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INHIBITORY EFFECTS OF A LUTEINIZING HORMONE-RELEASING HORMONE AGONIST ON BASAL AND EPIDERMAL GROWTH FACTOR-INDUCED CELL PROLIFERATION AND METASTASIS-ASSOCIATED PROPERTIES IN HUMAN EPIDERMOID CARCINOMA A431 CELLS

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The purpose of this study was to investigate the effects of a potent LHRH agonist, [D-Trp^6]LHRH on the basal and EGF-induced cell proliferation and the metastasis-associated properties in A431 human epidermoid carcinoma. [D-Trp^6]LHRH time-dependently inhibited the basal and EGF-stimulated growth of A431 cancer cells. It is assumed that phosphorylation/dephosphorylation of cellular proteins is highly related to cell growth. This study demonstrates that [D-Trp^6]LHRH decreased the basal and EGF-induced total cellular kinase activity, particularly the tyrosine phosphorylation of several cellular proteins including the EGFR. In contrast, [D-Trp^6]LHRH did not cause detectable changes in basal and EGF-stimulated serine/threonine phosphorylation of A431 cellular proteins. The inhibitory effect of [D-Trp^6]LHRH on A431 cell proliferation was associated with apoptosis as evidenced by the cell morphology and DNA integrity (ladder pattern), the expression of interleukin 1β-converting enzyme (ICE) and activation of caspase. Furthermore, EGF could rescue the remaining attached A431 cells following [D-Trp^6]LHRH treatment for 48 hr, which suggests that limited exposure to [D-Trp^6]LHRH did not channel all cells to irreversible apoptotic process. We also determined the effects of [D-Trp^6]LHRH on metastasis-associated properties in A431 cells. [D-Trp^6]LHRH reduced both basal and EGF-stimulated secretion of MMP-9 and MMP-2. In addition, [D-Trp^6]LHRH suppressed the basal and EGF-induced invasive activity of A431 cells based on an *in vitro* invasion assay. In conclusion, this study indicates that [D-Trp^6]LHRH may act partly through activating tyrosine phosphatase activity to inhibit cell proliferation and the metastasis-associated properties of A431 cancer cells. Our work suggests that [D-Trp^6]LHRH may be therapeutically useful in limiting the tumor growth and metastasis of some neoplasms.

Key words: *proliferation; [D-Trp^6]LHRH; Interleukin-1β converting enzyme; protein tyrosine phosphatase; protein tyrosine kinase; apoptosis; matrix metalloproteinase*

The life threatening characteristics of cancers lie in the uncontrolled cell mitogenic activity and the acquiring of metastatic capability. Therefore, the strategies for cancer therapy are aimed at controlling the cancer cell growth and/or inhibiting the cancer cell invasion/metastasis. Early reports contended that protein tyrosine kinase (PTK) regulatory mechanisms are clearly implicated in the growth of neoplastic cells, and inactivating specific PTKs could retard the growth of tumors.[1–3] Among the PTKs investigated, epidermal growth factor receptor (EGFR) tyrosine kinase has attracted much attention with regard to its role in transducing signal for promoting cell growth. Autophosphorylation of EGFR appears to be a potential target for cancer therapy because that would engender a blockade of the EGFR-mediated signaling.[3] In light of this, it is imperative to explore whether luteinizing hormone-releasing hormone (LHRH) analogues that possess antiproliferative activity could have any direct effects on the autophosphorylation of EGFR and its intrinsic tyrosine kinase activity.

More than 3 decades have passed since the isolation and characterization of the hypothalamic LHRH, which controls the anterior pituitary secretion of luteinizing hormone and follicle stimulating hormone.[4] Accumulated data have indicated that LHRH might also present in extra-hypothalamic tissues, such as ovary, testis, kidney and placenta.[5] Currently, more than 3,000 analogues of LHRH have been synthesized.[6] Agonists of LHRH have important clinical applications in gynecology and oncology.[6] Potent antagonists of LHRH, such as Cetrorelix, are also available for clinical use.[6] The actions of LHRH and its analogues are mediated by high-affinity receptors for LHRH found on pituitary gonadotrophs and various tumors.[6] The presence of high affinity binding sites for LHRH and the expression of mRNA for LHRH receptors have been shown in human prostatic, mammary, ovarian and endometrial cancers. These LHRH receptors on tumor cells can mediate direct effects of LHRH agonists and antagonists. Inhibitory effects of LHRH agonists and antagonists on prostatic, mammary, ovarian and endometrial cancer cells have been demonstrated *in vitro*.[6–11] Thus, a potent agonist [D-Trp^6]LHRH and LHRH antagonist Cetrorelix synthesized in the laboratory of one of us (A.V.S.)[6] was shown to inhibit the growth of human ovarian endometrial and mammary cancer cells[9] and LHRH agonists Zoladex and Buserelin suppressed the proliferation of human prostate cancer lines.[7,10] The effects of LHRH-analogues on mitogenic signal transduction in cancer cells were also the subjects of exten-

Abbreviations: DMSO, dimethyl sulphoxide; EDTA, ethylenediamine tetraacetic acid; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ICE, interleukin-1β converting enzyme; LHRH, luteinizing hormone-releasing hormone; MMP, matrix metalloproteinase; PARP, poly(ADP-ribose) polymerase; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

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sive investigations.[12–14] Thus, LHRH agonist triptorelin was shown to antagonize signal transduction and mitogenic activity of EGF in human ovarian and endometrial cancer cell lines.[12] This mitogenic effect of EGF on proliferation was nullified by triptorelin, without affecting the concentrations of EGF receptors.[12–14] Tyrosine phosphorylation induced by EGF was nearly completely suppressed by triptorelin. Exposure to EGF induced an increase in activity of mitogen-activated protein kinase (MAP-kinase)/extracellular signal regulated kinase (ERK), which was virtually nullified when the cells were exposed to triptorelin. The mechanism of action is probably an LHRH-induced activation of a phosphotyrosine phosphatase, counteracting the effects of receptor associated tyrosine kinase.[12–14] LHRH analogs blocked the EGF-induced MAP-kinase activity of ovarian and endometrial cancer cells.[12–14] LHRH agonists also interfered with the stimulatory actions of epidermal growth factor in human prostatic cancer cell lines LNCaP and DU 145.[15] Our earlier study showed that [D-Trp^6]LHRH decreased the basal and epidermal growth factor (EGF)-stimulated total kinase activity in MiaPaCa-2 human pancreatic carcinoma and HCPC (hamster cheek pouch carcinoma) cells, particularly the phosphorylation of certain membrane-associated proteins including the 170-, 65- and 60-kDa molecules.[16] The similarity in substrate specificity suggests that the dephosphorylation of these proteins may play an important role in inhibition of cell growth. Also, limited studies indicate that decrease in tyrosine phosphorylation of cellular proteins induced cell apoptosis.[17–19] However, other authors[20] report LH-RH and its agonists can induce nuclear factor kB-Activation and inhibit apoptosis in ovarian cancer cells. Various cellular signaling pathways involved in apoptosis are being intensively studied. The interleukin-1β converting enzyme (ICE)-related proteases or caspases, belonging to a multigene family encoding cysteine proteases, have been demonstrated to function in apoptotic execution,[21–24] mediating through specific targets as poly(ADP-ribose) polymerase (PARP), gelsolin, DFF-45 and the nuclear lamins.[25] PARP is a 116 kDa nuclear protein that is specifically cleaved by caspase-3 and caspase-6 into an 85- and a 25-kDa apoptotic fragment.[26] Analyzing PARP cleavage sheds light on the activation of caspase-3 or some other protease(s) that may be implicated in the given model of cell death.[25]

Spread of cancer through metastasis represents 1 of the gravest dangers of the disease.[27,28] In human cancers of the breast, liver, colon, lung and ovary, the production of certain matrix metalloproteinases (MMPs) correlates with the cancer invasion/metastasis.[28–34] The MMPs are encoded by at least 20 genes[35] and categorized into 4 subclasses based on structural organization and substrate specificity: collagenases, gelatinases, stromelysins and membrane-type MMPs.[29,36] Collectively, MMPs degrade most components of the extracellular matrix. Tumor cells probably need more than 1 MMP, as well as more general degradative enzymes to cross the tissue barriers they encounter. The activities of MMPs are regulated at multiple levels including expression and secretion of MMPs, and the activation processes of MMPs.[29,36,37] The secretion of MMPs is necessary for tumor invasion as indicated by the observations that treatment with antibodies or inhibitors against MMPs abolished the invasive behavior of certain tumor cells.[24,38] Therefore, the suppression of secretions and activities of MMPs in cancers would be expected to limit the metastatic potential of cancer cells.

The purpose of this study was to investigate the effects of [D-Trp^6]LHRH on basal and EGF-induced proliferation and the metastasis-associated properties of cancer cells. This study employed human epidermoid carcinoma A431 cells because of the high constitutive expression levels of EGF receptors (EGFR).[39] Also, our previous study shows that EGF stimulated the secretion of MMPs involved in invasion/metastasis in A431 cells.[40] There were 3 specific objectives in this study. First was to evaluate the effect of [D-Trp^6]LHRH on the basal and EGF-stimulated proliferation and phosphorylation of cellular proteins including EGFR in A431 cells. The second was to investigate the molecular mechanism of the inhibitory effect of [D-Trp^6]LHRH on cell growth. The third objective was to examine the effect of [D-Trp^6]LHRH on the basal and EGF-stimulated metastasis-associated properties in A431 cells, including the secretion of MMPs and cell invasive activity.

MATERIAL AND METHODS

Materials

The LHRH analogue, [D-Trp^6]LHRH (PGlu-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly-NH_2), was synthesized by solid-phase methods and purified by HPLC.[6] RPMI-1640, Dulbecco's modified Eagle medium (DMEM), penicillin and streptomycin were acquired from GIBCO Life Technology (Grand Island, NY). EGF and fetal bovine serum (FBS) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). [γ-^{32}P]ATP and enhanced chemiluminescence kit were obtained from Amersham (Buckinghamshire, UK). Mouse monoclonal anti-human EGFR, anti-phosphotyrosine and anti-PARP DNA binding domain antibodies were obtained from Transduction Laboratories (San Diego, CA). Mouse monoclonal anti-phosphoserine and anti-phosphothreonine antibodies were from Zymed Laboratories, Inc. (South San Francisco, CA). Rabbit polyclonal anti-human ICE antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-human MMP-9 and MMP-2 antibodies were obtained from Biogenesis (Sandown, NH). Unless otherwise indicated, all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Growth experiment

Human epidermoid carcinoma A431 cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained at 37°C in a humidified atmosphere (95% air and 5% CO_2) and grown as a monolayer in plastic culture flasks containing DMEM supplemented with 10% FBS according to the recommendation of ATCC. Cells at logarithmic growth phase were harvested using 0.25% trypsin-0.02% EDTA solution for 5 min, pelleted by centrifugation at 500*g* for 5 min, washed once with medium and resuspended at a concentration of 1×10^4 cells/ml DMEM medium. Cells of 1×10^4 or 1×10^5 were incubated in 24-well plate (for growth study) and 10 cm dish (for collecting cellular proteins), respectively. The cells were then incubated at 37°C for 24 hr to allow the attachment to plates. After that the culture media were changed, and [D-Trp^6]LHRH and/or EGF were added to obtain a final concentration of 100 nM and 10 nM respectively for various incubation time periods as previously described.[16,41] At the end of incubation, cells from triplicate wells for each treatment group were harvested with trypsin-EDTA solution, and cell numbers were determined using a Coulter Multisizer II Counter (Coulter Electronics, Luton, England). Cell viability was also determined using trypan blue dye exclusion method.

Preparation of cell lysates

A431 cells were collected by trypsinization and washed 3 times with PBS. The cells were then lysed in gold lysis buffer containing 20 mM Tris, pH 7.9, 137 mM sodium chloride, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM sodium orthovanadate, 1 mM EGTA, 1 mM sodium pyrophosphate, 100 μM β-glycerophosphate, 10 μg/ml leupetin, 10 μg/ml aprotinin and 2 mM phenylmethylsulfonyl fluoride (PMSF).[42] Cell lysates were cleared by centrifugation at 12,000*g* for 10 min at 4°C. The protein concentration of cell lysate was determined according to the method of Bradford[43] and adjusted to 100 μg/50 μl. The samples were then divided into 200 μl aliquots and stored at −70°C for kinase activity assay, immunoprecipitation and immunoblotting analyses.

Total kinase activity assay

The total kinase activity assays were performed as originally described by Cohen et al.[44] with slight modifications.[16] Briefly, the reaction mixtures in a final volume of 80 μl contained 50 μg of cellular proteins in 20 mM HEPES buffer, pH 7.4, 1.0 mM $MnCl_2$ and 100 nM [D-Trp^6]LHRH or 100 nM EGF, or the combination of [D-Trp^6]LHRH and EGF. The reaction mixtures were preincu-

bated at room temperature for 2 hr and then chilled at 4°C for 10 min. The reaction was initiated by the addition of [γ-^{32}P]ATP ($6–12 \times 10^6$ cpm) and unlabeled ATP (60 μM). After 5 min, the reaction was stopped by pipetting 50 μl aliquots of the reaction mixtures onto a Whatman 3MM filter paper of 2.3 cm in diameter, which was immediately dropped into a solution of 10% trichloroacetic acid containing 10 mM pyrophosphate. The filter papers were then washed sequentially with 10% trichloroacetic acid, 50% and 100% ethyl alcohol (10 ml each), dropped into a beaker with 50 ml diethyl ether for 2 min and then dried in a fume hood. The radioactivity was determined in a Beckman scintillation counter (Beckman Instruments, Fullerton, CA).

Gel electrophoresis and autoradiography

The kinase assay reaction mixtures described above were also subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine changes of phosphorylation levels of A431 cellular proteins in response to [D-Trp^6]LHRH and EGF. The kinase reactions were terminated by the addition of 50 μl sample buffer (0.5 M Tris-HCl, pH 6.9; 2% SDS; 20% glycerol and 0.1% bromophenol) followed by boiling for 3 min. The reaction products were then electrophoresed on a 3–18% gradient SDS-PAGE according to the method of Laemmli.[45] Proteins were electrophoretically transferred onto nitrocellulose membranes as previously described[46] and autoradiographed with Kodak X-Omat AR film (Kodak, Rochester, NY) between 2 intensifying screens at −70°C. The intensities of ^{32}P-labeled proteins were determined using a densitometer (Vilber Lourmat, France).

Immunoblotting analyses and immunoprecipitation

Samples were electrophoresed on a 3–18% gradient SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane as described above. The electrophoretic blots were soaked in 5% BSA in PBS for 2 hr at room temperature to saturate additional protein binding sites. The membrane was rinsed 3 times in PBS and incubated with primary antibody appropriately diluted in 1% BSA/PBS at room temperature for 2 hr. The nitrocellulose membrane was then extensively washed 3 times in PBS containing 0.1% Tween-20 (PBST) for 10 min and incubated with appropriate secondary antibodies conjugated with horseradish peroxidase for 1 hr. The membrane was subsequently washed 3 times with PBST and twice with PBS. Bands were detected with enhanced chemiluminescence reagents (Amersham Pharmacia, Uppsala, Sweden) and exposed to Kodak BioMax film. To ascertain whether the 170 kDa phosphotyrosyl protein was the EGFR, the same blot was stripped off bound primary anti-phosphotyrosine and secondary antibodies in Strip buffer (2% w/v SDS; 62.5 mM Tris-Cl pH 6.8; 100 mM β-mercaptoethanol) at 37 °C for 30 min and then reprobed with anti-EGFR antibodies. For immunoprecipitation of EGFR, cell lysates were first reacted with monoclonal anti-human EGFR antibody for 1 hr and then with protein A/G-agarose for 12 hr at 4°C. The immunoprecipitates were washed 3 times with PBS containing 1% Trixton-100 and then boiled in 50 μl Laemmli sample loading buffer[45] and subjected to immunoblotting analyses for phosphotyrosine and EGFR as described above.

Analysis of DNA fragmentation

Tumor cells at logarithmic growth phase were treated with 100 nM [D-Trp^6]LHRH or 10 nM EGF for 48 hr. Cells were then collected by cell scrapper and centrifuged at 500*g* for 5 min. The cell pellet was then washed twice with PBS, and DNA was isolated and quantified as previously described.[47] Ten micrograms of DNA was loaded into each well of 1.8% agarose gel and electrophoresed in a buffer containing 0.04 M Tris, 0.04 M sodium acetate and 1 mM EDTA, pH 8.0, at 100 V for 1 hr at room temperature. Gels were stained with ethidium bromide, visualized by UV fluorescence and photographed.

Gelatin zymography analyses and characterization of MMPs

A431 cells at logarithmic growth phase were treated with 100 nM [D-Trp^6]LHRH and/or 10 nM EGF in serum-free DMEM medium containing 0.1% lactalbumin hydrolysate for 24 hr. The conditioned media were collected and analyzed for MMPs using gelatin zymography,[40,48] and cell numbers were determined using Coulter counter. Cell viability was also determined by using trypan blue dye exclusion method. In brief, samples of conditioned media were subjected to SDS-PAGE on a 3–18% gradient gel containing 0.1% porcine skin gelatin. The loading volume of each conditioned medium sample was normalized according to the cell number. Electrophoresis was performed under denatured-and-nonreducing conditions in 25 mM Tris, 192 mM glycine and 0.1% SDS at 15 mA during stacking and 12 mA during separation. After electrophoresis, gels were first washed twice for 30 min each in 2.5% Triton X-100 to remove SDS and then in reaction buffer (50 mM Tris-HCl, pH 8.0 containing 5 mM $CaCl_2$, 0.02% NaN3) for an additional 30 min. The gels were then incubated in reaction buffer at 37°C for 18 hr, and then stained with 0.25% Coomassie brilliant blue R-250 in 10% acetic acid/20 % ethanol for 1 hr and destained in the same solution without dye. A clear zone on the gel indicates the presence of gelatinase activity. Gelatinase activity was quantified using a densitometer (Vilber Lourmat, France).

To characterize the gelatinases produced by A431 cancer cells according to their sensitivity to specific inhibitors, gels following electrophoresis and Triton X-100 wash were incubated in reaction buffer containing metalloproteinase inhibitors, 5 mM 1,10-phenanthroline or 10 mM EDTA,[49] and then processed as described above. In addition, the identities of 92 and 72 kDa gelatinases were further determined using immunoblotting analyses as described above.

In vitro *invasion assay*

In vitro invasion assay was carried out by the method of Saiki *et al*.[50] Briefly, 24-well transwell units with 8 μM porosity polycarbonate filters (Becton Dickinson, Franklin Lake, NJ) were coated with 0.1 ml of 0.8 mg/ml Englebreth-Holm-Swarm sarcoma tumor extract, named EHS matrigel, at room temperature for 1 hr. The EHS matrigel consists of reconstituted basement membrane substances. The EHS tumor extract was generously provided by Dr. M. M. IP (Roswell Park Cancer Institute, Buffalo, NY). A431 cells (2×10^5 cells/0.4 ml DMEM) were placed in the upper compartment and incubated with vehicle, 100 nM [D-Trp^6]LHRH and/or 10 nM EGF at 37°C for 48 hr in a humidified atmosphere with 95% air and 5% CO_2. The filters were then fixed with 3% glutaraldehyde in PBS and stained with crystal violet. Cells on the upper surface of the filter were removed by wiping with a cotton swab, and cells that penetrated through matrigel to the lower surface of the filter were counted under light microscope at ×200. Each treatment was assayed in triplicate, and 2 independent experiments were performed.

Statistics

Data are expressed as mean ± SEM of 2 to 3 independent experiments. Statistical significance between 2 groups was determined by unpaired Student's *t*-test. Data of invasion assay were analyzed by analysis of variance and multiple range test. A probability of $p \leq 0.05$ was considered significant.

RESULTS

Effects of [D-Trp^6]LHRH on basal and EGF-induced cell proliferation

In growth experiments, A431 cells were allowed to attach for 24 hr and then cultured in serum-free medium for 24 hr prior to the treatment with 100 nM [D-Trp^6]LHRH and/or 10 nM EGF for 24, 48 and 72 hr. At the end of culture, cells were detached with trypsin-EDTA solution and counted by a Coulter Counter. Consistent to our previous study,[40] EGF time-dependently stimulated the proliferation of A431 cells during the 3-day culture period (Fig. 1). This study clearly shows that [D-Trp^6]LHRH inhibited the growth of A431 cells in a time-dependent manner (Fig. 1). Interestingly, we noticed that [D-Trp^6]LHRH could counteract the stimulatory effect of EGF on the proliferation of A431 cells (Fig. 1).

Effects of [D-Trp^6]LHRH on basal and EGF-induced cellular protein phosphorylation

It is well known that EGF activates EGFR tyrosine kinase activity and promotes cell growth. Also consistent with our previous study,[40] EGF stimulated total kinase activities and increased phosphorylation of A431 cellular proteins (+21.7 ± 4.8%) compared to the control. This study demonstrates that EGF specifically stimulated the phosphorylation of 170, 125, 85, 65, 44 and 22 kDa cellular proteins (Fig. 2*a*, lane c; Table I). In contrast, [D-Trp^6]LHRH decreased total kinase activity and reduced phosphorylation of cellular proteins (−22.3 ± 5.3%) compared to the control. [D-Trp^6]LHRH was found to decrease the basal phosphorylation of cellular proteins described above (Fig. 2*a*, lane b; Table II). Furthermore, [D-Trp^6]LHRH could counteract the stimulatory effect of EGF on the total kinase activity (−7.6 ± 3.6%) and the phosphorylation of those cellular proteins (Fig. 2*a*, lane d; Table I), as well as the proliferation of A431 cells (Fig. 1). It is worth to note, the 125 kDa protein band was further identified as focal adhesion kinase (FAK) by immunoblotting analysis (unpublished data).

We further set out to determine the identity of phosphoproteins, phosphotyrosine or phosphoserine/threonine proteins; those are regulated by EGF and [D-Trp^6]LHRH treatment in A431 cells. To detect phosphotyrosyl proteins, immunoblotting analyses were performed using anti-phosphotyrosine protein antibodies. Incubation of A431 cell lysates with 100 nM EGF for 1 hr consistently stimulated tyrosine phosphorylation of 170, 125, 105, 85, 65, 55, 44, 38 and 35 kDa protein bands (Fig. 2*b*, lane c). Treatment with 100 nM [D-Trp^6]LHRH inhibited basal tyrosine phosphorylation of 170 kDa band (Fig. 2*b*, lane b). Furthermore, [D-Trp^6]LHRH suppressed all the EGF-stimulated tyrosyl-phosphorylated cellular proteins (Fig. 2*b*, lane d). Similar changes in phosphorylation levels of 170, 125, 85, 65 and 44 kDa protein bands were also observed in response to EGF and [D-Trp^6]LHRH using kinase activity assay as shown in Figure 2*a*.

In order to further determine whether the tyrosine phosphorylated 170 kDa protein is EGFR, 2 approaches were employed. A431 cells were treated for 24 hr with vehicle, [D-Trp^6]LHRH and/or EGF, and then cell lysates were analyzed. First approach using the immunoblotting technique, the same blot that immunoblotted with anti-phosphotyrosine antibody, was then reprobed with anti-EGFR antibody. The result showed that the 170 kDa protein band was immunoreactive with both antibodies (Fig. 2*b*,*c*). In the second approach, cell lysates were first immunoprecipitated with anti-EGFR antibodies and then immunoblotted with anti-phosphotyrosine or anti-EGFR antibodies. The degree of EGFR phosphorylation was expressed as the relative density ratio of phosphotyrosine level to EGFR level. In this EGFR overexpressed A431 cell line, a basal level of EGFR tyrosine phosphorylation was detectable (Fig. 3, lane a). Treatment with EGF increased tyrosine phosphorylation of EGFR, while [D-Trp^6]LHRH diminished both basal and EGF-stimulated tyrosine phosphorylation level of EGFR (Fig. 3, lanes b–d).

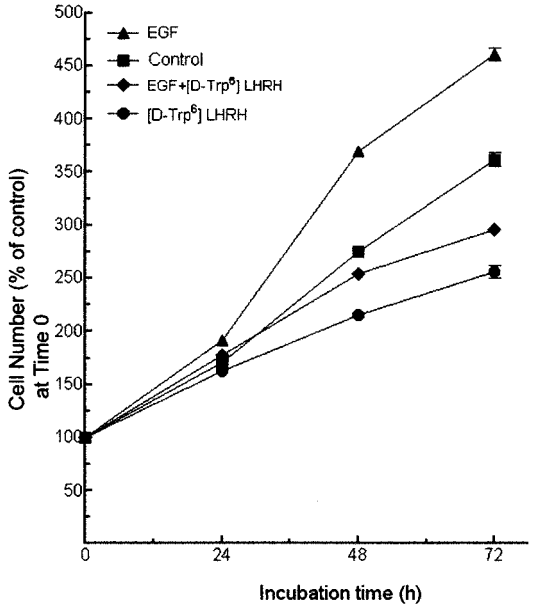

FIGURE 1 – Effects of [D-Trp^6]LHRH on basal and EGF-induced proliferation of A431 cells. Cells were treated with vehicle control, 100 nM [D-Trp^6]LHRH and/or 10 nM EGF for 24–72 hr. At the indicated time, cell numbers of the cultures were determined using Coulter counter. Each point represents the mean (± SEM) of triplicate wells from 1 of 3 independent experiments, all of which gave similar results.

To determine whether [D-Trp^6]LHRH might promote protein phosphatase activities other than protein tyrosine phosphatase, we performed experiments to detect the changes of phosphoserinyl and phosphothreoninyl protein levels in response to EGF and [D-Trp^6]LHRH. Immunoblotting analyses using anti-phosphoserinyl and phosphothreoninyl protein antibodies were performed. We observed that EGF elevated certain phosphoserinyl (120, 85, 83, 65, 44 and 42 kDa) and phosphothreoninyl (250, 130, 110, 80, 75 and 44 kDa) protein levels of A431 cellular extracts as well (Fig. 4*a*,*b*, lane b). In contrast to the inhibitory effect on phosphorylation of tyrosyl proteins, [D-Trp^6]LHRH treatment did not cause any detectable change in the basal and EGF-stimulated phosphoserinyl and phosphothreoninyl protein levels (Fig. 4*a*,*b*, lanes c,d).

Mechanism of [D-Trp^6]LHRH-induced inhibition of cell proliferation

Inhibition of cell proliferation could be the result of the induction of apoptosis and/or cell cycle arrest. We therefore investigated whether [D-Trp^6]LHRH could induce apoptosis in A431 cells. The cell morphology and the molecular integrity of DNA were examined to reveal [D-Trp^6]LHRH influence on cell homeostasis. A431 cells incubated with 100 nM [D-Trp^6]LHRH for 48 hr exhibited protuberant cytoplasmic blebs and progressively shrinking morphology (Fig. 5*c*). The cells shrank into a rounded configuration and then detached from the flasks. In addition, cells treated with [D-Trp^6]LHRH exhibited loss of DNA integrity, showing a ladder pattern of multiples of 180–200 base pairs, a typical characteristic of apoptosis (Fig. 6, lane c). To determine if cell growth inhibition by [D-Trp^6]LHRH could be reversed by EGF, A431 cells treated with [D-Trp^6]LHRH for 48 hr were then replaced with fresh medium containing 10 nM EGF and cultured for an additional 24 hr. The remaining attached cells started growing (Fig. 5*d*), and no significant nuclear fragmentation was observed in these cells (Fig. 6, lane d).

To investigate the mechanism of [D-Trp^6]LHRH-induced apoptosis of A431 cells, the expression of ICE, a known mediator of apoptotic process, was determined using immunoblotting analysis. The results of a typical experiment are shown in Figure 7*a*, and the relative density of the 20 kDa ICE protein band was determined by scanning densitometry. The induction of ICE protein (5.1 ± 1.0 fold) was observed when cells were treated with 100 nM [D-Trp^6]LHRH for 48 hr, while ICE was not detected in 48 hr EGF-treated cultures (Fig. 7*a*, lanes c, b). In addition, no ICE-immunoreactive protein was detected in the attached cells of the culture subjected to 48 hr [D-Trp^6]LHRH treatment followed by subsequent exposure to EGF alone for an additional 24 hr (Fig. 7*a*, lane d). We then further determined the effect of [D-Trp^6]LHRH on caspase activity, the executioner of apoptosis, using PARP as the substrate and analyzed by immunoblotting analysis with antibody against the N-termial domain of PARP. The dominant cleavage of 116 kDa PARP to fragments of approximately 25 and 85 kDa, typically an early event in apoptosis, was evident at 48 hr [D-Trp^6]LHRH-treated culture (Fig. 7*b*, lane c). It is worthy of notice that significantly less PARP fragments appeared in the attached cells of the culture subjected to 48 hr [D-Trp^6]LHRH treatment followed by subsequent exposure to EGF alone for an

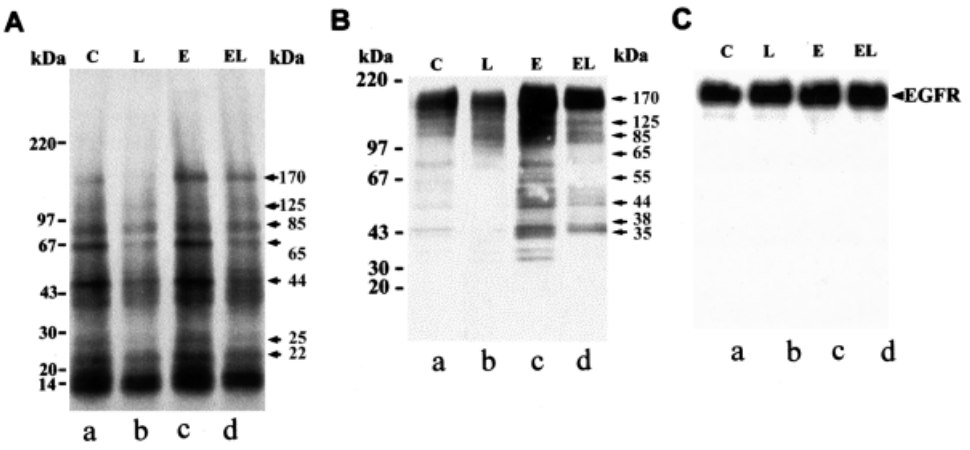

FIGURE 2 – Effects of [D-Trp^6]LHRH on basal and EGF-induced cellular protein phosphorylation in A431 cells. (*a*) A representative autoradiogram of A431 cellular proteins after phosphorylation/dephosphorylation induced by factors. Cell lysates of 50 μg proteins each were incubated with 100 nM [D-Trp^6]LHRH and/or 100 nM EGF in the presence of ^{32}P. Lanes: a, vehicle control (C); b, [D-Trp^6]LHRH (L); c, EGF (E); d, EGF plus [D-Trp^6]LHRH (EL). The reaction mixtures were subjected to SDS-PAGE and autoradiography. (*b*) Immunoblotting analysis of phosphotyrosine proteins in A431 cell lysates. The same procedure was used as in (*a*) except excluding [γ-^{32}P]ATP as the kinase substrate, anti-phosphotyrosine antibody and enhanced chemiluminescence were used to detect phosphotyrosyl proteins. (*c*) The same blot (*b*) was stripped off primary and secondary antibodies, and reprobed with anti-EGFR antibody. Note that [D-Trp^6]LHRH dramatically decreased the phosphorylation of most protein bands, including the 170 kDa EGFR band (*a*,*b*), while similar amounts of the EGFR band were observed in all lanes (*c*). Data shown are representatives of 3 separate experiments performed with duplicate samples of different cell preparations.

TABLE I – DENSITOMETRIC ANALYSIS OF CHANGES IN PHOSPHORYLATION OF CELLULAR PROTEINS IN RESPONSE TO [D-TRP^6]LHRH AND EGF IN A431 CELLS SHOWN IN FIGURE 2A[1]

Protein size (kDa)	^{32}P incorporated, % change *vs.* control		
	EGF	[D-Trp^6]LHRH	EGF + [D-Trp^6]LHRH
170	+163.1 ± 12.6*	−35.6 ± 7.8*	+48.4 ± 6.8*
125	+59.6 ± 2.0*	−42.4 ± 1.8*	−5.6 ± 3.9
85	+16.2 ± 4.5*	−47.6 ± 2.5*	−27.0 ± 3.2*
65	+27.9 ± 3.6*	−63.8 ± 3.0*	−47.9 ± 4.1*
44	+37.5 ± 9.7*	−32.2 ± 6.5*	−5.4 ± 1.2*
25	+17.5 ± 13.9	−57.3 ± 6.0*	−46.4 ± 2.3*
22	+53.5 ± 6.1*	−66.2 ± 5.6*	−22.6 ± 4.3*

[1]Quantitative analyses were performed using scanning densitometry. The percentage of optical autoradiographic density is compared to the control. Values are expressed as the mean percentage density change (± SEM) from samples of 3 separate experiments. All hormones were used at 100 nM for total kinase assays.–*$p < 0.05$.

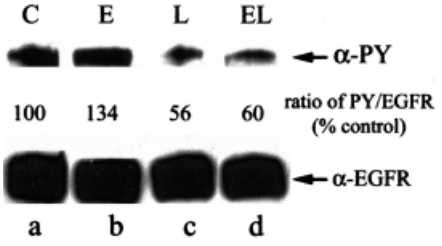

FIGURE 3 – Effects of [D-Trp^6]LHRH on EGF-induced autophosphorylation of EGFR in A431 cells. Cells were preincubated with 10 nM EGF for 20 min and then treated with vehicle, EGF and/or 100 nM [D-Trp^6]LHRH for an additional 24 hr. Cells lysates were immunoprecipitated with EGFR antibodies and subsequently analyzed for EGFR and its tyrosine phosphorylation by immunoblotting as described in the Material and Methods section. Lanes: a, vehicle control (C); b, EGF (E); c, [D-Trp^6]LHRH (L); d, EGF plus [D-Trp^6]LHRH (EL). Quantitative changes of tyrosine phosphorylation of EGFR were estimated based on the ratio of the density of phosphotyrosyl-EGFR to the density of EGFR. The immunoblots shown are representatives of 3 separate experiments performed with duplicate samples of different cell preparations.

additional 24 hr (Fig. 7*b*, lane d). This is consistent to the results of DNA fragmentation as shown in Figure 6. Together these results indicate that [D-Trp^6]LHRH-induced cell death may be transduced, at least in part, through ICE and caspase(s).

Effects of [D-Trp^6]LHRH on basal and EGF-induced metastasis-associated properties

To investigate the effect of [D-Trp^6]LHRH on basal and EGF-induced secretion of MMPs involved in invasion/metastasis of cancer cells. Culture conditioned media were collected from A431 cells treated with 100 nM [D-Trp^6]LHRH and/or 10 nM EGF for 48 hr. Gelatinase activities were assessed using gelatin zymography and scanning densitometry. A representative zymogram was shown in Figure 8*a*. The relative percentage change of gelatinase activity was determined based on the density value of the control group. Control A431 cells exhibited secreted activities of 2 major gelatinases of 92 and 72 kDa (Fig. 8*a*, lane a). [D-Trp^6]LHRH reduced the basal secreted activities of 92 kDa (−27.4 ± 6.5%) and 72 kDa (−18.3 ± 3.8%) gelatinases (Fig. 8*a*, lane b), while EGF increased the activities of both gelatinases (92 kDa, +53.0 ± 4.3%; 72 kDa, +16.2 ± 3.4%) (Fig. 8*a*, lane c). Also, [D-Trp^6]LHRH was able to offset the EGF-stimulated secreted activities of both gelatinases (Fig. 8*a*, lane d). The secreted gelatinases were further characterized according to the biochemical dependence of the enzyme activity and their immunoreactivities. Addition of metalloproteinase inhibitor 5 mM 1,10-phenanthroline (Fig. 8*b*) or 10 mM EDTA (data not shown) abolished the activity of all gelatinases. We also performed immunoprecipitation using anti-MMP-2 and MMP-9 antibodies to remove these MMPs prior to the zymography analysis, and no detectable MMP-2 and MMP-9 were observed in this analysis (data not shown). In addition, immunoblotting analysis demonstrated that the 92 kDa gelatinase was MMP-9 and the 72 kDa gelatinase was MMP-2 (Fig. 8*c*). Both MMP-9 and MMP-2 are type IV collagenases.

The inhibitory effect of [D-Trp^6]LHRH on secreted activities of MMPs led us to further determine the effect of [D-Trp^6]LHRH on the invasive behavior of A431 cancer cells. To this aim, we employed an *in vitro* invasion assay using a transwell chamber coated with a reconstituted basement membrane (EHS matrigel). A431 cells were treated with 100 nM of [D-Trp^6]LHRH and/or 10 nM EGF for 48 hr, and cells penetrated through the matrigel to the

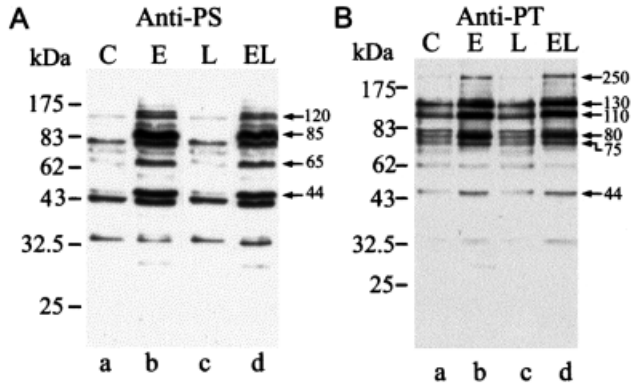

FIGURE 4 – Effects of EGF and [D-Trp^6]LHRH on the phosphorylation of serine and threonine residues of cellular proteins in A431 cells. The same reaction mixtures as described in Figure 2*b* were subjected to SDS-PAGE and immunoblotting analyses using anti-phosphoserine antibody (Anti-PS, *a*) and anti-phosphothreonine antibody (Anti-PT, *b*). Lanes: a, vehicle control (C); b, EGF (E); c, [D-Trp^6]LHRH (L); d, [D-Trp^6]LHRH and EGF (EL). The immunoblots shown are representatives of 3 separate experiments with duplicate samples of different cell preparations.

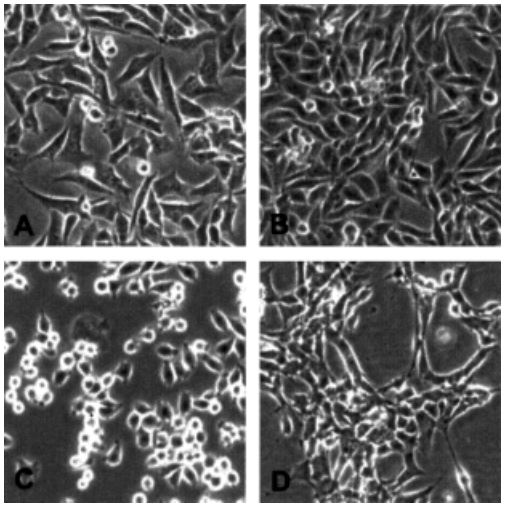

FIGURE 5 – Effects of [D-Trp^6]LHRH and EGF on the morphology of A431 cells. Cells were treated with 100 nM [D-Trp^6]LHRH or 10 nM EGF for 48 hr and photographed under phase-contrast microscope. (*a*) Vehicle control; (*b*) EGF; (*c*) [D-Trp^6]LHRH; (*d*) [D-Trp^6]LHRH for 48 hr and then EGF alone for an additional 24 hr. Original magnification ×200.

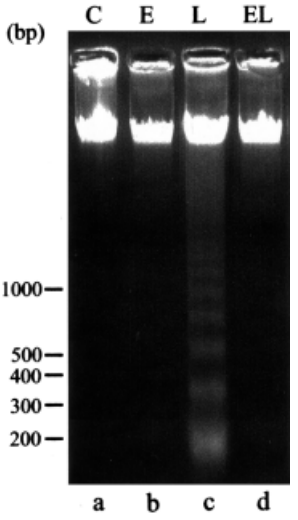

FIGURE 6 – Effects of [D-Trp^6]LHRH and EGF on DNA integrity of A431 cells. Cells were treated with 100 nM [D-Trp^6]LHRH or 10 nM EGF for 48 hr. Lanes: a, vehicle control (C); b, EGF (E); c, [D-Trp^6]LHRH (L); d, [D-Trp^6]LHRH for 48 hr and then EGF alone for an additional 24 hr (EL). At the end of the culture, approximately 2×10^6 cells were harvested. DNA was isolated from each sample and subjected to electrophoresis in a 1.8% agarose gel. This ladder pattern of DNA fragments is a characteristic of apoptosis. The gel shown is a representative of 3 separate experiments performed in duplicate.

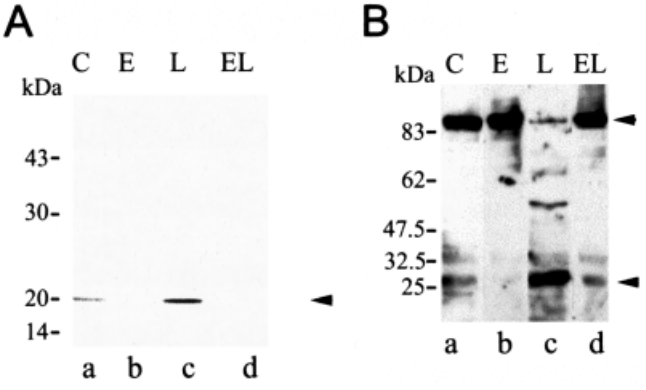

FIGURE 7 – Effects of [D-Trp^6]LHRH and EGF on ICE protein expression and caspase activity in A431 cells. Cells were treated with 100 nM [D-Trp^6]LHRH and/or 10 nM EGF for 48 hr. Cell lysates of 50 μg each were subjected to immunoblotting analyses using anti-ICE protein antibody (*a*) and anti-PARP antibody recognizing N-terminal domain of PARP (*b*). The formation of PARP fragments is an indication of casepase activity, the executioner of apoptosis. The lower arrowhead indicates the 25 kDa PARP fragment. Lanes: a, vehicle control (C); b, EGF (E); c, [D-Trp^6]LHRH (L); d, [D-Trp^6]LHRH for 48 hr and then EGF for an additional 24 hr. The immunoblots shown are representatives of three separate experiments performed in duplicate.

lower surface of the transwell filter were counted. Representative micrographs of transwell filters were shown in Figure 9*a*. Treatment with [D-Trp^6]LHRH significantly inhibited the invasive activity of A431 cells through matrigel by about 45% compared with the control, while EGF markedly promoted cell invasion by 7.67-fold (Fig. 9). In addition, [D-Trp^6]LHRH partially suppressed the EGF-induced invasive activity of A431 cells (Fig. 9).

DISCUSSION

Antiproliferative actions of agonists and antagonists of LHRH have been previously intensely investigated in ovarian, endometrial and prostatic cancer lines and other tumors[6–10,12–15] (for reviews see references 6, 12 and 14). Various studies also examined the signal transduction mechanisms involved in the mediation of the effects of LHRH analogs on cancer cells.[12–15,20,51–53] Thus, LHRH agonist [D-Trp^6]LHRH and related analogs were shown to antagonize signal transduction and mitogenic activity of EGF in human ovarian, endometrial and prostatic cancer lines.[10,12–15]

The present study demonstrates for the first time that [D-Trp^6]LHRH, a potent LHRH agonist, suppressed basal and EGF-stimulated cellular activities in EGFR overexpressing epidermoid carcinoma A431 cells including cell proliferation, total kinase activity (particularly tyrosine, but not serine/threonine phosphorylation of certain cellular proteins), and metastasis-associated properties. Our finding on the inhibitory effects of [D-Trp^6]LHRH on basal and EGF-induced cell proliferation in human epidermoid carcinoma A431 cells are in accord with reports that LH-RH

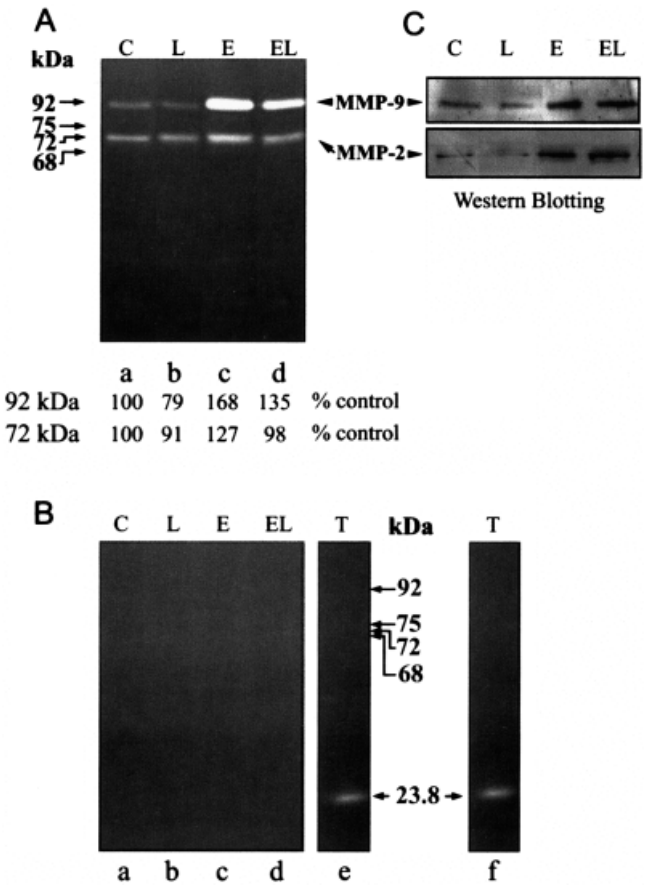

FIGURE 8 – Effects of [D-Trp^6]LHRH and EGF on the secretion of gelatinases in A431 cells. Cells were treated with 100 nM [D-Trp^6]LHRH and/or 10 nM EGF in serum free medium for 24 hr. The conditioned media were collected and normalized by cell numbers prior to gelatin zymography analysis. Representative zymograms in the absence (*a*) or presence (*b*) of a metalloproteinase inhibitor 5 mM 1,10-phenanthroline during substrate buffer incubation. Lanes: a, vehicle control (C); b, [D-Trp^6]LHRH (L); c, EGF (E); d, EGF plus [D-Trp^6]LHRH (EL). Trypsin, a serine protease (T; lane f), was not inhibited by 1,10-phenanthroline (lane e). (*c*) Immunoblotting analyses using anti-MMP-9 and MMP-2 antibodies indicated that the identity of 92 and 72kDa gelatinases were MMP-9 and MMP-2, respectively. The zymograms (*a*,*b*) and immunoblots (*c*) shown are representatives of 3 separate experiments performed in duplicate.

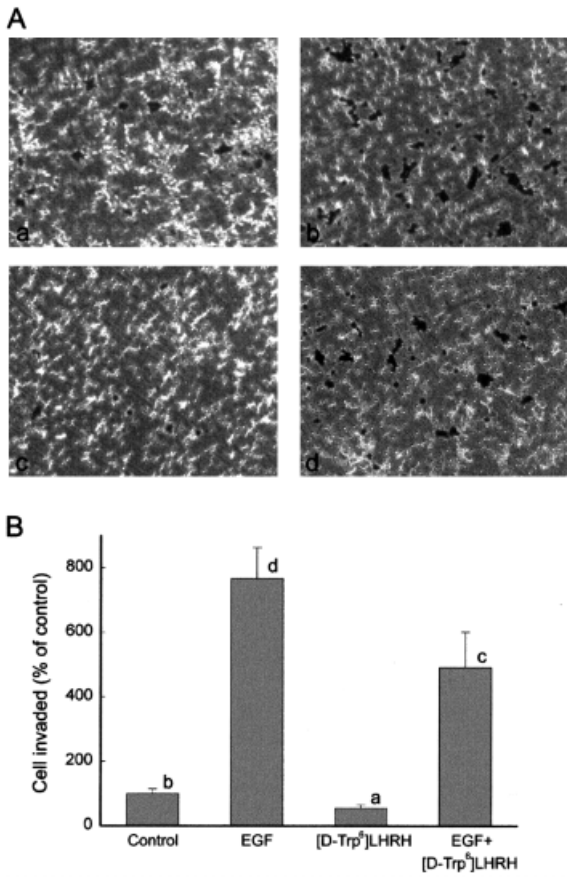

FIGURE 9 – Effects of [D-Trp^6]LHRH and EGF on invasive activity of A431 cells. *In vitro* invasion assay was performed using 24-well Transwell units with 8 μm porosity polycarbonate filter coated with EHS matrigel. A431 cells (2×10^5 cells/0.4 ml) were placed in the upper compartment and treated with 100 nM [D-Trp^6]LHRH and/or 10 nM EGF for 48 hr in the presence of serum. At the end of culture, filters of the Transwell units were fixed and stained with crystal violet. The numbers of cells that penetrated through the matrigel to the lower surface of the filter were determined under the microscope. (*a*) Representative photographs of the invaded cells: a, vehicle control; b, EGF; c, [D-Trp^6]LHRH; d, [D-Trp^6]LHRH plus EGF. (*b*) Each bar represents mean percentage (± SEM) of invaded cells relative to the control value expressed as 100%. Two independent experiments were performed in triplicate. Different lower-case letters (a–d) indicate significant differences among groups ($p < 0.05$).

analogs reduce expression of EGF receptors and their mRNA[15,53] and/or EGF-induced tyrosine kinase activity.[13,15,41,54] EGF-induced tyrosine phosphotyrosine is probably counteracted by LH-RH analogs through activation of a phosphotyrosine phosphatase.[12–15,41,52,54] Gründker *et al*.[52] were able to show that [D-Trp^6]LHRH greatly reduced EGF-induced autotyrosine phosphorylation of EGF receptors in ovarian and endometrial cancer cell lines. This inhibitory effect of [D-Trp^6]LHRH on phosphorylation of EGF receptors was completely blocked by the phosphotyrosine phosphatase inhibitor vanadate.

Our work suggests that the inhibitory effect of [D-Trp^6]LHRH on A431 cell proliferation may be induced partly through the activation of the apoptotic process as evidenced by the characteristics of the cell morphology and DNA integrity (ladder pattern), the expression of ICE protein and activation of caspase activity. In addition, we showed that A431 cells became rounded and some with blebing following [D-Trp^6]LHRH treatment for 48 hr, and when the culture medium were replaced with EGF for an additional 24 hr, the remaining attached cells began to spread and grew again. This suggests that limited exposure to [D-Trp^6]LHRH did not channel all cells to irreversible apoptotic process.

In a previous study,[19] *in vivo* treatment of experimental animals with agonist [D-Trp^6]LHRH, the LHRH antagonist Cetrorelix or somatostatin analog RC-160 also resulted in enhancement of apoptosis in MXT breast cancers in mice, Dunning prostate cancers in rats and nitrosamine-induced pancreatic cancers in hamsters. However, Grundker *et al*.[20] showed that in EFO-21 and EFO-27 ovarian cancer lines, treatment *in vitro* with [D-Trp^6]LHRH induced NFκB activation and inhibited apoptosis. Thus, LHRH analogs may protect ovarian cancer cells from programmed cell death[20] and the findings obtained in A431 cells may not be always representative of other tumor cells.

Several lines of evidence suggest that the induction of cellular protein tyrosine phosphatase (PTP) activity correlates with growth inhibition of several cancer cells. Activation of somatostatin receptors leads to activation of protein tyrosine phosphatase activities[55,56] and that somatostatin analogues inhibited proliferation and reduced phosphorylation level of membrane-associated proteins in human pancreatic cell line MiaPaCa-2 and hamster cheek pouch carcinoma cell line HCPC.[11,16,41] In addition, this study showed that [D-Trp^6]LHRH inhibited the basal and EGF-stimulated cell

proliferation and tyrosine phosphorylation of cellular proteins including EGFR in A431 cells. Also, [D-Trp^6]LHRH did not appear to affect basal or EGF-stimulated serine/threonine phosphorylation of cellular proteins in A431 cells. The inhibitory effects of LHRH analogues on cell growth and cellular protein phosphorylation was also observed in MiaPaCa-2 and ovarian tumor cells.[11,16,54] The similarity in analogue efficacy between repression of tyrosine phosphorylation and inhibition of cell proliferation suggests that net tyrosine phosphorylation of 1 (or more) signaling molecules may well be an important determining factor for growth control. Interestingly, our current and previous studies[11,16] indicated that the phosphorylation/dephosphorylation of 170 and 65 kDa proteins are the most reliable indicators of cell proliferation in response to EGF, [D-Trp^6]LHRH and somatostatin in A431, MiaPaCa-2 and HCPC cell lines. The phosphorylation of this 170 kDa protein was upregulated by EGF and downregulated by [D-Trp^6]LHRH. Furthermore, this study identified that the 170 kDa phosphotyrosyl protein band is the EGFR as determined by immunoprecipitation and immunoblotting analyses using anti-EGFR and anti-phosphotyrosine antibodies, whereas the identity of the 65 kDa phosphoprotein band awaits further investigation. In addition, changes occurred in the phosphorylation of some of these proteins were detected in solid tumors including breast, prostate, liver and oral cancer.[57]

The suppressive effect of [D-Trp^6]LHRH on the basal and EGF-stimulated cellular protein tyrosine phosphorylation and proliferation of A431 cells suggests that [D-Trp^6]LHRH might signal through the activation of protein tyrosine phosphatase (PTP) activity and the dephosphorylation of EGFR. The identity of the PTPs and their roles in signal transduction remains unclear. To further delineate the activated PTP induced by [D-Trp^6]LHRH, we determined PTP activities in both [D-Trp^6]LHRH treated- and untreated-A431 cell lysates by *in vitro* enzyme assay. It is worth noting that our preliminary data indicate that the cytosolic PTP-1D might be a putative PTP activated by [D-Trp^6]LHRH (unpublished data). The relationship between the [D-Trp^6]LHRH-induced PTP-1D activity and the specific intracellular regulatory pathways involved in the modulation of tumor cell growth awaits to be further clarified.

Expression of MMPs, such as MMP-2 and MMP-9, has been implicated in processes leading to the invasion/metastasis of cancer cells in several in vitro and *in vivo* systems.[21–24,27–38] Evidence suggests that MMPs may also play a major role in tumor angiogenesis.[27] These enzymes, therefore, appear to be appropriate targets for the development of anticancer and anti-metastatic agents. Of considerable interest in our study was that [D-Trp^6]LHRH significantly decreased the basal and EGF-stimulated secreted activities of the MMP-9 and MMP-2 from A431 cells. Recent studies show that MMP-9 and MMP-2 are expressed in different human epithelial cancers, and their levels seem to be related to metastatic potential and malignancy.[29–34] Furthermore, synthetic peptides that inhibit MMP activities can block cancer cell invasion and malignant tumor growth in animals.[34,58 59] Studies over the past decade showed that MMPs are regulated by a variety of growth factors including EGF.[60,61] The present study demonstrated that [D-Trp^6]LHRH suppressed not only the basal but also EGF-induced invasive activity in A431 cells, and that the inhibitory effect of [D-Trp^6]LHRH on tumor cell invasion might partially be attributed to the down-regulation of the expression of MMPs. Furthermore, limited studies suggest that repression of tyrosine phosphorylation may be the mechanism involved in metastatic process. It was reported that the EGFR tyrosine kinase inhibitor, genistein, inhibited the invasion of murine mammary carcinoma cells,[62] and *HER-2/neu* tyrosine kinase inhibitors repressed the secretion of gelatinase and invasive potential.[63] These findings suggest that repression of tyrosine phosphorylation may be the mechanism involved in metastasis.[63] In addition, this study shows that [D-Trp^6]LHRH induced the reduction of tyrosine phosphorylation of a spectrum of cellular proteins including 170 kDa EGFR and 125 kDa one. Our recent preliminary work further identified the 125 kDa phosphotyrosyl protein as focal adhesion kinase (FAK) using immunoblotting analysis (unpublished data). Recent works indicate that changes in FAK kinase activity is associated with cell invasion/metastasis.[64–66]

In conclusion, this study suggests that in A431 human epidermoid carcinoma cells, [D-Trp^6]LHRH may act partly through activating the tyrosine phosphatase activity that leads to growth inhibition and reduced cell invasion/metastasis. Agents such as LHRH analogs that inhibit EGFR tyrosine kinase activity may be therapeutically useful in the control of cancer metastasis.

ACKNOWLEDGEMENTS

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