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Apoptosis-related Gene Expression Affected by a GnRH Analogue Without Induction of Programmed Cell Death in LNCaP Cells

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Abstract. *Background: In this study we confirmed the ability of a Gonadotropin Releasing Hormone (GnRH) agonist, leuprorelin acetate (LA), to counteract or even suppress the 5α-dihydrotestosterone (DHT)-stimulated growth of androgen-sensitive prostate cancer cells (LNCaP). Since the cellular mechanisms mediating this effect are not well defined, we investigated the activity of LA, also in combination with DHT or with cyproterone acetate (CA), on the expression of genes (bcl-2, bax, c-myc) which may contribute to the proliferative behaviour of LNCaP cells. In addition, experiments aimed to evaluate the action of the analogue on apoptosis were performed. Materials and Methods: Gene expression was evaluated by RT-PCR and Western blotting on cells treated with LA (10^{-11} or 10^{-6} M), alone or combined with 10^{-9} M DHT or 10^{-7} M CA. The occurrence of apoptosis following treatment with LA (10^{-11}, 10^{-6} or 10^{-5} M), alone or combined with 10^{-9} M DHT, was assessed by DNA fragmentation analysis. Results: Both the mRNA and protein of the anti-apoptotic gene bcl-2 were induced (30-125%) by DHT after 24-144 h. LA decreased bcl-2 mRNA (10-40%), while it did not unequivocally affect protein expression. The analogue always reduced (13-74%) both mRNA and protein levels obtained under DHT treatment. The mRNA and protein of the pro-apoptotic gene bax were down-regulated by DHT (15-40%), while LA generally induced bax protein but not its mRNA. LA counteracted DHT activity, even increasing bax protein levels over the controls. c-myc mRNA and protein were enhanced by DHT (15-45%) but down-regulated by LA (10-40%). Once more, the androgen effect was antagonized by LA, sometimes reducing c-myc content below the controls. CA produced the most similar effects to those triggered by DHT. The hormonal treatment did not induce any DNA fragmentation. Conclusion: In spite of gene modulation, apoptosis was not observed under LA treatment, in agreement with the lack of a cell growth effect when the analogue was used alone. Nevertheless, the observed changes in gene expression may be directly or indirectly involved in the antiproliferative effect of LA on androgen-stimulated cells.*

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It is now well established that Gonadotropin Releasing Hormone (GnRH) affects a variety of normal and malignant human extrapituitary tissues (1, 2). A series of studies showed that GnRH and GnRH analogues may act as negative regulators of cell growth inhibiting proliferation of breast, endometrial, ovarian and prostate cancer cell lines which express GnRH mRNA, GnRH immuno- and bio-activity as well as GnRH receptor mRNA and protein (3-10). In contrast, some authors failed to detect any direct antitumour effects of GnRH analogues or observed them only at very high concentrations of these compounds (11-14). The discrepancies might be explained on the basis of the different culture and experimental conditions, types of GnRH analogues used or differences in cell lines studied.

In our experience, GnRH analogues are ineffective in regulating cell growth when used alone, but they counteract or suppress the stimulation of cell proliferation induced by estrogens in mammary and endometrial cancer cells (15-17) and by androgens in prostatic cancer cells (18). Moreover, GnRH analogues halt the increase in cell growth determined by Epidermal Growth Factor in androgen-insensitive PC-3 prostate cancer cells (18).

The mechanism by which GnRH analogues inhibit tumour cell proliferation *per se* or affect hormone-induced cell growth is not clear. Some data from literature suggest that

analogues have a cytostatic action. In fact, flow cytometric DNA analysis of the cell cycle has demonstrated that the agonist buserelin causes a marked increase in the percentage of mammary cancer cells in the G0-G1 resting phase of the cell cycle and a corresponding decrease in the percentage of cells in both the S- and G2M-phase of the cell cycle (19).

On the other hand, both GnRH agonists and antagonists have been reported to induce apoptosis in human uterine leiomyoma, human endometrial and ovarian cancer cells, and in porcine and human ovary (20-23). In addition, these compounds have been shown to inhibit proliferation and enhance apoptotic death of human prostate carcinoma PC-82 transplanted in male nude mice (24) and to induce apoptosis in Dunning R-3327 hormone-dependent prostate tumours (25). To the contrary, Gruendker *et al.* reported that GnRH protects ovarian cancer cells from programmed cell death (26).

In the present paper, we investigated the basal expression of some genes related to apoptosis (bcl-2, bax and c-myc) in androgen-sensitive prostate cancer cells (LNCaP) and also the variation in gene expression induced by exposure of the cells to different treatment, including the GnRH analogue, leuprorelin acetate (LA), alone or in association with 5α-dihydrotestosterone (DHT) or cyproterone acetate (CA). In parallel, proliferation experiments using LA, DHT and CA were performed and the effect of the GnRH analogue on apoptosis was studied.

Materials and Methods

Hormones. The GnRH analogue, [*D*-Leu^{6}-(des-Gly^{10}-NH$_2$)]LH-RH ethylamide (leuprorelin acetate, LA), was kindly donated by Takeda Italia Farmaceutici SpA, Roma, Italy. It was dissolved in saline solution and stored at 4˚C. 5α-dihydrotestosterone (DHT) and the synthetic steroidal anti-androgen, cyproterone acetate (CA), were purchased from Sigma-Aldrich (St. Louis, MO, USA). They were dissolved in absolute ethanol and stored at 4˚C.

Antibodies. Mouse monoclonal antibody (mAb) to human bcl-2 (clone 124) was from DAKO (Glostrup, Denmark); mouse mAb to human bax (B-9) and mouse mAb to human c-myc (C-33) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); mouse mAb to β-actin (clone AC-15) was from Sigma-Aldrich. The secondary Ab, horseradish peroxidase (HRP)-labeled goat anti-mouse IgG, was obtained from Vector Laboratories (Burlingame, CA, USA).

Cell lines and culture conditions. The hormone-sensitive human prostate carcinoma cell line LNCaP was kindly provided by Professor E. Petrangeli (Institute of Biomedical Technology, CNR, Rome, Italy) and used between passages 43 and 51. The cells were cultured in RPMI-1640 medium (EUROBIO, Les Ulis Cedex B, France) supplemented with 10% (v/v) fetal bovine serum (FBS, ICN Biomedicals, Costa Mesa, CA, USA), antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin, EUROBIO), 2 mM glutamine (EUROBIO), 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES buffer, EUROBIO) and 2.5 μg/ml amphotericin B (IS_{TM} Irvine Scientific, Santa Ana, CA, USA).

Jurkat cells, derived from human acute T-cell leukaemia, were included in the study as a positive control for bcl-2, bax and c-myc protein expression. They were obtained from the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia (Brescia, Italy), used at passage 21 and maintained in RPMI-1640 medium supplemented with 10% (v/v) FBS (ICN Biomedicals), antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin, EUROBIO), 2 mM glutamine (EUROBIO) and 10 mM HEPES buffer (EUROBIO).

HL-60 cells, derived from human acute promyelocytic leukaemia, were kindly provided by Dr. E. Vigneti (Biologia Cellulare, CNR, Rome, Italy) and used in the DNA fragmentation assay as control of the ladder pattern. Cells were maintained in RPMI 1640 supplemented with 10% (v/v) FBS (ICN Biomedicals), antibiotics (EUROBIO), 2 mM glutamine (EUROBIO) and 10 mM HEPES buffer (EUROBIO). All the cell lines were subcultured weekly and maintained in humidified air: CO_2 atmosphere (95%:5%) at 37˚C.

Cell proliferation studies. Proliferation of LNCaP cells was tested with various concentrations of DHT, CA and LA, alone or in combination. In all cell growth experiments, LNCaP cells were plated out at a density of 50,000 cells/ml of standard culture medium in 60-mm plastic plates. The cells were allowed to adhere and, 48 h after plating, the seeding medium was changed with fresh RPMI-1640 supplemented with 5% charcoal-treated FBS (CH-FBS) and the different agents, alone or in combination. Medium without hormones and with DHT or CA was changed every 48 h and LA was added daily to the cells.

In the experiments performed in order to assess the sensitivity of LNCaP cells to the single agents, DHT, CA or LA were used at concentrations ranging from 10^{-11} M to 10^{-5} M. In the combination experiments, aimed to establish the effect of LA on the action of DHT and/or CA, cells were treated with 10^{-9} M DHT or 10^{-7} M CA, either in the absence or in the presence of the same concentrations of LA tested in the experiments in which the analogue was used alone.

In all the culture plates the final ethanol concentration was 0.1%. After 48, 96, 144 and 192 h of treatment, cells were harvested by trypsinization and counted using a hemocytometer. Triplicate cultures were set up for each drug concentration and control dishes (cells without hormones) were run in parallel. Cell viability was assessed by trypan blue dye exclusion.

The evaluation of bcl-2, bax and c-myc expression by RT-PCR and Western blot analysis, based on the results from the above growth experiments, was performed using the same culture conditions described for the proliferation assays. Cells were exposed to the following hormones: 10^{-9} M DHT, 10^{-7} M CA, 10^{-11} M and 10^{-6} M LA. LA, at the indicated concentrations, was also used in combination with 10^{-9} M DHT or 10^{-7} M CA. The treatment was stopped after 24, 48, 96 and 144 h by removing the culture medium.

RT-PCR analysis. At each of the above treatment times, LNCaP cells were trypsinized, washed with phosphate-buffered saline (PBS) and centrifuged. Cell pellets were snap-frozen in liquid nitrogen and stored at -80˚C until used. Total RNA from LNCaP cells was extracted using the acid guanidinium thiocyanate-phenol-chloroform technique (27).

An aliquot of 1 μg of total RNA was reverse transcribed to complementary DNA (cDNA) and then used in the PCR-based assay. Briefly, cDNA synthesis was performed with 200 U Moloney

murine leukaemia virus reverse transcriptase (M-MLV RT, Gibco, Life Technologies, Paisley, UK) primed with 2.5 μM random hexamers (Gibco, Life Technologies) in a 20 μl volume containing a final concentration of 10 mM dithiothreitol, 10 U RNasin ribonuclease inhibitor (Promega, Madison, WI, USA), 1 mM of each dNTP and 1x RT buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$). The samples were incubated at 45˚C for 45 min and then at 72˚C for 10 min. The product of the RT reaction (1/10) was used for amplification by PCR in a total volume of 25 μl with a final concentration of 1x Gold buffer (Perkin Elmer, Norwalk, CT, USA), 1.5 mM $MgCl_2$, 100 μM of each dNTPs, 10 pmol of each specific primer and 1.5 U of Taq polymerase (AmpliTaq Gold, Perkin Elmer). Analysis of the mRNA of bcl-2, bax and c-myc was carried out by PCR amplification of fragments of 459 bp, 323 bp and 217 bp, respectively, using the following primers: bcl-2: 5'-GGTGCCACCTGTGGTCCACCTG-3' and 5'-CTTCACTTGTG GCCCAGATAGG-3', bax: 5'-ATGGACGGTC CGGGGAGCGC-3' and 5'-CCCCAGTTGAAGTTGCCGTC AG-3', c-myc: 5'-CAA GAGGCGAACACACAACGTC-3' and 5'-CTGTTCTCGTCGTT TCCGCAAC-3'. The cDNA of the housekeeping gene aldolase was used as internal control for the amount of mRNA and for its integrity and coamplified, in the same reaction tube, with each cDNA using 1 pmol of primers Ald 1 (5'-CGCAGAAGGGGTC CTGGTGA-3'), Ald 2 (5'-CAGCTCC TTCTTCTGCTCCGGGG-3') and Ald 3 (5'-GGTTCTCC TCGGTGTTCTCG-3'). The Ald 1/Ald 2 primer pair was designed to amplify a 181 bp fragment in coamplification with bcl-2 and bax, while the Ald 1/Ald 3 couple, designed to amplify a longer (300 bp) region of aldolase cDNA, was used in the coamplification with c-myc. An initial denaturation at 94˚C for 7 min was followed by 28 cycles of PCR (each cycle: 94˚C for 45 sec, 60˚C for 45 sec and 72˚C for 45 sec) and a final extension at 72˚C for 7 min.

Each PCR reaction product was separated on a 1.8% agarose gel and visualized by ethidium bromide staining, and the relative intensity against the respective aldolase was measured by a densitometer. The ratio between the values of treated and control cells was calculated, with the controls set as 1.

Western blot analysis. At the end of each period of treatment described above, LNCaP cells in 100-mm dishes were placed on ice, washed twice with ice-cold PBS, detached using a cell scraper, pelleted at 1,200 rpm for 5 min and lysed in RIPA buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% sodium dodecyl sulphate, SDS, 1% Nonidet P40, 0.5% sodium deoxycholate, 100 μg/ml phenylmethylsulfonylfluoride, 1 mM Na_3VO_4, 30 μg/ml aprotinin). Lysates were kept on ice for 30 min and then cleared at 10,000 g for 10 min at 4˚C. The total protein concentration of the supernatants was determined by a modification of the Lowry method (28). Jurkat cells, used as positive control for the expression of all the protein studied, were lysed as described previously (29). Samples were equalized to 40 μg/lane and run on a 12% (for bcl-2 and bax analysis) or 8% (for c-myc analysis) polyacrylamide gel. Proteins were then electroblotted onto a polyvinylidene difluoride membrane (Immobilon P, Millipore, Bedford, MA, USA) which was probed (1 h, room temperature, r.t.) with the following primary Abs in PBS containing 0.02% Tween 20 (PBS-T) and 1% casein (blocking buffer): anti-bcl-2 mAb, 1:1,500; anti-bax mAb, 1:150, anti-c-myc mAb, 1:300. After four washes in PBS-T, the blot was overlaid with the HRP-labelled secondary Ab diluted 1:1,000 for bcl-2 and c-myc analysis, 1:1,800 for bax analysis, for 40 min, at r.t. in blocking buffer. At the end of the incubation time, the blot was washed four times in PBS-T. The protein bands were detected using an enhanced chemiluminescence system (ECL, Amersham, Buckinghamshire, UK) and visualised on Hyperfilm ECL (Amersham). The membrane was stripped using the standard method and rehybridized (using the same procedures described above) with an anti β-actin mAb (1:5,000) used as an internal control for protein loading. The signals were quantitated by densitometric scanning (Chemi Doc Documentation System/Quantity One quantitation software, Bio-Rad Laboratories, Hercules, CA, USA). Densitometric units of the protein of interest were then corrected for the densitometric units of β-actin. The specific protein/β-actin ratio from each treated sample was then divided by the value obtained under control conditions to obtain the fold enhancement or reduction of the protein.

DNA fragmentation assay. The effect of LA (10^{-11}, 10^{-6} M and 10^{-5} M), alone or combined with 10^{-9} M DHT, on DNA integrity of LNCaP cells was also evaluated. Cells, plated out at an initial density of 150,000 cells/ml of standard culture medium in 100-mm plastic plates, were allowed to reach subconfluence and then exposed to the above drugs for 96 and 144 h. At the end of the treatment, cells were harvested to carry out the DNA fragmentation assay. As control of the ladder pattern, HL-60 cells treated for 12 h with 10 μM etoposide, a chemotherapeutic agent, were used.

DNA, released from LNCaP and HL-60 cells, was extracted and separated by electrophoresis in agarose, as described by Kravtsov *et al.* (30).

Statistical analysis. In all the experiments, the significance of the difference between two groups was determined by an unpaired two-tailed Student's *t*-test.

Results

Effect of hormone treatment on the growth of LNCaP cells. Data obtained from the experiments in which the androgen-sensitivity of LNCaP cells was studied by DHT (10^{-11}-10^{-5} M) treatment confirmed our previous results and those of other authors, concerning the biphasic action of androgens (18, 31). In our preceding study (18), the DHT-induced proliferation occurred at notably low androgen doses (from 10^{-11} to 10^{-9} M). In the present experiments, a significant ($p<0.01$, Student's *t*-test) stimulation of cell growth was evident starting from the concentration of 10^{-10} M DHT and persisting at concentrations ranging from 10^{-9} to 10^{-7} M. This may be justified by the different culture conditions and the higher cell passages used. Higher doses of the androgen determined a diminution in cell numbers below the values of the controls. It is important to note that 10^{-9} M DHT was the lowest concentration that gave the highest enhancement of cell proliferation at all times tested. This stimulation reached the maximum (about 50%, compared with the control, $p<0.01$) after 144 and 192 h of treatment (not shown). For this reason the concentration of 10^{-9} M, which is close to the dissociation constant (Kd) value of the androgen receptor, was used in the experiments aimed at evaluating whether the androgen-induced cell proliferation could be influenced by LA.

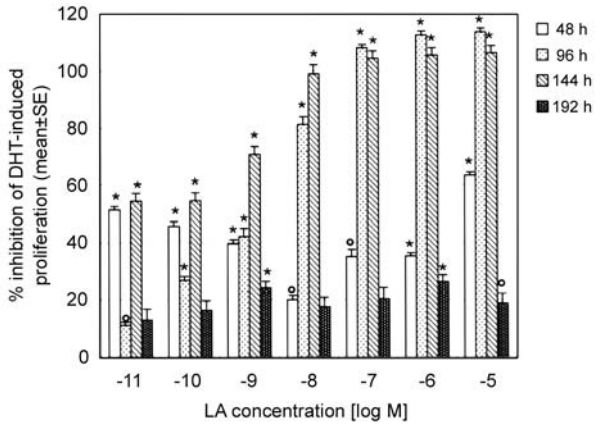

Figure 1. *Effect of increasing concentrations of LA on the DHT-stimulated growth of LNCaP cells after 48, 96, 144 and 192 h. Each column represents the mean±SE (n=6) of the data obtained from two independent experiments run in triplicate. °p<0.05 versus DHT alone, Student's t-test; *p<0.01 versus DHT alone, Student's t-test.*

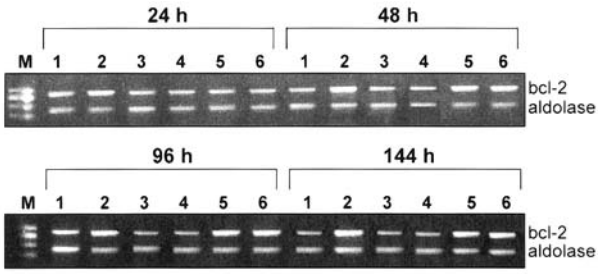

Figure 2. *RT-PCR analysis of bcl-2 mRNA in LNCaP cells treated with hormones for 24-144 h. Total RNA was extracted from the cells and then reverse-transcribed by specific priming to single-stranded cDNA. The cDNA was amplified by PCR as described in "Materials and Methods". PCR products were electrophoresed on a 1.8% agarose gel, stained with ethidium bromide, visualized under a UV illuminator and the relative intensity against aldolase was measured by a densitometer. A band of mRNA for bcl-2 was detected at the expected position (459 bp) in untreated cells (lane 1), cells treated with 10^{-9} M DHT (lane 2), 10^{-6} M LA (lane 3), 10^{-11} M LA (lane 4), 10^{-6} M LA plus 10^{-9} M DHT (lane 5), 10^{-11} M LA plus 10^{-9} M DHT (lane 6). A molecular weight marker (Puc Mix Marker 8, MBI Ferments, Vilnius, Lithuania) is present in lane M. One experiment representative of two is reported.*

Table I. *Bcl-2 gene expression in LNCaP cells treated with hormones as determined by RT-PCR.*

	24*	48	96	144
Control	1	1	1	1
DHT, 10^{-9} M	1.45±0.04[a]	1.65±0.07[b]	1.85±0.07[b]	1.95±0.07[b]
LA, 10^{-6} M	0.90±0.03[a]	0.85±0.04[a]	0.75±0.07[a]	0.60±0.07[a]
LA, 10^{-11} M	0.75±0.07[a]	0.80±0.03[a]	0.90±0.03[a]	0.75±0.07[a]
LA, 10^{-6} M + DHTτ	1.15±0.04[a,c]	1.20±0.03[a,c]	1.55±0.07[b,c]	1.50±0.07[a,c]
LA, 10^{-11} M + DHT	1.20±0.03[a,c]	1.35±0.04[b,c]	1.50±0.03[b,c]	1.70±0.03[b,c]

τ10^{-9}M DHT was used in association with LA.
The intensity of the bands was quantified by densitometric analysis and normalized to the coamplified aldolase cDNA fragment.
The numbers represent the ratio between values of treated samples and controls (set at 1).
Data shown as mean±SE (n=4) of two independent experiments, each performed in duplicates.
[a] $p<0.05$ and [b] $p<0.01$ *versus* control (Student's *t*-test); [c] $p<0.05$, *versus* DHT-treated cells (Student's *t*-test).
*Hours of treatment.

LA (10^{-11}-10^{-5} M) proved inactive in regulating the proliferation of LNCaP cells when used alone (not shown), but counteracted the stimulatory action of DHT, in agreement with our previous data (18), even reducing cell growth below the control values. This effect was already evident after 48 h (20-63% reduction in DHT-induced cell growth), reaching the maximum (from 80 to 110%) after 96 and 144 h of treatment, at concentrations ranging from 10^{-8} M to 10^{-5} M, when a certain dose-dependency was observed. The effectiveness of LA in counteracting DHT mitogenic activity sharply declined after 192 h of treatment (Figure 1).

The proliferation curves, performed in order to assess the sensitivity of LNCaP cells to the anti-androgen CA (10^{-11}-10^{-5} M), showed a similar trend to that displayed after DHT treatment. LNCaP cells responded with a significant ($p<0.01$) stimulation of cell growth at CA concentrations ranging from 10^{-9} to 10^{-6} M, while the dose of 10^{-5} M determined a reduction in cell numbers, with slightly lower values than those observed in controls (not shown). A remarkable and time-dependent enhancement of LNCaP cell proliferation (25-129%, compared with the control, $p<0.01$) was observed after 48-192 h with 10^{-7} M CA (not shown), in agreement with other data from the literature (32, 33), and this concentration was deemed suitable for use in combination experiments. In the latter the GnRH analogue (10^{-11}-10^{-5} M) reduced the mitogenic effect of CA, but its action was less pronounced than that observed when associated with the androgen (not shown).

In all cell growth experiments cell viability was always higher than 90%.

Basal expression of bcl-2, bax and c-myc genes. With regard to the basal mRNA and protein levels of the three apoptosis-related genes in LNCaP cells, significant differences were observed 24, 48, 96 and 144 h after cell adhesion to the culture plates. Bcl-2 showed the weakest expression,

Table II. *Bcl-2 protein expression in LNCaP cells treated with hormones and anti-hormones as determined by Western blotting.*

	24*	48	96	144
Control	1	1	1	1
DHT, 10^{-9} M	1.30±0.04[b]	2.25±0.07[b]	1.90±0.04[b]	1.90±0.25[a]
CA, 10^{-7} M	1.55±0.07[b]	1.55±0.07[b]	2.25±0.21[a]	1.75±0.04[b]
LA, 10^{-6} M	0.65±0.07[a]	1.40±0.07[a]	1.35±0.07[a]	0.60±0.07[a]
LA, 10^{-1}1 M	0.75±0.07[a]	1.30±0.07[a]	1.30±0.07[a]	0.55±0.07[a]
LA, 10^{-6} M + DHTτ	0.55±0.07[a,d]	1.45±0.07[a,d]	1.20±0.02[b,d]	0.50±0.07[a,c]
LA, 10^{-11} M + DHT	0.60±0.04[b,d]	1.55±0.07[b,c]	0.85±0.07[d]	1.45±0.07[a,c]
LA, 10^{-6} M + CAυ	0.45±0.07[b,d]	0.90±0.14[c]	1.15±0.07[c]	1.40±0.00[b,c]
LA, 10^{-11} M + CA	0.65±0.07[a,d]	1.30±0.00[b,c]	1.60±0.00[b,c]	1.50±0.00[b,c]

τ10^{-9} M DHT was used in association with LA.
υ10^{-7} M CA was used in association with LA.
Bcl-2 content was assessed by densitometric analysis and normalized against the β-actin protein level as a loading control. The numbers represent the ratio between values of treated samples and controls (set at 1).
Data shown as mean±SE (n=4) of two independent experiments, each performed in duplicates.
[a]$p<0.05$ and [b]$p<0.01$, *versus* control (Student's *t*-test); [c]$p<0.05$ and [d]$p<0.01$, *versus* DHT- or CA-treated cells (Student's *t*-test).
*Hours of treatment.

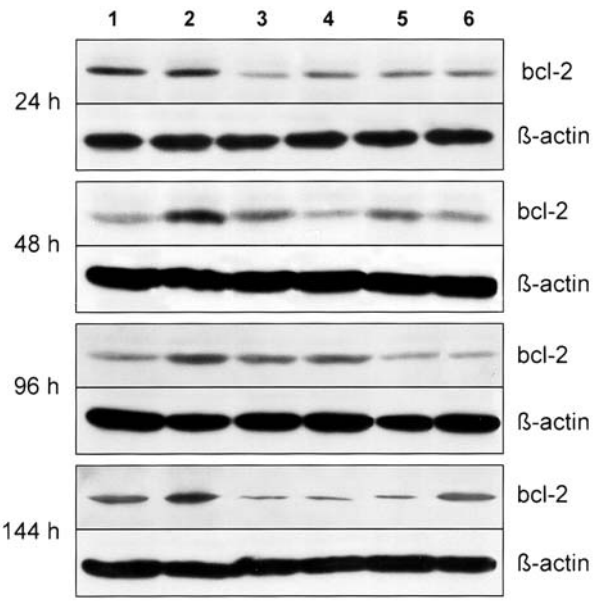

Figure 3. *Western blot analysis of bcl-2 expression in LNCaP cells treated with hormones for 24-144 h. The cell extracts were electrophoresed (40 μg of total protein/lane) on a 12% polyacrylamide gel and blotted onto an Immobilon P membrane, which was probed with a monoclonal antibody against bcl-2. The specific bands were visualized using the ECL chemiluminescence detection system. The same blots were stripped and then probed with an anti-β-actin antibody for loading control. The intensities of bcl-2 signals in Western blot autoradiographs were quantified by densitometric scanning and the results normalized for β-actin. Untreated cells (lane 1), cells treated with 10^{-9} M DHT (lane 2), 10^{-6} M LA (lane 3), 10^{-11} M LA (lane 4), 10^{-6} M LA plus 10^{-9} M DHT (lane 5), 10^{-11} M LA plus 10^{-9} M DHT (lane 6). One experiment representative of two is reported.*

followed by bax, while c-myc generally gave the most intense signal. For each gene no time-dependent variations in mRNA or protein levels were seen (not shown).

Effect of hormone treatment on bcl-2 expression. When LNCaP cells were treated over various time intervals (24-144 h) with 10^{-9} M DHT, a time-dependent enhancement (from 45 to 95%, compared with the control) in the levels of bcl-2 mRNA was observed. LA alone, at both concentrations used (10^{-11} or 10^{-6} M), always down-regulated bcl-2 mRNA expression (from 10 to 40% reduction, compared with the control). The analogue (10^{-11} or 10^{-6} M) counteracted the stimulatory effect produced by the androgen on bcl-2 mRNA expression (from 13 to 27% reduction in the values obtained in the presence of DHT) at all the times stated (Table I). Figure 2 shows the results of a representative experiment in which the effect of 24 to 144-h treatment with the above two hormones (alone or in combination) on bcl-2 mRNA expression was evaluated.

The expression of the anti-apoptotic bcl-2 protein was also increased in LNCaP cells maintained in the presence of 10^{-9} M DHT. After 24 h a 30% enhancement was observed, while a strong bcl-2 increase became clearly evident after a 48-h-treatment, when it reached the maximum (125%, compared with the control), and persisted until 144 h. Variations in bcl-2 protein expression produced by LA alone, at both concentrations used (10^{-11} or 10^{-6} M), did not have a unequivocal trend. The analogue (10^{-11} or 10^{-6} M) combined with 10^{-9} M DHT counteracted the stimulatory effect produced by the androgen on bcl-2 protein expression, often determining its reduction below the control values (from 24 to 74% reduction in the values obtained in the presence of DHT) (Table II, Figure 3).

Treatment with the anti-androgen CA (10^{-7} M) led to an increase in bcl-2 mRNA and protein levels, very similar to that induced by 10^{-9} M DHT. Moreover, the combination of the analogue (10^{-11} or 10^{-6} M) with CA determined an effect overlapping that triggered by the association of LA with DHT both at the mRNA (not shown) and protein levels (Table II).

Table III. *Bax protein expression in LNCaP cells treated with hormones and anti-hormones as determined by Western blotting.*

	24*	48	96	144
Control	1	1	1	1
DHT, 10^{-9} M	0.60 ± 0.00^{b}	0.60 ± 0.00^{b}	0.85 ± 0.04^{a}	0.85 ± 0.04^{a}
CA, 10-7 M	0.95 ± 0.07	0.90 ± 0.03	1.05 ± 0.07	0.95 ± 0.04
LA, 10^{-6} M	1.25 ± 0.07^{a}	1.10 ± 0.01^{b}	1.00 ± 0.00	1.05 ± 0.01^{a}
LA, 10^{-11} M	1.00 ± 0.14	1.10 ± 0.01^{a}	1.10 ± 0.01^{a}	1.30 ± 0.00^{b}
LA, 10^{-6} M + DHTτ	$1.35\pm0.07^{a,d}$	$1.30\pm0.07^{a,d}$	$1.50\pm0.14^{a,c}$	$1.35\pm0.07^{a,c}$
LA, 10^{-11} M + DHT	$1.25\pm0.07^{a,d}$	$1.15\pm0.02^{a,d}$	$1.05\pm0.03^{a,c}$	$1.40\pm0.07^{a,c}$
LA, 10^{-6} M + CAυ	$1.25\pm0.03^{b,c}$	$1.15\pm0.03^{a,d}$	$1.60\pm0.14^{a,c}$	$1.10\pm0.01^{a,c}$
LA, 10^{-11} M + CA	$1.35\pm0.04^{b,c}$	1.00 ± 0.00^{c}	$1.55\pm0.07^{b,c}$	$1.15\pm0.01^{b,c}$

τ10^{-9} M DHT was used in association with LA.
υ10^{-7} M CA was used in association with LA.
Bcl-2 content was assessed by densitometric analysis and normalized against the β-actin protein level as a loading control. The numbers represent the ratio between values of treated samples and controls (set at 1).
Data shown as mean±SE (n=4) of two independent experiments, each performed in duplicates.
$^{a}p<0.05$ and $^{b}p<0.01$, *versus* control (Student's *t*-test); $^{c}p<0.05$ and $^{d}p<0.01$, *versus* DHT- or CA-treated cells (Student's *t*-test).
*Hours of treatment.

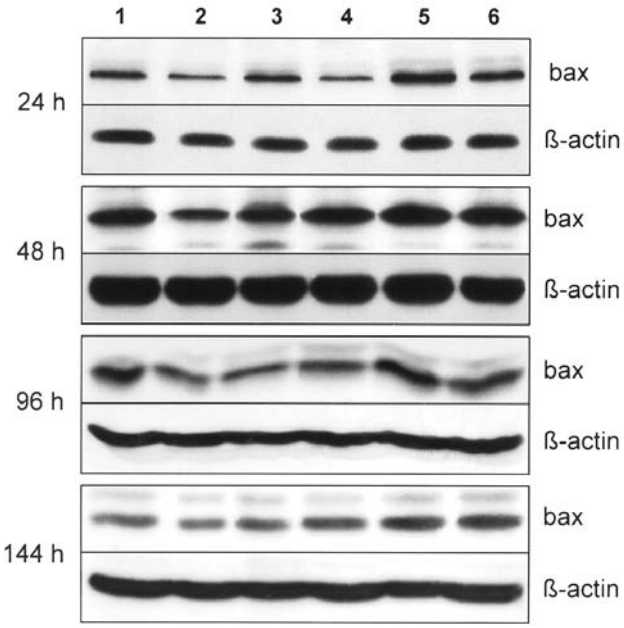

Figure 4. *Western blot analysis of bax expression in LNCaP cells treated with hormones for 24-144 h. The cell extracts were electrophoresed (40 μg of total protein/lane) on a 12% polyacrylamide gel and blotted onto an Immobilon P membrane, which was probed with a monoclonal antibody against bax. The specific bands were visualized using the ECL chemiluminescence detection system. The same blots were stripped and then probed with an anti-β-actin antibody for loading control. The intensities of bax signals in Western blot autoradiographs were quantified by densitometric scanning and the results normalized for β-actin. Untreated cells (lane 1), cells treated with 10^{-9} M DHT (lane 2), 10^{-6} M LA (lane 3), 10^{-11} M LA (lane 4), 10^{-6} M LA plus 10^{-9} M DHT (lane 5), 10^{-11} M LA plus 10^{-9} M DHT (lane 6). One experiment representative of two is reported.*

Effect of hormone treatment on bax expression. The transcript of the pro-apoptotic gene bax was moderately down-regulated by DHT (10^{-9} M) treatment after 24-144 h ($p<0.05$) (not shown). LA alone did not produce any significant variation in bax mRNA levels at all the concentrations used (10^{-11} or 10^{-6} M), (not shown). When LA (10^{-11} or 10^{-6} M) was combined with 10^{-9} M DHT, the inhibitory action of the androgen was only sporadically antagonized (not shown). At the protein level, DHT still reduced the bax expression (from 15 to 40%, compared with the control), while LA generally increased the bax protein signal when used alone (from 10 to 30%, compared with the control). When combined with the androgen, LA counteracted the DHT effect and, in most cases, increased the bax protein levels over the controls (from 24 to 125% increase in the values obtained in the presence of DHT) (Table III, Figure 4).

No significant variation in the bax mRNA or protein was observed after treatment with CA alone, while an increase in the bax expression, often above the control values, was seen when the analogue was combined with the anti-androgen, particularly at the protein level (Table III).

Effect of hormone treatment on c-myc expression. The c-myc protein expression showed an increase after 24-144 h of DHT (10^{-9} M) treatment (15-45%, compared with the control; $p<0.05$). To the contrary, the exposure of LNCaP cells to LA alone (10^{-11} or 10^{-6} M) produced a reduction of c-myc protein (10-30%, compared with the control; $p<0.05$). At both the concentrations tested, the analogue not only antagonized the effect of DHT on c-myc protein but sometimes reduced the expression below the control levels ($p<0.05$). Figure 5 shows the above results from a typical immunoblot relative to a 48-h treatment, when the effect is more evident. Similar effects, even less pronounced, were observed on c-myc mRNA as well (not shown).

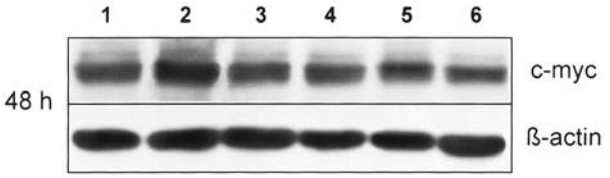

Figure 5. *Western blot analysis of c-myc expression in LNCaP cells treated with hormones for 48 h. The cell extracts were electrophoresed (40 μg of total protein/lane) on a 8% polyacrylamide gel and blotted onto an Immobilon P membrane, which was probed with a monoclonal antibody against c-myc. The specific bands were visualized using the ECL chemiluminescence detection system. The same blots were stripped and then probed with an anti-β-actin antibody for loading control. The intensities of c-myc signals in Western blot autoradiographs were quantified by densitometric scanning and the results normalized for β-actin. Untreated cells (lane 1), cells treated with 10^{-9} M DHT (lane 2), 10^{-6} M LA (lane 3), 10^{-11} M LA (lane 4), 10^{-6} M LA plus 10^{-9} M DHT (lane 5), 10^{-11} M LA plus 10^{-9} M DHT (lane 6). One experiment representative of two is reported.*

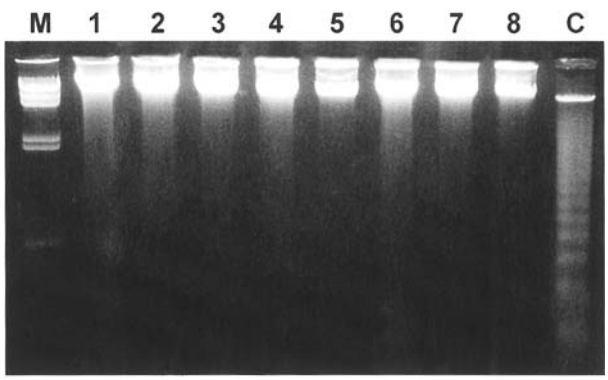

Figure 6. *DNA laddering analysis in LNCaP cells after 144 h of treatment with hormones. The cells were lysed and DNAs (1 μg/lane) were analyzed by 1.5% agarose gel electrophoresis. Molecular weight markers (lane M); untreated cells (lane 1), cells treated with 10^{-9} M DHT (lane 2), cells treated with 10^{-11} M LA (lane 3), 10^{-6} M LA (lane 4), 10^{-5} M LA (lane 5), 10^{-11} M LA plus 10-9 M DHT (lane 6), 10-6 M LA plus 10^{-9} M DHT (lane 7), 10^{-5} M LA plus 10^{-9} M DHT (lane 8), positive control for DNA fragmentation (HL-60 cells treated for 12 h with 10 μM etoposide, lane C). Similar results were obtained in two independent experiments.*

A constant enhancement of c-myc mRNA was seen in LNCaP cells after exposure to CA (10^{-7} M), which did not trigger any effect at the protein level. The analogue, at both the concentrations used, generally reduced or abolished the CA-elicited expression of c-myc mRNA during the treatment and determined a certain decrease in the protein levels obtained in the presence of CA (not shown).

Jurkat cells, used in Western blot analysis as a positive control for bcl-2, bax and c-myc protein expression, gave the expected signal (not shown).

DNA fragmentation. Electrophoretic analysis of genomic DNA extracted from LNCaP cells treated with DHT (10^{-9} M) and LA (10^{-11}, 10^{-6} M and 10^{-5} M), alone or in combination for 96 and 144 h, did not reveal any DNA fragmentation. A ladder pattern of oligonucleosomal length DNA fragments, characteristic of apoptosis, was observed in etoposide-treated HL-60 cells which were used as control (Figure 6).

Discussion

Proliferation experiments confirmed our previous results concerning the androgen-sensitivity of LNCaP cells and the inefficacy of LA in modifying cell growth at all concentrations tested (18). In addition, the analogue essentially counteracted the androgen-stimulated cell proliferation. Cell viability was always higher than 90% in cells treated with LA or the combination DHT/LA and DNA laddering was absent in all the conditions studied. At the same time, we monitored the expression of genes such as bcl-2, bax and c-myc, as their interplay may affect the proliferative response of the cells to the hormonal treatment.

The expression of bcl-2 was increased at all the times considered at both mRNA and protein levels by DHT treatment. On the contrary, down-regulation of bcl-2 mRNA and protein by DHT has recently been reported in LNCaP-FGC cells (34). However, our results are in accordance with other experimental evidence regarding androgen action on bcl-2 in LNCaP cells (35) and with the antiapoptotic role played by this gene (36).

Our findings are also consistent with the observation that rat prostatic epithelium undergoes apoptosis within hours of castration as a consequence of androgen withdrawal (37). Nevertheless, it is necessary to point out that *in vivo* the androgen-independent state of prostatic cancer is found to be associated with bcl-2 overexpression in humans and in rodent models (38-40). In such a context, it seems conceivable that up-regulation of bcl-2 could bypass the signal for apoptosis that is normally generated by androgen ablation (41). Further studies are needed to provide the understanding of the mechanism by which this bypass pathway interacts with AR signalling.

In our model the androgen effect on bcl-2 parallels the action exerted on cell growth. Similarly, other authors found that DHT down-regulates bcl-2 mRNA and protein in ZR-75-1 breast cancer cells and inhibits their proliferation (42).

The mechanism underlying the DHT-dependent regulation of bcl-2 remains to be clarified. The existence of both a direct and an indirect pathway(s) has been hypothesized. In particular, the lack of a perfect consensus sequence for an androgen response element in the bcl-2 gene sequence has

been demonstrated (43), thus supporting the possibility that an indirect mechanism may be involved. LA alone determined a reduction in bcl-2 mRNA, which did not always result in a parallel decrease in protein expression. This might justify, to some extent, the lack of an antiproliferative effect of the analogue. Nevertheless, when it was combined with DHT, LA induced a reduction of the androgen-stimulated bcl-2 expression or even decreased bcl-2 values below the controls, particularly at the protein level. This is in complete agreement with data obtained in cell proliferation experiments.

Bax expression was reduced under DHT treatment and slightly up-regulated by LA at the protein level in LNCaP cells in compliance with its role as an apoptosis-promoting gene (44). The DHT-induced decrease in the bax expression was counteracted by LA when the cells were exposed to both the hormones; an even higher increase of the protein level was evident than that induced by LA alone.

The opposite behaviour of the two above-mentioned genes in response to agents regulating cell growth and/or apoptosis is widely known (45-48). Nevertheless, the LA-induced variations in the expression of the two apoptosis regulatory genes seem less obvious in the absence of cell death, a phenomenon that has been described by other authors (49, 50) in various models and with different agents. On the contrary, induction of apoptosis has been reported without any changes in bcl-2 and/or bax expression (51-53). It can be hypothesised that different pathways for induction of apoptosis exist in the different models and that the involvement of the bcl-2/bax machinery occurs in some, but not all, cell types.

The fact that the variations in bcl-2 and bax expression under hormonal treatment always parallel the proliferative response of the cells led us to consider a direct or indirect role of the above genes in the growth modulating activity of DHT and LA.

As described in literature and according to its role of cell-cycle regulator, the proto-oncogene c-myc underwent variations similar to those which occurred in bcl-2, mostly at the protein level (54). In fact, it was up-regulated by DHT treatment and reduced by LA. Also in this case, LA induced a reduction in DHT-stimulated c-myc expression, the c-myc levels sometimes being below that of controls.

The anti-androgen CA elicited an enhancement in LNCaP cell proliferation similar to that induced by DHT. This finding is in agreement with other data from literature (32, 55) and can be ascribed to the presence of a mutated androgen receptor in LNCaP cells that illicitly binds to progestins, oestrogens and anti-androgens which act as agonists (33). Particularly interesting is the observed increase in bcl-2 gene expression produced by the anti-androgen, which completely overlaps the androgen-elicited effect. On the other hand, a slight up-regulation of bcl-2 and c-myc mRNA or protein has been observed in LNCaP cells by other authors using androgen antagonists such as bicalutamide and CA (46, 55, 56).

In conclusion, the described effects produced by LA on apoptosis-related genes can be included in the direct actions of the GnRH analogues. These changes triggered by LA do not allow the death program to be completed, suggesting that other apoptosis-leading signals may be missing in our model. This might explain the lack of a cytotoxic effect when the analogue is used alone, even at high concentrations. Nevertheless, it cannot be excluded that the above variations in gene expression, particularly those concerning bcl-2, account for the inhibitory effects of LA on LNCaP cell growth in the presence of androgens.

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