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Antiproliferative Effects of Luteinizing Hormone-releasing Hormone (LHRH) Agonists on Human Androgen-independent Prostate Cancer Cell Line DU 145: Evidence for an Autocrine-inhibitory LHRH Loop¹

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ABSTRACT

The therapeutic options for the treatment of androgen-independent prostatic cancers are rather limited; this is mainly because our understanding of the local mechanisms involved in the control of androgen-independent proliferation of the tumor is still very poor. The present experiments have been performed to verify whether luteinizing hormone-releasing hormone (LHRH) agonists may possess a direct effect on the growth of the human androgen-independent prostate cancer cells DU 145 and whether a LHRH growth regulatory system may be present in these cells. The data have shown that two potent LHRH agonists (Zoladex and Buserelin) exert a significant and dose-dependent antiproliferative action on DU 145 cells, after 4 days of treatment. The inhibitory action of Zoladex and Buserelin is completely counteracted by the simultaneous treatment of the cells with a potent LHRH antagonist, suggesting that the action of the LHRH agonists may be mediated by specific receptors. This hypothesis has been confirmed by the demonstration that low-affinity binding sites for ¹²⁵I-Buserelin are present on DU 145 cell membranes, particularly when cells are cultured in serum-free conditions. By using the reverse transcription-polymerase chain reaction technique, in the presence of a pair of specific oligonucleotide primers complementary to the human LHRH complementary DNA, it has been demonstrated that a mRNA for LHRH is expressed in DU 145 cells. Taken together, these data seem to indicate that an autocrine/paracrine LHRH (or LHRH-like) loop is present in androgen-independent prostate cancer cells, and may participate in the regulation of tumor cell growth. To verify this hypothesis, DU 145 cells have been cultured in serum-free conditions, and treated with a LHRH antagonist for 4 days. The treatment resulted in a significant increase of cell proliferation, suggesting an inhibitory role for the LHRH system in the local regulation of cell growth.

In conclusion, these data demonstrate that: (a) LHRH agonists exert a specific antiproliferative action on the human androgen-independent DU 145 cells; (b) an autocrine/paracrine LHRH (or LHRH-like) loop, which seems to be inhibitory on cell proliferation, is expressed in DU 145 cells.

INTRODUCTION

Prostatic cancer is usually androgen dependent in its early stages, and consequently testosterone withdrawal represents initially the therapy of choice (1-5). LHRH³ agonists are widely and successfully used for the treatment of this pathology on the basis of their ability to suppress the activity of the pituitary-testicular axis and, therefore, to reduce testosterone secretion (2, 6).

In later stages, prostatic carcinoma may progress to a condition in

which androgen dependence is lost (1, 7) and cell proliferation is mainly stimulated by locally synthesized growth factors. A variety of growth stimulatory factors have been shown to be produced by human androgen-independent prostatic tumors (8-14). In these conditions, conclusive indications for successful therapy are still lacking. A better understanding of the local mechanisms involved in the control of the growth of androgen-independent tumors will certainly be of help in improving therapeutic options.

The present experiments have been performed to verify whether LHRH agonists might also affect the proliferation of prostatic carcinoma when the tumor has lost its androgen dependence. Advantage has been taken of the existence of the DU 145 cell line, which was derived from a brain metastasis of a human prostatic adenocarcinoma; this cell line retains the androgen independence of the original tumor (15-17), and does not possess androgen receptors (18). The expression of a functional LHRH-like system (LHRH mRNA and LHRH receptors) as well as the possible role played by this system in the local mechanisms regulating DU 145 cell proliferation have also been investigated.

MATERIALS AND METHODS

Materials

LHRH agonists Zoladex (D-Ser(tBu)⁶Aza-Gly-LHRH) and Buserelin (D-Ser(tBu)⁶Des-Gly¹⁰-LHRH-N-ethylamide) were kindly donated by Zeneca (Milano, Italy) and Hoechst A. G. (Frankfurt, Germany), respectively. LHRH antagonist (NaI-Arg-LHRH) was kindly provided by Dr. W. V. Vale (The Salk Institute, La Jolla, CA).

Animals

Adult male Sprague-Dawley rats were obtained from Charles River (Calco, Como, Italy). Animals were maintained on a 14-h light/10-h dark schedule, with standard pellet food and water available *ad libitum*. All rats were killed by decapitation; hypothalami and pituitaries were quickly collected and frozen at -70°C until membrane preparation for LHRH receptor assays, and RNA extraction for the RT-PCR studies.

Cell Culture

The cell line DU 145 was obtained from American Type Culture Collection (Rockville, MD). Cells (passages 60-70) were routinely grown in RPMI-1640 medium (Seromed, Biochrom KG, Berlin, Germany) supplemented with 5% FCS (Gibco, Paisley, Scotland), glutamine (1 mM), and antibiotics (100 units/ml penicillin G sodium, 100 µg/ml streptomycin sulfate) in a humidified atmosphere of 5% CO₂/95% air. In these conditions the duplication period of the cells is 36 h.

LHRH Agonists and DU 145 Cell Proliferation

DU 145 cells were plated at a density of 500-800 cells/cm² in 100-mm dishes in RPMI-1640 medium supplemented with 5% FCS. Cells were allowed to attach and start growing for 3 days; the seeding media were then changed to experimental media.

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³ The abbreviations used are: LHRH, luteinizing hormone-releasing hormone; ANT, LHRH antagonist; RT-PCR, reverse transcription-polymerase chain reaction; FCS, fetal calf serum; cDNA, complementary DNA.

Experiment 1. Cells were treated daily, for 4 days, with Zoladex or Buserelin (10^{-11} – 10^{-6} M, 500 μ l), while the medium was changed every 2 days. At the end of the treatment, cells were harvested and counted by hemacytometer.

Experiment 2. To verify the specificity of the effect of LHRH agonists on DU 145 cell proliferation, cells were treated daily with Zoladex or Buserelin (10^{-8} M, 500 μ l) either in the absence or presence of ANT (10^{-8} M, 500 μ l). After 4 days of treatment, cells were harvested and counted.

LHRH Receptors

DU 145 cells were grown in serum-free conditions (RPMI-1640 medium supplemented with 6.25 μ g/ml of insulin and transferrin, 6.25 ng/ml of selenious acid, and 1.25 mg/ml of bovine serum albumin) (10). The LHRH radioreceptor assay was performed as previously described (19, 20), by using 125 I-Buserelin (specific activity, 800–1000 μ Ci/ μ g) as the specific ligand. Briefly, displacement curves were performed by incubating 100- μ l cell membrane aliquot with 125 I-Buserelin (~200,000 cpm) either in the absence or presence of increasing concentrations of Buserelin (10^{-9} – 10^{-5} M). Nonspecific binding was determined in the presence of 10^{-4} M unlabeled Buserelin. Displacement curves allowed the determination of binding characteristics for LHRH receptors (K_d = dissociation constant; B_{max} = maximum concentration of binding sites) on DU 145 cells. LHRH receptors have also been analyzed on male rat pituitaries used as controls. The protein content of each membrane preparation was evaluated according to the method of Bradford (21).

RNA Extraction and RT-PCR

Total RNA was extracted from DU 145 cells as well as from rat hypothalamus and pituitaries (positive and negative controls, respectively) according to a modification of the guanidium thiocyanate/cesium chloride method (22). RT-PCR was performed as previously described (23). Briefly, RNAs (1 μ g) extracted from DU 145 cells, rat hypothalamus and pituitaries were reverse transcribed into cDNAs with an oligodeoxythymidylate [oligo(dT) $_{16}$] as a primer, using the Gene AMP kit (Perkin Elmer Cetus, Norwalk, CT). The cDNAs obtained were then amplified in the presence of Taq polymerase and a pair of specific primers (23), according to the instructions of the manufacturer. The primers were synthesized according to the reported human and rat LHRH cDNA sequences (24). The sequence of the primers has been chosen to span two introns of the LHRH genomic structure, in order to avoid contamination by genomic DNA. Amplification was carried out for 35 cycles (1-min denaturation at 94°C, 1-min primer annealing at 50°C, and 3-min primer extension at 72°C). According to the sequence of the primers, a 228-base pair fragment was expected after RT-PCR amplification of the LHRH message. As negative controls, samples containing no mRNA were subjected to the same RT-PCR procedure; these samples did not give rise to any detectable product.

Aliquots of 10 μ l of the RT-PCR products were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide and Southern blotted as described (25). Blots were then hybridized with a 32 P-labeled oligonucleotide (17 mer) probe complementary to a sequence (nucleotides 159/175) which is common to the human and rat LHRH cDNA (23, 24).

LHRH Antagonist and DU 145 Cell Proliferation in Serum-free Conditions

DU 145 cells were plated at a density of 2.5×10^3 cells/cm 2 in 100-mm dishes in serum-free conditions, as described for LHRH receptor studies. Cells were treated daily with ANT (10^{-12} – 10^{-6} M, 500 μ l); the medium was changed every 2 days. After 4 days of treatment the cells were harvested and counted.

Statistical Analysis

The data from experiments involving cell proliferation were analyzed according to Dunnett's test (26) after one-way analysis of variance. The data from displacement curves for LHRH receptors were analyzed by the Ligand program (27) which allows the determination of K_d and B_{max} values for the receptors under study.

RESULTS

LHRH Agonists and DU 145 Cell Proliferation

Experiment 1. The effects of a 4-day treatment with the two LHRH agonists Zoladex and Buserelin (10^{-11} – 10^{-6} M) on DU 145 cell proliferation are reported in Fig. 1. Both analogues inhibited cell growth in a dose-dependent fashion. The antiproliferative action of both LHRH agonists was significant *versus* controls at doses ranging from 10^{-9} to 10^{-6} M; Buserelin was significantly effective also at the dose of 10^{-10} M (Fig. 1b).

Experiment 2. To verify the specificity of the antiproliferative action of LHRH agonists on DU 145 cells, it has been investigated whether the inhibitory effect of Zoladex and Buserelin could be counteracted by the simultaneous treatment of the cells with an LHRH antagonist. In preliminary experiments, the effects of a treatment with ANT on DU 145 cell proliferation have been studied. Cells were treated for 4 days with ANT at different doses (10^{-12} – 10^{-6} M). None of the doses used proved able to modify cell proliferation (data not shown). The intermediate dose of 10^{-8} M was then selected for the following experiments. Fig. 2a confirms that ANT (10^{-8} M) is ineffective and that Zoladex (10^{-8} M) significantly inhibits cell proliferation. Fig. 2a also shows that, when the two compounds are given together, ANT completely counteracts the inhibitory effect of Zoladex. Similar results have been obtained by treating the cells with Buserelin, at the same doses, either in the absence or presence of ANT (Fig. 2b).

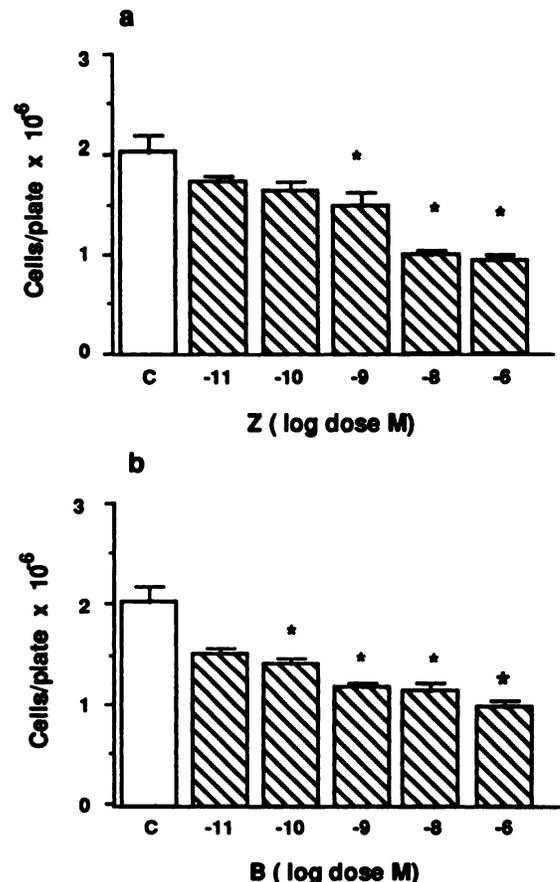


Fig. 1. Effects of two LHRH agonists Zoladex [Z(a)] and Buserelin [B(b)] on the proliferation of DU 145 cells, after 4 days of treatment. Results are expressed as the mean cell number per plate \pm SE. C, controls without drugs. *, $P < 0.05$ *versus* C.

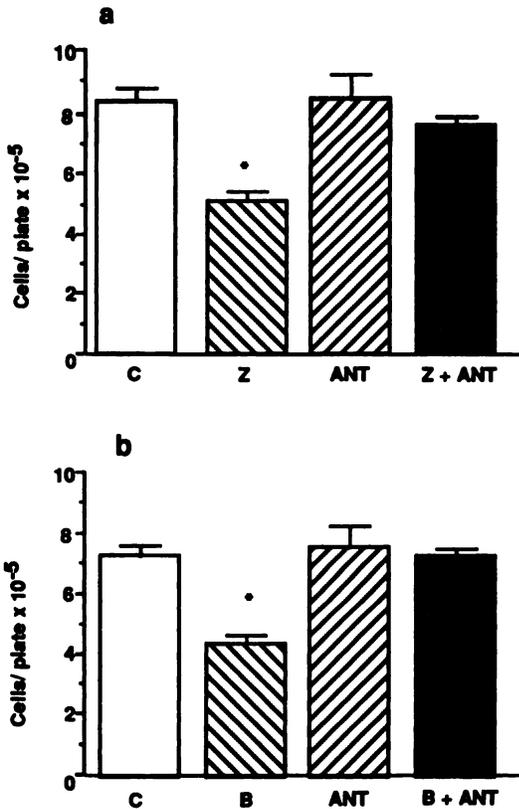


Fig. 2. Effects of ANT (10^{-8} M) on the inhibition of DU 145 cell proliferation induced by the LHRH agonists Zoladex [Z(a)] and Buserelin [B(b)] (10^{-8} M). Results are expressed as the mean cell number per plate \pm SE. C, controls without drugs. *, $P < 0.05$ versus C.

LHRH Receptors

Fig. 3 shows that binding sites for the LHRH analogue 125 I-Buserelin are present on the membranes of DU 145 cells when these are cultured in serum-free conditions. Computer analysis of the data of the displacement curves revealed the presence of a single class of low-affinity ($K_d = 3\text{--}10\ \mu\text{M}$), high-capacity ($B_{\text{max}} = 425\text{--}668$ pmol/mg protein) binding sites. Analysis of LHRH receptors on male rat pituitaries, used as controls, showed the presence of a single class of high-affinity ($K_d = 1$ nM), low-capacity ($B_{\text{max}} = 150\text{--}170$ fmol/mg protein) binding sites, confirming previous data of this and other laboratories (28, 29).

Expression of LHRH mRNA

After the RT-PCR procedure, the cDNAs obtained from DU 145 cells and from rat hypothalami and pituitaries were separated on a 1.5% agarose gel, Southern blotted, and hybridized with a 32 P-labeled LHRH oligonucleotide (17 mer) probe. A 228-base pair cDNA fragment was obtained from DU 145 cells as well as from rat hypothalamus; no amplification product of this size could be obtained from rat pituitaries. Fig. 4 shows that the cDNA fragments obtained from two samples of DU 145 cells (Lanes 2 and 3), and from one sample of rat hypothalamus (Lane 4) specifically hybridize with the 32 P-labeled LHRH probe; no hybridization band is present in the pituitary lane (Lane 1).

LHRH Antagonist and DU 145 Cell Proliferation in Serum-free Conditions

To verify whether the LHRH (or LHRH-like) system expressed in DU 145 cells (LHRH receptors, LHRH mRNA) might play an auto-

crine role on cell proliferation, the effects of ANT on the proliferation of DU 145 cells cultured in serum-free conditions were studied. Cells were treated daily with different doses of ANT (10^{-12} – 10^{-6} M) for 4 days. Fig. 5 shows that the treatment with ANT at two dose levels (10^{-8} and 10^{-6} M) is followed by a significant increase of cell proliferation; lower concentrations of ANT (10^{-12} and 10^{-10} M) proved inactive.

DISCUSSION

The present results show that the growth of the human androgen-independent prostatic cancer cell line DU 145 is significantly inhibited by treatment with two potent LHRH agonists (Zoladex and Buserelin). The antiproliferative action of the two drugs is dose dependent and specific, since it is completely counteracted by the simultaneous treatment of the cells with a potent LHRH antagonist. In contrast with these observations, Qayum *et al.* (30) have reported that a treatment with various doses (from 10^{-10} to 10^{-7} M) of Buserelin does not affect the proliferation of DU 145 cells. Differences in the experimental design adopted (*i.e.*, cell culture conditions, length of the

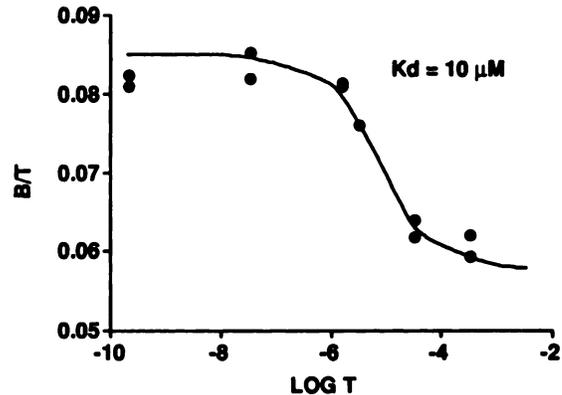


Fig. 3. Binding of 125 I-Buserelin to DU 145 cell membranes. The binding assay was performed as described in "Materials and Methods." Data were analyzed according to the Ligand program. One representative curve, out of four binding experiments, is reported. B/T, bound/total ratio. K_d , dissociation constant.

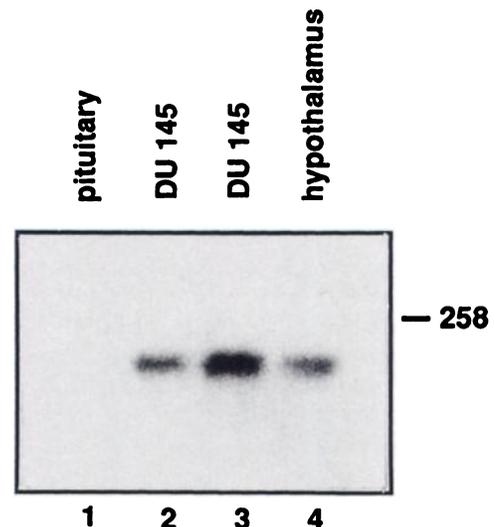


Fig. 4. Autoradiography of the RT-PCR products obtained from rat pituitary (Lane 1), DU 145 cells (Lanes 2 and 3), and rat hypothalamus (Lane 4) after gel electrophoresis, Southern blotting, and hybridization with a specific 32 P-labeled oligonucleotide LHRH probe.

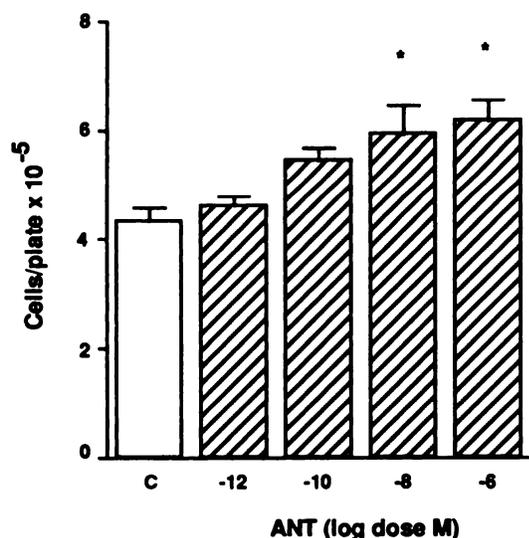


Fig. 5. Effects of ANT (10^{-12} – 10^{-6} M) on the proliferation of DU 145 cells cultured in serum-free conditions, after 4 days of treatment. Results are expressed as the mean cell number per plate \pm SE. C, controls without drug. *, $P < 0.05$ versus C.

treatment, etc.) might account for the discrepancies between these results.

Recently, it has been shown in the authors' laboratory (20) that Zoladex and Buserelin specifically inhibit the proliferation of the androgen-dependent prostatic cancer cell line LNCaP. The suggestion was advanced that, when utilized for the treatment of prostatic carcinoma, LHRH agonists might inhibit tumor growth not only by suppressing the activity of the pituitary-testicular axis, and therefore by inhibiting testosterone secretion, but also by acting directly at the level of the tumor. The present data seem to suggest that LHRH agonists might also exert this direct inhibitory action on the growth of prostatic carcinoma when this has progressed to an androgen-independent stage. It seems then possible to speculate that LHRH agonists might be utilized also for the treatment of androgen-independent prostatic tumors, because of their direct effect on prostatic cell proliferation.

The fact that the inhibitory action of LHRH agonists on DU 145 cell proliferation is counteracted by the simultaneous treatment of the cells with a LHRH antagonist led us to investigate whether receptors for LHRH agonists might be expressed on these cells. By using ^{125}I -Buserelin as the specific ligand, low-affinity LHRH binding sites have been detected on DU 145 cells, particularly when cultured in serum-free conditions. These observations are in line with those reported by Qayum *et al.* (30) and are germane to previous observations obtained in the authors' and other laboratories on the presence of similar binding sites in the androgen-dependent prostate tumor cell line LNCaP (20, 30). The presence of low-affinity LHRH binding sites has also been reported for other human extrapituitary tissues, both normal (gonads and placenta) (31–33) and tumoral (breast cancer cells) (34, 35). The fact that, in the present studies, the concentration of Buserelin required to displace ^{125}I -Buserelin ($K_d = 10 \mu\text{M}$) is higher than the doses of LHRH agonists able to elicit a biological response on cell proliferation (10^{-10} – 10^{-6} M), appears intriguing. However, a similar dichotomy has been previously reported by Limonta *et al.* (20), using LNCaP cells, and by Miller *et al.* (34), using the human breast cancer cell line MCF-7. As pointed out also by Miller *et al.* (34), the different experimental conditions (*e.g.*, culture conditions, time, temperature, etc.) adopted for the binding assay and for the evaluation of the biological effects might be responsible for this discrepancy.

The presence of LHRH receptors on DU 145 cells suggested that LHRH, or a LHRH-like factor, might be produced by these cells. This hypothesis has been confirmed by showing that a mRNA for LHRH is expressed in DU 145 cells as well as in the rat hypothalamus used as positive control. The observation that no mRNA for LHRH is expressed in the rat pituitary gland is in line with previous observations of the authors' (23) and other (36) laboratories. A mRNA for LHRH is also expressed in the androgen-responsive prostate cancer cell line LNCaP (23). In agreement with the present data, Qayum *et al.* (30, 37) have shown the presence of LHRH immunoreactive material in the culture media of DU 145 cells. Taken together, all these data suggest that an autocrine LHRH or LHRH-like loop (mRNA, LHRH peptide, LHRH receptors), similar to the one expressed in LNCaP cells, might be expressed also in androgen-independent prostatic cancer cells.

To verify this hypothesis, and to clarify the possible role played by this system in the local mechanisms regulating tumor growth, the effects of potent ANT on the proliferation of DU 145 cells cultured in serum-free conditions have been investigated. The treatment with ANT resulted in a significant increase of cell proliferation. In the authors' opinion, several major considerations can be drawn from these data. First of all, DU 145 cells seem to express a functional LHRH or LHRH-like system endowed with autocrine or paracrine inhibitory properties on cell proliferation. Second, this LHRH-like system seems to be expressed and/or active when the cells are grown in the absence of FCS. Actually, as described in the present paper (see "Results"), a similar treatment with ANT is completely devoid of any effect on cells grown in standard conditions (RPMI-1640 medium supplemented with 5% FCS). In this context, it is also interesting to emphasize that LHRH receptors are detectable only on DU 145 cells grown in serum-free conditions. These data seem to indicate that the activity of the autocrine/paracrine LHRH-like inhibitory loop in DU 145 cells is regulated in a negative way by one or more factors present in serum. Further experiments are now in progress in the authors' laboratory to verify whether serum-derived growth factors might be responsible for this negative regulation.

Recently, it has been reported that a similar LHRH-like inhibitory loop is active in controlling the growth of the androgen-dependent human prostatic cancer cell line LNCaP (20, 23). Moreover, receptors for LHRH, and LHRH-like immunoreactive material, have been demonstrated to be present in specimens of human benign and malignant prostatic tumor tissues (30, 37, 38), as well as in samples of experimental prostatic cancers (39, 40). Finally, it has been recently reported that a mRNA for LHRH as well as a bioactive and immunoreactive LHRH are also present in the normal prostate of the adult rat (41). Taken together, these observations clearly point to LHRH as a local growth regulatory factor, involved, possibly with an inhibitory activity, in the mechanisms regulating the proliferation of both normal and malignant prostatic tissues.

The fact that, under specific experimental conditions, LHRH antagonists may facilitate cell proliferation in prostatic cancer cell lines (LNCaP, DU 145, etc.) suggests a word of caution in the clinical utilization of these drugs. The authors do not want to overemphasize their results, which might occur only in cells cultured in particular conditions; however, the present and previous findings (23), suggest that the relationships between LHRH antagonists and normal and pathological cell proliferation in the prostate should be given particular attention in the future.

In conclusion, the present data indicate that: (a) LHRH agonists exert a direct antiproliferative action on the human androgen-independent prostate cancer cell line DU 145; (b) a LHRH or LHRH-like growth inhibitory system is functionally expressed in these cells.

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