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# Expression of gonadotropin-releasing hormone (GnRH) and GnRH receptor mRNA in prostate cancer cells and effect of GnRH on the proliferation of prostate cancer cells

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**Abstract** The purpose of this study was to determine the production of gonadotropin-releasing hormone (GnRH), the co-occurrence of GnRH receptors in prostate cancer cells, and the effect of GnRH on prostate cancer cell proliferation. Four human prostate cancer cell lines were studied. LNCaP is an androgen sensitive prostate cancer cell line, DU-145 and PC-3 are androgen resistant, and TSU-Pr1 is uncharacterized. The expression of GnRH and GnRH receptor mRNAs were assessed by in situ hybridization and the effect of exogenous GnRH on proliferation of prostate cancer cells was measured by thymidine incorporation assay. GnRH mRNA expression, determined by in situ hybridization, was found in 83.48% of the LNCaP, 89.7% of the TSU-Pr1, 86.2% of the PC-3 and 95.3% of the DU-145. Signals of GnRH receptor mRNA were detected in more than 95% of the cells of all four cell lines. The proliferation of the prostate cancer cells grown in media supplemented with peptide hormone lacking charcoal-stripped serum was significantly ($P < 0.05$) suppressed. No significant effect of GnRH on the proliferation of all four prostate cancer cells was observed. In summary, prostate cancer cells produced GnRH and its receptors, and exogenous GnRH treatment did not affect the prostate cancer cell proliferation. The existence of GnRH and GnRH receptor mRNA in the same cell suggests that the role of GnRH produced by prostate cancer cells would be autocrine.

**Key words** GnRH · GnRH receptor · mRNA · Prostate cancer · In situ hybridization

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## Introduction

Since Harris [11] proposed the neurohumoral hypothesis in 1955, gonadotropin-releasing hormone (GnRH) has been believed to be produced exclusively in the hypothalamus and secreted to the hypophysis via the portal system. Decades after that proposal, it had been reported that the production of GnRH or GnRH-like materials occurred in several organs other than the hypothalamus. GnRH or GnRH-like materials have been detected in the spinal ganglia [14], the pancreatic islet cell [7], the ovary [28] and the male genital organs including testis [3]. Furthermore, GnRH-degrading activity has been found in soluble and particulate fractions obtained from the hypothalamus [24], the pituitary gland [16] and the gonad [5]. Besides the main role of GnRH in the regulation of pituitary gonadotropes, it is now widely accepted that GnRH has a role in several organs. GnRH analogues have been used as a hormonal agent for the treatment of prostatic cancer [10, 14]. Recently, there have been reports concerning the production of GnRH [2] and the existence of the GnRH receptor [25] in prostate cancer cells. However, there is still controversy about the production GnRH in prostate cancer cells.

Therefore, the present study was designed to confirm the production of GnRH and its receptor in prostate cancer cells. To this end, GnRH and its mRNA expression was examined by in situ hybridization in four different human prostate cancer cell lines. And we attempted to observe the direct effects of the exogenous GnRH on the proliferation of human prostate cancer cells.

## Materials and methods

### Cell lines

Four prostatic cancer cell lines, established from metastatic human prostate cancers, were used in this study. Hormone sensitive LNCaP [12], and hormone refractory DU-145 [22] and PC-3 [15]

were purchased from the American Type Culture Collection. The TSU-Pr1 [13] was generously gifted by Iizumi (Teikyo University, Japan). Three cell lines, LNCaP, DU-145 and TSU-Pr1, were cultured using RPMI-1640 (Sigma, St Louis, Mo.) and the remaining cell line PC-3 was cultured using HAM's F-12K (Sigma) in a $CO_2$ incubator as usual.

### cRNA probes for GnRH and GnRH receptor

Partial cDNA fragments (204 bp) from monkey GnRH, subcloned into pGEM bluescripts (a generous gift from Dr S. Ojeda, USA) were used to synthesize the GnRH cRNA probe. In the case of the GnRH receptor, the GnRH receptor cDNA fragments (generously given by Dr K. Kim, Korea) were reconstructed. Partial GnRH receptor cDNA fragments (441 bp from 721 to 1162) were inserted into pGEM-4Z (Promega) vector.

In the case of the antisense cRNA probes on GnRH and GnRH receptors, the plasmids were linearized with EcoRI (GnRH) and Hind III (GnRH receptor) and cRNAs were synthesized using SP6 RNA polymerase. For the sense probes, plasmids were linearized with Hind III (GnRH) and EcoRI (GnRH receptor) and cRNAs were synthesized using T7 RNA polymerase. A total of 20 µl of reaction solution was made with 4 µl of 5× transcription buffer (200 mM TRIS-HCl, pH 7.9, 30 mM $MgCl_2$, 10 mM spermidine), 2 µl of 100 mM DTT (Dithiotheitol, Promega, USA), 0.33 µl of 10 mM RNasin (60 units/µl, Boehringer Mannheim), 1 µl of 10 mM GTP (Guanocine Triphospate, Promega, USA), 1 µl of 10 mM CTP (Cytosine Triphosphate, Promega, USA), 1 µl of 0.1 mM UTP (Uracil Triphospate, Promega, USA), 200 ng linearized DNA template, 1 µl of SP6 or T7 RNA polymerase (15 units/µl, BRL), 5 µl $\alpha^{35}$S-UTP (10 mCi/µl) Sulfur-uracil Triphosphate, Amersham, USA and distilled water. Reaction solutions were incubated for 1 h at 37°C (T7) and 40°C (SP6) and then 1 µl of DNase I (10 units/µl, Sulfur-Uracil Triphosphate, Amersham, USA) was added to digest the template cDNAs. The GnRH and GnRH-receptor cRNA probes were then purified using Sephadex G-50 RNA grade column and eluted with SET buffer (0.1% SDS, 1 mM EDTA, 10 mM TRIS, 10 mM DTT). The collected tubes were measured by liquid scintillation counter. Polyacrylamide electrophoresis of the purified probes was done to confirm the full-length transcript of GnRH and the GnRH-receptor. The specific activity of the cRNA probes was approximately $1 \times 10^9$ cpm/µl.

### In situ hybridization

In situ hybridization of GnRH and GnRH-receptor mRNA were performed as previously described [1]. The specific activity of the probes was greater than $1 \times 10^9$ cpm/µl. In order to perform in situ hybridization with each cRNA probe, cultured cells of different cell lines were fixed and embedded into the OCT compound (Embedding material from Miles, USA). Tissue blocks were sectioned at the thickness of 10 µm and slides were washed in 2× sodium chloride-sodium citrate buffer (SSC). Subsequently, sections were covered with prehybridization buffer and incubated at 37°C for 1 h. After the removal of the prehybridization buffer, hybridization with the sense or antisense probes were carried out in the same solution with the addition of 500 µl/ml yeast tRNA, and $1 \times 10^5$ cpm/slides of RNA probes. Slides were cover-slipped and incubated at 60°C for 20–24 h. Tissue slides were then washed using posthybridization buffer. To exclude the possibility of mismatch, sections were treated with RNase A (50 µg/ml). Slides were then transferred to washing buffer containing 0.1 × SSC, and washed twice in 2 × SSC buffer (50°C, high-stringency). Slides were then dipped in Kodak $NTB_2$ emulsion, exposed for 2 weeks at 4°C, developed in a Kodak D19 developer (1:1 dilution, 15°C) and counterstained with methyl green. Slides were observed under a bright-field microscope and then photographed.

### Northern blot analysis

Total cellular RNA was isolated from prostate cancer cells by lysis with 7.5 M guanidine HCl, followed by extraction in phenol-chloroform. For hybridization, total RNA was fractionated by denaturing agarose gel electrophoresis, visualized by staining with ethidium bromide, and transferred to Zeta-Probe membrane (Bio-Rad Laboratories, Richmond, Calif.) by capillary diffusion in 10 × SSC (1.5 M NaCl and 0.15 M Na citrate). Membranes were prehybridized in buffer containing 50% deionized formamide, 270 mM NaCl, 15 mM Na phosphate, 1.5 mM EDTA, 0.5% nonfat dry milk, 1% SDS, and 0.1 µg/ml denatured sonicated salmon sperm DNA for 2 h at 42°C. Membranes were then hybridized using $1 \times 10^6$ cpm/ml GnRH cDNA probes at 42°C for 16 h. Membranes were rinsed in 2× SSC and washed in succession with 2× SSC-0.1% SDS, 0.5× SSC-0.1% SDS and 0.1× SSC-0.1% SDS at 55°C. Membranes were exposed to X-ray film (Amersham, UK) at −70°C.

### Thymidine incorporation assay

Thymidine incorporation assay was conducted using the procedure of Boreham et al. [6]. Two media, one supplemented with 10% of normal bovine calf serum (media A) and the other supplemented with 10% of charcoal-stripped serum (media B), were prepared. Cells were divided into five groups; two control groups in media A and B, and three experimental groups each treated individually with GnRH $10^{-7}$ M, $10^{-9}$ M and $10^{-11}$ M in media B. Five groups were seeded with $1 \times 10^6$ cells/ml in 75 $cm^2$ culture flasks with media A and incubated for 24 h. The media had changed as grouped; one with media A (control for normal serum) and four with media B after two gentle washings of each group of cells in the flask with specific media (10 ml). The GnRH (Sigma) in the experimental groups was treated according to schedule. Immediately after the GnRH treatments, 2 µCi $^3$H-thymidine was added to all five groups. In LNCaP, the media change, GnRH and $^3$H-thymidine treatments were repeated once at 96 h after seeding and the assay was done on the seventh day. In the other three cell lines, assays were done 96 h after seeding. Briefly, the flasks were washed three times with cold PBS and then washed twice with ice-cold 5% trichloroacetate and after rinsing, 3 ml of 0.25 M NaOH was added to each flask, resulting in complete cell lysis. From the cell lysate, 1 ml solution was taken and mixed with 10 ml scintillation cocktail (PPO 5.5 g, POPOP 0.1 g, Toluene 667 ml and Triton X-100 333 ml) for the measurement of the incorporated radioactivity. All samples were measured using a scintillation counter (Packard, Tri-Carb 1500).

### Statistical analysis

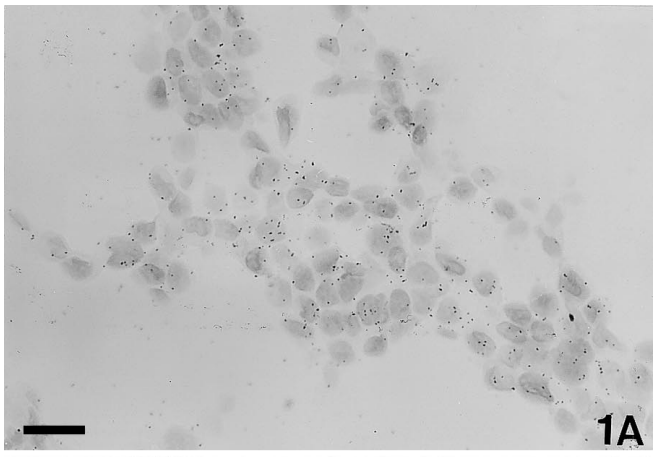
Statistically significant differences between various experimental groups were proved using Student's *t*-test ($P \leq 0.05$).

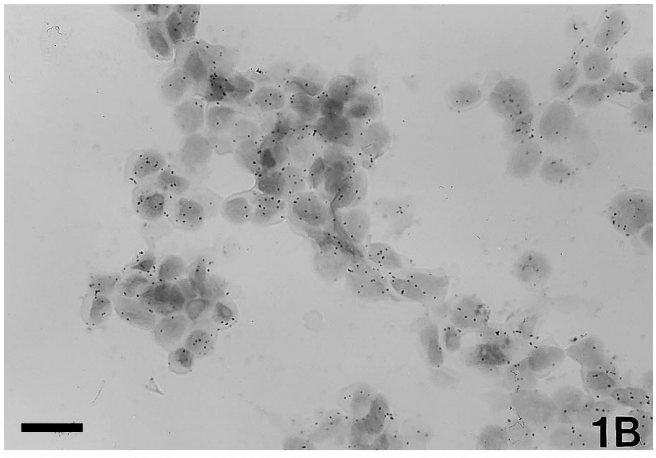
## Results

### In situ localization of GnRH and GnRH receptor mRNAs

GnRH mRNA was localized in the majority of cells in four cell lines (Fig. 1). GnRH mRNA signals were observed in 83.48% LNCaP cells and 95.3% DU-145 (Fig. 1A, B). The sense probe did not show any signals for GnRH mRNA (Fig. 1C). The number of signals within each cell varied from zero to eleven. Mean signal numbers of the GnRH mRNA were 4.1, 6.95, 7.12 and 7.31, respectively for LNCaP, Du-145, TSU-Pr1 and PC-3.

Signals for the GnRH receptor mRNA were detected in the majority of cells (Fig. 2). The majority of the cultured LNCaP and Du-145 cell lines hybridized with



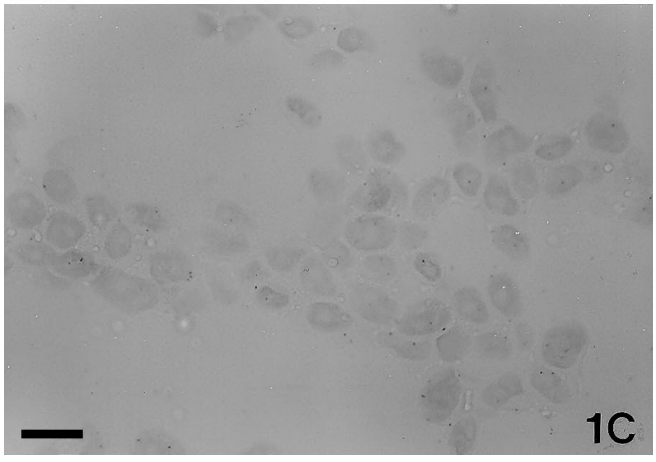
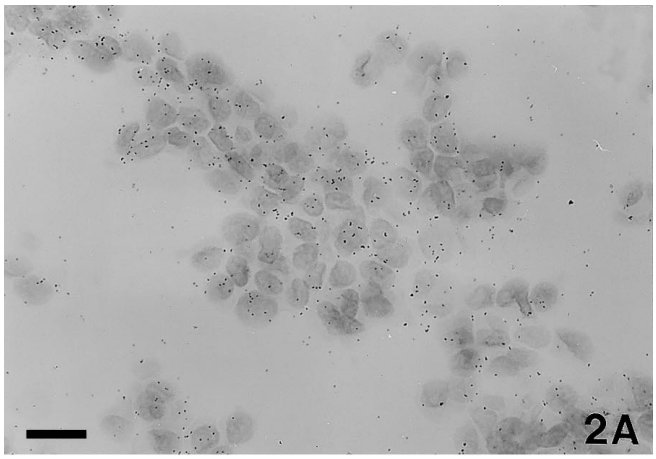
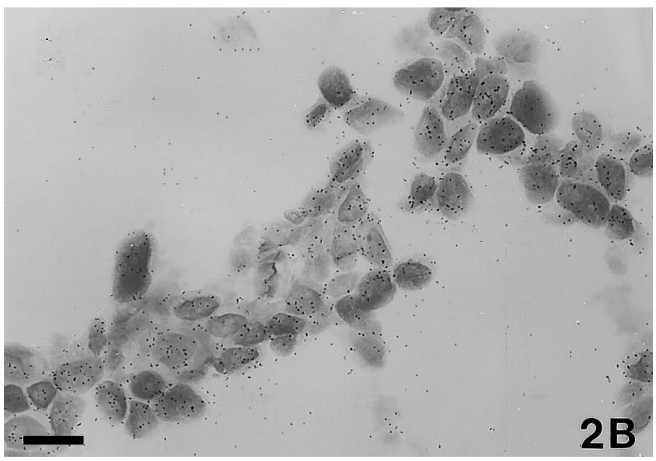




**Fig. 1** In situ localization of GnRH mRNA in cultured prostate cancer cell lines, LNCaP (A) and DU-145 (B and C). Antisense (A and B) and sense (C) RNA probes of GnRH were used for in situ hybridization. Cultured cells were collected and embedded with OCT compound, and blocks were sectioned to 10 μm thickness. Signals for the GnRH mRNA were identified as black dots in the figures. Mean signal numbers of GnRH mRNA in a cell of LNCaP and DU-145 were 2.75 and 4.1 respectively. The bar represents 50 μm



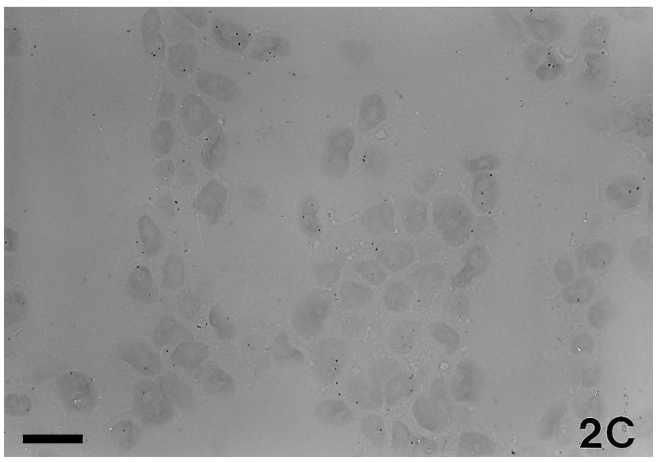




**Fig. 2** In situ localization of GnRH receptor mRNA in cultured prostate cancer cell lines, LNCaP (A) and DU-145 (B and C). Antisense (A and B) and sense (C) RNA probes of GnRH receptor were used for in situ hybridization. Cultured cells were collected and embedded with OCT compound, and blocks were sectioned to 10 μm thickness. Signals for the GnRH receptor mRNA were identified as black dots in the figures. Mean signal numbers of GnRH receptor mRNA in a cell of LNCaP and DU-145 were 4.15 and 6.95 respectively. The bar represents 50 μm

the antisense probe represented as black dots (Fig. 2A, B). However, the sense probe failed to show any signals (Fig. 2C). The number of signals for the GnRH receptor mRNA in all cell lines was greater than that of GnRH mRNA.

## Expression of GnRH mRNA in prostate cancer cell lines

Northern blot hybridization showed that the prostate cancer cell lines expressed the same GnRH mRNA as

that expressed in the hypothalamus (Fig. 3). The level of GnRH transcripts in each prostate cancer cell line appeared to almost even, when the same amount of total RNA (20 μg) was loaded on to each lane. All prostate cancer cell lines contained a considerable amount of GnRH mRNA compared with the hypothalamus, which served as a positive control. The cortex served as a negative control.

### Thymidine incorporation assay

The growth of control groups in media A and B was significantly different ($P < 0.05$; Fig. 4) in all four cell lines. The proliferation of control cells grown in media B was significantly lower than in media A. The proliferation of hormone sensitive LNCaP cells showed some fluctuations depending on the concentration of the GnRH, but had no significance ($P > 0.05$). The proliferation of the hormone refractory cells was not consistent either. Variations in proliferation after the treatment of GnRH in prostate cancer cell obtained from the hormone-resistant cell lines were statistically insignificant ($P > 0.05$).

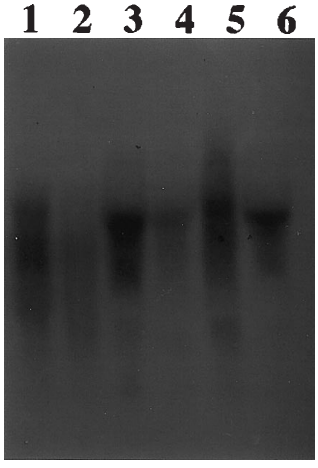



**Fig. 3** Northern blot analysis of GnRH transcripts. Total cytoplasmic RNA (20 *μg* each) derived from each prostate cancer cell lines were applied. RNA blotted membranes were hybridized with $^{32}$P-labeled GnRH cDNA probes. Note that the size of GnRH mRNA derived from each prostate cancer cell line was the same as that of the hypothalamus. Lane 1: hypothalamus (positive control); 2: cortex (negative control); 3: DU-145; 4: LNCaP; 5: PC-3; 6: TSU-Prl

## Discussion

There have been several reports concerning the extrahypothalamic production of GnRH [2, 3, 10, 14, 27, 28]. These studies suggested the additional role of locally produced GnRH rather than hypothalamic GnRH. Recently, prostate has been shown to produce GnRH [2, 27]. Furthermore, GnRH or GnRH-like material was also produced in normal and malignant prostate cells [18, 26]. In the present study, we confirmed by in situ hybridization and Northern blot analysis of GnRH mRNA that GnRH is produced in human prostate cancer cells. Although the role of this GnRH produced from the prostate cancer cells has not been fully defined, it is noteworthy that GnRH or its analog may useful for prostate cancer therapy. Few reports have determined the direct effect of GnRH or GnRH-related products on the proliferation of prostate cancer cells [7, 17, 19, 25]. Both proliferative [25] and antiproliferative [7, 17, 19] effects of GnRH on prostate cancer cells have been previously reported. Qayum et al. [25] stated that GnRH has a direct proliferative effect on prostate epithelium. He discovered that GnRH analog-binding sites exhibited a high affinity for the androgen-sensitive LNCaP with proliferation of prostate epithelium, while possessing a low affinity for the androgen-resistant DU-145 without

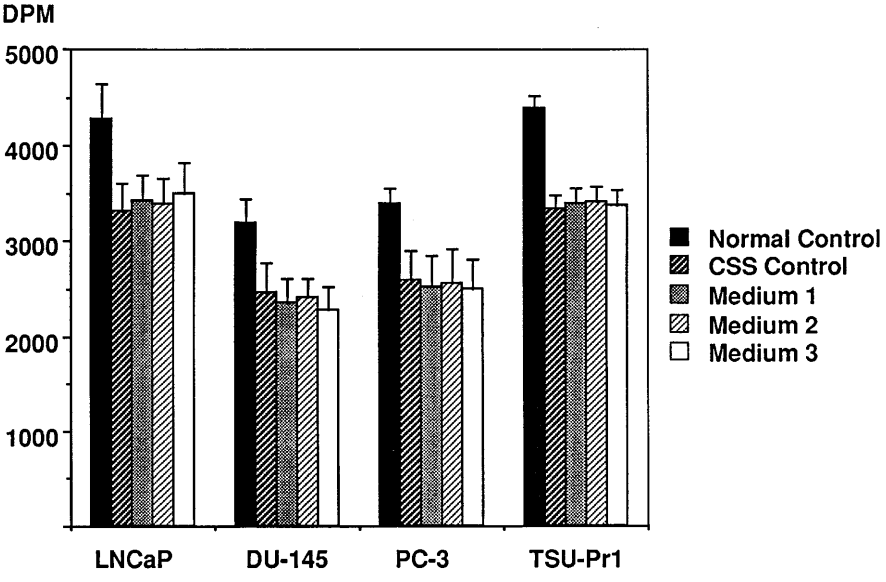



**Fig. 4** The uptake of tritium ($^3$H)-labeled thymidine. There was no significant difference in the uptake of thymidine between the control and GnRH-treated experimental groups grown in medium containing charcoal-stripped serum (CSS), regardless of cell lines. The uptake of thymidine from the cultured cells grown in medium containing normal serum was significantly different from that grown in the medium containing CSS. DPM disintegrations per minute, *medium 1* medium containing 10%-CSS treated with $10^{-11}$ M of GnRH, *medium 2* medium containing 10% CSS treated with $10^{-9}$ M of GnRH, *medium 3* medium containing 10% CSS treated with $10^{-7}$ M of GnRH

any biologic role. Numerous reports [7, 17, 19] have documented data comparative to Qayum et al. [25], stating that the GnRH analog demonstrates antiproliferative effects on prostate cancer cells. Loop et al. [19] proposed that antiproliferative effects of GnRH were due to competitive binding between the GnRH analog and the receptor complex in the prostate. Our data using native GnRH did not show any significant effect of suppression or promotion on the proliferation of prostate cancer cells. Others [8, 19] have reported that the small effect of proliferation on prostate cancer cells is due to the lower affinity binding of native GnRH in prostate cancer cells. Compared with the effect of the single hormone GnRH, the lack of the multiple steroid hormones in the serum of the culture media significantly reduced the proliferation of prostate cancer in all four cell lines. Although it is not well known which hormones are related to prostate cancer cell growth, the finding that downregulation of the proliferation of prostate cancer cells occurred in steroid hormone-deficient media suggests that the lack of some steroid hormone could be responsible for the downregulation.

The antiproliferative effect of the GnRH analog on human breast carcinoma cells [23] and prostate cancer cells [19] does not seems to be related to direct cytotoxicity. The antiproliferative role against native ligands in the GnRH analog seems to be due to the competitive high-affinity binding to prostate cancer cells against native ligands [19]. Qayum et al. [25] detected the existence of GnRH-specific receptors in LNCaP but not in DU-145. He also determined high-affinity GnRH analog binding in more than 85% of benign and malignant human prostatic tumors and GnRH-like peptides in 68% of benign and 27% of malignant tumors [25]. Others [9] have reported that 86% of malignant tumors and 91% of benign prostatic tumors possess the high-affinity receptor for GnRH. In the present study, expressions of GnRH mRNA were found in more than 83% of whole prostate cancer cells of four human cell lines. In addition, GnRH receptor mRNAs were detected in more than 95% of cancer cells, regardless of hormone-sensitive or refractory cells.

In conclusion, prostate cancer cells produce GnRH, the pivotal hormone on the hypothalamopituitary gonadal axis, as well as its receptor. The treatment of prostate cancer cells with exogenous GnRH does not exert any direct effect on the proliferation of these cells. But the proliferation of prostate cancer cells was downregulated by the absence of the steroid hormones in the serum, although it is not well known which hormone is responsible for the suppression of the proliferation. Although, it is difficult to sate definitely for all four cell lines, when we combine our data with Maggi and coworkers' report that the GnRH-degrading enzyme is present in the prostate cancer cells, it can be assumed that some prostate cancer cells possess a system comprising production of GnRH and GnRH receptor, as well as GnRH metabolic enzymes within them. We hypothesized that the role of the GnRH produced in the prostate cancer cell is autocrine and that the peripheral mechanism for the GnRH analog in the treatment of prostate cancer should be studied in greater detail in addition to their central mechanism.

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