analyzed as described [12]. Briefly, the number of binding sites and androgen affinity were analyzed on whole cells using [^{3}H]DHT (Amersham, Buckinghamshire, GB). For western blots, 0.4 M KCl extracts (100 mg proteins) were separated by SDS-PAGE (10% polyacrylamide, 0.27% bis acrylamide) and receptors were detected by a double antibody method. The first monoclonal antibody (F39.4.1) was from Sambio (Unden, Netherlands) ant the second polyclonal antibody labelled with alkaline phosphatase was from Immunotech (Luminy, France). The sequencing was performed after PCR amplification according to Joly et al. [12] with some modifications. Briefly, the primers described by Lubahn et al. [19] and the Taq Dye Deoxyterminator kit reagents (Applied Biosystems, Fostere City, CA) were used for PCR amplification and labeling. The PCR products (0.5 pmol) were separated by electrophoresis (10 h, 1400 V, 20 mA) on a 24-well polyacrylamide gel (6% acrylamide, 0.36 bis acrylamide, 8 M urea, 1 × TBE) with the 373A DNA sequencer (Applied Biosystems). The sequence data were analyzed with the 373A Analysis and Sequed software (Applied Biosystems). The CAG repeat of exon 1 was analyzed as follows. DNA was amplified and labelled by PCR using the unlabelled primer 5′-CAGCCTGTTGAACTCTTCTGAGC-3′ and the primer 5′-GCGGCTGTGAAGGTTGCTGTTCCTC-3′ with the Joe NHS ester fluorophore from Applied Biosystems. The labelled amplicon was digested with MboI (New England Biolabs, Hitchin, UK). The fluorescent DNA (240–280 bp) was mixed with fluorescent molecular weight standard Genescan 2500 P (Applied Biosystems) and analyzed by electrophoresis as described for sequencing. The size of the CAG repeat-containing fragment was calculated with the 672 Genescan software (Applied Biosystems).

2.6. RT-PCR

Total RNA from control MOP cells was used. The reverse transcription was performed using hexamer primers and the kit Superscript™ (Gibco BRL, Life Technologies). PCR was performed with Taq Polymerase (Eurobio) in the presence of 1.5 mM $MgCl_2$. The primers for progestin receptors were: TGGCACACAACATCCAAACT and CAACGCCTCCTCCTCTAGG. The primers for estrogen receptor α were: AGGCTGCGCGTTCGGC and AGCCATACTTCCCTTGTCAT. Those for estrogen receptor β were: TTC CCA GCA ATG TCA CTA ACT and CTC TTT GAA CCT GGA CCA GTA. After 10 min denaturation at 94°C, 35 cycles were performed for the progestin receptor, 45 for the estrogen receptors. For each cycle: denaturation 20 s at 94°C, hybridization 40 s at 50°C, elongation 1 min at 72°C. The last elongation was performed for 10 min.

2.7. PSA determinations

PSA was measured using the T-PSA(equi) kit from Immunotech (Luminy, France) according to the recommendations of the manufacturer. The lowest limit of detection was 0.1 ng/ml. CV was 7% around 1 ng/ml and 5% above 12 ng/ml.

2.8. Statistical analysis

Values were expressed as mean ± S.D. Significance of differences was analyzed with one-way ANOVA using Statview for Macintosh. A P value of < 0.05 was considered statistically significant.

3. Results

3.1. Inhibition of the cell growth and DNA synthesis in culture

R 1881 and the natural androgen DHT decreased in a dose-dependent manner the number of MOP cells recovered after 7 days of culture (Fig. 1A). The concentration of a compound which allows the recovery of 70% of the number of control cells (EC_{70}) was ≈ 50 pM for R 1881 and 0.8 nM for DHT. A plateau (at $\approx 40\%$ of control) was observed from 1 to 10 nM. Other compounds known to bind to mutated AR [9] and to other receptors were less potent inhibitors than R 1881 at low concentrations but they caused inhibition similar to that of R 1881 at high concentrations (Fig. 1A and B). The EC_{70} was 50 nM for cyproterone acetate, 50 nM for E2, 2 nM for progesterone and 10 nM for the synthetic progestin R 5020. Note that the EC_{70} corresponds to the mean of the concentrations

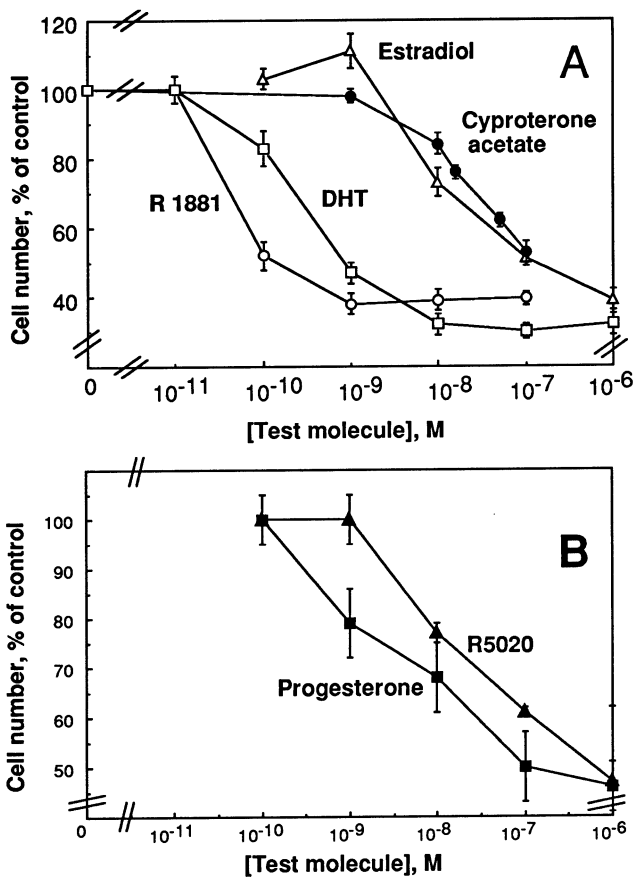

Fig. 1. Dose-dependent inhibition of the proliferation of MOP cells by R 1881, 5α dihydrotestosterone (DHT), cyproterone acetate (CYPA) and estradiol (E2) (A), and R 5020 and progesterone (B). MOP cells were seeded in 96-well plates. Twenty-four hours later test compounds were added or not to the culture medium and 7 days later cells were counted. Results are means ± S.D. (four experiments with triplicates).

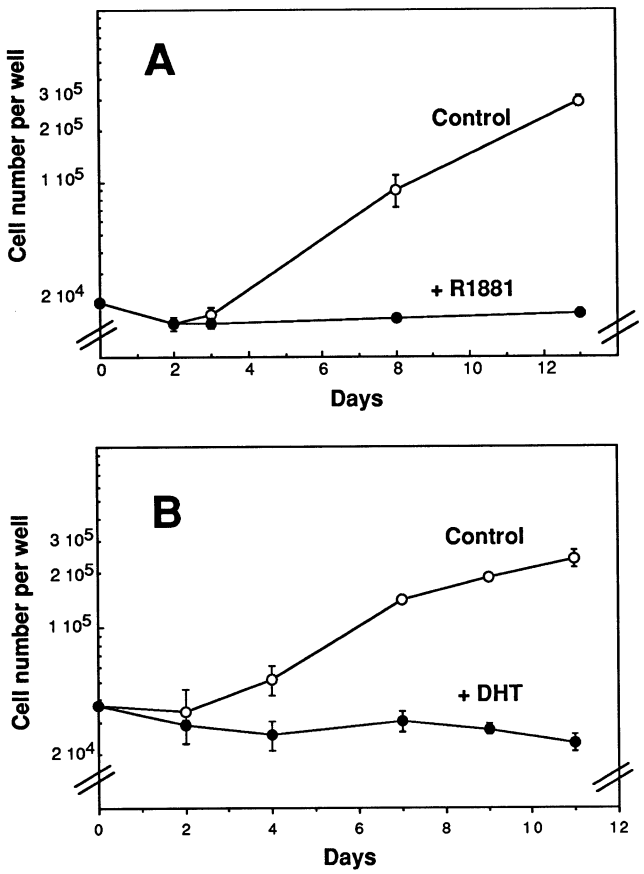

Fig. 2. Inhibition of growth rate by R 1881 (A) and 5α dihydrotestosterone (DHT) (B). MOP cells were seeded in 24-well plates. Twenty-four hours later R 1881 or DHT (100 nM) was added (●) or was not added (○) to the culture medium and cells were counted at various times. Results are means ± S.D. of triplicates (a typical experiment).

required to produce half the maximum decrease and that the number of cells counted after treatment with an androgen (R 1881 and DHT) at high concentration was similar to that of seeded cells (Fig. 2A and B). There is some controversy about the role of SHBG in controlling the effects of the androgens known to bind to this protein. Accordingly, the consequences of the addition of SHBG (2.5 μg/ml) on the dose response of MOP cells to DHT were examined. The inhibitory effect of DHT was totally prevented (not shown) suggesting that the SHBG-bound DHT was inert in this model and that the concentration of unbound DHT was too low to be active. Cell proliferation was insensitive to 100 nM diethyl stilbestrol and dexamethasone (not shown). The growth rate was dramatically repressed by R 1881 and DHT (Fig. 2). The doubling time of control cells in the exponentially growing phase was 50–60 h and that of R 1881-treated cells increased so much that it was not measurable within the time frame used in these experiments. [^{3}H]Thymidine incorporation into DNA of R 1881-treated cells decreased in a dose-dependent manner (Fig. 3). These results sug-

gested that R 1881 blocked cell cycle progression and/or killed cells. These two hypotheses were therefore verified. There is evidence for both effects. Evidence for a reversible block of the cell cycle came from the following observation: 8 days after the arrest of a 4-day treatment with 100 nM R 1881, cells grew again at the rate of control cells (not shown). Evidence for an increase of the percentage of G0–G1 cells and apoptosis is presented in paragraph 3.

3.2. Presence of classical AR and increase of PSA secretion

Since the control of the proliferation of MOP cells by androgens was so different to that expected in prostate cells one looked for abnormalities of AR and of androgen control of PSA. AR gene and AR were characterized in MOP cells and compared to those of LNCaP taken as a reference for androgen stimulated cells. The same polymorphism was found in the two types of cells in the region encompassing the CAG repeat of the exon 1 of AR gene (Fig. 5) indicating that in MOP cells there

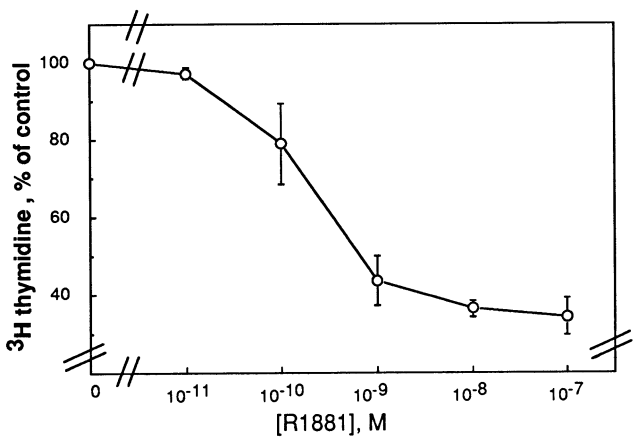

Fig. 3. Dose-dependent inhibition of [^{3}H]thymidine incorporation. MOP cells were seeded in 24-well plates. R 1881 was added 24 h later and after a further 24 h cells were pulse-labelled 2 h with [^{3}H]thymidine (1 μCi/ml). Radioactivity was counted after precipitation with trichloroacetic acid (TCA). Results are means ± S.D. ($n = 3$ with triplicates).

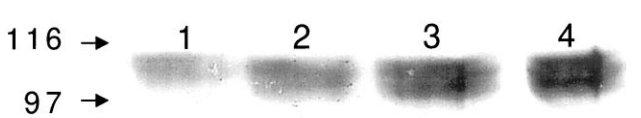

Fig. 4. Western blots of androgen receptors of MOP and LNCaP cells. LNCaP (lanes 1 and 2) and MOP (lanes 3 and 4) cells were cultured in basal conditions and disrupted by sonication in buffer containing 0.4 M KCl. One hundred μg proteins of cell extract cleared by centrifugation were separated by electrophoresis in SDS-polyacrylamide gel. Androgen receptors were detected by a double antibody method. The extracts were prepared from cells at different subcultures. Protein/DNA ratio in both cells: ≈20. At the left, molecular weight markers expressed in kDa.

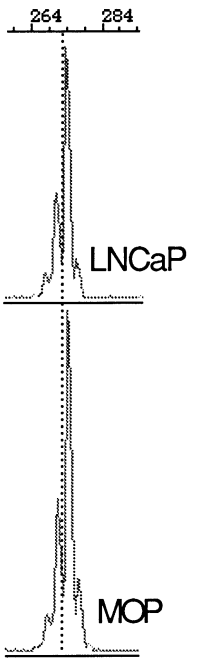

Fig. 5. Poly CAG polymorphism of androgen receptor gene of the exon 1. DNA was extracted from LNCaP and MOP cells. A locus of the AR gene encompassing the poly CAG repeat of LNCaP and MOP cells was amplified by PCR. The product of amplification was digested with MboI and anlayzed by electrophoresis on polyacrylamide gel in denaturing conditions. The sizes (shown on the top of the figure in bp) were determined by comparison with markers run simultaneously.

was no particular genome instability at this locus. The sequences of exons 2–8 were identical and the T876A mutation reported in LNCaP cells was also found in MOP cells. These observations therefore provide good evidence that MOP is effectively a LNCaP variant. MOP cells bound ^{3}H-DHT with high affinity ($K_D \approx 0.5$ nM) and limited capacity (≈ 100 000 binding sites/cell vs. 60 000 in LNCaP cells [12]). A band (some times a doublet) of 110 kDa proteins was shown by western blot (Fig. 4); the signals were stronger in MOP cells (lanes 3 and 4) than in LNCaP (lanes 1 and 2), in agreement with the binding assay. Thus, there is good evidence that the inhibition of growth by androgens is not due to major qualitative or quantitative modification of AR. Since several compounds used are able to bind other steroid receptors one looked for progestin and estrogen receptors. RT-PCR allowed us to demonstrate the presence of mRNA coding progestin receptors but not those coding estrogen receptors α and β (not shown). MOP cells produced immunoreactive PSA (Table 1). The cumulative amount of PSA recovered in the culture medium after 2 and 6 days of culture increased 25- and 65-fold after treatment with 100 nM R 1881. Under the same conditions, CYPA and E2 caused ≈10- and ≈20-fold increases. The androgen-induced increase of PSA (Table 1) was much more sustained than that of the protein/DNA ratio (Table 2).

Table 1
PSA accumulation in culture medium[a]

	2 days	6 days
Control	2.6 ± 0.7	10.8 ± 1.8
R 1881	64.8 ± 4.0	696.0 ± 54.1
Cyproterone acetate	26.6 ± 2.0	210 ± 13.8
Estradiol	33.0 ± 1.4	216 ± 3.6

[a] MOP cells were cultured for 2 or 6 days in RPMI 1640 medium containing 2.5% CT-FCS in the absence (control) or presence of 100 nM test compound. PSA was measured in the culture medium clarified by centrifugation. Results (means ± S.D., $n = 3$) are expressed in ng PSA per 10^6 cells.

Thus R 1881 clearly enhanced the synthesis or the secretion of PSA in MOP cells.

3.3. Effects of R 1881 and CYPA on cell shape, mitoses, apoptosis and distribution of cells in the cell cycle

The consequences of treatments by R 1881 and CYPA on cell biology were examined. The size, shape and behavior of living MOP cells were modified in a dose-dependent manner by R 1881 and CYPA (Fig. 6). The two compounds led to the same alterations as seen on phase contrast microscopy: they increased the size of attached cells, the number of spherical cells either floating or loosely attached to the plastic and the number of fragmented cells. Simultaneously they decreased cell density. Cells were more sensitive to R 1881 than to CYPA (Fig. 6, compare B–E to F–I) as previously seen for cell proliferation and PSA secretion. The effects of R 1881 on cell size were confirmed on examining fixed and colored cells (Fig. 7). The cell enlargement caused by R 1881 might be a true cell hypertrophy since the protein/DNA ratio increased (Table 2).

The mitotic index was markedly decreased as early as the second day of treatment with 100 nM R 1881 while the apoptotic index increased dramatically (Table 2). Cells were taken as apoptotic when they showed fragmented nuclei in the measure of Hoechst 33258 fluorochrome. According to these morphological features, 37% of cells were apoptotic after 6 days of treatment versus $< 0.4\%$ in control cells. Simultaneously, $> 80\%$ cells excluded the vital dye trypan blue versus 97% in control cells. Under the same experimental conditions, R 1881 caused internucleosomal DNA breaks (Fig. 8): DNA of mono nucleosome and oligo nucleosome size was detected in the supernatant of the cell lysate from R 1881-treated cells (lane 3) and not in that from control cells (lane 2). The increase of DNA breaks was confirmed by TdT-mediated dUTP nick end-labeling (TUNEL) and flow cytometry (Fig. 9A–D). CYPA and E2 had similar effects (Fig. 9E–H). In these experiments, two measures were performed simultaneously: the propidium fluorescence, related to the DNA content (y axis) and the FITC fluorescence, related to the amount of biotinylated nucleotide incorporated at the DNA breaks (x axis). TUNEL-positive cells are depicted as dots to the right of the threshold established on the control cells and is shown on each dot plot. An increase in cell debris with low DNA content was found in some experiments (Fig. 9E–H).

The distribution of cells in the cell cycle was modified by the three compounds (Fig. 9 and Table 3). After 6 days of treatment with R 1881 (1–100 nM) the cells in G0–G1 phase increased from ~ 75 up to $\sim 92\%$ while the percentage of S cells decreased from ~ 13 to $\sim 2\%$ and that of G2 + M cells decreased from ~ 11 to $\sim 6\%$. Similar modifications were observed after treatment with 100 nM CYPA and E2 except that there was no evidence for any decrease of the percentage of G2 + M cells.

3.4. Inhibition of the growth of MOP cells in nude mice by testosterone enanthate

Two types of experiments were performed to examine the response of MOP cells to androgens in vivo. In both, MOP cells were xenotransplanted sc. in female Swiss nude mice, one group received sesame oil and the second received testosterone enanthate (Androtardyl®) sc. every 10 days. The concentration of testosterone in the blood 10 days after the injection was 120 ± 20 versus 11 ± 4 nM in control males and 0.5 ± 0.3 nM in control females ($n = 10$). In the first type of experiment, a group of mice was treated from the day of transplantation. Fig. 10 shows the results of a particular experi-

Table 2
Action of R 1881 on mitotic index, apoptotic index and protein/DNA ratio[a]

	2 days			4 days			6 days		
	MI	AI	Prot/DNA	MI	AI	Prot/DNA	MI	AI	Prot/DNA
Control	38 ± 2	4 ± 2	23.4 ± 5.8	53 ± 4	0	21.3 ± 1.0	70 ± 20	0	19.0 ± 3.3
R 1881 0.1 nM	Nd	nd	26.8 ± 1.1	nd	nd	28.2 ± 0.8	nd	nd	36.0 ± 1.9
R 1881 100 nM	2 ± 2	104 ± 12	32.7 ± 1.9	0	118 ± 19	37.4 ± 3.5	0	371 ± 32	44.2 ± 1.0

[a] MOP cells were cultured in the absence (control) or in the presence of R 1881 for 2, 4 or 6 days. The mitotic (MI) and apoptotic index (AI) are expressed per 1000 cells. Protein/DNA ratios (w/w) were calculated from assays on cell lysates. Results are the means ± S.D. of triplicates.

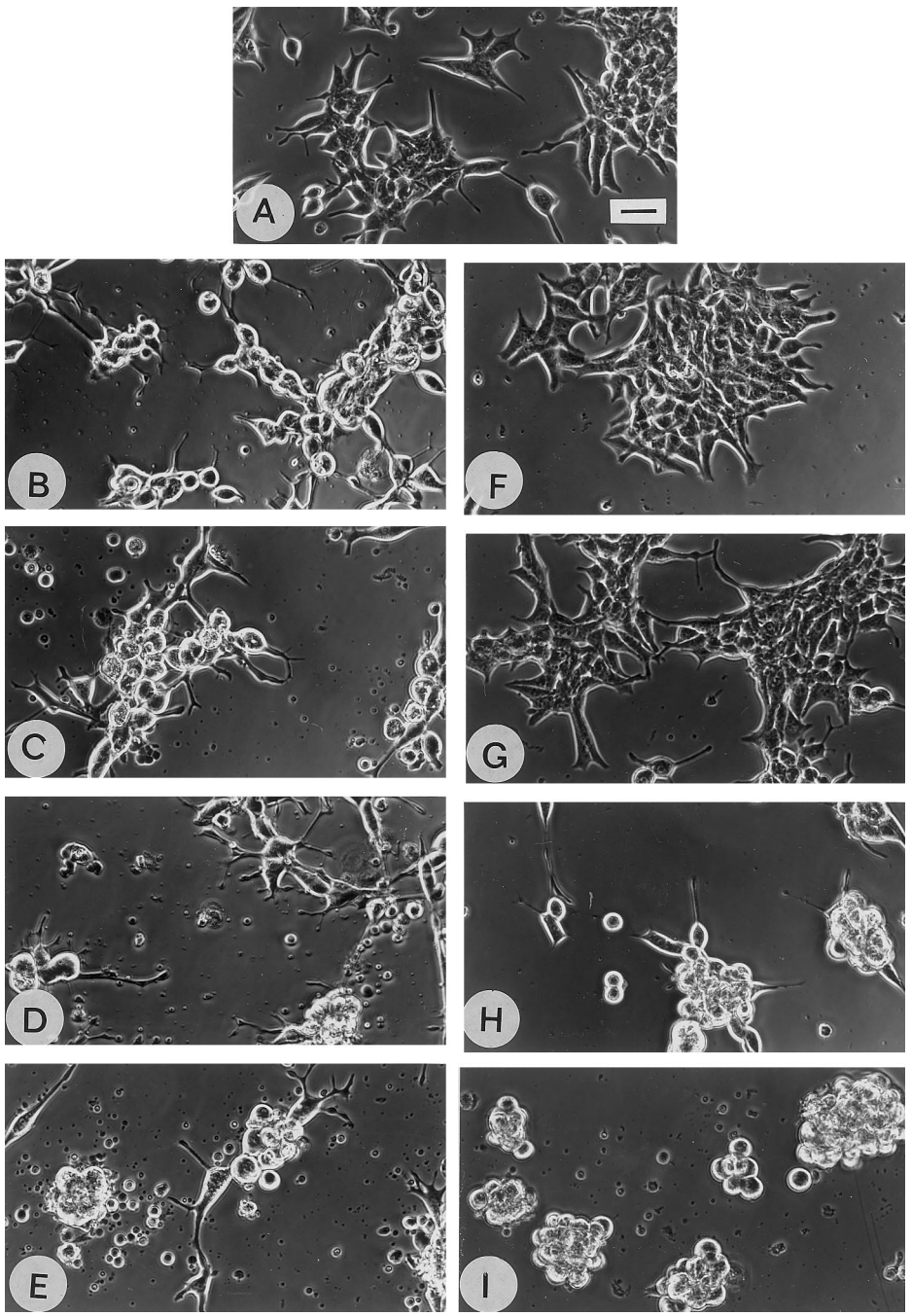

Fig. 6. Morphological changes caused by R 1881 and cyproterone acetate (CYPA) in MOP cells: phase contrast microscopy. MOP cells were seeded in T flasks. R 1881 (B–E: 0.1, 1, 10, 100 nM), CYPA (F–I: 0.1, 1, 10, 100 nM) and vehicle (A) were added 24 h later. Cells were examined 7 days later. The bar: 30 μm.

ment in which 2×10^6 cells were injected. In control mice the first palpable tumors were detected at 3 weeks and the incidence at 10 weeks was 6/10. In the testosterone-treated group no tumor was palpable up to 12 weeks and tumors appeared after the arrest of treatment; 3 weeks after the last testosterone injection the first palpable tumors occurred and the tumor incidence in this group, 12 weeks later was similar to that of the control group (5/10). In the second type of experiment, testosterone administration was delayed until the tumors had developed (Fig. 11). Mice received 3×10^6 cells and the tumor incidence was 100% at 3 weeks. The treatment was started either early (28 days after cell injection) when tumors were small (tumor volume 285 ± 17 mm^3, range 210–365) or late (84 days after cell injection) when tumors were large (1340 ± 44 mm^3, range 300–3400). In both cases tumors regressed ($P < 0.05$) from 2 weeks after the beginning of treatment and continued to regress with time. However, the volume of small tumors which were followed-up for a long period

of time decreased till 56 days post-treatment (85 ± 17 mm^3, $P < 0.05$; three of nine tumors were no longer palpable) and grew again from day 63 post-treatment (all mice had a palpable tumor). The tumors recovered the volume they had at the beginning of treatment, 70 days later. The shoulder observed on the graph of the tumor volume of control mice (Fig. 11) was due to the split of the group at day 84: half of the mice received testosterone enanthate the other half received sesame oil. The tumor volumes at the end of the experiment were: 2720 ± 560, 300 ± 55, 490 ± 160 mm^3 in control, early-treated mice and late treated mice respectively. Tumor regression and regrowth under treatment were confirmed in another experiment under the same conditions. Similar effects were observed (not shown) when mice received 1/10th of the dose of testosterone enanthate; testosterone in blood 10 days after injection was 13 ± 2 nM. It was suspected that the 7-day treatment with testosterone might be increasing tumor necrosis. However, there was so much variation in the area and location of necrosis in control tumors that this potential effect of testosterone requires further investigation. Nevertheless these treatments led to a near total disappearance of mitotic figures in the necrotic areas but with no evidence for an increase in the apoptotic index.

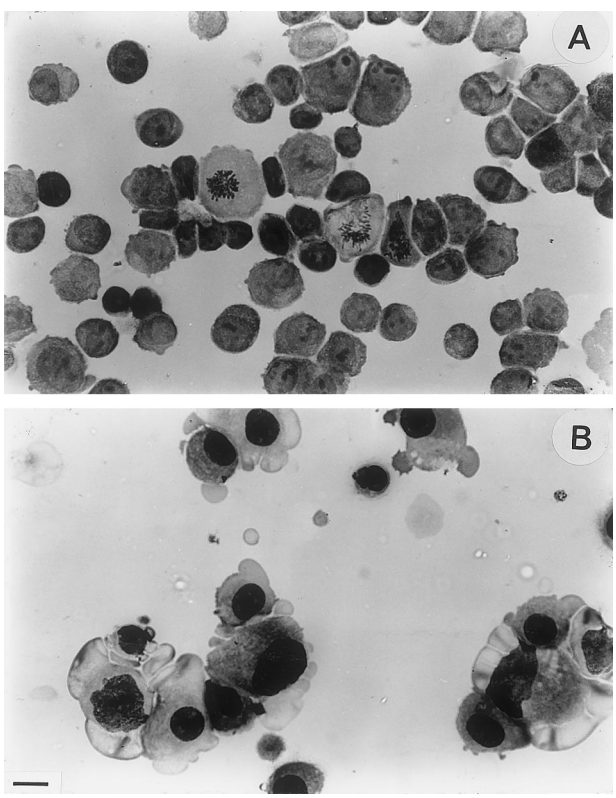

Fig. 7. Morphological changes caused by R 1881: fixed and colored cells. MOP cells were seeded in T flasks. Vehicle (A), or 100 nM R 1881 (B) were added 24 h later. Seven days later cells were detached with trypsin-EDTA, fixed on microscope slides and colored with May Grünwald-Giemsa staining. The bar: 30 μm.

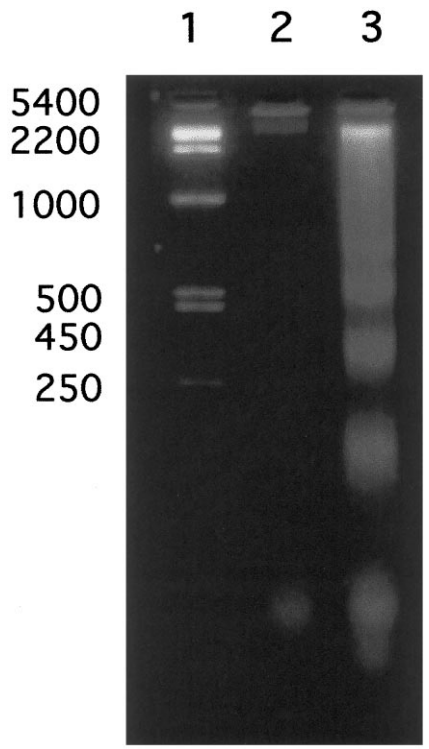

Fig. 8. Internucleosomal DNA breaks induced by R1881. MOP cells were seeded in T flasks. Vehicle (lane 2) or 100 nM R 1881 (lane 3) were added 24 h later. Six days later cells were detached with trypsin-EDTA and incubated in Triton-X 100-containing buffer. Cell extracts were cleared by centrifugation, treated first with DNAse-free RNAse and finally with proteinase K. DNA was purified with phenol and analyzed by 2% agarose gel electrophoresis. Lane 1: DNA size markers.

4. Discussion

Long term culture of LNCaP cells in low-androgen medium has allowed us to establish MOP, a new subline whose growth is repressed by androgens, CYPA, E2, progesterone and R 5020. R 1881, CYPA and E2 slowed cell proliferation in culture by limiting the entry of cells into S phase and by killing some cells. Thirty seven percent of cells were in apoptosis after treatment for 6 days with R 1881. In nude mice, testosterone enanthate delayed the take of tumors from MOP cells and caused the regression of established MOP tumors. This work extends those previously reported by this laboratory [12] and by others [13,14], it outlines that growth inhibition by androgens of CaP cells is not a rare and casual event. In addition it demonstrates, for the first time, that androgens are able to induce apoptosis in prostatic cells not transfected with complementary DNA of the AR.

Although LNCaP cells are often taken as a model of androgen stimulated cells the reality is not so simple: in medium containing charcoal-treated fetal calf serum the dose response curve is bell-shaped [8] and more surprisingly, in medium containing fetal calf serum not treated

with charcoal, androgens are indeed potent inhibitors [20] and unpublished results. Let one focus on cells inhibited by androgens in CT-FCS containing medium. It was reported previously [12] that androgens inhibit the growth of R2 cells, a spontaneous variant of LNCaP cells [15]. While the present report was delayed to verify the stability of the MOP phenotype (more than 80 passages) because LNCaP variants are known to be unstable, two other groups reported on the inhibitory effects of androgens in culture and in vivo. Liao's group established the LNCaP 104-R sub-line [14,21] from a clone of LNCaP which had been isolated after serial dilution. The response of these cells to androgens was 'biphasic', i.e. the cell proliferation was stimulated at low concentration and inhibited at high concentration. This response is clearly different to that of MOP cells reported here: androgens at low concentration did not stimulate cell proliferation. In nude mice, endogenous or exogenous androgens prevented tumor growth from LNCaP 104-R and caused regression of established tumors [13] similar to the observations reported here for MOP cells. Chung's group established the ARCaP cell line from a clone of cells isolated from the ascites fluid of a patient with widely disseminated carcinoma of the prostate [14]. The patient, who had been castrated 11 months before, was presumed to have a hormone-refractory disease because the tumors progressed rapidly despite castration. Androgens and estradiol suppressed the growth of ARCaP cells in culture and in vivo. Thus, androgen repression is not restricted

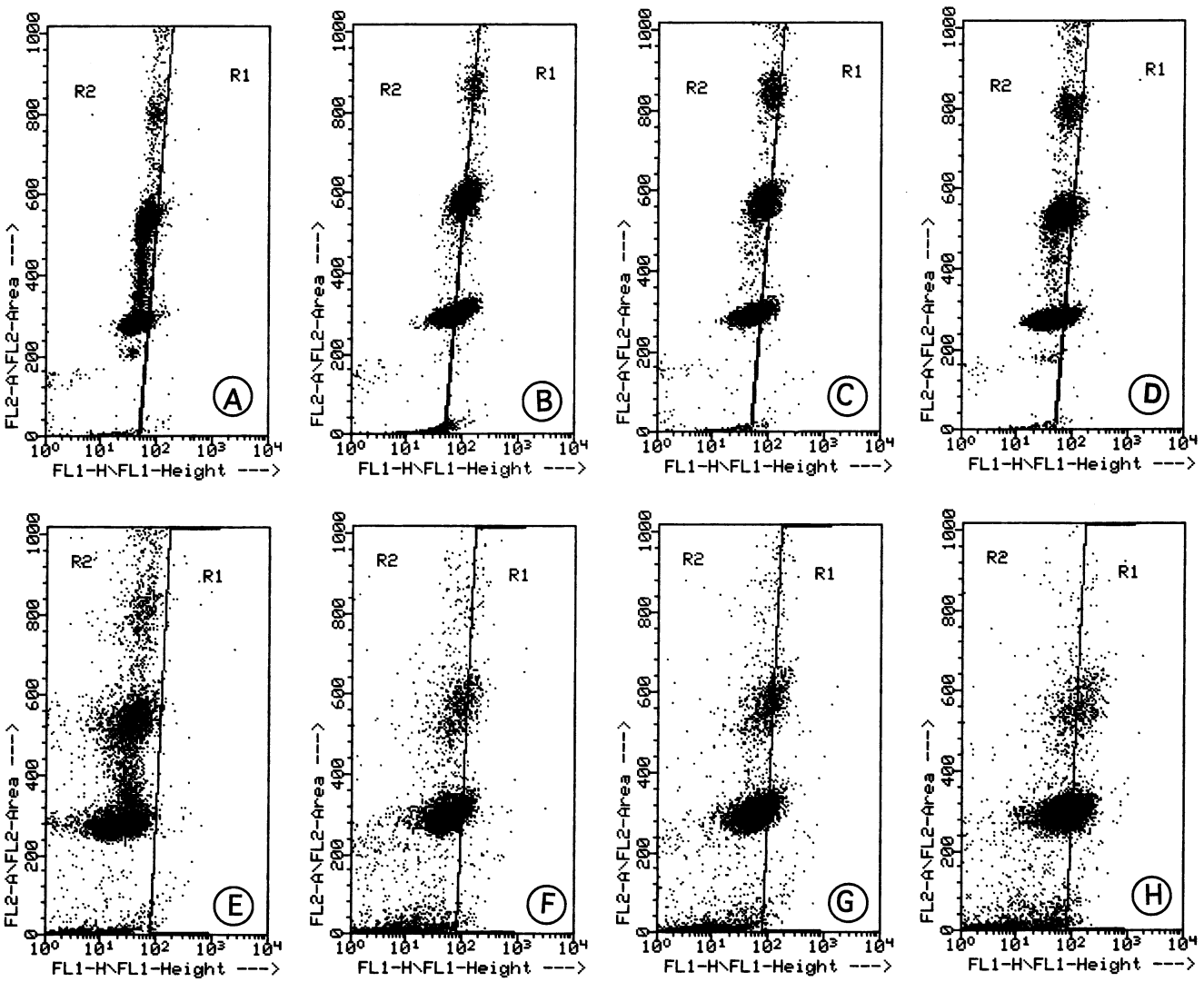

Fig. 9. Cell cycle block and DNA breaks induced by R 1881, cyproterone acetate (CYPA) and estradiol (E2). Results of two independent experiments carried out with MOP cells. Upper row, dose response to R 1881: 100 nM (B), 10 nM (C) and 1 nM (D); control (A). Lower row: effects at 100 nM E2 (F), CYPA (G) and R 1881 (H); control (E). Cells cultured for 6 days with either test compound (B–D, F–H) or vehicle (A, E) were detached, fixed, processed for TUNEL and labelled with propidium iodide. Ten thousand cells were examined by flow cytometry. Each dot corresponds to a cell characterized by the intensity of fluorescence of the FITC-avidin used in TUNEL (x axis, FL1) and of propidium iodide (y axis, FL2). The oblique line in each dot plot permits TUNEL-positive cells in R1 to be discriminated from TUNEL-negative cells in R2.

Table 3
Effects of 6 day-treatments with R 1881, CYPA and E2 on cell cycle[a]

	G0–G1	S	G2+M
Control (6)	75.1 ± 2.8	13.4 ± 10.8	11.5 ± 2.6
R 1881 1 nM (3)	90.8 ± 2.1	2.8 ± 1.1	6.4 ± 2.0
R 1881 10 nM (3)	90.9 ± 2.9	2.4 ± 0.3	6.7 ± 2.7
R 1881 100 nM (6)	91.9 ± 1.6	2.4 ± 2.0	5.7 ± 2.5
CYPA 100 nM (3)	87.8 ± 0.4	1.8 ± 0.4	10.3 ± 0.8
E2 100 nM (3)	84.6 ± 1.6	3.4 ± 0.4	11.6 ± 1.8

[a] MOP cells were cultured for 6 days in the absence (control) or in the presence of the test compounds at the concentration indicated. Cell cycle was analyzed by flow cytometry as described in the legend of Fig. 9. Results shown are the means of the percentage of the cells in each phase ± S.D. The number of experiments is shown in brackets.

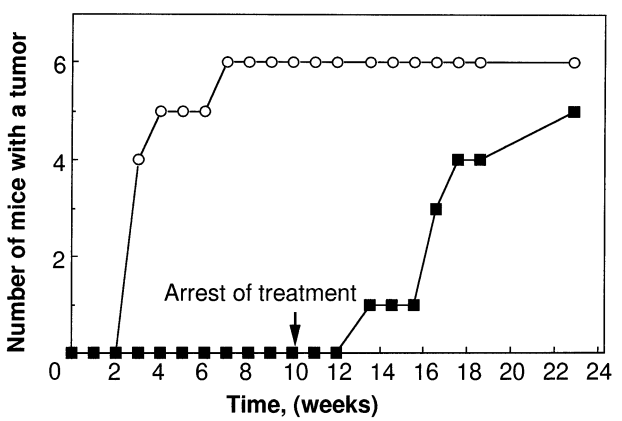

Fig. 10. Inhibition in vivo of the proliferation of MOP cells by testosterone enanthate. Twenty mice received MOP cells (2×10^6 in Matrigel) sc. and were distributed randomly in two groups of ten. Mice of one group (○) received sesame oil sc. every 10 days, mice of the other group (■) received testosterone enanthate until week 10 and sesame oil thereafter. Results are expressed as the number of mice with a palpable tumor at increasing times after cell injection.

to the LNCaP model and a low-androgen environment appears to be a critical step for the selection or isolation of androgen-repressed cells in culture and in vivo. Although R2, MOP, LNCaP 104-R and ARCaP are repressed by androgens (at least above 0.01 nM R 1881) in culture in the presence of charcoal-treated serum, they differ from each other by their androgen sensitivity and/or the patterns of responses to other hormones, e.g. R2 cells are less sensitive than MOP cells to androgens and they are not induced in apoptosis by androgens [12], in culture, estradiol stimulates R2 and ARCaP while it represses MOP cells, in vivo, estradiol represses ARCaP and stimulates LNCaP 104-R; cyproterone acetate represses MOP cells while it activates R2 cells. Thus, androgens are potent inhibitors of various CaP cells and the mechanisms of inhibition may be cell specific. Indeed, cells inhibited by androgens exhibited different patterns of responses to various AR ligands and while some are induced in apoptosis (MOP cells) other under the same conditions, are not (LNCaP and R2 cells). Interestingly, DHT (1 and 10 nM) was shown to increase the expression of the antiapoptotic bcl-2 protein in LNCaP cells [22]. Finally, it was also shown that androgens inhibit the proliferation of PC-3 [23] and MCF7 [24] cells transfected with complementary DNA of the human AR.

While it may be reasonably concluded that DHT and R 1881 control MOP cell proliferation and tumor growth via their binding to AR, the mechanisms of action of other compounds (CYPA, progesterone, R 5020 and estradiol) have to be discussed. The authors favor the hypothesis that these compounds act at least in part via their binding to AR. Indeed, their ED_{70} paralleled the relative binding affinity for the AR of LNCaP cells and R_2 cells [12]. The unusual affinity and biological efficiency of such compounds in various cells have been related to the T876A mutation in the ligand binding domain of the AR [25–28]. This reasoning may be extended to MOP cells in wich the same mutation has been found. As an example, it was reported [12] that the relative binding affinity of progesterone for the AR with the T876A mutation is 10-fold higher than that of R 5020, in agreement with the fact that MOP cells are more sensitive to progesterone than R 5020. The fact that the classical antiandrogen CYPA has agonist activity is not restricted to MOP cells: it has already been described for in LNCaP cells [29,30] and Hela cells [26] transfected with expression vectors containing the mutated AR sequence. Progesterone was

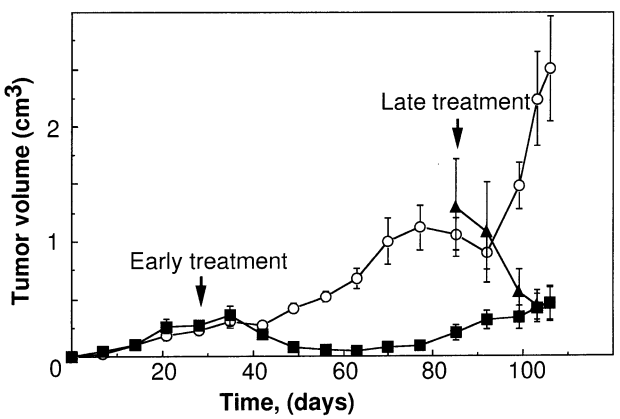

Fig. 11. R 1881 caused the regression of MOP tumors. Twenty mice received sc. MOP cells (3×10^6, in Matrigel) and were distributed randomly in two groups of ten. Mice of the group I (■) received testosterone enanthate every 10 days from day 28 after cell injection, when the tomor volume was ≈ 250 mm^3. Mice of the group II (○) received sesame oil every 10 days. From day 84 after injecting cells, when the tumor volume was ~ 1 cm^3, five mice of this group (▲) received testosterone enanthate every 10 days, the other five continued to be treated with sesame oil. Means ± S.D. Differences between the control vs. treated in each group: $P < 0.05$ at day 14 after the beginning of treatment, < 0.01 thereafter. Differences, within the group I, between the tumor volume at day 63 vs. that at day 84: $P < 0.05$ and vs. that at day 91: $P < 0.01$.

also shown to be a potent stimulator of transcription in CV1 and PC3 cells transfected with the same sequences [27,28]. The T876A mutation may explain the unusual steroid binding and some unusual biological responses of MOP cells but other partners such as the co-factors of nuclear receptors have to be taken into account to explain the cell specific responses to AR binding ligands. Although one favors the hypothesis that estradiol, CYPA, progesterone and R 5020 act through AR one has to consider the putative role of estrogen and progestin receptors. The possibility that estradiol acts in MOP cells through binding to the classical estrogen receptors may be excluded for two reasons: (a) diethyl stilbestrol known to bind estrogen receptors with a higher affinity than estradiol did not inhibit cell proliferation; and (b) the messenger RNAs of α and β estrogen receptors were not detected after RT-PCR under conditions where the AR, progestin receptors, VEGF, c myc mRNA were detected (to be published). However, as MOP cells do contain messenger RNAs for the progesterone receptor it cannot be excluded that these receptors may be involved in the biological effects of progestins and CYPA in these cells.

All the compounds tested led, at high concentrations (>100 nM) to a similar decrease of the recovered cells: 40% of control. These results suggest that these compounds share a common limiting step. The decrease in the percentage of remaining cells was dependent on the duration of culture (Fig. 2) because the number of treated cells remained practically constant while the number of control cells increased with time. These results, regardless of the ratio of control to treated cells, suggest that at the end of treatment, cells may be resistant to androgens and/or may be arrested at a point in the cell cycle which does not trigger apoptosis. Indeed, when MOP cells treated for 24 days with R 1881 (100 nM) were subsequently cultured without androgens they then grow like untreated cells in that they displayed the same responses to androgens (growth inhibition with a plateau at 40% of control cells and apoptosis) as cells not treated chronically (not shown). Furthermore, as the population of MOP cells was heterogeneous in shape under basal conditions (Fig. 7A) it is conceivable that this population also displayed heterogeneous androgen sensitivity. Since all the sublines raised after cloning of MOP cells (7/7) were shown to be inhibited by androgens it is suggested that the percentage of androgen resistant cells is low and/or that apparent resistance is indeed a kind of cell dormancy in the G0 phase, or elsewhere in the cell cycle upstream to the block induced by androgens.

Androgen deprivation and/or androgen blockade is known to induce apoptosis in the normal prostate, in some prostate carcinomas in humans and in experimental models [31,32]. In contrast it is reported here that, in culture, R 1881, CYPA and E2 induced apoptosis of MOP cells and blocked cell cycle between G0/G1 and S phases. Similar results were reported in PC-3 cells transfected with AR cDNA cloned from a human prostate library [33]. Very recently it has been reported that androgens accelerate thymocyte apoptosis [34]. Thus, the auxiliary factors which determine a positive or a negative response to androgens remain to be found.

In nude mice, testosterone delayed the take of palpable tumors at the point of injection of MOP cells. However, following the arrest of treatment, palpable tumors appeared with a delay similar to that observed in control mice. These results are in agreement with those obtained in culture in that they show that testosterone reversibly blocked the cell cycle and did not kill all the cells before they were organized in tumors. In contrast, when cells were organized in tumors, testosterone killed a large number of cells since it induced a dramatic tumor regression but the apoptotic index was not found to be modified by androgens. Two hypotheses may be proposed to explain this observation. First, apoptosis occurred but either methodological artifacts did not allow one to show it or it led quickly to necrosis. Second, apoptosis did not occur because some factors required for apoptosis might be deficient, e.g. ATP since the apoptosis is an active process [35]. The molecular mechanisms of tumor regression require further investigations. The regression of tumors under treatment with testosterone was not complete and tumors escape treatment. These results indicate that some cells were able to grow again in vivo in the presence of androgens and in the absence of androgens. The investigations into the mechanisms which are involved in the switch have not been completed yet but preliminary results indicate that tumors which escaped treatment contain cells which display an unusual response to androgens. Indeed, two cell lines established from tumors which escaped in vivo were not induced in apoptosis by R 1881 while their growth was inhibited by this compound. It would therefore seem reasonable to put forward, as a working hypothesis, that, in vivo, androgens enrich the tumors in cells whose growth is insensitive to androgens, but sensitive to stimulatory factors secreted by neighboring cells. These factors would be absent when tumors are grown in culture.

In conclusion, it is reported that androgens, and several other compounds, inhibit the proliferation of a new variant of a human prostate cancer cell line. In addition, it is demonstrated for the first time in culture but not in vivo, that androgens are able to induce apoptosis of prostate cancer cells non-transfected with AR. Although the factors responsible for the positive and negative control of cell proliferation by androgens are not yet identified it has been shown that it is unlikely that abnormalities of AR are responsible for the inhibitory effect on MOP cells. These results allow

one to suggest that some tumors may grow in castrated patients because they do not have enough androgens to inhibit or kill some of the tumor cells. In addition, these results taken with reports from others [13,14], provide a further rational for intermittent treatment of prostate cancer, i.e. intermittent androgen privation [36] and intermittent antiandrogen or estrogen administration.

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