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Luteinizing Hormone-Releasing Hormone Agonists Interfere with the Mitogenic Activity of the Insulin-Like Growth Factor System in Androgen-Independent Prostate Cancer Cells*

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ABSTRACT

We have previously shown that LHRH agonists exert a direct and specific inhibitory action on the proliferation of the androgen-independent DU 145 prostate cancer cell line; however, the cellular mechanisms mediating this antiproliferative action are not well defined. It is well known that the insulin-like growth factor (IGF) system plays a crucial role in the local regulation of the growth of androgen-independent prostate cancer. The present experiments were performed to evaluate whether LHRH agonists might exert their antimitogenic effect by interfering with the activity of the locally expressed IGF system. To this purpose, the effects of the LHRH agonist Zoladex (LHRH-A) on 1) the mitogenic action of IGF-I, 2) the tyrosine phosphorylation of type 1 IGF-I receptor (IGF-IR), 3) the concentration of IGF-IR, and 4) the secretion of IGF-binding protein-3 were studied. The results obtained show that in DU 145 cells, LHRH-A 1) counteracts the mitogenic action of IGF-I in a dose-dependent manner, 2) prevents the IGF-I-induced tyrosine phosphorylation of the IGF-IR, 3) reduces the concentration of IGF-IR without affecting its K_d value, and 4) does not affect the secretion of IGF-binding protein-3 in the conditioned medium from these cells.

These data suggest that LHRH agonists may inhibit the proliferation of human androgen-independent prostate tumor cells by interfering with some of the cellular mechanisms mediating the stimulatory action of the IGF system. (*Endocrinology* **140:** 329–334, 1999)

PROSTATE adenocarcinoma has become the second leading cause of death from malignancies in men (1). In its initial stages, the development and the progression of prostate cancer are mainly regulated by androgens (2, 3); however, this pathology may eventually progress to a condition in which hormone dependence is lost. Growth factors, either exogenous or locally produced, have been shown to be involved in the regulation of growth of prostate carcinoma (1). In particular, the insulin-like growth factor (IGF) system has been reported to exert a strong mitogenic action on prostate cancer (4); moreover, serum IGF-I levels have recently been correlated with prostate cancer risk (5). Androgen-independent prostate cancer cells produce IGFs peptides (6–9), express type 1 IGF receptors (IGF-IR) (8–13), which are known to mediate the biological activities of IGFs (14), and respond to the mitogenic action of IGFs (7–11, 15, 16). IGF-binding proteins (IGFBPs), which bind IGFs and modulate their actions, have also been shown to be produced by steroid-unresponsive prostate cancer cells (9, 17–20).

The clinical utility of LHRH agonists for the treatment of androgen-responsive prostate cancers is now well recognized (2, 3). These compounds act mainly by suppressing the activity of the pituitary-testicular axis (21); in addition, a direct inhibitory action at the level of the tumor has been found in our (22, 23) as well as other (24, 25) laboratories. Recently, we demonstrated that LHRH agonists may also exert a direct antiproliferative action on DU 145 cells, an androgen-independent prostate cancer cell line, either in culture (26) or when inoculated into nude mice (27).

The present study was performed to investigate whether LHRH agonists might exert their antiproliferative action on DU 145 cells by interfering with the activity of the IGF system. To this purpose, the effects of the LHRH agonist Zoladex (LHRH-A) on 1) the proliferative action of IGF-I, 2) the tyrosine phosphorylation of IGF-IR, 3) the concentration of IGF-IR, and 4) the expression and secretion of IGFBP-3 were studied.

Materials and Methods

Materials

The LHRH agonist Zoladex [D-Ser(tBu)^{6}Aza-Gly-LHRH; LHRH-A] was donated by Zeneca (Milan, Italy). Human recombinant IGF-I and [^{125}I]IGF-I (250 mCi/mg) were purchased from Amersham (Milan, Italy). IGF-IRα, a mouse monoclonal antibody to IGF-IR, was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antiphosphotyrosine mouse monoclonal antibody (IgG2b$_k$) and IGFBP-3 rabbit polyclonal antiserum were obtained from Upstate Biotechnology (Lake Placid, NY). The enhanced chemiluminescence (ECL) Western blotting kit (Amersham) was used for the detection of immunoprecipitated tyrosine-phosphorylated IGF receptor and IGFBP-3.

Cell culture

The cell line DU 145 was obtained from American Type Culture Collection (Manassas, VA). These cells were derived from a brain me-

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tastasis of a human androgen-unresponsive prostate carcinoma; they retain the androgen independence of the original tumor and do not express the androgen receptor (28). DU 145 cells (passages 60–70) were routinely grown in RPMI 1640 medium (Seromed Biochrom, Berlin, Germany) supplemented with 5% FCS (Life Technologies, Paisley, Scotland, UK), glutamine (1 mmol/liter) and antibiotics (100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate) in a humidified atmosphere of 5% CO_2-95% air. Under these conditions, the doubling time was 36 h.

Cell proliferation studies

Cell growth studies were performed on exponentially growing cells. DU 145 cells were plated at a density of 2×10^4 cells in 60-mm dishes. After 3 days, the seeding media were changed to RPMI 1640 supplemented with 2% FCS. Under these conditions, DU 145 cells have been reported to positively respond to the mitogenic action of growth factors (11). Cells were treated every day with IGF-I (10 ng/ml) in either the absence or presence of different doses of LHRH-A (10^{-10}–10^{-6} M). After 7 days of treatment, cells were harvested and counted, in a blind manner, by hemocytometer. Previous studies from our laboratory have shown that LHRH-A does not affect prostate cancer cell viability but, rather, exerts an effect on cell proliferation (22).

Tyrosine phosphorylation of IGF-IR

Analysis of the tyrosine phosphorylation of IGF-IR has been performed as previously described by Neuenschwander *et al.* (29), with some modifications.

In preliminary experiments, cells were treated with IGF-I (75 ng/ml) for different time periods (1–10 min) to evaluate the time course of the stimulation of tyrosine kinase activity of IGF-IR. As the maximum level of activity was found as early as 3 min in DU 145 cells (see *Results*), this time period has been used for subsequent studies. For these preliminary experiments, immunoprecipitation of IGF-IR and analysis of receptor tyrosine phosphorylation were performed as follows.

To study the possible interaction of LHRH-A with the IGF-I-induced tyrosine phosphorylation of IGF-IR, DU 145 cells were plated at a density of 1×10^6 cells in 100-mm dishes in RPMI 1640 medium supplemented with 5% FCS. After 2 days, cells were refed with 10 ml serum-free medium for 24 h and then pretreated for 30 or 60 min with LHRH-A (10^{-6} M final concentration in the cells) before IGF-I stimulation (3 min). At the end of the treatment, the experimental medium was removed, the cell layer was rinsed with PBS, and cells were then harvested in 1 ml RIPA buffer [0.05 M Tris-HCl (pH 7.7), 0.15 M NaCl, 0.8% Triton X-100, 0.8% sodium deoxycholate, 0.08% SDS, 10 mM EDTA, 100 μM Na_3VO_4, 50 mM NaF, 0.3 mM phenylmethylsulfonylfluoride, and 5 mM iodoacetic acid] on ice for 10 min. Cell lysates were centrifuged at $15{,}000 \times g$ for 20 min; supernatants were immunoprecipitated with 2 μg/20 μl of the monoclonal antibody IGF-IRα, raised against IGF-IR, for 15 min in the presence of 10 μl rabbit antimouse IgG. Protein A-Sepharose (75 μl) was added at room temperature for 30 min, and samples were then centrifuged at $4000 \times g$ for 5 min. Precipitated proteins were electrophoresed by SDS-PAGE on a 7.5% polyacrylamide gel (30) and blotted onto a nitrocellulose filter. Filters were incubated with antiphosphotyrosine monoclonal antibody (1 μg/ml) for 1 h at room temperature and then with an antimouse IgG at the final concentration of 1:5000. Antibody bound to phosphotyrosine was detected with the ECL-Western blotting detection system after a 5- to 10-min exposure to a Hyperfilm-ECL x-ray film (Amersham, Milan, Italy) at room temperature.

IGF-IR binding studies

DU 145 cells, plated at a density of 1×10^6 cells in 100-mm dishes and grown in standard culture conditions, were treated with LHRH-A (10^{-6} M final concentration in the cells) for 3 or 6 h. At the end of the treatment, cells were washed with PBS, scraped, and resuspended in Tris-HCl. The IGF-IR receptor assay was performed on cell membrane preparations. [^{125}I]IGF-I (50,000 cpm; 100 μl) was incubated with 100-μl aliquots of cell membranes and 100 μl unlabeled IGF-I (10^{-7} M). After 1-h incubation at 22 C, 1 ml ice-cold buffer (10 mM Tris-HCl containing 1 mM dithiothreitol and 0.15% BSA, pH 7.6) was added, and the tubes were immediately centrifuged at $48{,}000 \times g$ for 25 min. Supernatants were discarded, and pellets were counted in a γ-counter (Packard Instruments, Milan, Italy). The maximum binding capacity of each membrane preparation was calculated as previously described (31).

The dissociation constant (K_d) of IGF-IR for each experimental group was determined by means of displacement curves. [^{125}I]IGF-I was incubated with 100 μl cell membranes in either the absence or presence of different doses of unlabeled IGF-I (10^{-10}-10^{-6} M). Incubations were performed as described above. The protein content of each membrane preparation was determined according to the method of Bradford (32).

Western blot analysis of IGFBP-3

DU 145 cells were plated at a density of 5×10^4 cells in 60-mm dishes and grown under standard conditions. After 2 days, culture media were changed to serum-free RPMI 1640 medium for 24 h; cells were then treated with LHRH-A (10^{-8} or 10^{-6} M) or with medium alone for 48 h. Conditioned media from treated or control cells were concentrated before electrophoresis through Centriplus-10 microconcentrators (Amicon, Beverly, MA) after acidification with 1 M acetic acid to dissociate IGFs from IGFBPs. Concentrates were lyophilized and submitted to gel electrophoresis on 12% SDS-polyacrylamide gels. Separated proteins were transferred to nitrocellulose filters overnight at 4 C with the IGFBP-3 polyclonal antibody at a final concentration of 1:1000. Filters were then incubated with goat antirabbit IgG (1:3000). Antibody bound to IGFBP-3 was detected with the ECL-Western blotting detection system after 5- to 10-min exposure to a Hyperfilm-ECL x-ray film (Amersham) at room temperature as described above.

Statistical analysis

The data from proliferation studies were analyzed according to Dunnett's test (33) after one-way ANOVA. The data from displacement curves for IGF-IR receptors were analyzed by the Ligand computerized curve-fitting program (34), supplied by the Biochemical Computing Technology Center (Nashville, TN).

Signals from tyrosine phosphorylation of IGF-IR and IGFBP-3 secretion were quantitated by densitometric analysis and then expressed as a percentage of the value of IGF-I-induced tyrosine phosphorylation of IGF-IR and of the control value, respectively.

Results

Cell proliferation studies

DU 145 cells were treated for 7 days with IGF-I (10 ng/ml) in either the absence or presence of different doses (10^{-10}-10^{-6} M) of LHRH-A. Figure 1 shows that, as expected, IGF-I exerted a significant stimulatory action on DU 145 cell proliferation. LHRH-A was able to antagonize, in a dose-dependent way, the mitogenic action of this growth factor; it significantly counteracted the stimulatory action of IGF-I when used at either 10^{-8} or 10^{-6} M (Fig. 1). On the basis of these observations, the dose of 10^{-6} M was selected for the subsequent experiments.

Tyrosine phosphorylation of IGF-IR

Preliminary experiments were performed to establish the timing of the activation of the IGF-IR induced by IGF-I. DU 145 cells were treated with the growth factor for different time intervals up to 10 min. As shown in Fig. 2, the phosphorylated IGF-IR β-subunit was detected in a band of approximately 97 kDa. It appears that IGF-I significantly activates its receptor at all time intervals considered, starting 1 min after the stimulus. The first time at which maximal activation occurred (3 min) was used for the subsequent experiments.

DU 145 cells were treated with LHRH-A (10^{-6} M) for either 30 or 60 min before being exposed to the 3-min stimulus with

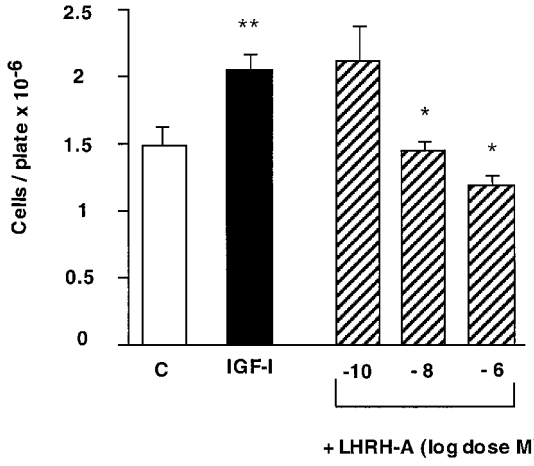

FIG. 1. Effect of LHRH-A (10^{-10}-10^{-6} M) on the proliferative action of IGF-I (10 ng/ml) in DU 145 cells. Each experimental group consisted of seven replicates. Each experiment was repeated three times with identical results. Results are expressed as the mean cell number per plate ± SE. □, Controls (C) without drug; ■, IGF-I; ▨, IGF-I plus LHRH-A. **, $P < 0.05$ *vs.* controls; *, $P < 0.05$ *vs.* IGF-I.

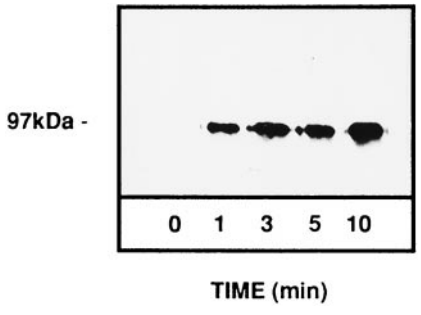

FIG. 2. Time-dependent stimulation of the IGF-I-induced tyrosine phosphorylation of the IGF-IR in DU 145 cells. Cells were treated with IGF-I (75 ng/ml) for 1–10 min. The IGF-IR was immunoprecipitated and resolved on 7.5% polyacrylamide gels. The amount of phosphotyrosine protein on the gel was determined by Western blot using a specific antibody against phosphotyrosine. One representative of three experiments is reported.

IGF-I. Figure 3A confirms that IGF-I induces the tyrosine phosphorylation of the IGF-IR β-subunits (lane 2 *vs.* lane 1). LHRH-A, when given in the absence of IGF-I, was completely devoid of any effect (Fig. 3A, lanes 3 and 4). The pretreatment of DU 145 cells with LHRH-A for 30 or 60 min substantially counteracted the IGF-dependent phosphorylation of IGF-IR (Fig. 3A, lanes 5 and 6). Figure 3B provides the densitometric evaluation of the same results. The inhibitory effect exerted by the treatment with LHRH-A was very clear (Fig. 3B, lanes 5 and 6 *vs.* lane 2).

IGF-IR binding studies

These studies were performed to verify whether LHRH-A might affect the number and/or the K_d value of IGF-IR in DU 145 cells. Figure 4 shows that LHRH-A, when given for either 3 or 6 h, induced a significant decrease in the concentrations of IGF-IR at both time intervals considered. The K_d values of IGF-IR were not affected by the treatment with LHRH-A (K_d = 1–1.5 nM range in control and treated groups).

Western blot analysis of IGFBP-3

These studies were performed to estimate whether LHRH-A might change IGFBP-3 secretion from DU 145 cells. The amount of protein secreted was evaluated by Western blot analysis in the media of cells treated with LHRH-A (10^{-8} or 10^{-6} M) for 48 h. Figure 5A (lane 1) shows that a protein band of 45 kDa, corresponding to IGFBP-3, is present in the culture media of control DU 145 cells. The amount of IGFBP-3 secreted by these cells does not seem to be affected by the treatment with LHRH-A (Fig. 5A, lanes 2 and 3 *vs.* lane 1). Figure 5B provides the densitometric analysis of the same results.

In agreement with this observation, we also observed that the messenger RNA levels of IGFBP-3 in DU 145 cells are not modified by the treatment with LHRH-A (10^{-6} M) for 6–48 h (data not shown). These experiments were performed using Northern blot analysis with the probe pHBP3–502 (donated by Dr. S. Shimasaki) (35).

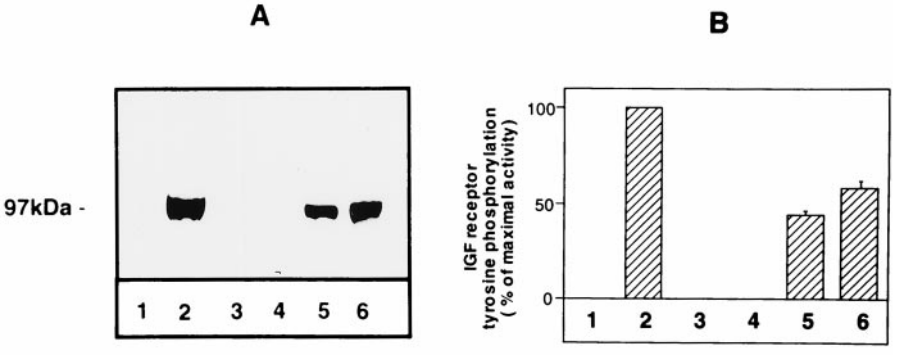

FIG. 3. Effect of LHRH-A (10^{-6} M) on the IGF-I-induced tyrosine phosphorylation of the IGF-IR in DU 145 cells. LHRH-A was added to the experimental medium either 30 or 60 min before treatment with IGF-I (75 ng/ml). Lane 1, Controls without drug; lane 2, IGF-I (3 min); lane 3, LHRH-A (30 min); lane 4, LHRH-A (60 min); lane 5, IGF-I plus LHRH-A (30 min); lane 6, IGF-I plus LHRH-A (60 min). A, Representative Western immunoblot of the tyrosine-phosphorylated IGF-IR. B, Densitometric analysis of tyrosine phosphorylation of IGF-IR. The results are expressed as a percentage of the value of IGF-I induced tyrosine phosphorylation of IGF-IR and are the mean ± SE of four separate experiments.

Discussion

We have previously shown that LHRH agonists exert a direct antiproliferative action on androgen-independent DU 145 prostate cancer cells (26). The present studies have been performed to gain additional information on the mechanism of this antimitogenic action.

IGFs and their binding proteins have been reported to play a crucial role in the proliferation of androgen-independent DU 145 prostate cancer cells (see references in introduction). The results here reported have shown that LHRH-A counteracts the proliferative effect of IGF-I, antagonizes the autophopshorylation of IGF-IR, and reduces the concentration of IGF-IR on DU 145 cell membranes. On the contrary, LHRH-A does not affect either the secretion or the expression of IGFBP-3. These observations indicate, then, that LHRH agonists inhibit the growth of androgen-independent prostate cancer cells at least partially by interfering with some of the mechanisms mediating the stimulatory action of IGF.

At partial variance with these data, Pinski and co-workers (24) have shown that LHRH agonists do not affect the concentration of IGF-I receptors in the androgen-independent Dunning R-3327-AT-1 rat prostate cancer *in vivo*. The different experimental conditions adopted (*in vitro vs. in vivo* studies, human *vs.* rat prostate cancer cells, LHRH agonist used) might be responsible for this discrepancy. On the other hand, a significant interaction between LHRH analogs and the IGF system has been previously reported for different types of tumors. Yano and co-workers (36) have shown that the LHRH agonist D-Trp^6-LHRH exerts a significant antitumoral activity on the MCF-7 MIII human breast cancer in nude mice; this effect is accompanied by a decrease in the number of IGF-I binding sites. Moreover, Yano and co-workers (37) have further shown that the same LHRH agonist is able to inhibit the growth of OV-1063 human epithelial ovarian cancer xenografts by reducing the concentration of IGF-I receptors. Finally, Hershkovitz *et al.* (38) have reported that a LHRH antagonist (SB-75), which has been shown to inhibit the *in vitro* proliferation of MCF-7 breast cancer cells, counteracts the proliferative action of IGF-II on these cells.

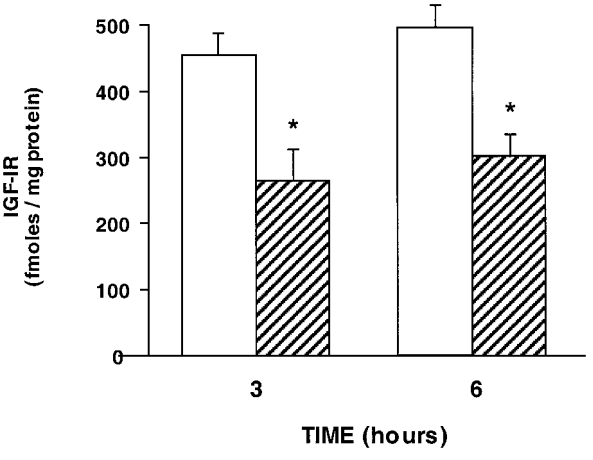

FIG. 4. Effect of LHRH-A (10^{-6} M) on the concentrations of IGF-IR in DU 145 cells. Each experimental group consisted of seven replicates. □, Controls without drug; ▨, LHRH-A. *, $P < 0.05$ *vs.* controls.

The observation that LHRH-A does not affect the secretion of IGF-BP3 is intriguing. As quoted in the introduction, different IGFBPs are secreted by prostate cancer cells and participate in the local regulation of tumor growth by modulating the actions of IGFs. It is then possible that LHRH agonists might regulate the secretion of IGFBPs different from IGFBP-3, a possibility that is at present under investigation in our laboratory.

As mentioned in the introduction, we have previously shown that LHRH agonists exert a direct antiproliferative action not only on androgen-independent cells, but also on the androgen-dependent prostate cancer cell line LNCaP (22, 23). An IGF system is also expressed in these cells, although their ability to respond to the mitogenic action of this growth factor seems to be lower than that of steroid-unresponsive cells (9, 16, 39). Interestingly, preliminary results obtained in our laboratory indicate that in LNCaP cells, LHRH-A does not interfere with the activation or the concentration of IGF receptors; on the contrary, the compound significantly stimulates the secretion of IGFBP-3 in the culture medium. These results confirm that LHRH

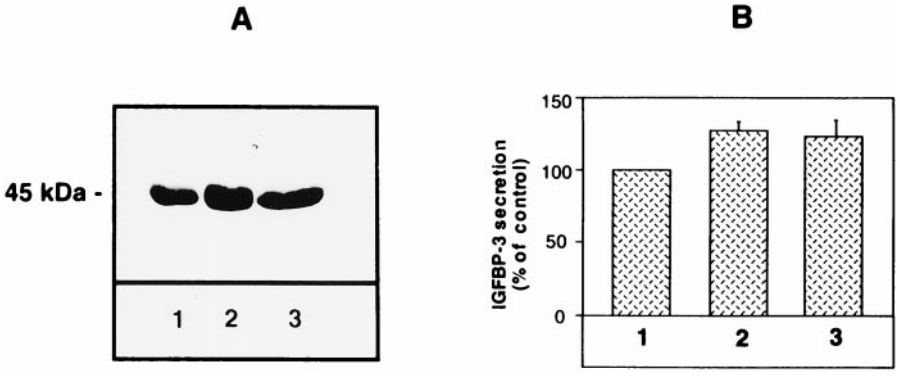

FIG. 5. Effect of LHRH-A on IGFBP-3 secretion in the culture medium of DU 145 cells treated with LHRH-A. Cells were grown in conditioned medium for 24 h and then treated with LHRH-A (10^{-8} or 10^{-6} M) for 48 h. Conditioned media from untreated and treated DU 145 cells were assayed for IGFBP-3 as described in *Materials and Methods*. Lane 1, Controls without drug; lane 2, LHRH-A (10^{-8} M); lane 3, LHRH-A (10^{-6} M). A, Representative Western immunoblot of IGFBP-3. B, Densitometric analysis of IGFBP-3 levels in culture medium of DU 145 cells. The results are expressed as a percentage of the control value and are the mean ± SE of four separate experiments.

agonists may inhibit prostate cancer cell proliferation by interfering with the local action of the IGF system; the molecular mechanisms of this interaction appear to be different in androgen-dependent and androgen-independent cells.

The observation that at the level of prostate cancer, the mechanism of the antiproliferative action of LHRH-A might be different according to the androgen dependence or androgen independence of the cells is in agreement with previous data from our laboratory (31). We have reported that LHRH-A interferes with the stimulatory action of the epidermal growth factor (EGF)/transforming growth factor-α system, which has been previously shown to participate in the local regulation of the growth of this tumor (40–42). In particular, we have shown that in DU 145 cells, LHRH-A may counteract the mitogenic action of EGF, inhibit the tyrosine autophosphorylation of the EGF receptor, and reduce the concentration of EGF binding sites without modifying the expression of the c-*fos* protooncogene that follows treatment with the growth factor. Interestingly, in the androgen-dependent LNCaP cells, LHRH-A antagonized the proliferative action of EGF by reducing the concentration of EGF receptors and suppressing the EGF-induced c-*fos* protooncogene expression without affecting receptor autophosphorylation (31). These observations further confirm that LHRH agonists exert a significant antimitogenic action on prostate cancer cells by interfering with the activity of locally expressed growth factor systems. However, the molecular mechanisms of this antiproliferative action differ according to the androgen dependence or androgen independence of the cells and on the type of growth factor investigated.

References

1. **Gittes RF** 1991 Carcinoma of the prostate. N Engl J Med 324:236–245
2. **Motta M, Serio M (eds)** 1988 Hormonal Therapy of Prostatic Diseases: Basic and Clinical Aspects. Medicom Europe, Amsterdam
3. **Crawford ED, De Antonio EP, Labrie F, Schroder FH, Geller J** 1995 Endocrine therapy of prostatic cancer: optimal form and appropriate timing. J Clin Endocrinol Metab 80:1062–1078
4. **Byrne RL, Leung H, Neal DE** 1995 Peptide growth factors in the prostate as mediators of stromal epithelial interaction. Br J Urol 77:627–633
5. **Chan JM, Stampfer MJ, Giovannucci E, Gann PH, Ma J, Wilkinson P, Hennekens CH, Pollak M** 1998 Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study. Science 279:563–566
6. **Pietrzkowski Z, Mulholland G, Gomella L, Hameson BA, Weinike D, Baserga R** 1993 Inhibition of growth of prostatic cancer cell lines by peptide analogues of insulin-like growth factor I. Cancer Res 53:1102–1106
7. **Angelloz-Nicoud P, Binoux M** 1995 Autocrine regulation of cell proliferation by the insulin-like growth factor (IGF) and IGF binding protein-3 protease system in a human prostate carcinoma cell line (PC-3). Endocrinology 136:5485–5492
8. **Figueroa JA, Lee AV, Jackson JG, Yee D** 1995 Proliferation of cultured human prostate cancer cells is inhibited by insulin-like growth factor (IGF) binding protein-1: evidence for an IGF-II autocrine growth loop. J Clin Endocrinol Metab 80:3476–3482
9. **Kimura G, Kasuya J, Giannini S, Honda Y, Mohan S, Kawachi M, Akimoto M, Fujita-Yamaguchi Y** 1996 Insulin-like growth factor (IGF) system components in human prostatic cancer cell lines: LNCaP, DU145, and PC-3 cells. Int J Urol 3:39–46
10. **Iwamura M, Sluss PM, Casamento JB, Cockett ATK** 1993 Insulin-like growth factor I: action and receptor characterization in human prostate cancer cell lines. Prostate 22:243–252
11. **Connolly JM, Rose DP** 1994 Regulation of DU145 human prostate cancer cell proliferation by insulin-like growth factors and its interaction with the epidermal growth factor autocrine loop. Prostate 24:167–175
12. **Burfeind P, Chernicky CL, Rininsland F, Ilan J** 1996 Antisense RNA to the type I insulin-like growth factor receptor suppresses tumor growth and prevents invasion by rat prostate cancer cells in vivo. Proc Natl Acad Sci USA 93:7263–7268
13. **Jungwirth A, Schally AV, Pinski J, Halmos G, Groot K, Armatis P, Vadillobuenfil M** 1997 Inhibition of in vivo proliferation of androgen-independent prostate cancers by an antagonist of growth hormone-releasing hormone. Br J Cancer 75:1585–1592
14. **Rechler MM, Nissley SP** 1985 The nature and regulation of the receptors for the insulin-like growth factor. Annu Rev Physiol 47:425–442
15. **Ritchie CK, Andrews LR, Thomas KG, Tindall DJ, Fitzpatrick LA** 1997 The effects of growth factors associated with osteoblasts on prostate carcinoma proliferation and chemotaxis: implications for the development of metastatic disease. Endocrinology 138:1145–1150
16. **Reyes-Moreno C, Koutsilieris M** 1997 Glucocorticoid receptor function possibly modulates cell-cell interactions in osteoblastic metastases on rat skeleton. Clin Exp Metastasis 15:205–217
17. **Conover CA, Perry JE, Tindall DJ** 1995 Endogenous cathepsin D-mediated hydrolysis of insulin-like growth factor-binding proteins in cultured human prostatic carcinoma cells. J Clin Endocrinol Metab 80:987–993
18. **Marcelli M, Haidacher SJ, Plymate SR, Birnbaum RS** 1995 Altered growth and insulin-like growth factor-binding protein-3 production in PC3 prostate carcinoma cells stably transfected with a constitutively active androgen receptor complementary deoxyribunucleic acid. Endocrinology 136:1040–1048
19. **Rajah R, Katz L, Nunn S, Solberg P, Beers T, Cohen P** 1995 Insulin-like growth factor binding protein (IGFBP) proteases: functional regulators of cell growth. Prog Growth Factor Res 6:273–284
20. **Srinivasan N, Edwall D, Linkhart TA, Baylink DJ, Mohan S** 1996 Insulin-like growth factor-binding protein-6 produced by human PC-3 prostate cancer cells: isolation, characterization and its biological action. J Endocrinol 149:297–303
21. **Belchetz PE** 1983 Gonadotropin regulation and clinical applications of GnRH. Clin Endocrinol Metab 12:619–640
22. **Limonta P, Dondi D, Moretti RM, Maggi R, Motta M** 1992 Antiproliferative effects of luteinizing hormone-releasing hormone agonists on the human prostatic cancer cell line LNCaP. J Clin Endocrinol Metab 75:207–212
23. **Limonta P, Dondi D, Moretti RM, Fermo D, Garattini E, Motta M** 1993 Expression of luteinizing hormone-releasing hormone mRNA in the human prostatic cancer cell line LNCaP. J Clin Endocrinol Metab 76:797–800
24. **Pinski J, Reile H, Halmos G, Groot K, Schally AV** 1994 Inhibitory effects of analogs of luteinzing hormone-releasing hormone on the growth of the androgen-independent Dunning R-3327-AT-1 rat prostate cancer. Int J Cancer 59:51–55
25. **Loop SM, Gorder CA, Lewis SM, Saiers JH, Drivdahl RH, Ostenson RC** 1995 Growth inhibition of human prostate tumor cells by an agonist of gonadotrophin-releasing hormone. Prostate 26:179–188
26. **Dondi D, Limonta P, Moretti RM, Montagnani Marelli M, Garattini E, Motta M** 1994 Antiproliferative effects of luteinizing hormone-releasing hormone (LHRH) agonists on human androgen-independent prostate cancer cell line DU 145: evidence for an autocrine-inhibitory loop. Cancer Res 54:4091–4095
27. **Dondi D, Moretti RM, Montagnani Marelli M, Pratesi G, Polizzi D, Milani M, Motta M, Limonta P** 1998 Growth inhibitory effects of luteinizing hormone-releasing hormone (LHRH) agonists on xenografts of the DU 145 human androgen-independent prostate cancer cell line in nude mice. Int J Cancer 76:506–511
28. **Stone KR, Mickey DD, Wunderli H, Mickey GH, Paulson DF** 1978 Isolation of a human prostate carcinoma cell line (DU 145). Int J Cancer 21:274–281
29. **Neuenschwander S, Roberts Jr CT, LeRoith D** 1995 Growth inhibition of MCF-7 breast cancer cells by stable expression of an insulin-like growth factor I receptor antisense RNA. Endocrinology 136:4298–4303
30. **Laemmli UK** 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
31. **Moretti RM, Montagnani Marelli M, Dondi D, Poletti A, Martini L, Motta M, Limonta P** 1996 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 Metab 81:3930–3937
32. **Bradford MM** 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
33. **Dunnett W** 1955 A multiple comparison procedure for comparing several treatments with a control. J Am Stat Assoc 50:1096–1121
34. **Munson PJ, Rodbard D** 1980 LIGAND: a versatile computerized approach for characterization of ligand-binding systems. Anal Biochem 107:220–239
35. **Shimasaki S, Ling N** 1991 Identification and molecular characterization of insulin-like growth factor binding proteins (IGFBP-1, -2, -3, -4, -5, -6). Prog Growth Factor Res 3:243–266
36. **Yano T, Korkut E, Pinski J, Szepeshazi K, Milovanovic S, Groot K, Clarke R, Comaru-Schally AM, Schally AV** 1992 Inhibition of growth of MCF-7 MIII human breast carcinoma in nude mice by treatment with agonists or antagonists of LH-RH. Breast Cancer Res Treat 21:35–45

37. **Yano T, Pinski J, Halmos G, Szepeshazi K, Groot K, Schally AV** 1994 Inhibition of growth of OV-1063 human epithelial ovarian cancer xenografts in nude mice by treatment with luteinizing hormone-releasing hormone anatgonist SB-75. Proc Natl Acad Sci USA 91:7090–7094
38. **Hershkovitz E, Marbach M, Bosin E, Levy J, Roberts Jr CT, LeRoith D, Schally AV, Sharoni Y** 1993 Luteinizing hormone-releasing hormone antagonists interfere with autocrine and paracrine growth stimulation of MCF-7 mammary cancer cells by insulin-like growth factors. J Clin Endocrinol Metab 77:963–968
39. **Joly-Pharaboz MO, Soave MC, Nicolas B, Mebarki F, Renaud M, Foury O, Morel Y, Andre JG** 1995 Androgens inhibit the proliferation of a variant of the human prostate cancer cell line LNCaP. J Steroid Biochem Mol Biol 55:67–76
40. **Connolly JM, Rose DP** 1990 Production of epidermal growth factor and transforming growth factor-α by the androgen-responsive LNCaP human cancer cell line. Prostate 16:209–218
41. **Connolly JM, Rose DP** 1991 Autocrine regulation of DU145 human prostate cancer cell growth by epidermal growth factor-related polypeptides. Prostate 19:173–180
42. **Limonta P, Moretti RM, Dondi D, Montagnani Marelli M, Motta M** 1994 The EGF/TGFα system as an autocrine growth stimulatory loop in LNCaP cells. Endocr Relat Cancer 3:5–13