Luteinizing Hormone-releasing Hormone Agonist Limits DU-145 Prostate Cancer Growth by Attenuating Epidermal Growth Factor Receptor Signaling

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ABSTRACT

Purpose: Advanced prostate cancer is treated initially by central suppression of androgen production by luteinizing hormone-releasing hormone (LHRH) agonists. Interestingly, even hormone-independent cancers often show some, if only slight, growth retardation when these agonists are delivered in pharmacological doses. Previous studies have shown in cell lines and animal xenograft models that activation of peripheral LHRH receptors on prostate carcinoma cells lead to growth suppression. In parallel, there is a decrease of epidermal growth factor receptors (EGFRs) and activity. Because autocrine EGFR stimulation exists in most, if not all, prostate carcinomas and is required for cell proliferation, we asked whether LHRH signaling cross-attenuated EGFR to limit tumor growth. One possible mechanism was suggested by LHRH receptors triggering phospholipase-C (PLC) to activate protein kinase C (PKC) because PKC activation limits EGFR tyrosine kinase activity by phosphorylating EGFR at threonine 654.

Experimental Design: To determine the role of this cross-attenuation mechanism, we mutated the threonine 654 amino acid to an alanine (A654) to abrogate this inhibition. DU-145 cells stably expressing wild-type and A654 EGFR were grown as xenografts in the s.c. space of athymic mice.

Results: DU-145 cells, overexpressing wild-type EGFR, formed tumors in athymic mice that were inhibitable by goserelin acetate (Zoladex). Tumors expressing the A654 EGFR were resistant to this growth inhibition. These results paralleled in vitro studies in which goserelin acetate blocked proliferation of the WT DU-145 but not A654 DU-145 cells.

Conclusions: These data support the model of LHRH agonists preventing EGFR-mediated tumor growth through a PKC pathway. This suggests new targets of modulatory intervention to limit the growth of androgen-independent prostate carcinomas.

INTRODUCTION

LHRH agonists have been introduced in clinical practice to effect medical castration in patients with prostate cancer. Because ~85% of human prostate cancers are initially sensitive to hormonal ablation, LHRH agonists are now widely used for the management of this disease. However, prostate cancer eventually becomes hormone-independent, and disease progression ensues. It was noted that a number of androgen-independent tumors responded, although slightly, to these agonists. Thus, it was postulated that a direct effect on the tumor cells might be operative.

LHRH agonists (1–3) and an antagonist (4) have been shown to produce antiproliferative actions on the human, androgen-independent prostate cell lines DU-145 and PC-3. Initially, the effectiveness of LHRH in prostate cancer treatment was thought to be exclusively through suppression of steroid hormone levels. However, the identification of LHRH binding sites on prostate cells highlights a second and probable direct route through which LHRH exerts negative regulation (1, 4–9). How the LHRH analogues might produce their secondary direct effect has been suggested by long-term treatment of human prostate cell line xenografts in athymic mice (2, 4). In both studies, tumor growth of androgen-independent DU-145 cells was retarded concomitant with decreased levels of EGFRs in these tumors. Still, this correlation was not conclusive and could be an epiphenomenon, because the mechanism by which EGFR attenuation occurs was not determined.

We hypothesized that the antiproliferative effects of LHRH agonists are mediated through negative attenuation of the EGFR, which is inactivated by phosphorylation by PKC. Although there is little data on LHRH signaling pathways in the prostate cells, there is evidence that LHRH agonists stimulate

4 The abbreviations used are: LHRH, luteinizing hormone-releasing hormone; EGF, epidermal growth factor; EGFR, EGF receptor; PKC, protein kinase C; PLC, phospholipase-C; WT, wild type; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PMA, phorbol 12-myristate 13-acetate.
PLC activity in mammary tumors (10) in a manner similar to that of LHRH in the pituitary (11, 12). In human DU-145 prostate carcinoma cells, LHRH triggers a pertussis-sensitive Gi protein to increase PLC (7). A central aspect of the LHRH signaling pathway is PLC activity, which generates diacylglycerol (DAG) and mobilizes intracellular Ca²⁺. These second messengers activate PKC; PKC then phosphorylates certain enzymes that are responsible for the final biological effects of the hormone (11, 12). This is highly suggestive because EGFR-signal cell responses are subject to PKC-mediated negative transmodulation as PKC phosphorylates EGFR at threonine 654 to both down-regulate EGFR (13) and prevent EGF-induced signaling (14–16). Furthermore, prostate cancer cell proliferation depends on a functional EGFR autocrine loop (17–20), and DU-145 cell growth and xenograft tumor invasion are mediated through the EGFR (21, 22).

Herein, we approached this question in vivo by inoculating DU-145 cells that expressed either WT or PKC-resistant (A654) EGFR. Fibroblasts expressing the A654 constructs are unaffected by PKC activators such as phorbol esters (15, 16). Growth of tumors expressing the WT EGFR was retarded by pharmacological doses of goserelin acetate (Zoladex) whereas the A654 tumors were unaffected. These results suggest that prostate tumor growth can be modulated by down-regulation of EGFR signaling and that this can be accomplished by triggering physiological attenuation mechanisms. These provide for new targets for rational intervention strategies.

MATERIALS AND METHODS

Animals. Male and female athymic BALB/c nu/nu mice, 6–8 weeks of age, were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research and Development (Frederick, MD) and housed in suitable conditions. Mouse weights ranged from 20 to 30 g at the onset of experiments. All of the animal experiments and protocols were approved by the Birmingham and Pittsburgh Veteran Administration Medical Centers Institutional Animal Care and Use Committees.

Cell Culture. WT DU-145 and A654 DU-145 prostate carcinoma cells were generated as described previously (21). These are DU-145 human prostate carcinoma derivatives (23) that overexpress exogenously encoded EGFRs. Briefly, WT EGFRs or a clone with threonine at amino acid 654 replaced by alanine (A654) were cloned into a murine leukemia virus-based retroviral expression plasmid (16). Ecotropic viruses were generated by transfection in ρ26 cells and used to infect the DU-145 cells. Retroviral transduction was used to generate polyclonal isolates and avoid the pitfalls of cell-cell variability in monoclonal selections (16). Furthermore, each experiment was performed on two independent polyclonal cell lines; for one of the independent polyclonal sets, densitometry of immunoblotting for EGFR on subconfluent cell-cell variability in monoclonal selections (16). Furthermore, each experiment was performed on two independent polyclonal isolates with concordant results. The exogenously encoded EGFR represent approximately one-half of the total EGFR on the transductant lines; for one of the independent polyclonal sets, densitometry of immunoblotting for EGFR on subconfluent cells (21) revealed EGFR levels on WT DU-145 at 160% and on A654 DU-145 at 210% of the levels on the parental DU-145 cells.

The parental cells were maintained in DMEM (4.5 g/ml glucose) containing 10% FCS and supplemented with l-glutamine (2 mm), penicillin/streptomycin (100 units/ml), nonessential amino acids (0.1 mm), and sodium pyruvate (1 mm). For stable selection of WT or A654 EGFR, cells were grown in G418 (1000 µg/ml).

Immunoblotting. Cells were grown in 6-well plates to near-confluence, washed with PBS, and lysed with Laemmli buffer. The lysate was separated by SDS-PAGE and immunoblotted. Primary antibodies used included anti-EGFR (Zymed Diagnostics, South San Francisco, CA) and antiphosphotyrosine PY-20 (Transduction Laboratories, Lexington, KY). The staining was visualized by a secondary antimouse IgG antibody linked to alkaline phosphatase.

In Vitro Growth Assay. Cytotoxicity and cell proliferation were evaluated by assessing mitochondrial reduction of MTT (Sigma Chemical Co., St. Louis, MO) as previously described (22). Briefly, cells were plated at 5000 cells/well in 96-well microtiter plates in 200 µl of growth medium (7.5% FBS in DMEM) and allowed to attach for 24 h. Serum-containing medium was removed, and the cells quiesced for 2 days in 0.5% dialyzed FBS in DMEM. For time-course and dose-response studies, this medium was removed and replaced with 7.5% FBS in DMEM with or without the appropriate effectors (Zoladex, PMA, and/or chelerythrine chloride). Medium was changed on day 2 of the 4-day experiment. At harvest, medium was removed from the appropriate wells, replaced with 50 µl of MTT solution (2 µg MTT/ml PBS) and incubated for 4 h at 37°C. After incubation, the MTT solution was carefully aspirated and replaced with 100 µl of DMSO. Cell growth was analyzed on days 2, 3, and 4 on a plate reader using the Soft Max program (Molecular Devices Corp., Menlo Park, CA). Experiments were performed in quadruplicate and repeated at least three times.

Tumor Cell Inoculations. We used the s.c. tumor growth model to follow tumor size during treatment by caliper measurement. Briefly, 2 million cells in 100 µl of PBS were mixed with Matrigel (100 µl each; Becton Dickinson and Company, Franklin Lakes, NJ). Matrigel provides the local microenvironment for prostate cancer cell growth in a s.c. site (24). Each mouse received one 200-µl injection in the s.c. tissue at the flank region.

Seven to 10 days after inoculation, tumors were measured and mice were stratified into two groups with equivalent tumor size; tumor penetrance was greater than 90%. Treatment with LHRH agonist goserelin acetate (Zoladex; 50 µg/kg/injection diluted in 10% DMSO in PBS, s.c. at a distant site) or diluent (10% DMSO in PBS) was initiated at that time (25).

Testosterone Measurement. Blood (100 µl) was collected from the heart at the time of euthanasia; plasma was obtained by centrifugation. Total testosterone was measured by RIA kit (Coat-A-Count; Diagnostic Products Corporation, Los Angeles, CA).

RESULTS

A654 EGFRs Are Resistant to Cross-attenuation by PKC. EGFR constructs that present alanine in place of threonine at amino acid 654 are resistant to PKC abrogation of signaling (15, 16). However, these studies were performed in...
PKC activation prevents DU-145 cell growth in vitro. Proliferation of DU-145 cells over-expressing exogenously encoded WT (□) or A654 (□□) EGFR was determined over a 4-day period, in the presence of PMA (10 μM) and chelerythrine chloride (10 μM). PMA caused a significant inhibition of proliferation only in WT DU-145 cells, which was reversed by chelerythrine chloride (both, P < 0.05). Chelerythrine chloride treatment alone had no significant effect on the proliferation of either cell line (data not shown). The slight decrement in A654 DU-145 proliferation did not reach significance (P > 0.05). Shown is the average ± SE of four experiments, each performed in quadruplicate.

Fig. 2

NR6 fibroblasts that lack endogenous EGFR. The question was still open as to whether the WT and A654 DU-145 cell sublines would be responsive and resistant to PKC attenuation because DU-145 express significant levels of endogenous EGFR (21). Two different polyclonal isolates of each DU-145 subline were generated. Cells were exposed to EGF (10 nM for 5 min) in the presence of the PKC activator PMA (10 nM, 5 min before EGF). The EGFR phosphotyrosine content is significantly increased by EGF (Fig. 1A). In the WT DU-145 cells, PMA cotreatment eliminates this EGF increase, whereas, in the A654 DU-145 cells, a significant amount of phosphotyrosine remains. The slight decrease from EGF alone in the A654 DU-145 is expected because the endogenous EGFR should be responsive to PKC transmodulation.

To further demonstrate that this attenuation is caused by PKC activity, we tested a pan-PKC inhibitor, chelerythrine chloride. Preexposure of the cells to chelerythrine chloride (10 μM simultaneously with PMA) blocked the ability of PMA to inhibit EGF-induced tyrosine phosphorylation (Fig. 1B). These findings provide a model system to test whether LHRH agonists limit EGFR signaling through PKC cross-attenuation.

PKC Activation Inhibits DU-145 Cell Growth in Vitro. Dose-response and time-course studies were used to determine whether the PKC activator, PMA (10^{-5} to 10^{-9} M), was capable of inhibiting cell proliferation of the WT DU-145 cell line through PKC attenuation of EGFR signaling. By day 4, PMA treatment resulted in a decrease in the WT DU-145 subline cell growth when compared with nontreated controls (data not shown). This growth inhibition was significant at the highest nontoxic concentration used (10^{-7} M; P < 0.05). Because PKC has numerous targets, it was critical to determine whether this growth inhibition was attributable to cross-attenuation of EGFR signaling (16). This was supported by the failure of PKC treatment to cause a significant inhibition of growth in the A654 DU-145 subline from which the PKC target site is removed (Fig. 2).

If PMA was capable of inhibiting WT DU-145 cell growth via PKC activation, it was predictable that a PKC inhibitor should in turn be able to overcome this inhibition, thus restoring growth to non-PMA-treated levels. WT and A654 DU-145 sublines were exposed to PMA alone, at the concentration that caused significant growth inhibition (10^{-5} M), or in conjunction with the PKC inhibitor, chelerythrine chloride (10 μM; Fig. 2). As observed previously, PMA significantly inhibited cell growth of WT DU-145 cells. However, when WT DU-145 cells were cotreated with PMA and chelerythrine chloride, the growth inhibitory effects caused by PMA alone were alleviated. As expected, A654 DU-145 subline demonstrated no significant inhibition of growth in cells exposed to either PMA or chelerythrine alone, or in combination.

LHRH Agonist Goserelin Acetate Limits EGFR Signaling in Vitro. If LHRH agonists limit prostate cancer cell growth through limiting PKC down-regulation of EGFR signaling, then the A654 cells should be resistant. We determined whether goserelin acetate limited EGFR kinase activity, but this was not the case. Although goserelin acetate blocked the ability of PMA to inhibit EGFR phosphorylation, it did not inhibit EGFR signaling. In contrast, goserelin acetate did not affect the ability of PMA to inhibit EGFR phosphorylation. Therefore, goserelin acetate did not limit EGFR signaling through PKC cross-attenuation.
cell growth. High doses of Zoladex (10⁻⁵ and 10⁻⁶ M) significantly retards the proliferation of WT DU-145 cells (Fig. 3). In support of the concept that this occurs via PKC cross-attenuation of EGFR signaling, various Zoladex doses had no antiproliferative effect on DU-145 subline A654.

**Tumor Growth Is Limited by Goserelin Treatment.** The true test for effects on tumorigenesis is in vivo xenograft growth. We found that parental DU-145 cells formed tumors in the s.c. space of BALB/c nu/nu mice (Fig. 4A). Daily treatment with 50 μg/kg goserelin significantly retarded tumor growth (P < 0.05 after day 17), whereas treatment every 4th day had no effect. The final tumor sizes, in absolute size as opposed to relative-to-initial-tumor size displayed in the graph, averaged 111 mm³ for the tumors daily treated, 274 mm³ for the control group and 314 mm³ for those treated every 4th day. These results mirrored those reported by others (2, 4). That the Zoladex treatment was functional was determined by measuring testosterone levels in the mice. Mean testosterone level was 21.78 ng/dl in the goserelin group versus 298 ng/dl in the control group (P < 0.01). However, there was still the concern that, although DU-145 cells are androgen insensitive, the LHRH effect could be secondary to central hormonal suppression. Thus, the ensuing studies were performed in female mice. Here, too, daily goserelin treatment limited tumor growth (Fig. 4B). At the end of the experiment, mean tumor size was 73.27 mm³ in control group, versus 18.35 mm³ in the treated group (P < 0.01). This was not caused by central androgen suppression, because the testosterone level was below 20 ng/dl in both groups.

To further stress this effect, tumors were established for 3 weeks before starting goserelin treatment. Again, daily goserelin injections limited tumor growth compared with diluent-alone treated mice (Fig. 4C). Although significant (P < 0.05), the magnitude of the diminution was less under this regimen.

**LHRH Inhibition of Tumor Growth Depends on PKC Site in EGFR.** To determine whether the LHRH inhibition of tumor growth used PKC-mediated attenuation of EGFR, we established tumors with DU-145 cells expressing A654 EGFR. These cells, and their WT EGFR-expressing counterparts, formed progressive tumors in the s.c. space of the mice (Fig. 5A). However, when these mice were treated daily with 50 μg/kg goserelin, only the tumors from cells expressing the WT
EGFR were inhibited. The tumor expressing A654 EGFR were impervious to the LHRH agonist. At the completion of the experiment, the A654 DU-145 tumors were of similar size (744% ± 104% diluent versus 777% ± 235% treated; P, not significant), whereas the WT DU-145 tumors were retarded by goserelin treatment (1160% ± 747% diluent versus 308% ± 69% treated, P < 0.05). A second experimental series was identical to the first (Fig. 5B). Again, the A654 DU-145 tumors were of similar size, whereas the WT DU-145 tumors were retarded by goserelin treatment. These results suggest that EGFR lacking the site for PKC-induced phosphorylation confers LHRH resistance to DU-145 cells.

**DISCUSSION**
This study provides further in vivo evidence supporting the contention that LHRH agonists exert a direct, testosterone-independent gross inhibitory effect on human prostate cell line DU-145 xenografts in nude mice. It must be acknowledged that, similarly to earlier studies (2, 4), the DU-145 xenografts were ectopically grown in the s.c. space. However, the need to serially follow tumor growth necessitates this location; one that is not noted in human prostate cancer. Our study suggests that there is a signaling cross-talk of LHRH and EGFR in prostate cancer involving PKC. This may turn out to be an important target for prostate cancer treatment in a subpopulation of tumors that are no longer responsive to conventional hormonal treatment.

LHRH direct inhibition of prostate cancer growth acts by attenuating a well-documented autocrine proliferation signaling loop. Most, if not all, carcinomas present an autocrine EGFR signaling system (27, 28). This is particularly true for prostate cancers in which EGFR signaling is present throughout normal development and all of the stages of carcinogenesis (20, 29–32). EGFR signaling has been shown to be required for the proliferation of a variety of prostate cell lines (20, 32–35). Thus, it is not surprising that LHRH targets this signaling access to prevent cell proliferation. Two prior reports demonstrated that LHRH agonist-mediated inhibition of prostate cancer growth resulted in decreased EGFR levels (2, 4) suggesting a direct negative attenuation. Herein, we confirm these findings and extend them to demonstrate that the major molecular inhibition occurs by targeting the regulatory threonine 654 of the EGFR. DU-145 prostate cancer cells that express EGFR engineered to lack this site, proliferate and form tumors in the face of an LHRH agonist.

Our finding that PKC-mediated cross-attenuation of EGFR is the operative mechanism for growth inhibition appears at odds with an earlier suggestion that LHRH agonists limit EGFR signaling by up-regulating tyrosine phosphatases (36). Our data do not exclude such a mechanism as also occurring in these cells; in fact, EGFR signaling is likely regulated in both normal and neoplastic prostate epithelial cells by the activity of prostatic acid phosphatase (37–39). However, the finding that replacement of threonine 654 with alanine enables escape from LHRH agonist inhibition argues strongly that PKC transmodulation is the predominant mechanism. Furthermore, the finding that LHRH exposure reduces EGFR levels (2, 4, 40) indirectly supports this mechanism because PKC phosphorylation at threonine 654 leads to receptor down-regulation secondary to internalization and degradation (13), whereas dephosphorylation should lead to receptor sparing and normal or increased levels (41, 42). Thus, although there may be multiple mechanisms for growth attenuation, negative transmodulation by phosphorylation at threonine 654 appears to be the predominant means whereby LHRH blocks DU-145 cell proliferation.

One has to recognize the fact that current LHRH therapy does not result in long-term remission of prostate cancers despite our, and others, finding in xenograft models (2, 4). This could be secondary to levels of either LHRH receptors or downstream signaling molecules being insufficient to overcome strong autocrine signaling or LHRH-mediated receptor down-regulation (40). We favor the second option because recent reports demonstrate significant levels of LHRH receptors on human prostate carcinomas (8). A third option lies in the pharmacokinetics of the LHRH agonists; the mode of delivery in the xenograft studies is significantly different to prevent direct
comparison to treatment levels in patients. However, whichever of these three options, or even a fourth, is the reason for limited efficacy, the clinical experience suggests that one should either augment LHRH signaling or directly trigger the attenuation mechanism. For this reason, ongoing studies that lie beyond the scope of this article are examining how PKC is activated downstream of LHRH and which isoform of PKC is critical. As for the activating mechanism, one might presume it would be PLC by analogy to pituitary physiology, although this might not occur in prostate cells (7, 11). These future studies may highlight new and selective targets for intervention aimed at this currently intractable stage of prostate cancer.

In sum, this secondary effect of LHRH agonists on prostate cancer growth is in addition to the well-documented central androgen-deprivation mechanism that causes partial remission in hormone-dependent prostate cancers (25). However, these tumors soon relapse as hormone-refractory aggressive cancers with few therapeutic options (43, 44). Thus, although at present it is only a partial and secondary response (45), the direct effect of LHRH agonist holds promise for future developments aimed at this segment of prostate cancer that causes the morbidity and mortality. Understanding the pathway(s) by which LHRH signaling down-regulates EGFR signaling of prostate cancer proliferation provides new targets for rational therapies.

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