

ORIGINAL ARTICLE

# Effect of Leuprolide and Cetrorelix on Cell Growth, Apoptosis, and GnRH Receptor Expression in Primary Cell Cultures from Human Prostate Carcinoma

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## ABSTRACT

Contradictory data have been reported regarding the effect of GnRH agonists and antagonists on cell growth and survival, using prostate cancer-derived cell lines expressing either endogenous or exogenous GnRH receptors. We addressed the issue studying the effect of leuprolide (agonist) and cetrorelix (antagonist) on cell growth, apoptosis and GnRH receptor expression using a primary cell coculture system. Also, binding characteristics of prostate GnRH receptor in this culture system are described. Epithelial and stromal cells were obtained from prostate adenocarcinoma samples and cocultured in a bicameral system. Expression of GnRH receptors was evaluated by semiquantitative RT-PCR (transcript level) and Western blot (protein level). Cell growth was estimated by MTT method and apoptosis by DNA fragmentation using COMET assay. Saturation and competition binding studies were carried out using <sup>125</sup>I-GnRH as radioligand. GnRH receptors from cell cultures of prostate cancer exhibited a single class of binding sites with a K<sub>d</sub> of  $1.11 \pm 0.28$  nM and a B<sub>max</sub> of  $2.81 \pm 0.37$  pmol/mg of membrane protein for GnRH. Leuprolide and cetrorelix showed no effect on GnRH receptor expression. Both analogues showed a significant reduction in cell growth rate and an increase in DNA-fragmented cell number. These effects were dependent on the analogue concentrations (from 5–20 ng/mL). Considering that the culture system used in this work represents more closely the in vivo conditions of tumor cells than metastatic derived cell lines, we conclude that GnRH analogues have a significant inhibitory effect on cell viability of cells expressing GnRH receptors. In addition, GnRH receptors expressed in tumor prostatic cells seem not discriminate between agonist and antagonist, both analogues activating these receptors. Also, leuprolide and cetrorelix treatments did not influence GnRH receptor expression in our culture system. These differences with pituitary receptors may be explained by differences in affinity, transduction mechanism and molecular context in prostatic tissue.

## INTRODUCTION

In most countries, prostate adenocarcinoma is among the main causes of cancer deaths in men. The treatment for prostatic cancer depends on the state at which pathology is detected. In its initial stages, development of prostate cancer is androgen dependent and most early treatments point to diminish androgen levels both surgically (orchiectomy) and pharmacologically (antiandrogens at proper doses) (1, 2). GnRH analogues have been extensively used to inhibit hypothalamic-pituitary axis in order to obtain a decrease in androgen level and the inhibition of tumor growth. Additionally, it has been observed that GnRH receptors are present both in prostatic malignant tissue and carcinoma

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derived cell lines, suggesting a local action (3). Several studies have shown that normal prostatic tissue does not express or express few GnRH receptors. Tissue from benign prostate hyperplasia (BPH) expresses these receptors in less than 1 percent of cases, whereas tissue from prostatic adenocarcinoma always expresses GnRH receptors (4–6). In humans, only Type I GnRH receptor is expressed. This receptor lacks C-terminal tail and therefore is resistant to desensitization by phosphorylation and internalizes very slowly (7). In pituitary cells, down regulation of gonadotrophin secretion under sustained treatment with GnRH agonist is rather due to desensitization of internal IP3 receptors (8). On the other hand, it has been found in prostatic derived cell lines DU 145 (androgen independent) and LNCaP (androgen dependent) an inhibition of proliferative activity in presence of potent GnRH agonists such as goserelin and buserelin (9, 10, 11). However, when these cells were treated with a GnRH antagonist, an increase in cell proliferation was observed (9). The opposite was found in patients and animal models treated with potent antagonists such as antide and the recently developed cetrorelix (SB-75) (12–15). The later antagonist efficiently blocks LH and testosterone secretion in treated patients (16) and produces a significant reduction of tumor size both in BPH and cancer patients. Also, this antagonist induces down regulation of GnRH receptors both in rat pituitary cells and animal models of experimental prostate cancer (17, 18). In the same models, it has been observed that cetrorelix induces apoptosis in malignant prostatic cells (12). Recently, more contradictory data on the effect of GnRH analogues on prostate cells have been reported using an exogenously expressing GnRH receptor cell line (19). Considering that most prostatic cell lines derives from metastasis and may not represent the characteristics of the actual prostate tumor, we study the effect of leuprolide and cetrorelix on cell growth, apoptosis, and GnRH receptor expression in a primary bicameral co-culture system in which epithelial and stromal cells were grown in connected compartments in order to preserve, at least in part, the paracrine interaction between these cell types from human adenocarcinoma. We recently have characterized this system regarding PSA secretion and androgen response (20).

## MATERIALS AND METHODS

### *Reagents*

All reagents used in this study, unless otherwise indicated, were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

### *Prostatic samples*

Prostatic samples were obtained from radical prostatectomy due to adenocarcinoma, from our Institutional Hospital. Prostate segments were received in sterile culture medium containing RNase inhibitors and hyperplastic tissue was separated from harder malignant nodes. Small pieces of each sample of tissue were processed by routine histological technique for Gleason classification (21). This procedure allowed us to know the actual classification of the samples used for cell isolation, which was always confirmed by our institutional pathologists. In this study

were included 6 prostate samples with a Gleason score range of 5–8. Culture systems were obtained separately from each sample. This proposal was approved by Bioethics Committee of our Institution.

### *Isolation of epithelial and stromal cells*

Prostatic cells were isolated and cultured essentially as described previously (20).

*Epithelial cells:* Prostatic tissue was cut in small pieces (1 mm<sup>3</sup>) and incubated for 45 minutes at 37°C in culture medium to eliminate blood cells. After washing, pieces were digested in collagenase (2.5 mg/mL), hyaluronidase (1 mg/mL) and deoxyribonuclease (0.01 mg/mL), for 2–3 hours at 37°C in a shaking water bath. Dispersed stromal cells were separated from digesting fragments and pooled. Resulting tight and large epithelial cell aggregates were washed and further digested with collagenase for another 8–12 hours in the same conditions as before. Resulting cell aggregates were mechanically dispersed, washed, and plated in cell culture inserts with polyethylene terephthalate (PET) bottom, 1 µm pore size, 12-well format (Falcon, Becton Dickinson Co. NJ, USA) at a density of  $0.5 \times 10^6$  cells/mL of Dulbecco's Modified Eagle's and HAM F-12 (1:1) media mixture supplemented with 5 percent fetal bovine serum (FBS) (Gibco, Invitrogen Co., CA, USA). Inside volume was 1.0 mL and outside volume was 2.0 mL.

*Stromal cells:* Pooled stromal cells were washed and plated on 12-well culture plates (Falcon, Becton Dickinson Co., NJ, USA) at a density of  $10^6$  cell/mL in 2 mL of Dulbecco's Modified Eagle's and HAM F-12 (1:1) media mixture supplemented with 5 percent of FBS.

### *Cell co cultures in bicameral system and GnRH analogues treatments*

After first medium change of both cultures, cells were washed and fresh medium without FBS and supplemented with human transferrin 5 mg/L, insulin 2 mg/L, epidermal growth factor 10 µg/L, vitamin A and E 200 µg/L, hydrocortisone 10 nM, sodium selenite 2 µg/L, and dihydrotestosterone (DHT) 10 nM was added. Then, epithelial and stromal cell cultures were set together, media were collected every 48 hrs and cocultures maintained for 2 weeks. GnRH analogues treatments were carried out during Day 4 and 6 of co culture. Both leuprolide (Sigma) and cetrorelix (Serono, Novartis Chile) were used in a concentration range of 1–20 ng/mL.

### *Immunocytochemistry*

Cellular composition of the different cultures was determined immunocytochemically using specific rabbit antibodies against prostate carcinoma tumor antigen PCTA-1 (epithelial cells) and against vimentin (stromal cells) (Santa Cruz Biotechnology Inc., CA, USA). Antirabbit-FIT-C (Santa Cruz Biotechnology Inc., CA, USA) was used as a secondary antibody. For that purpose cells were grown on coverslips, washed with PBS and fixed in 3 percent paraformaldehyde (Merck, Germany) - 2 percent sucrose in PBS for 30 minutes at room temperature and blocked

with PBS containing 20 mM glycine and one percent bovine serum albumin (BSA). Cell cultures were evaluated in a Zeiss Fluorescence Microscope. PCTA-1 is a specific membrane antigen of prostate epithelial cells which is overexpressed cancer tumors (22, 23).

### **Reverse transcription-polymerase chain reaction of GnRH receptor mRNA**

Total RNA was isolated from prostate epithelial cell using Trizol Reagent (Gibco BRL, Rockville, MD, USA) according to the manufacturer's instructions. RNA (2  $\mu$ g) from each sample was used in a RT reaction. Total RNA was reverse transcribed and cDNA was subjected to polymerase chain reaction (PCR). The specific primers used to amplify Type I GnRH receptor DNA fragments were: sense 5'-GCTTGAAGCTCTGTCTGGGA-3' (-25 to -5) and antisense 5'-CCTAGGACATAGTAGGG-3' (884-860) (24, 25). The amplification was carried out using a Thermal Cycler (PT-100, MJ Research, Inc., Watertown, MA, USA) with 2.5 U of DNA polymerase (Biotools), in a buffer of PCR 1  $\times$ , 2 mM  $MgCl_2$  and 0.25  $\mu$ M dNTPs.  $\beta$ -actin was used as an internal standard for GnRH receptor mRNA expression. Amplification was performed for 39 cycles for GnRH receptor PCR products and 28 cycles for  $\beta$ -actin. The cDNA of interest was amplified linearly between 33 and 45 cycles of PCR. The PCR products were electrophoretically resolved on 1.2 percent agarose gel and stained with ethidium bromide. The bands were evaluated using an image analyzer UN-SCAN-IT gel version for Windows 4.1 and Kodak Digital Science 1D Software (Rochester, NY, USA) and normalized relative to  $\beta$ -actin product. Data were calculated as the ratio between mRNAs of the GnRH receptor gene/ $\beta$ -actin and expressed as arbitrary units (UA).

### **Western blot analysis**

Cell were lysed in buffer containing 500 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1  $\mu$ g/mL PMSF and 3  $\mu$ g/mL aprotinin. Lysate was homogenized and centrifuged at 13,000 rpm for 15 minutes. Sixty  $\mu$ g of protein from supernatant was separated by 10 percent SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad, CA, USA). After blocking with fatless milk during one hour at room temperature, the membrane was incubated with a goat polyclonal antibody against human GnRH receptor (Santa Cruz Biotechnology, CA, USA) at 1:500 dilution for one hour at room temperature, washed and incubated with an anti-goat polyclonal antibody coupled to HRP. Detection of antibodies complex was carried out using  $H_2O_2$ , coumaric acid and luminol as substrates. Using a similar protocol,  $\beta$ -actin was identified using a rabbit polyclonal antibody against human protein (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and an anti-rabbit IgG as secondary antibody.

### **Receptor binding assays**

Membrane preparation procedure and binding assay conditions were similar to those described previously (26). Briefly,

semi confluent epithelial cultures were used for membrane preparation. Cells were detached and homogenized. Crude membrane preparations were ultracentrifuged at 70,000 g for 50 minutes at 4°C. The pellets were washed, resuspended in homogenization buffer, and stored at -80°C until assay. Protein concentration was determined by the Bradford method (27). For saturation assay, membrane homogenates containing 40–60  $\mu$ g of protein were incubated in triplicate with six concentrations of  $^{125}I$ -Tyrs-GnRH (Perkin Elmer, Life Sci. Inc., Boston, MA, USA), ranging from 0.05 to 1.60 nM in the absence or presence of 100-fold excess of noniodinated GnRH in a final volume of 300  $\mu$ L of binding buffer (10 mM Tris/HCl, pH 7.5, 1 mM dithiothreitol, 0.1 percent BSA). After 90 minutes of incubation at 4°C, reaction was stopped by immersing the tubes in ice-water and transferring 270  $\mu$ L of suspension onto cold propylene microfuge tubes. Aliquots were centrifuged at 12,000 g for 2 minutes at 4°C, the supernatants were aspirated and the pellets were counted for radioactivity. For competition assays, membrane preparations containing 50–60  $\mu$ g of protein were incubated in triplicate with 0.15 nM of  $^{125}I$ -GnRH in absence or presence of increasing concentrations ( $10^{-12}$ – $10^{-7}$  M) of unlabeled GnRH, leuprolide or cetrorelix. A nonlinear regression analysis (PRISM, GraphPad Software, Inc., San Diego, CA, USA) was used to determine dissociation constant (Kd) and binding capacity (Bmax).

### **Proliferative activity**

Proliferative activity was evaluated by a spectrophotometric method based in mitochondrial dehydrogenases assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (28). Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple MTT formazan crystals which can be dissolved in acid isopropanol and spectrophotometrically measured. Cell number is proportional to absorbance (OD) at 570 nm depending on cell type. For calibration, separate cultures at different cell densities were assayed by MTT protocol and cell counting. A correlation of cell density versus absorbance was established (starting cell density was  $10^5$  cells/mL).

### **Single cell gel electrophoresis (COMET assay)**

Apoptosis was estimated by DNA fragmentation using COMET assay (29). Cells were detached from culture plates with 0.1 percent trypsin solution. Aliquot of cell suspension were mixed with low melting point agarose and carefully layered on glass slide precoated with regular agarose. The slides were covered with a coverslip to obtain a thin layer of gel, and put on a cold tray kept on ice. After coverslip removing, a new layer of regular agarose was layered on cell-containing gel. Then, slides were dipped in a lysis solution containing 2.5 M NaCl, 100 mM  $Na_2EDTA$ , 10 mM Tris(hydroxymethyl)aminomethane, one percent sodium N-lauroylsarcosine, 10 percent DMSO, one percent Triton X-100, pH 10 for 1 hour at 4°C. After lysis, slides were placed on horizontal electrophoresis chamber and electrophoresis buffer (300 mM NaOH, 1 mM  $Na_2EDTA$ , pH 13)



was added. Electrophoresis was run at 25V/300 mA during 30 minutes. Then, slides were carefully washed and stained with 2  $\mu\text{g/mL}$  ethidium bromide. Cells were evaluated and counted in a Zeiss fluorescence microscope.

### Statistic analysis

ANOVA analysis and non parametric test of Kruskal-Wallis followed by Dunn's post test was performed for statistic evaluation.  $P < 0.05$  was considered significant. All experiments were repeated at least 3 times and results are expressed as means  $\pm$  standard deviations.

## RESULTS

### Cell culture composition

In epithelial cultures more than 90 percent of cells were strongly positive for PCTA-1 (specific for epithelial prostatic cells and overexpressed in cancer). In stromal cultures less than 5 percent of cells were positive for PCTA-1, whereas more than 80 percent were positive for vimentin.

### Expression of GnRH receptors

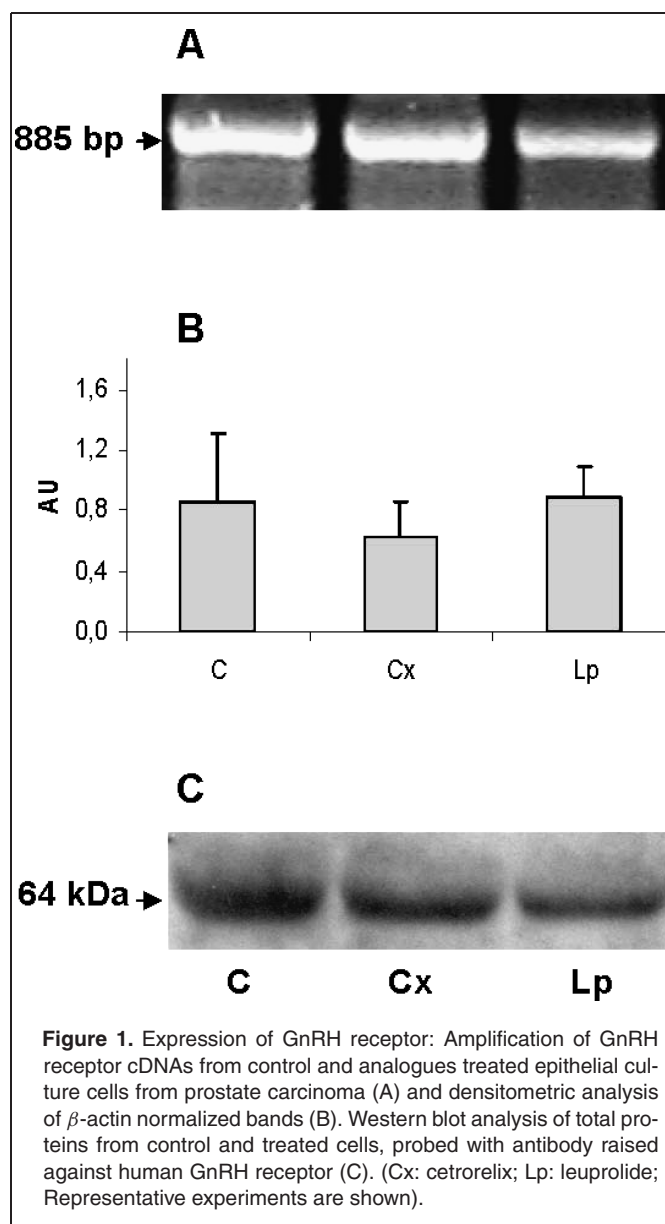
Epithelial cells showed Type I GnRH expression both at the level of mRNA (RT-PCR) and protein (Western blot). Treatment with leuprolide and cetrorelix (20 ng/mL) did not significantly change the GnRH receptor at none level (Figure 1). Stromal cells showed no detectable expression of GnRH receptor at any level (data not shown).

### Radioligand binding studies

Nonlinear regression analysis of saturation experiments (Figure 2A) showed that the one-site model provided the best fit, indicating the presence of a single class of high-affinity GnRH receptors in membrane preparations of prostate cancer cells. These receptors showed a dissociation constant (Kd) of  $1.11 \pm 0.28$  nM with a maximal binding capacity of  $2.81 \pm 0.37$  pmol/mg of membrane protein for GnRH. Displacement experiments (Figure 2B) showed the specificity of the receptor binding. The binding of  $^{125}\text{I}$ -GnRH was completely displaced by increasing concentration ( $10^{-12}$ – $10^{-7}$  M) of unlabeled GnRH, leuprolide, and cetrorelix.

### Effect of leuprolide and cetrorelix on proliferative activity

Both analogue treatments showed a significant and concentration dependent (from 5 to 20 ng/mL) decrease in cell number in epithelial cultures compared with untreated controls. The effect of cetrorelix was slightly more pronounced than leuprolide (Figure 3). Both analogues have no effect on proliferative activity in stromal cells (data not shown).



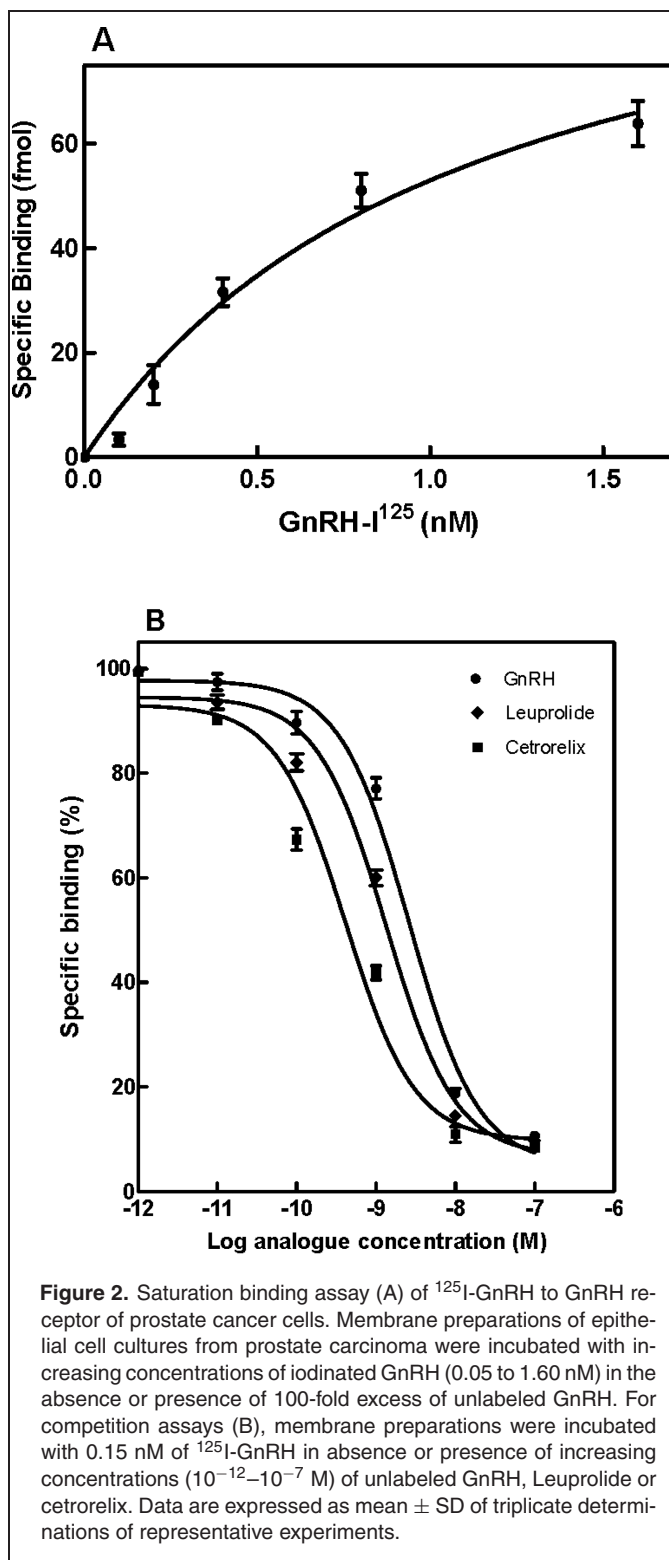
**Figure 1.** Expression of GnRH receptor: Amplification of GnRH receptor cDNAs from control and analogues treated epithelial culture cells from prostate carcinoma (A) and densitometric analysis of  $\beta$ -actin normalized bands (B). Western blot analysis of total proteins from control and treated cells, probed with antibody raised against human GnRH receptor (C). (Cx: cetrorelix; Lp: leuprolide; Representative experiments are shown).

### Effect of leuprolide and cetrorelix on apoptotic activity

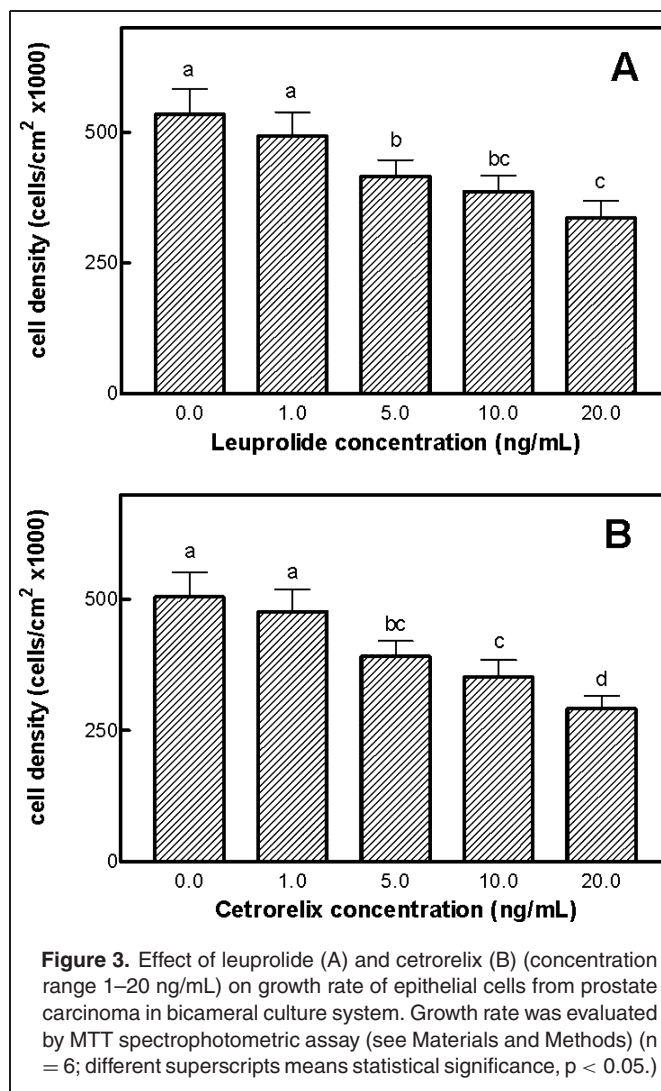
Both analogues treatment showed a significant and concentration dependent (from 5 to 20 ng/mL) increase in DNA-fragmented cells in epithelial cultures compared with untreated controls. Cetrorelix also showed a slightly more pronounced effect (Figure 4). Both analogues have no effect on apoptotic activity in stromal cells (data not shown).

## DISCUSSION

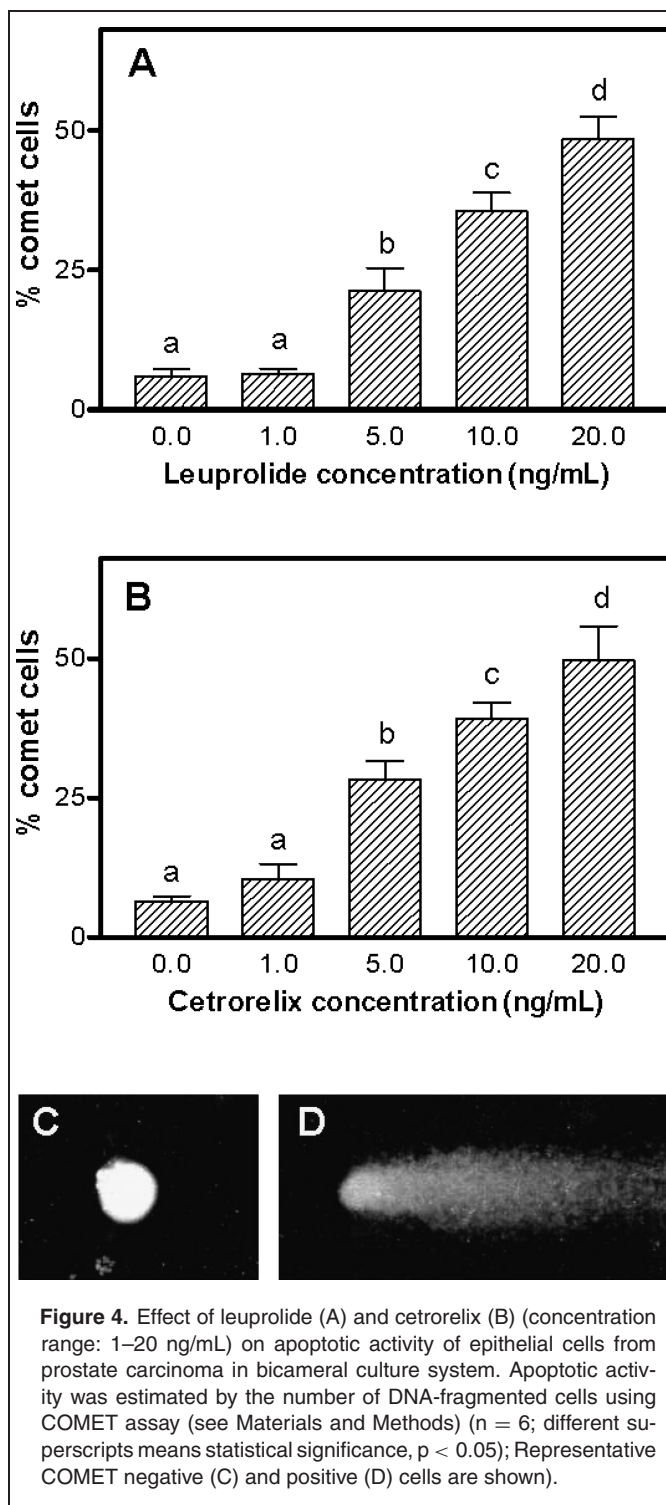
GnRH-analogue therapy, widely used to diminish androgen levels in prostate cancer, shows also a significant decrease in malignant lesions. In addition, GnRH receptors have been reported both in prostatic malignant tissue and carcinoma derived



cell lines, suggesting a local action for GnRH analogues (3). On the other hand, several GnRH analogues have been shown to have effect on proliferative and apoptotic activities in cell lines cultures (10, 11). However, contradictory effects have been reported depending on cell line and experimental model used (10,



14, 15). Recently, we have characterized a primary bicameral culture system in which epithelial prostatic cells from cancer tumors were cocultured with their corresponding stromal cells (20). The main advantage of this system is the maintenance of paracrine relationship between stroma and epithelium which is thought to be crucial in prostate cancer development (30, 31). In the present work, using the same coculture system, we have evaluated the effect of leuprolide and cetrorelix on proliferative and apoptotic activities, as well as GnRH receptor expression. We have found that epithelial cells express Type I GnRH receptor both at the level of transcript and protein which were not significantly affected by leuprolide or cetrorelix treatments. The opposite is found in pituitary cells where agonists stimulate the expression of GnRH receptor and antagonists block such effect (32). Although, the same pituitary Type I GnRH receptor mRNA has been found in extrapituitary sites, the action mechanism seems different. In pituitary, GnRH receptor acts via G protein coupled to phospholipase C, and is activated by agonists and blocked by antagonists, while in prostate and other extrapituitary sites, GnRH is coupled to Gi (inhibitory) protein



(24). This different transduction mechanism may not be coupled with regulation of GnRH receptor gene in prostate cancer cells.

Interestingly, most data available indicate that agonist and antagonist have the same effect, mainly antiproliferative and/or apoptotic (33–35). It has been found that GnRH receptors from endometrial cancer have lower affinity for goserelin (GnRH ag-

onist) than pituitary receptors suggesting that these differences might account for the similar effects of agonist and antagonist (36). In addition, the agonist triptorelin had no effect on androgen unresponsive PC-3 prostate cell line and had a double stimulatory-inhibitory effect on the proliferation of androgen responsive LNCaP cell line (37). The same authors reported a single class of low affinity GnRH receptor for PC-3 cells and 2 classes (moderately high and low affinity) of receptor for LNCaP cells. However, in our primary culture system, GnRH receptors from epithelial cancer cells showed a rather high affinity binding for GnRH, leuprolide and cetrorelix. These data are in accordance with those reported in tissue samples of prostate cancer (6). Our results suggest that receptor affinity may be a key issue in GnRH antiproliferative and apoptotic effects. Recently, it has been shown that PC-3 cells infected with adenovirus expressing GnRH receptor display affinity and transduction mechanism similar to pituitary cells (19). In this PC-3 infected cells, buserelin and GnRH cause and increase in cytoplasmic  $Ca^{+2}$  and inhibition of growth rate. Both effects were blocked by cetrorelix and antide antagonists. We consider that overexpression of GnRH receptor in a prostate cell line that, according to the authors, did not express endogenous receptor may not represent the best model for studying the effect of GnRH analogues, because molecular context and transduction system involved may be quite different to those expressed in epithelial cells obtained directly from the prostate tumor. However, the fact that PC-3 cells do not express GnRH receptors (19) or express only low affinity receptors (37) is interesting because they represent a more malignant form of prostate cancer than LNCaP. In addition, a negative correlation between the concentration of GnRH receptors and cancer grade has been reported using tissue samples of prostate tumors (6), but in this case, the few receptors found in more malignant tumors present medium to high affinity. Our cultures come from tumor samples of medium grade and showed an important amount of high affinity receptors. Assuming that the number of GnRH receptors decreases with Gleason score, but maintain or increase the affinity (6), it is still possible a receptor-based therapy, although the low number of high affinity receptor may limit the therapeutic effects of GnRH analogues on patients with malignant metastatic prostate cancer. Data presented in this work reinforce the effect of GnRH (agonist and antagonist) on survival of cells from prostate cancer and suggest that local treatments with these analogues would be an interesting approach for the therapeutic management of this pathology. For high grade tumors, treatments intended to increase the expression of GnRH receptors in prostate cells may have important clinical implications in order to improve the effectiveness of GnRH therapies.

## CONCLUSIONS

We conclude that in the culture system used in the present work, both leuprolide and cetrorelix showed a similar antiproliferative and apoptotic effect on epithelial cells. These effects were concentration dependent in a wide range. Our results are in agreement with some previous reports in cell lines, but in

contradiction with those reported in PC-3 infected cells expressing exogenous GnRH receptor. We think that primary cocultures in which paracrine effects are preserved may be more representative of in vivo conditions. Further investigation is required to explain the activation of GnRH receptor in prostate cancer by both agonists and antagonists.

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