Decrease in the level and mRNA expression of LH-RH and EGF receptors after treatment with LH-RH antagonist Cetrorelix in DU-145 prostate tumor xenografts in nude mice

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Abstract. Using radioligand binding, RT-PCR, and Southern blot analyses, we evaluated whether agonist [D-Trp^6]LH-RH and antagonist Cetrorelix could affect the levels of receptors for LH-RH and EGF and expression of mRNA for these receptors in DU-145 human androgen-independent prostate cancers xenografted into nude mice. Radioligand binding studies showed the presence of specific high affinity receptors for LH-RH and EGF in DU-145 prostate tumors. Cetrorelix, but not [D-Trp^6]LH-RH significantly inhibited tumor growth. The concentration of LH-RH receptors was reduced by 22% ($p<0.05$) and 67% ($p<0.01$) after 4 weeks of treatment with [D-Trp^6]LH-RH and Cetrorelix respectively. The concentration of EGF receptors fell by 48% ($p<0.05$) in the [D-Trp^6]LH-RH group, whereas Cetrorelix led to a 66% reduction ($p<0.01$). The expression of LH-RH and EGF receptor mRNA was investigated by RT-PCR analysis followed by Southern blotting. Densitometric analysis of the developed bands showed that the antagonist Cetrorelix decreased the expression of LH-RH receptor mRNA by 55% ($p<0.01$) compared to control group while the 20% reduction after treatment with the LH-RH agonist was non-significant. Treatment with [D-Trp^6]LH-RH and Cetrorelix also reduced the expression of EGF receptor mRNA by 35% and 68% respectively (both, $p<0.01$) compared to control group. In conclusion, these data demonstrate that growth inhibition of DU-145 prostate tumors induced by prolonged administration of LH-RH antagonist Cetrorelix is accompanied by a marked decrease in the concentration of LH-RH and EGF receptors as well as in their mRNA levels.

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Abbreviations: LH-RH, luteinizing hormone-releasing hormone; LH-RHR, luteinizing hormone-releasing hormone receptor; EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Key words: LH-RH antagonist, androgen-independent prostate cancer, RT-PCR, Southern blotting

Introduction

Hypothalamic LH-RH plays a key role in the reproductive process in mammals. After its release from the hypothalamus, LH-RH binds to its specific receptors on the pituitary gonadotropes. Activation of these receptors stimulates the secretion of gonadotropins, which in turn regulate the reproductive functions and secretion of steroid hormones (1). Specific high affinity binding sites for LH-RH are also found in extrapituitary tissues including prostate cancers (2). Clinical applications of LH-RH agonists and antagonists in the treatment of prostatic carcinoma are mainly based upon the ability of these analogs to suppress testosterone secretion through the desensitization of the gonadotropes and down-regulation of the pituitary LH-RH receptors (3,4). In the case of the antagonists, a competitive LH-RH receptor occupancy is possibly also involved (4). Repeated administration of LH-RH agonists is required to induce suppression of LH and FSH release and a reduction in the levels of sex steroids. Similar effects can be obtained after a single injection of LH-RH antagonists (3,4). Recent studies support the view that antagonists down-regulate pituitary LH-RH receptors (5-9). Both LH-RH agonists and antagonists decrease mRNA levels for pituitary LH-RH receptors (10-13). It has also been shown that LH-RH analogs exert direct inhibitory effects on tumor cells *in vitro* (2-4,14). Clinical trials in normal subjects and patients with benign prostatic hyperplasia and prostate cancer demonstrated protracted suppression of LH and testosterone levels by Cetrorelix (15-17). It was also shown that administration of 10 mg loading doses of Cetrorelix, followed by daily maintenance doses of 1-2 mg can produce a persistent suppression of serum testosterone in normal men, suggesting a down-regulation of LH-RH receptors in men (17).

Recently we reported that LH-RH antagonist Cetrorelix down-regulates LH-RH and EGF receptors on tumor membranes and inhibits the growth of human androgen-independent DU-145 prostate cancers xenografted into nude

Table I. Oligonucleotide primers and probes used for RT-PCR and Southern blotting for LH-RH receptors, hEGF receptors, and hGAPDH mRNA expression in DU-145 prostate tumors.

mRNA product	Primers, probes	Sequences (5'-3')	Position	Target size (bp)	Refs.
hLH-RHR	Sense	GAC CTT GTC TGG AAA GAT CC	93-112	319	21
	Antisense	CAG GCT GAT CAC CAC CAT CA	392-411		
	Target probe	ATG ACA ATC AGA GTC TCC AAC AGG TTG GCT	254-284		
hEGFR	Sense	ACG CAG ATA GTC GCC CAA AGT TCC	3047-3070	400	22
	Antisense	AGG AAG GTG TCG TCT ATG CTG TCC	3423-3446		
	Target probe	TGG ATG AAG AAG ACA TGG ACG ACG TGG TGG	3191-3220		
hGAPDH	Sense	TCC TCT GAC TTC AAC AGC GAC ACC	907-930	207	23
	Antisense	TCT CTC TTC CTC TTG TGC TCT TGG	1091-1114		
	Target probe	TGT CAA GCT CAT TTC CTG GTC TGA CAA CGA	981-1010		

mice (18). The agonist [D-Trp^6]LH-RH produced a lesser receptor down-regulation and no significant tumor inhibition. Based on these observations, it is hypothesized that treatment with LH-RH agonists and antagonists might also result in decrease in mRNA expression for LH-RH and EGF receptors on tumors. In this study, we investigated the effect of *in vivo* treatment with antagonist Cetrorelix and agonist [D-Trp^6]-LH-RH on the levels of receptors for LH-RH and EGF and expression of their mRNA in DU-145 prostate tumors xenografted into nude mice.

Materials and methods

Peptides. LH-RH antagonist Cetrorelix (SB-75), originally synthesized in our laboratory (3,4), was made and supplied by ASTA Medica (Frankfurt/Main, Germany). Agonist [D-Trp^6]LH-RH was provided by Debiopharm (Lausanne, Switzerland). [D-Trp^6]LH-RH was dissolved in 0.1% dimethyl sulfoxide (DMSO) in 0.9% saline. Cetrorelix was dissolved in distilled water containing 5% mannitol.

Cell culture. The human androgen-independent prostatic carcinoma cell line DU-145 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). It was grown in RPMI 1640 medium (Gibco, Grand Island, NY) with supplements. Xenografts were initiated by s.c. (subcutaneous) injection of $1x10^7$ cells into the right flanks of 5 male nude mice.

Animals. Male athymic NCR/c (nu/nu) 6-week-old mice were obtained from the National Cancer Institute (Bethesda, MD) and housed and fed as described previously (18). All animal studies were conducted in accord with institutional guidelines for the care and use of experimental animals.

Experimental protocol. Tumors resulting after 8 weeks were aseptically dissected; 3 mm^3 pieces of tumor tissue were transplanted s.c. by trocar needle into male mice as described (18). Four weeks after transplantation, the mice bearing 70-90 mm^3 tumors were divided into various experimental groups of 8 animals each. The 3 groups analyzed in this study have received the following treatments: group 1 (controls), saline only; group 2, agonist [D-Trp^6]LH-RH at a dose of 100 µg/day/animal s.c.; group 3, Cetrorelix, 100 µg/day/animal s.c. The treatment was continued for 4 weeks. At the end of the experiment, mice were anaesthetized with methoxyflurane (Metofane; Pitman-Moore, Mundelein, IL), sacrificed by decapitation, and the tumor samples for receptor and molecular biology studies were placed immediately in liquid nitrogen.

Radioligand binding studies. Receptors for LH-RH and EGF on tumor cell membranes were measured by ligand competition assays. Preparation of membrane fractions and receptor binding studies were performed as previously described (9,19). Radioiodinated [D-Trp^6]LH-RH was prepared by chloramine-T method as described (9), while ^{125}I-labeled EGF was purchased from Amersham (Arlington Height, IL, USA). The ligand-PC computerized curve fitting program of Munson and Rodbard (20) was used to determine the type of receptor binding, dissociation constant (K_d) and maximal binding capacity of receptors (B_{max}).

RNA extraction. Total RNA was extracted from human DU-145 tumors using RNAZol™ B (Tel-Test Inc, Friendswood, TX) according to the manufacturer's instructions. The RNA pellets were suspended in 50 µl of 10 mM Tris, 1 mM EDTA buffer (pH 8.0) and quantified spectrophotometrically at 260 nm.

Reverse transcription and polymerase chain reaction. One µg of total RNA extracted from DU-145 prostate tumors was reverse transcribed into single strand cDNA using Moloney murine leukemia reverse transcriptase according to the manufacturer's instructions (Perkin Elmer, Norwalk, CT). After an initial 5-min denaturation step at 94°C, the RT-reaction mixture was subjected to 28 cycles of PCR amplification using specific primers for human LH-RH receptors, hEGF receptors and hGAPDH (21-23) (Table I). The number of cycles was determined previously to be within

the exponential range of PCR product amplification necessary for quantitative densitometry. Each cycle consisted of 1 min denaturation step at 94°C, a 1 min annealing step at 60°C and 2 min elongation at 72°C. The last cycle was followed by 10 min elongation at 72°C using a thermal cycler (Stratagene, La Jolla, CA). Negative controls were run in parallel to control for cross-contamination of samples. A reaction was performed as described above, but without the addition of reverse transcriptase as a test for the presence of contaminating genomic DNA in the RNA preparation from these tumors. Ten microlitres of PCR-amplified product was resolved by electrophoresis on 1.8% agarose gel, stained with ethidium bromide and visualized under ultraviolet light.

Southern blot analysis. After electrophoresis, the gel was treated with denaturation buffer and neutralization buffer, the PCR products were transferred to Hybond N$^+$ membrane by capillary transfer, and the DNA was linked onto it by baking for 2 h at 80°C. Sample blots were prehybridized at 60°C for 16 h in buffer containing 4X SSC, 2X Denhardt's solution, 0.1% SDS, 5 mM EDTA and 100 µg/ml denatured salmon sperm DNA. After prehybridization, the sample blots were hybridized at 60°C for 20 h in hybridization buffer containing 5X SSC, 0.5X Denhardt's solution, 0.02 M Tris-HCl, 100 µg/ml sonicated salmon DNA and 150 ng of [^{32}P]-5'-end-labeled oligonucleotide probes (Table I). The blots were washed under stringent conditions and the signals from samples were scanned and quantified using an imaging densitometer (Model GS-700, BioRad, CA).

Statistical analyses. All values are expressed as the mean ± SEM, and statistical analysis were performed using Duncan's multiple range test as described previously (5,7-9,12,18,19).

Results

Radioligand binding studies. Receptor binding studies ascertained the presence of LH-RH and EGF receptors in membrane fractions of DU-145 human androgen-independent prostate tumors xenografted into nude mice. Biochemical parameters established the identity of specific binding sites by determining the characteristics of LH-RH and EGF binding to cell membranes of DU-145 tumors. The displacement of radiolabeled [D-Trp^6]LH-RH by the same unlabeled peptide and the Scatchard analysis of these data (Fig. 1A) indicated that in membrane fractions of DU-145 tumors, labeled [D-Trp^6]LH-RH was bound to one class of high affinity binding sites with a mean dissociation constant (K_d) of 6.13±1.1 nM and with a mean maximal binding capacity (B_{max}) of 1019.9±38.7 fmol/mg membrane protein. After treatment with Cetrorelix the concentration of receptors for LH-RH was significantly ($p<0.01$) decreased by 67% (B_{max} = 335.9±37.9 fmol/mg membrane protein) compared with controls. The agonist [D-Trp^6]LH-RH also reduced the concentration of LH-RH receptors, but by only 22% (B_{max} = 795.8±10.2 fmol/mg membrane protein) ($p<0.05$) (Table II).

Specific binding sites for EGF were also found in membranes of DU-145 tumors. In samples from the control DU-145 tumors, the computerized curve fitting and the Scatchard plot analyses of the EGF binding data (Fig. 1B) demonstrated that the radiolabeled peptide was bound to a single class of high affinity, low capacity binding sites (K_d = 0.92±0.01 nM; B_{max} = 292.5±20.9 fmol/mg membrane protein). A highly significant 66% reduction ($p<0.01$) in maximal binding capacity of EGF receptors was observed after *in vivo* treatment with Cetrorelix (B_{max} = 99.6±0.5 fmol/mg membrane protein) (Table II). Therapy with [D-Trp^6]-LH-RH decreased the levels of EGF receptors by 48% to 153.7±22.2 fmol/mg membrane protein ($p<0.05$) in membranes of DU-145 tumors (Table II). Treatment with LH-RH agonist or antagonist did not have a significant effect on the affinity of receptors for LH-RH or EGF in either groups.

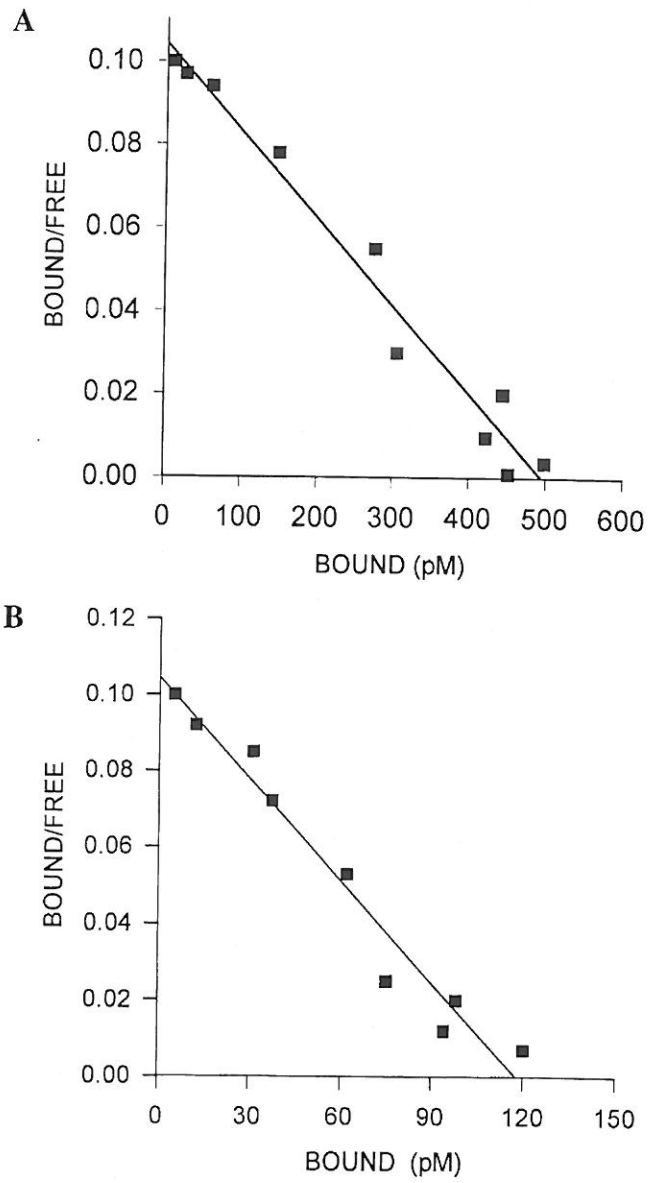

Figure 1. A, Representative example of Scatchard plots of [^{125}I][D-Trp^6]LH-RH binding to the membrane fraction isolated from untreated DU-145 human prostate cancer samples. Specific binding was determined as described. Each point represents mean of triplicate determinations; B, Representative example of Scatchard plots of [^{125}I]EGF binding to the membrane fraction isolated from untreated DU-145 human prostate cancer samples. Specific binding was determined as described. Each point represents mean of triplicate determinations.

Table II. Binding characteristics of EGF and LH-RH receptors in membranes of DU-145 human prostate cancer cell line xenografts in nude mice after *in vivo* treatment with antagonist Cetrorelix and agonist [D-Trp^6]LH-RH.

Treatment	LH-RH receptors		EGF receptors	
	K_d (nM)	B_{max} (fmol/mg protein)	K_d (nM)	B_{max} (fmol/mg protein)
Control	6.13±1.1	1019.9±38.7	0.92±0.01	292.5±20.9
[D-Trp^6]LH-RH	5.05±1.1	795.8±10.2[a]	0.85±0.18	153.7±22.2[a]
Cetrorelix	8.52±1.68	335.9±37.9[b]	0.65±0.03	99.6±0.5[b]

[a]$p<0.05$ vs. control; [b]$p<0.01$ vs. control. Binding characteristics were obtained from 12-point displacement experiments. All values represent mean ± SE of 2-3 independent experiments, each done in duplicate or triplicate. Significance was calculated using Duncan's new multiple range test.

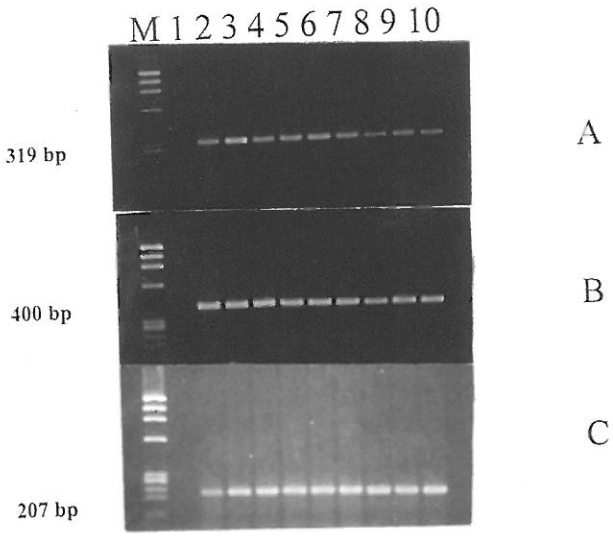

Figure 2. RT-PCR analysis of human LH-RH receptors (A), hEGF receptors (B) and hGAPDH (C) mRNA expression. PCR products were separated by 1.8% agarose gel electrophoresis and stained with ethidium bromide. The sizes of expected PCR products were 319, 400 and 207 bp for hLH-RHR, hEGFR, and hGAPDH, respectively. Lane M (molecular weight marker): ΦX174 HaeIII digest; lane 1, negative control; lanes 2-4, tumor samples from untreated animals; lanes 5-7, tumor samples from animals treated with agonist [D-Trp^6]LH-RH; lanes 8-10, tumor samples from animals treated with LH-RH antagonist Cetrorelix.

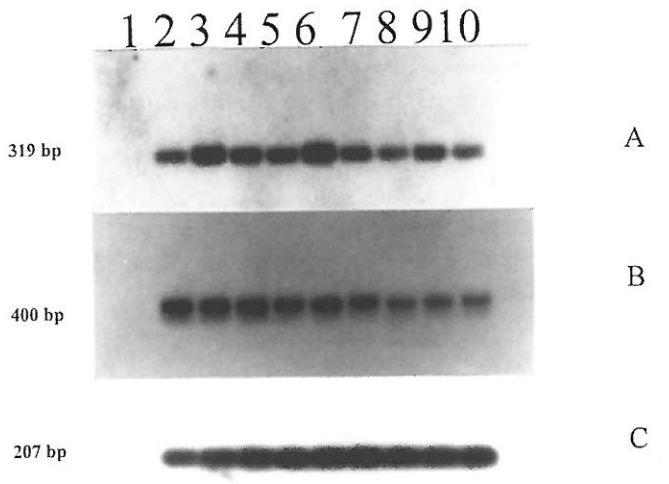

Figure 3. Southern blot analysis of complementary cDNA for hLH-RH receptors, hEGF receptors and hGAPDH obtained after RT-PCR. The PCR products were separated by 1.8% agarose gel electrophoresis and transferred to Hybond N$^+$ membranes. Hybridizations were performed with the oligonucleotide probes specific for hLH-RH receptors (A), hEGF receptors (B) and hGAPDH (C). The blots were washed under stringent conditions and exposed to autoradiography. Numbering is the same as in the Fig. 2.

Molecular biology analysis. To investigate whether the decrease in the concentration of LH-RH and EGF receptors observed by radioligand binding assays is associated with the inhibition of mRNA expression for EGF receptors and LH-RH receptors, RT-PCR analysis was performed. The PCR products of 319 and 400 bp for LH-RH receptors and EGF receptors were respectively detected after ethidium bromide staining under UV light (Fig. 2A and B). The specificity of PCR products was confirmed by Southern blotting using oligonucleotide probes (Fig. 3A and B). No PCR products were amplified from the negative controls, ruling out the possibility of any genomic contamination. Amplification with hGAPDH specific primers produced a single product of 207 bp from all samples, confirming that there was no degradation in the RNA preparation (Figs. 2C and 3C). Semi-quantitative analysis of the developed bands by densitometry showed that the mRNA levels for the LH-RH receptors were non-significantly reduced to 79.5±5.9% in the group treated with [D-Trp^6]LH-RH compared with controls accepted as 100±12.9% (Fig. 4A) while Cetrorelix strongly suppressed the LH-RH receptor mRNA expression, reducing it to 45.0±6.2% ($p<0.01$). We also investigated the effect of [D-Trp^6]LH-RH and Cetrorelix on the expression of mRNA for EGF receptors. After treatment with the agonist, the expression of EGF receptors mRNA was reduced to 65.1±4.6% ($p<0.01$), while following Cetrorelix the levels of mRNA for EGF receptors were more powerfully decreased to 31.5±4.5% ($p<0.01$) compared with controls accepted as 100±11.5% (Fig. 4B).

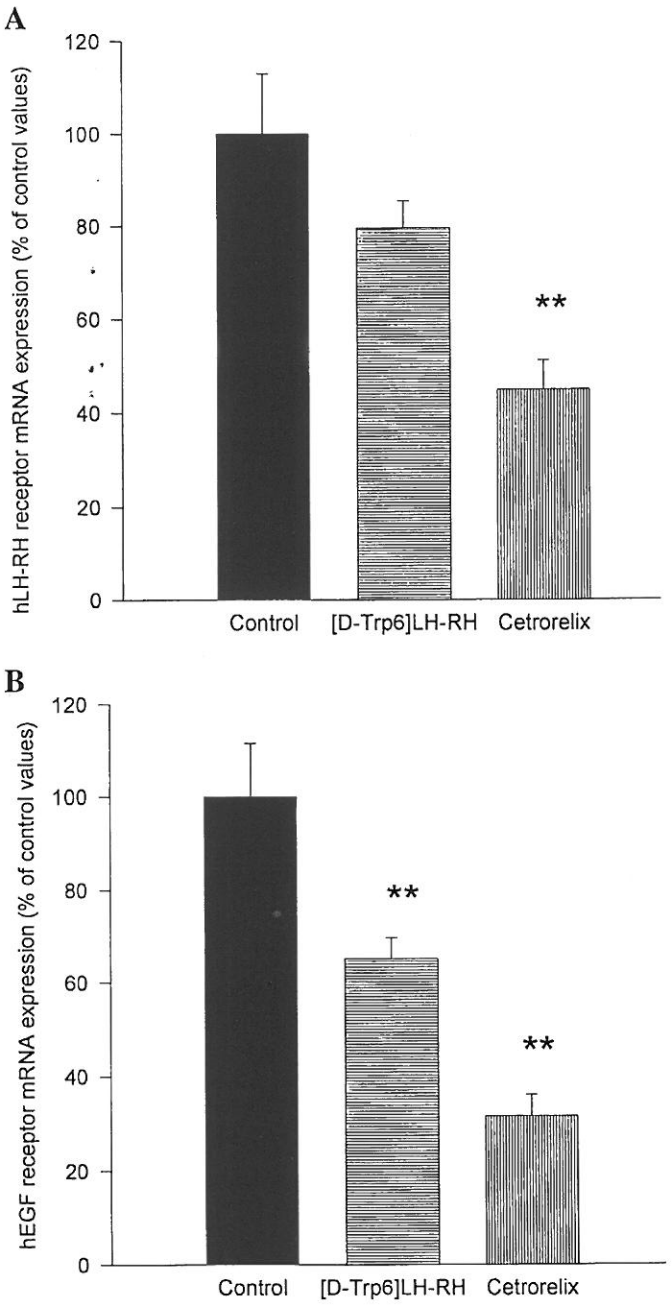

Figure 4. Densitometric analysis of hEGF receptors, hLH-RH receptors and hGAPDH mRNA from DU-145 prostate tumors of control (untreated) mice and animals treated with agonist [D-Trp^6] LH-RH or antagonist Cetrorelix. The levels of mRNA for LH-RH receptor (A) and EGF receptor (B) were standardized according to the levels of hGAPDH mRNA and are expressed as a percentage of control value. The results are presented as mean values ± standard error of the mean of two independent experiments. The significance was calculated using Duncan's new multiple range test. $^{**}p<0.01$.

Discussion

Previous investigations have shown that LH-RH antagonists and agonists exert their therapeutic effects through inhibition of release of LH, FSH, and sex steroids (3,4,13,17) which result from desensitization of gonadotropes, down-regulation of LH-RH receptors, and reduction in the mRNA for LH-RH receptors in the pituitary (10-13,24,25). In addition, LH-RH analogs may exert direct effects on tumors (2-5). Recently, Dondi *et al* (26), have shown that LH-RH agonists inhibit the proliferation of DU-145 human prostate cancer cell line *in vitro*. This was attributed to a direct effect on LH-RH receptors expressed in the membranes of this tumor (26). We have recently shown that [D-Trp^6]LH-RH agonist and antagonist Cetrorelix produced a significant decrease in the levels of serum LH and testosterone, and a dramatic fall in the concentration of receptors for EGF and LH-RH in DU-145 human prostate carcinomas xenografted into nude mice (18). Cetrorelix also strongly inhibited the growth of these tumors (18). In addition, Moretti *et al* (27), reported that LH-RH agonists antagonize the proliferative action of EGF and reduce the concentration of EGF receptors in both androgen-dependent LNCaP and androgen-independent DU-145 prostate cancer cell lines. Irmer *et al* (28), detected mRNA for LH-RH receptors in human ovarian cancer specimens and in ovarian cancer lines. It has also been reported that LH-RH agonist Triptorelin and the antagonist Cetrorelix inhibit the proliferation *in vitro* of human ovarian cancer cell lines EFO-21 and EFO-27 as well as of human endometrial cancer cell lines Ishikawa and HEC-1A (14).

The present study confirms and extends our previous findings (18) that [D-Trp^6]LH-RH and Cetrorelix reduce significantly the concentration of EGFR and LH-RHR in DU-145 human prostate cancers xenografted into nude mice. In addition, using RT-PCR and Southern blot analyses, we showed the expression of LH-RH and EGF receptor mRNA in DU-145 tumors. The PCR products obtained were of the expected sizes. The treatment with Cetrorelix led to significant reductions in mRNA for LH-RH and EGF receptors while [D-Trp^6]LH-RH decreased significantly only the mRNA expression for EGF receptors. Cetrorelix also inhibited the growth of PC-3 human prostate tumors and this inhibition was associated with a significant decrease in the concentration of EGF receptors in tumor membranes (29). Similarly, we demonstrated that Cetrorelix, but not [D-Trp^6]LH-RH, inhibited the growth of OV-1063 human epithelial ovarian cancers in nude mice and reduced the levels of EGF receptors and their mRNA (30).

DU-145 human prostate cancer cells express about 10 times more EGF receptors than androgen-sensitive LNCaP cells (31), and also secrete both immunoreactive TGF-α and EGF into serum-free medium (32). Androgens represent the major exogenous stimulus for the growth of androgen-dependent LNCaP prostate cancer cells and regulate the expression and activity of the EGF receptors (33-35). In androgen-independent DU-145 prostate tumors, the down-regulation of EGF receptors after treatment with LH-RH analogs could be the result of a direct effect of LH-RH analogs on the level and expression of EGF receptors.

The exact mechanisms involved in LH-RH and EGF receptor down-regulation are not known. It has been shown that LH-RH and LH-RH agonists aggregate on the cell surface and are rapidly internalized in response to ligand binding, accumulating first in Golgi apparatus and later in secretory granules and lysosomes (36,37). LH-RH may initiate a series of intracellular signals in the gonadotropes that result in phosphatidylinositol hydrolysis and mobilization of Ca^{2+} from intracellular stores as well as production of

diacylglycerol and activation of protein kinase C (38). The similarity of LH-RH receptors in the pituitary and in the peripheral tumors suggests that LH-RH signal-transduction pathways in some cancers might be the same as those functioning in the pituitary, such as phospholipase C and protein kinase C (14). On the other hand, LH-RH analogs might counteract growth factor-induced tyrosine phosphorylation in tumors through activation of a phosphotyrosine kinase, which is probably coupled to the LH-RH receptor through a Gi protein (19). There are various pathways by which LH-RH receptors could be affected by LH-RH analogs: i) Modulation of the receptor binding due to changes in the receptor conformation, degradation or recycling of binding sites; ii) Internalization of the receptor (38); iii) Modulation of receptor synthesis at transcriptional level (10-13).

EGF receptors could also be affected by similar mechanisms (40-43). It has also been reported that LH-RH agonists suppress the EGF-induced activation of mitogen-stimulated protein kinase in ovarian and endometrial cancer cells (14) and expression of c-*fos* gene in prostate cancer cells (27). There are differences in the magnitude of the effect of LH-RH antagonists as compared with that of agonists. In some cells, LH-RH antagonists activate only phospholipase A2 (30). The lack of stimulatory effect on phospholipase C by LH-RH antagonists might result in the failure to activate protein kinase C. In epithelioid tumor lines, the activation of protein kinase C leads to a 5-fold induction of EGFR gene transcription (reviewed in ref. 30). Thus, our observation that LH-RH antagonist Cetrorelix greatly reduced the levels of EGFR mRNA might result from lack of stimulation pathways that activate protein kinase C, which could increase *de novo* transcription of the EGFR gene. Cetrorelix may also inhibit cancer cell growth by down-regulation of the EGFR through suppression of its phosphorylation on a tyrosine residue. Other mechanisms, such as alteration of its rate of degradation, might also be involved in modifying pools of EGFR mRNA.

Our results clearly demonstrate for the first time that the reduction in the concentration of LH-RH and EGF receptors in DU-145 human androgen-independent tumors after treatment with LH-RH antagonist Cetrorelix correlates with the decrease in mRNA levels for these receptors. This observation may suggest that loss of receptors is the result of down-regulation of gene transcription or a change in the stability of mRNA for the receptor. Cetrorelix appears to act directly on tumors, and its net effect is the reduction in available binding sites for LH-RH and EGF on tumors. A complete elucidation of the molecular mechanism through which LH-RH antagonists like Cetrorelix reduce the proliferation of prostate cancers and other carcinomas is important for the clinical use of these drugs. The use of antagonistic analogs of LH-RH for the treatment of cancers including prostate would avoid the transient stimulation of the release of gonadotropins and sex steroids that occurs initially in response to LH-RH agonists, thus preventing the temporary clinical flare-up of the disease (4).

In conclusion, our findings reported herein and in another study (18) suggest that growth of androgen-independent DU-145 human prostate tumors xenografted in nude mice can be strongly inhibited by LH-RH antagonist Cetrorelix. This effect was associated with a reduction in the concentration of LH-RH receptors and EGF receptors on the membranes of those tumors and significant decrease in the expression of mRNA for LH-RH receptors and EGF receptors. Taken together, these results suggest that the down-regulation of both EGF and LH-RH receptors occur at transcriptional level, but other mechanisms could be involved in modifying the pools of those receptors. The reduction in the levels of EGFR and their mRNA after treatment with Cetrorelix is of clinical relevance since a high expression of EGFR may be related to tumor aggressiveness.

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References

1. Clayton RN: Gonadotropin-releasing hormone: its actions and receptors. J Endocrinol 120: 9-11, 1989.
2. Qayum A, Gullick W, Clayton RC, Sikora K and Waxman J: The effects of gonadotropin hormone analogues in prostate cancer are mediated through specific tumor receptors. Br J Cancer 62: 96-99, 1990.
3. Schally AV and Comaru-Schally AM: Rational use of agonists and antagonists of luteinizing hormone-releasing hormone (LH-RH) in the treatment of hormone-sensitive neoplasms and gynæcologic conditions. Adv Drug Deliv Rev 28: 157-169, 1997.
4. Schally AV and Comaru-Schally AM: Hypothalamic and other peptide hormones. In: Cancer Medicine 4M. Holland JF, Frei E, Bast RC, Kufe DW, Morton DL and Weichselbaum RR (eds). Williams & Williams, Baltimore, MD, pp1067-1086, 1997.
5. Srkalovic G, Bokser L, Radulovic S, Korkut E and Schally AV: Receptors for luteinizing hormone-releasing hormone (LH-RH) in Dunning R-3327 prostate cancers and rats anterior pituitaries after treatment with a sustained delivery system of LH-RH antagonist SB-75. Endocrinology 127: 3052-3060, 1990.
6. Perheentupa A and Huhtaniemi I: Gonadotropin gene expression and secretion in gonadotropin-releasing hormone antagonist-treated male rats: effects of sex steroid replacement. Endocrinology 126: 3204-3209, 1990.
7. Bokser I, Srkalovic G, Szepeshazi K and Schally AV: Recovery of pituitary-gonadal function in male and female rats after prolonged administration of a potent antagonist of luteinizing hormone-releasing hormone. Neuroendocrinology 54: 136-145, 1991.
8. Pinski J, Yano T, Groot K, Milovanovic S and Schally AV: Comparison of biological effects of a sustained delivery system and noncapsulated LH-RH antagonist SB-75 in rats. Peptides 13: 905-911, 1992.
9. Halmos G, Schally AV, Pinski J, Vadillo-Buenfil M and Groot K: Down-regulation of pituitary receptors for luteinizing hormone-releasing hormone (LH-RH) in rats by LH-RH antagonist Cetrorelix. Proc Natl Acad Sci USA 93: 2398-2402, 1996.
10. Kaiser UB, Jakubowiak A, Stainberger A and Chin WW: Regulation of rat gonadotropin-releasing hormone receptor mRNA levels *in vivo* and *in vitro*. Endocrinology 133: 931-934, 1993.

11. Lerrant Y, Kottler ML, Bergametti F, Moumni M, Blumberg-Tick J and Counis R: Expression of gonadotropin-releasing hormone (GnRH) receptor gene is altered by GnRH agonist desensitization in a manner similar to that of gonadotropin ß-subunit genes and castrated rat pituitary. Endocrinology 136: 2803-2808, 1995.
12. Pinski J, Lamharzi N, Halmos G, Groot K, Jungwirth A, Vadillo-Buenfil M, Kakar SS and Schally AV: Chronic administration of the luteinizing hormone-releasing hormone (LH-RH) antagonist Cetrorelix decreases gonadotrope responsiveness and pituitary LH-RH receptor messenger ribonucleic acid levels in rats. Endocrinology 137: 3430-3436, 1996.
13. Wu JC, Sealfon SC and Miller WL: Gonadal hormones and gonadotropin-releasing hormone (GnRH) alter messenger ribonucleic acid levels for GnRH receptors in sheep. Endocrinology 134: 1846-1850, 1994.
14. Emons G, Ortmann O, Schulz K-D and Schally AV: Growth-inhibitory actions of analogues of luteinizing hormone-releasing hormone on tumor cells. Trends Endocrinol Metab 8: 355-362, 1997.
15. Gonzales-Barcena D, Vadillo-Buenfil M, Gomez-Orta F, Fuentes-Garcia M, Cardenas-Cornejo I, Graef-Sanchez A, Comaru-Schally AM and Schally AV: Responses to the antagonistic analog of LH-RH (SB-75, Cetrorelix) in patients with benign prostatic hyperplasia (BPH) and prostatic cancer. Prostate 24: 84-92, 1994.
16. Gonzalez-Barcena D, Vadillo-Buenfil M, Cortez-Morales A, Fuentes-Garcia M, Cardenas-Cornejo I, Comaru-Schally AM and Schally AV: LH-RH antagonist SB-75 (Cetrorelix) as primary single therapy in patients with advanced prostatic cancer and paraplegia due to metastatic invasion of spinal cord. Urology 45: 275-281, 1995.
17. Behre H, Kliesch S, Pühse G, Reissmann T and Nieschlag E: High loading and low maintenance doses of a gonadotropin-releasing hormone antagonist effectively suppress serum luteinizing hormone, follicle-stimulating hormone, and testosterone in normal men. J Clin Endocrinol Metab 82: 1403-1408, 1997.
18. Jungwirth A, Pinski J, Galvan G, Halmos G, Szepeshazi K, Cai R-Z, Groot K, Vadillo-Buenfil M and Schally AV: Inhibition of growth of androgen-independent DU-145 prostate cancer *in vivo* by luteinizing hormone-releasing hormone antagonist Cetrorelix and bombesin antagonists RC-3940-II and RC-3950-II. Eur J Cancer 7: 1141-1148, 1997.
19. Halmos G and Schally AV: Reduction in receptors for bombesin and epidermal growth factor in xenografts of human small-cell lung cancer after treatment with bombesin antagonists RC-3095. Proc Natl Acad Sci USA 94: 956-960, 1997.
20. Munson PJ and Rodbard DA: A versatile computerized approach for characterization of ligand binding system. Anal Biochem 107: 220-239, 1980.
21. Kakar SS, Musgrove LC, Devor CD, Sellers JC and Neill ND: Cloning, sequencing and expression of human gonadotropin releasing hormone (GnRH) receptor. Biochem Biophys Res Commun 189: 289-295, 1992.
22. Xu Y-H, Richert N, Ito S, Merlino GT and Pastan I: Characterization of epidermal growth factor receptor gene expression in malignant and normal cell lines. Proc Natl Acad Sci USA 81: 7308-7313, 1984.
23. Gordon AW, Pegues PC, Johnson GR, Kannan B, Auesperg N and Stromberg K: mRNA phenotyping of the major ligands and receptors of the EGF superfamily in human ovarian epithelial cells. Cancer Lett 89: 63-71, 1995.
24. Mason DR, Arora KK, Mertz LM and Catt KJ: Homologous down-regulation of gonadotropin-releasing hormone receptor sites and messenger ribonucleic acid transcripts in αT3-1 cells. Endocrinology 135: 1165-1170, 1994.
25. Kakar S, Nath S, Bunn J and Jennes L: The inhibition of growth and down-regulation of gonadotropin releasing hormone (GnRH) receptor in αT3-1 cells by GnRH agonist. Anti-Cancer Drugs 8: 369-375, 1997.
26. Dondi D, Limonta P, Moretti RM, Marelli MM, Garattini E and Motta M: Antiproliferative effects of luteinizing hormone-releasing hormone (LH-RH) agonists on the human androgen-independent prostate cancer cell line DU-145: evidence for an autocrine-inhibitory LHRH loop. Cancer Res 54: 4091-4095, 1994.
27. Moretti MR, Marelli MM, Dondi D, Poletti L, Martini L, Motta M and Limonta P: Luteinizing hormone-releasing hormone agonists interfere with the stimulatory actions of epidermal growth factor in human prostatic cancer cell lines, LNCaP and DU-145. J Clin Endocrinol Met 81: 3930-3937, 1996.
28. Irmer G, Bürger C, Müller R, Ortmann O, Peter U, Kakar S, Neill J, Schulz K-D and Emons G: Expression of the messenger RNAs for luteinizing hormone-releasing hormone (LH-RH) and its receptor in human ovarian epithelial carcinoma. Cancer Res 55: 817-822, 1995.
29. Jungwirth A, Galvan G, Pinski Y, Halmos G, Szepeshazi K, Cai R-Z, Groot K and Schally AV: Luteinizing hormone-releasing hormone antagonist Cetrorelix and bombesin antagonist RC-3940-II inhibit the growth of androgen-independent PC-3 prostate cancer in nude mice. Prostate 32: 164-172, 1997.
30. Shirahige Y, Cook CB, Pinski J, Halmos G, Nair R and Schally AV: Treatment with luteinizing hormone-releasing hormone antagonist SB-75 decreases levels of epidermal growth factor receptor and its mRNA in OV-1063 human epithelial ovarian cancer xenografts in nude mice. Int J Oncol 5: 1031-1035, 1994.
31. MacDonald A and Habib FK: Divergent responses to epidermal growth factor in hormone sensitive and insensitive human prostate cancer cell lines. Br J Cancer 651: 177-182, 1992.
32. Schuurmans ALG, Bolt J and Mulder E: Androgens stimulate both growth rate and epidermal growth factor receptor activity of the prostate tumor cell LNCaP. Prostate 12: 55-63, 1988.
33. Berns EMJJ, deBoer W and Mulder E: Androgen-dependent growth regulation and the release of specific proteins by the androgen receptor containing human prostate tumor cell line LNCaP. Prostate 9: 247-259, 1983.
34. Henttu P, Liao S and Vihko P: Androgen up-regulate the human prostate specific antigen messenger ribonucleic acid (mRNA), but down-regulate the prostatic acid phosphatase mRNA in the LNCaP cell line. Endocrinology 130: 766-772, 1992.
35. Liu X-U, Wiley HS and Meikle AW: Androgens regulate proliferation of human prostate cancer cells in culture by increasing transforming growth factor-α (TGF-α) and epidermal growth factor (EGF)/TGF-α receptor. J Clin Endocrinol Metab 77: 1472-1478, 1993.
36. Hazum E, Cuatrecasas P, Marian J and Conn PM: Receptor-mediated internalization of fluorescent gonadotropin-releasing hormone by pituitary gonadotropes. Proc Natl Acad Sci USA 77: 6692-6695, 1980.
37. Pelletier G, Dubé D, Guy J, Séguin C and Lefebvre FA: Binding and internalization of a luteinizing hormone-releasing hormone agonist by rat gonadotrophic cells. A radioautographic study. Endocrinology 111: 1068-1076, 1982.
38. Conn PM: The molecular basis of gonadotropin-releasing hormone action. Endocr Rev 7: 3-10, 1986.
39. Hazum E and Conn PM: Molecular mechanism of gonadotropin-releasing hormone (GnRH) action. I. The GnRH receptor. Endocr Rev 9: 379-386, 1988.
40. Rosner MR: Regulation of the epidermal growth factor receptor by growth modulating agents. Biotech Appl Biochem 12: 473-477, 1990.
41. Ewing TM, Murphy LJ and Ng M-L: Regulation of epidermal growth factor receptor by progestins and glucocorticoids in human breast cancer cell lines. Int J Cancer 44: 744-752, 1989.
42. Bertics PJ, Weber W, Cochet C, Pang GYN, Lee CSL, Watts KW and Sutherland RL: Regulation of the epidermal growth factor receptor by phosphorylation. J Cell Biochem 29: 195-208, 1985.
43. Lund KA, Lazar CS, Chen WS, Walsh BJ, Welsh JB, Herbst JJ, Walton GM, Rosenfeld MG, Gill GN and Wiley HS: Phosphorylation of the epidermal growth factor receptor threonine 654 inhibits ligand-induced internalization and down-regulation. J Biol Chem 265: 20517-20523, 1990.