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Inverse Relationships Between Cell Proliferation and Basal or Androgen-stimulated Apolipoprotein D Secretion in LNCaP Human Prostate Cancer Cells

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We have recently demonstrated that the biphasic action of androgens on LNCaP cell proliferation is opposite to their effect on apolipoprotein D (apo-D) secretion, the stimulation of apo-D secretion being associated with a steroid-induced inhibition of cell proliferation. To further characterize the control of apo-D expression in LNCaP cells, we studied basal as well as androgen-induced apo-D secretion in slowly proliferating, low-passage (LP; 20–29th) and rapidly proliferating high-passage (HP; 111–117th) cell cultures. For comparison, the androgen-induced stimulation of prostate specific antigen secretion was also investigated in LP and HP cell cultures. In the absence of androgens, basal cell proliferation of HP cells was significantly higher than that of LP cells, whereas apo-D secretion was higher in LP cells than in HP cells. Furthermore, the biphasic action of dihydrotestosterone and of the synthetic androgenic compound R1881 on apo-D release and cell proliferation was observed in both LP and HP cells. The stimulation of apo-D secretion was inversely related to that of cell proliferation and influenced by cell density. The inhibition of basal and androgen-induced cell proliferation by the calcium channel blocker nifedipine was also associated with an increase in apo-D secretion. The amount of PSA released and the sensitivity of its response to R1881 were increased in LP cells compared with HP cells. The present study thus demonstrates, for the first time, that apo-D secretion is inversely correlated to cell proliferation and cell density in the absence as well as in the presence of androgens in both LP and HP LNCaP human prostate cancer cells. This finding suggests that apo-D expression can be modulated not only by steroid hormones, but also by other factors involved in the control of cell proliferation.

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INTRODUCTION

Androgens are well known to play a crucial role in the regulation of cell growth and specific protein synthesis in hormone-sensitive prostate cancer [1–5], the most frequently occurring cancer in men [6]. Proteins induced by steroids in human prostate cancer cells provide potential markers of hormone action and for monitoring the response to endocrine therapy. The apolipoprotein D (apo-D) has recently been characterized as a new biochemical marker of steroid action in the LNCaP human prostatic adenocarcinoma cell line [7].

Apo-D is a glycoprotein associated mainly with high-density lipoproteins (HDL) in the plasma [9,10]. It shares no sequence similarity with other apolipoproteins but has a predicted structural homology with members of the superfamily of lipophilic–ligand carrier proteins or lipocalins [11–15]. Although the physiological role of apo-D has yet to be elucidated (see Ref.[16] for review), apo-D, together with apo-A1, lecithin:cholesterol acyltransferase (LCAT), and cholesteryl ester transfer protein (CETP) are present

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in a pre-β-HDL subclass, which is believed to be involved in the reverse cholesterol transport from peripheral tissues to the liver [17,18]. More recently, it has been demonstrated that in both HDL and plasma, the apo-D–apo-AII disulfide-linked heterodimer corresponds to the major form of apo-D [19]. However, apo-D corresponds to the major protein found in human breast cystic disease fluid, namely, gross cystic disease fluid protein-24 (GCDFP-24) [20], which was first identified for its binding specificity for progesterone and pregnenolone [21–23]. The expression of apo-D has been demonstrated in several tissues in humans and other mammals [11,14,24–29]. In LNCaP cells, the changes induced by steroids on apo-D secretion are opposite to those on cell proliferation and cell-cycle kinetic parameters, its stimulation being associated with an inhibition of cell proliferation [7,30]. Similarly, in ZR-75-1 human breast cancer cells, the modulation of apo-D gene expression by androgens, glucocorticoids, and estrogens is also inversely related to their respective action on cell proliferation [31,32]. In addition, in MCF-7 human breast cancer cells, apo-D secretion is inhibited by estrogens [31]. In breast carcinoma cytosol, the concentration of apo-D is higher in well-differentiated carcinomas than in more aggressive, poorly differentiated tumors [33]. Provost *et al.* [34] demonstrated that apo-D gene expression in fibroblasts, the major cell type expressing apo-D in normal tissues [27] increases specifically in both growth-arrested and senescent cultures [34].

In order to further understand the control of apo-D expression and its association with basal and steroid-regulated cell proliferation in hormone-sensitive cancer cells, we investigated basal and androgen-regulated apo-D secretion in relation to cell proliferation in slowly proliferating low passage (LP; 20–29th) and rapidly proliferating high-passage (HP; 111–117th) cultures of LNCaP human prostate cancer cells. LNCaP cells offer the opportunity to discriminate between positive- [1 to ~100 pM 5α-dihydrotestosterone (DHT)] and negative- (1 to 1000 nM DHT) modulated cell growth processes [30]. For comparison, regulation of the secretion of the well-recognized prostate-specific antigen (PSA) [3–5,35–37 and refs therein] was also measured under similar experimental conditions. We have also studied the effect of the antiproliferative action of the calcium channel-blocker nifedipine on apo-D secretion and on cell proliferation.

MATERIALS AND METHODS

Steroid hormones and chemicals

DHT was purchased from Steraloids (Wilton, NH), 17β-hydroxy-17α-methyl estra-4,9,11,-trien-3-one (R1881) from New England Nuclear-Dupont (Boston, MA) and nifedipine from Calbiochem (Missisauga, Ontario).

Maintenance of stock cell cultures

All media and supplements for cell culture were obtained from Sigma Chemical Co. (St Louis, MO) except fetal bovine serum (FBS), which was purchased from Hyclone (Logan, UT). The LNCaP human prostatic cancer cell line (derived from a fast-growing colony of a lymph node carcinoma) [38] was obtained from the American Type Culture Collection (Rockville, MD) at passage 19. Cells were routinely cultured in phenol red-free RPMI-1640 medium supplemented with 5% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU penicillin/ml, and 50 μg streptomycin/ml in a water-saturated atmosphere of 95% air: 5% CO_2 at 37°C. Cell cultures were used between passages 20–29 and 107–125. Cells were subcultured once a week by digestion in a solution of 0.08% pancreatin in *n*-2-hydroxyethyl-piperazine-*n*'-2-ethanesulfonic acid buffer containing 3 mM EDTA (pH 7.2).

Studies of cell proliferation

LNCaP cells harvested in their logarithmic growth phase were plated on poly-L-lysine-coated 24-well plates (2 cm^2/well) at the indicated density in RPMI-1640 medium containing 2% dextran-coated charcoal-treated FBS as described previously [7,30]. Plating density was confirmed by Coulter Counter (model ZM Coulter Electronics, Hialeah, FL). After 1 day in culture, the medium was replaced with fresh medium containing the indicated concentrations of compounds. Cells were grown for the indicated periods, with medium changes every 48 h. At the end of the incubation, the medium was carefully removed from the wells, and 150 μl of methanol was added. Plates were left to dry at room temperature. The number of cells was determined by measurement of DNA content as described previously [7,30–32].

Apo-D and PSA radioimmunoassays

The amounts of apo-D released into medium during the last 24 or 48 h of incubation were measured using duplicate 100 to 200 μl of cell culture medium, 30,000 dpm ^{125}I-labeled apo-D and rabbit anti-apo-D diluted to a final concentration of 1:60,000 in 6% normal rabbit serum in phosphate-buffered saline plus 0.05 M sodium EDTA, pH 7.1, in a final volume of 500 μl as described previously [7,31]. The amount of PSA released during the last 48 h of incubation was measured using duplicate 100 ml of cell culture medium as described previously [37]. Radioimmunoassay (RIA) data were analyzed using a program based on model II of Rodbard and Lewald [39]. Dose–response curves and EC_{50} as well as IC_{50} values were calculated using a weighted iterative nonlinear least-squares regression [40]. Each data point was obtained from duplicate radioimmunoassay measurements of triplicate well samples. Statistical significance was determined according to the multiple range test of Kramer [41].

RESULTS

To characterize androgen responsiveness as well as the growth rate of LNCaP cells from LP and HP cultures, we first studied the time course of DHT action on cell proliferation. As illustrated in Fig. 1(A–C), basal cell proliferation was significantly higher in HP cells than in LP cells, as indicated by the 1.6-, 2.5-, and 3.1-fold higher ($P<0.01$) amounts of DNA per well after 4, 6, and 8 days of incubation, respectively. It can also be seen in Fig. 1(A–C) that incubation with increasing concentrations of DHT showed a biphasic effect on cell proliferation in both LP and HP LNCaP cells. For example, an 8-day exposure to 0.05 nM DHT caused a maximal 1.8- and 2.0-fold increase ($P < 0.01$) in cell proliferation in LP and HP cells, respectively, whereas incubation with higher concentrations of DHT led to a progressive inhibition of cell growth, inducing a further 40% decrease in DNA/well below the level measured in both LP and HP control cells [Fig. 1(C)].

We then measured the modulation of apo-D secretion under the same experimental conditions. As illustrated in Fig. 1(D–F), the amount of apo-D released in the absence of androgen during the last 24 h of incubation in HP cells was 50, 55, and 40% lower ($P<0.01$) than that measured in LP cells after 4, 6, and 8 days in culture, respectively. It can also be seen in Fig. 1(D–F) that the sensitivity of the apo-D response was approximately one order of magnitude higher in LP cells than in HP cells.

To further understand the inverse relationship between cell proliferation and modulation of apo-D secretion by DHT, we next investigated the effect of a 10-day incubation with increasing concentrations of DHT in both LP and HP cells plated at four different initial densities (Fig. 2). As illustrated in Fig. 2(B and D), cell density had an influence on basal apo-D secretion in both LP and HP cells. For example, the amount of apo-D released per microgram of DNA in HP cells was 8.3-, 5.0- and 2.2-fold higher after plating at an initial density of 5000, 10,000, and 25,000 cells per well, respectively, compared with 50,000 cells per well [Fig. 2(D)]. Similar data were obtained in LP cells [Fig. 2(B)].

Exposure to increasing concentrations of DHT led to the biphasic pattern of cell proliferation at all plating densities, although the concentrations of DHT required

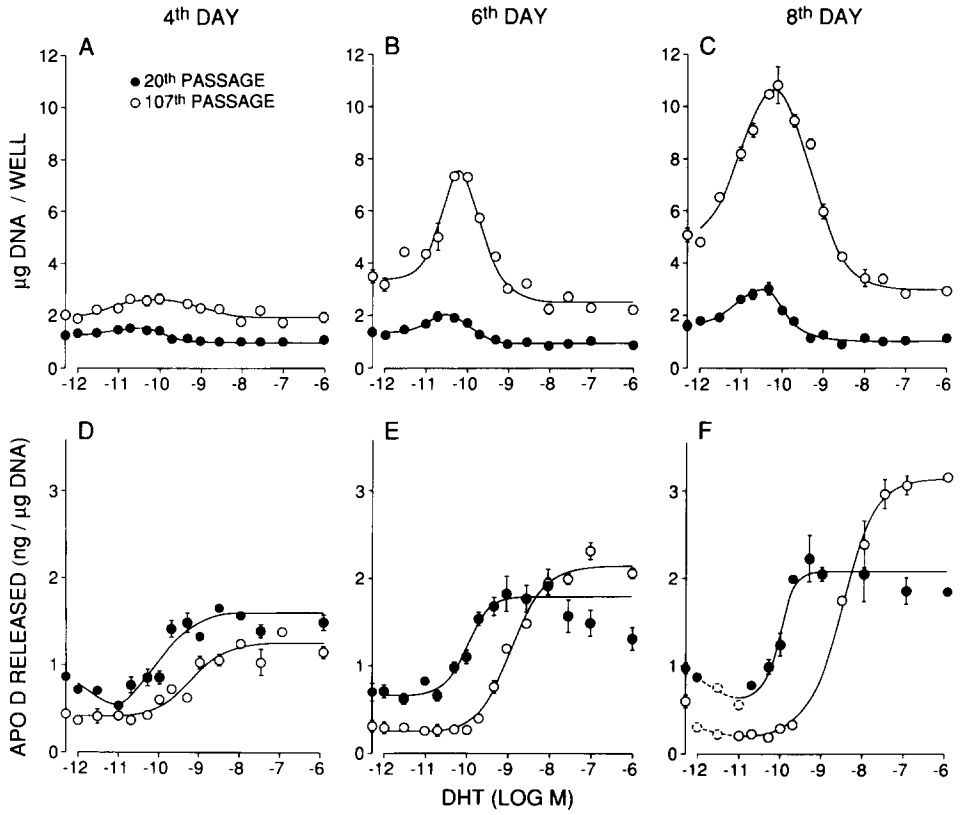

Fig. 1. Time course of the effect of increasing concentrations of DHT on cell proliferation (A–C) and apo-D secretion (D–F) in LNCaP human prostate cancer cells from LP and HP cell cultures. One day after plating at a density of 10×10^3 cells/well, the cells were incubated for specified times with the indicated concentrations of DHT. Medium was changed every second day. At the end of the incubation period, cell number was determined by measurement of DNA content. Apo-D release during the last 24 h of incubation was measured by RIA. Data are expressed as the mean ± SEM of triplicate wells. When SEM overlaps with the symbol used, only the symbol is shown. When the amount of apo-D was below the limit of detection of the RIA, the limit detection value was divided by micrograms of DNA per well and is illustrated as a dotted symbol. Data obtained in the absence of the steroid are indicated on the Y axis.

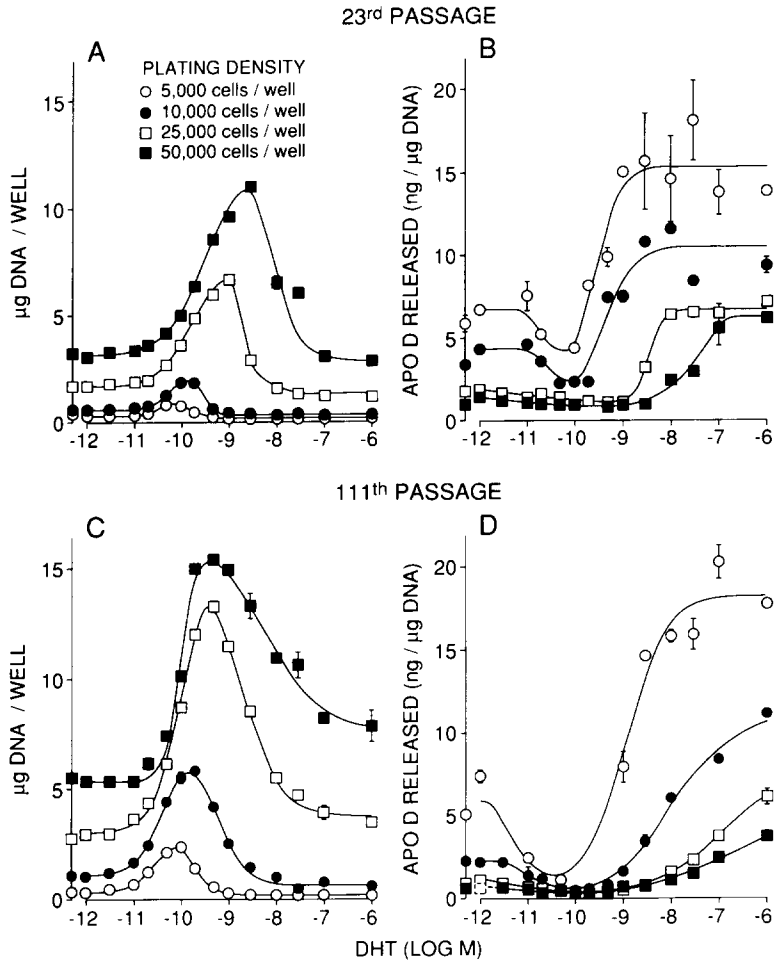

Fig. 2. Effect of increasing concentrations of DHT on cell proliferation (A, C) and apo-D secretion (B, D) in LNCaP human prostate cancer cells from LP and HP cell cultures. One day after plating at a density of 5, 10, 25, and 50×10³ cells/well, the cells were incubated for 10 days with the indicated concentrations of DHT. Medium was changed every second day. At the end of the incubation period, cell number was determined by measurement of DNA content. Apo-D release during the last 48 h of incubation was measured by RIA. Data are expressed as the mean ± SEM of triplicate wells. When SEM overlaps with the symbol used, only the symbol is shown. When the amount of apo-D was below the limit of detection of the RIA, the limit detection value was divided by micrograms of DNA per well and is illustrated as a dotted symbol. Data obtained in the absence of the steroid are indicated on the Y axis.

to cause a stimulation as well as an inhibition of cell proliferation increased with cell density (Fig. 2). Furthermore, the sensitivity of the DHT-induced stimulation of apo-D secretion was also inversely related to cell density, the stimulatory effect being measured at EC_{50} values of 0.3, 0.5, 3.6, and 18 nM after plating at an initial density of 5000, 10,000, 25,000, and 50,000 LP cells per well, respectively [Fig. 2(B)]. Similar data were also found in HP cells where the EC_{50} values of the apo-D response shifted from 1.3 to 52 nM by increasing cell density [Fig. 2(D)].

To investigate whether such a decrease in sensitivity to DHT as a function of cell density could be explained by a higher rate of metabolism of DHT [2,42], we studied the effect of increasing concentrations of the highly stable synthetic androgenic compound R1881 (methyltrienolone) [2,43,44] on cell proliferation and apo-D secretion in LP and HP cells after plating at an initial density of 25,000 or 75,000 cells/well (Fig. 3). As illustrated in Fig. 3(A and B), basal cell proliferation of HP cells was 7.6-fold higher compared with that of LP cells 10 days after plating at an initial density of 25,000 cells per well, the amount of apo-D released per microgram of DNA being 4.2-fold higher in LP cells than in HP cells. Similar differences were obtained after plating at an initial density of 75,000 cells [Fig. 3(C and D)]. It can be seen that exposure to R1881 caused a maximal stimulation of cell proliferation in LP and HP cells at the same concentration of the steroid (0.05 nM) at both plating densities. As seen with DHT, exposure to higher concentrations of R1881 progressively decreased cell proliferation, the inhibitory effect being exerted at IC_{50} values of 0.09 and 0.12 nM in LP cells after plating at 25,000 and 75,000, respectively, whereas the stimulatory effect of R1881 on apo-D secretion was exerted at respective EC_{50} values of 0.08 and 0.11 nM

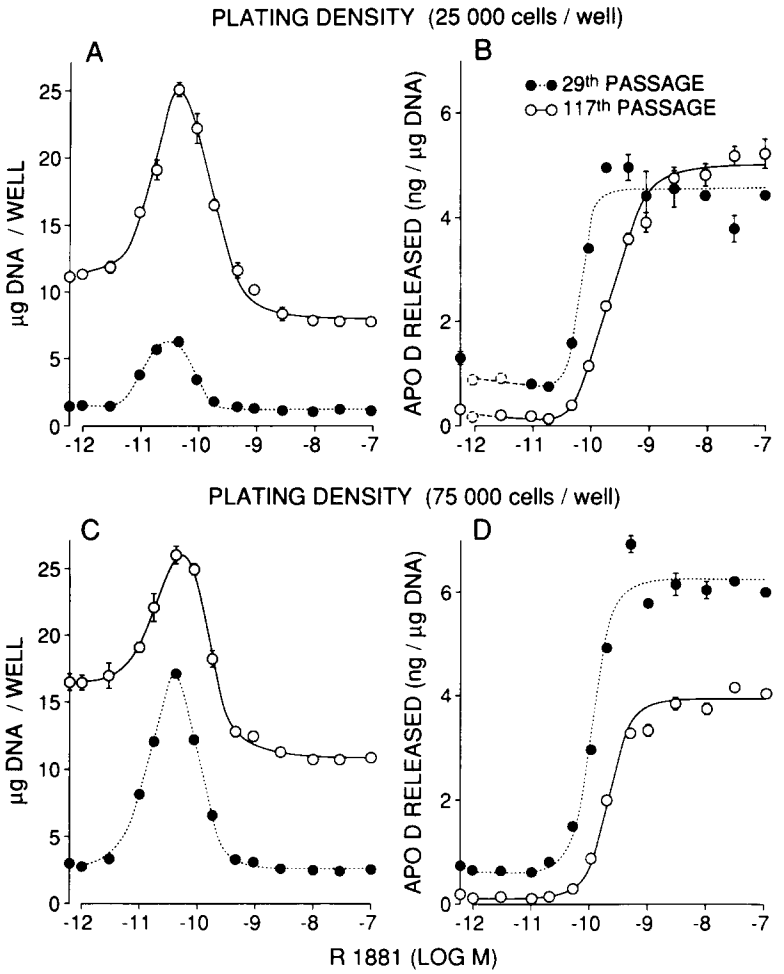

Fig. 3. Effect of increasing concentrations of the synthetic androgen R1881 on cell proliferation (A, C) and apo-D secretion (B, D) in LNCaP human prostate cancer cells from LP and HP cell cultures. One day after plating at a density of 25×10^3 (A, B) or 75×10^3 (C, D) cells/well, the cells were incubated for 10 days with the indicated concentrations of R1881. Medium was changed every second day. At the end of the incubation period, cell number was determined by measurement of DNA content. Apo-D release during the last 48 h of incubation was measured by RIA. Data are expressed as the mean ± SEM of triplicate wells. When SEM overlaps with the symbol used, only the symbol is shown. When the amount of apo-D was below the limit of detection of the RIA, the limit detection value was divided by micrograms of DNA per well and is illustrated as a dotted symbol. Data obtained in the absence of the steroid are indicated on the Y axis.

[Fig. 3(B and D)]. Moreover, the sensitivity of the apo-D response to R1881 in HP cells was also superimposable to that on cell proliferation; the action on apo-D secretion was observed at EC_{50} values of 0.23 and 0.22 nM after plating at 25,000 and 75,000 cells, respectively, whereas the inhibitory effect of this compound on cell proliferation was observed at the respective IC_{50} values of 0.19 and 0.18 nM. These results strongly suggest that the changes in the function of cell density after incubation with DHT are likely due to its more rapid metabolism.

We then compared the effect of R1881 on the secretion of the well-recognized prostatic tumor marker PSA and cell proliferation in both LP and HP cells. As illustrated in Fig. 4, a 7-day exposure of LP cells to R1881 increased PSA release by 110-fold, this effect being exerted at an EC_{50} value of 0.035 nM. The basal PSA release in HP cells was 2.4-fold ($P<0.01$) lower than that in LP cells, while R1881 caused a 68-fold increase of the value of this parameter at an EC_{50} value of 0.221 nM (Fig. 4).

Finally, we studied the effect of the calcium channel blocker nifedipine (10 μM), a compound described as an inhibitor of cell growth [45–47]. An 8-day incubation with 10 μM nifedipine decreased basal cell proliferation by 33%, whereas the same treatment decreased the amplitude of the maximal DHT-induced stimulation of cell proliferation by 40% [Fig. 5(A)]. Moreover, in the presence of nifedipine, the inhibitory effect of DHT on cell proliferation was exerted at an IC_{50} value of 0.4 nM compared with 1.4 nM in its absence [Fig. 5(A)]. As illustrated in Fig. 5(B), exposure to nifepidine increased basal apo-D release by 2.0-fold. While the sensitivity of the apo-D response to DHT was superimposable to that observed on cell proliferation, the stimulatory effect

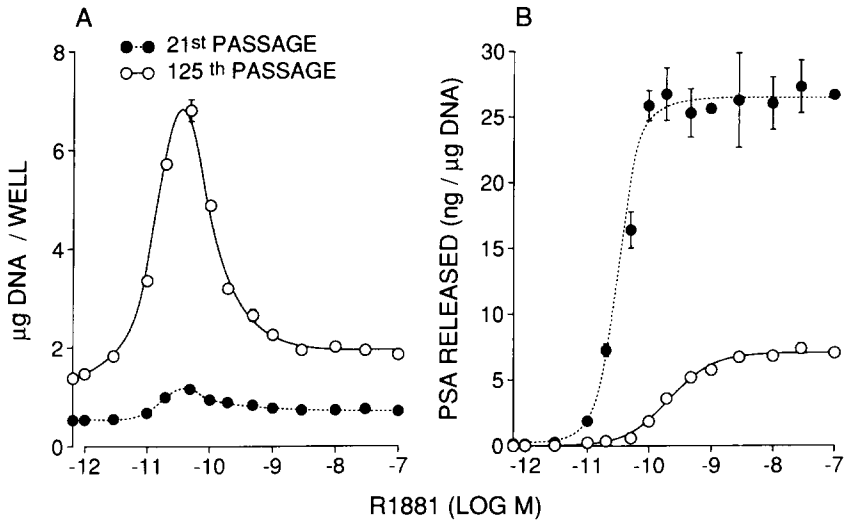

Fig. 4. Effect of increasing concentrations of the synthetic androgen R1881 on cell proliferation (A) and PSA secretion (B) in LNCaP human prostate cancer cells from LP and HP cell cultures. One day after plating at a density of 20×10^3 cells/well, the cells were incubated for 7 days with the indicated concentrations of R1881. Medium was changed every second day. At the end of the incubation period, cell number was determined by measurement of DNA content. PSA release during the last 48 h of incubation was measured by RIA. Data are expressed as the mean ± SEM of triplicate wells. When SEM overlaps with the symbol used, only the symbol is shown. Data obtained in the absence of the steroid are indicated on the Y axis.

being exerted at EC_{50} values of 0.4 and 1.22 nM in the presence and absence of nifedipine, respectively.

DISCUSSION

The present study demonstrates for the first time that LNCaP human prostate cancer cell apo-D secretion, in the absence of steroid hormones, is inversely correlated with the basal cell proliferation rate. This conclusion follows the observation of a higher amount of apo-D released per cell in (1) slowly proliferating LP cells compared with rapidly proliferating HP cells; (2) lower cell density cultures in both LP and HP cells; and (3) effect of the calcium channel blocker nifedipine which reduces basal cell proliferation rate. These findings strongly suggest that apo-D expression in LNCaP cells can be modulated not only by steroid hormones, but also by other factor(s) involved in the control of cell proliferation. In agreement with this conclusion, it has

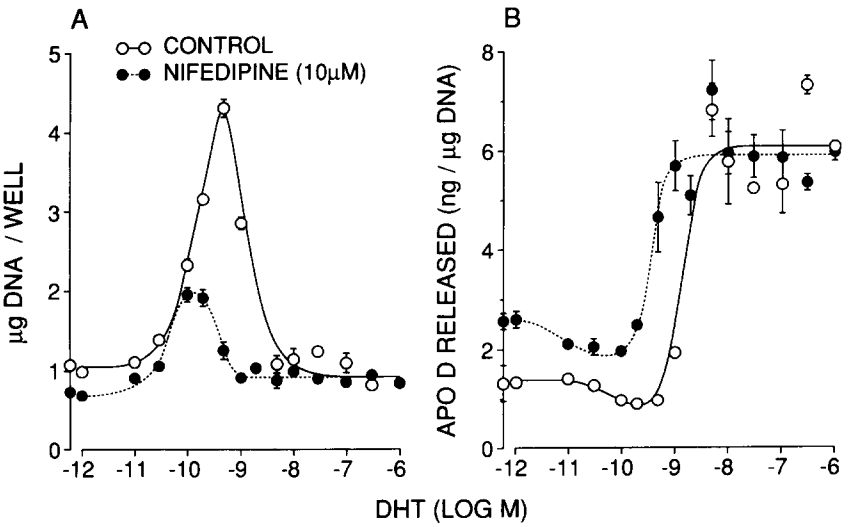

Fig. 5. Effect of increasing concentrations of DHT in the absence or presence of the calcium channel blocker nifedipine (10 µM) on cell proliferation (A) and apo-D secretion (B) in LNCaP human prostate cancer cells. One day after plating at a density of 20×10^3 cells/well, the cells were incubated for 8 days with the indicated concentrations of DHT. Medium was changed every second day. At the end of the incubation period, cell number was determined by measurement of DNA content. Apo-D release during the last 48 h of incubation was measured by RIA. Data are expressed as the mean ± SEM of triplicate wells. When SEM overlaps with the symbol, only the symbol is shown. Data obtained in the absence of the steroid are indicated on the Y axis.

been shown that in early passage cultured human diploid fibroblasts, no apo-D mRNA is detected in replicating cells in sparse culture, while the gene is expressed in quiescent cells in confluent and serum-starved cultures; however, at late-passages, fibroblasts did express apo-D mRNA in sparse culture, but the level increased at confluence [34]. These findings also suggest that apo-D expression could be a characteristic of growth arrest, at least under some conditions.

The present study clearly shows that the stimulation of apo-D secretion coincides with the androgen-induced inhibition of cell proliferation in both LP and HP LNCaP cells, thus extending our initial observation in LP LNCaP cells [7]. Analysis of cell kinetic parameters of LNCaP cells has shown that high concentrations of DHT cause an increase in the G_0–G_1 fraction at the expense of the S and especially the G_2+M fractions [30]. High concentrations of DHT (100 nM), thus decrease the percent of cycling LNCaP cells, probably by blocking the cells from entering into the S phase [30]. This latter study revealed that control plates and plates treated with high concentrations of DHT contained approximately the same number of cells, but the cells had different cell kinetic patterns. Furthermore, high concentrations of androgens have been shown to cause a marked inhibition of colony formation in soft agar and down-regulated c-*myc* RNA levels in LNCaP cells [48]. It is thus possible that the stimulatory effect of androgens on apo-D secretion is mediated through an indirect mechanism associated with the blockade of cells into G_0/G_1 phases. This hypothesis is also in agreement with the data on apo-D expression in human fibroblasts in culture, as mentioned above [34].

The association of increased apo-D gene expression and steroid-induced inhibition of cell proliferation was also observed in human breast cancer cells [31,32]. In fact, in ZR-75-1 human breast cancer cells, the potent antiproliferative effect of DHT and of the synthetic glucocorticoid dexamethasone was coupled with a marked stimulation of apo-D expression, whereas the mitogenic action of 17β-estradiol coincided with an inhibition of apo-D expression. The inhibitory effect of 17β-estradiol on apo-D secretion was also observed in MCF-7 cells [31]. However, in the T-47D human breast cancer cell line, 17β-estradiol failed to regulate both cell proliferation and apo-D secretion [49], a finding which could be explained by the presence of phenol red in the culture medium and/or by the presence of a mutant estrogen receptor in this cell line [50].

In LNCaP cells, DHT is rapidly metabolized in conjugated and nonconjugated metabolites [2,42,51]. At higher cell densities, the degradation rate of DHT was probably more rapid, thus explaining the shift to higher concentrations in the EC_{50} values of the DHT-induced stimulatory effect on apo-D secretion as well as the biphasic pattern of DHT action on cell proliferation (Fig. 2). This hypothesis is well supported by the results obtained with the stable synthetic androgenic compound R1881 showing an identical sensitivity to R1881 at both cell densities tested for cell proliferation and apo-D response (Fig. 3). This experiment also clearly demonstrated that the sensitivity of the R1881-induced stimulation of apo-D secretion was superimposable to its inhibitory effect on cell proliferation in both LP and HP cells.

The present findings also demonstrate that after more than 2 years in culture, the HP LNCaP cells remain responsive to androgens on the three parameters tested, namely cell proliferation and the secretion of apo-D as well as PSA. As observed for apo-D, PSA secretion was higher in LP cells compared with HP cells, in both the presence or absence of androgen. The androgenic induction of PSA secretion in LNCaP cells is in agreement with previous reports [4,48,8].

In conclusion, apo-D secretion is inversely correlated with cell proliferation and cell density in the absence as well as in the presence of androgens in both LP and HP LNCaP cells. The present study thus strongly suggests that, in addition to steroid hormones, other factor(s) modulating cell proliferation are involved in the regulation of apo-D expression.

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