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Context: GnRH is known to directly regulate prostate cancer cell proliferation, but the precise mechanism of action of the peptide is still under investigation.

Objective: This study demonstrates differential effects of GnRH-I and GnRH-II on androgen-independent human prostate cancer cells.

Results: Both GnRH-I and GnRH-II increased the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_i\)]), either through Ca\(^{2+}\) influx from external Ca\(^{2+}\) source or via mobilization of Ca\(^{2+}\) from internal Ca\(^{2+}\) stores. Interestingly, the [Ca\(^{2+}\)\(_i\)] increase was mediated by activation of the ryanozide receptor but not the inositol trisphosphate receptor. Trporelix-1, a novel GnRH-II antagonist but not cetrorelix, a classical GnRH-I antagonist, completely inhibited the GnRH-II-induced [Ca\(^{2+}\)\(_i\)] increase. Concurrently at high concentrations, trporelix-1 and cetrorelix inhibited GnRH-I-induced [Ca\(^{2+}\)\(_i\)] increase, whereas at low concentrations they exerted an agonistic action, inducing Ca\(^{2+}\) influx. High concentrations of trporelix-1 but not cetrorelix-induced prostate cancer cell death, probably through an apoptotic process.

Conclusions: This study suggests the existence of a novel GnRH-II binding protein, in addition to a conventional GnRH-I receptor, in prostate cancer cells. These data may facilitate the development of innovative therapeutic drugs for the treatment of prostate cancer.

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in the regulatory actions of GnRH on prostate cancer cells. It has been suggested that, in prostate cancer cells, GnRH activates a G<sub>i</sub>-linked signaling cascade (15). The exact nature of such a discrepancy in the signaling pathways from the same receptor is unclear. Alternatively, it may be ascribed to the presence of uncharacterized GnRH receptors in prostate cancer cells that may trigger a different signaling cascade.

Another question raised from these studies is whether GnRH-II could be involved in the regulation of prostate cancer cell growth because it is likely to have diverse functions in reproductive and immune tissues (6–12). It is noteworthy that, in human endometrial and ovarian cancer cells, GnRH-II appears to be a more potent inhibitor of cell proliferation than GnRH-I (17). GnRH-II exhibits a differential hormonal regulation pattern in human granulosa-luteal cells (11). Recently it was found that GnRH-II, produced by human T cells, triggers laminin receptor gene expression and cell migration (12). Interestingly, GnRH-II-induced laminin receptor gene expression is not blocked by the GnRH-I antagonist cetrorelix (12), suggesting the existence of an uncharacterized receptor for GnRH-II in human. However, involvement of GnRH-II and its novel receptor in the regulation of prostate cancer cell growth has not been carefully studied.

Recently we and others identified the receptors having a higher sensitivity/affinity for GnRH-II than GnRH-I in nonmammalian species, referred to as nonmammalian GnRH receptors (18, 19). Furthermore, the functional receptor for GnRH-II was cloned from the monkey and called mammalian type 2 GnRH receptor (20–22). In humans, the genes for the mammalian type 2 GnRH receptor are localized in chromosomes 1 and 14 and are expressed in a variety of tissues including brain (23). However, these genes are thought to be nonfunctional pseudogenes due to the introduction of a premature stop codon (23, 24). Although alternative splicing patterns to circumvent the frame-shift that allows the production of a two-membrane domain protein has been suggested, the functionality of such a protein is still in question (25).

Although genetics-based studies suggest the absence of a functional type 2 GnRH receptor in humans, many physiological and pharmacological findings raise the possibility of novel human receptors for GnRH-II (11, 12, 17). Based on the characterization of GnRH-II receptors in both nonmammalian and mammalian species (26–28), we designed specific GnRH-II analogs (22, 29, 30). In the present study, using these GnRH-II analogs, we provide evidence for the possible involvement of a novel GnRH-II-binding protein in cell signaling and death of androgen-independent prostate cancer cells. We also demonstrate that a novel GnRH-II antagonist, tptorelix-1, induces prostate cancer cell death probably via an apoptotic process.

Materials and Methods

GnRH and its analog

GnRH-I (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH<sub>2</sub>) and [des-Gly<sub>10</sub>, p-Trp<sub>1</sub>]GnRH-I (GnRH-Ia) were purchased from Sigma (St. Louis, MO). Cetrorelix (Ac-o-Nal (2)-p-Phe(4Cl)-p-Pal (3)-Ser-Tyr-p-Cit-Trp-Trp-Pro-o-Ala-NH<sub>2</sub>) trptorelix-1 (Ac-o-Nal (2)-p-Phe(4Cl)-p-Pal (3)-Ser-Tyr-p-Cit-Trp-Trp-Pro-o-Ala-NH<sub>2</sub>) tptorelix-2 (Ac-o-Nal (2)-p-Phe(4Cl)-p-Pal (3)-Ser-His-o-Cit-Trp-Trp-Pro-o-Ala-NH<sub>2</sub>) [azidobenzoyl-o-Lys]-GnRH-I, and [azidobenzoyl-o-Lys]-GnRH-II were synthesized by AnyGen (Gwangju, Korea).

Cell culture

DU-145 and LNCaP cells were obtained from Korea Cell Bank (Seoul, Korea) and American Type Culture Collection (Manassas, VA), respectively. PCC-1 (31), ALVA-41 (32), and TSU-Prl (33) cells were obtained from Dr. J. Y. Bahk (Gyeongsang National University, Chinju, Korea) with the permissions of their original developers. DU-145, ALVA-41, TSU-Prl, PCC-1, MCF-7, CV-1, HEK-293, and GH<sub>4</sub> cells were cultured at 37°C in DMEM containing 10% heat-inactivated fetal bovine serum (FBS), 1 mm glutamate, 100 U penicillin, and 100 μg/ml streptomycin. LNCaP cells were grown in RPMI 1640 medium containing 10% FBS at 37°C.

Establishment of stable cell lines using retroviral expression vectors

The establishment of stable cell lines expressing bullfrog GnRHR (bfGnRHR)-3 or rat GnRH has been previously described (22, 34). Briefly, the entire open reading frames of bfGnRHR-3 and rat GnRHR receptor (GnRHR) were cloned into the retrovirus expression vector, pLXSN (Clontech, Palo Alto, CA) at the EcoRI and Xhol sites. The pLXSN retroviral vectors containing either bfGnRHR-3 or rat GnRHR were transfected in the dual-tropic packaging cell line PT67 using the Superfect transfection kit (Qiagen, Chatsworth, CA). The cells were treated with 25 mm chloroquine (Sigma) 1 h before transfection, and the media were replaced (DMEM, 10% FBS). After 24 h, the cells were treated with 800 μg/μl G418 (Clontech) for 2 wk. Large healthy colonies were selected and expanded. The presence of the stably expressed genes was confirmed by RT-PCR and inositol phosphate (IP) assay. The culture media soup was used for infection of GH<sub>4</sub> cells with retroviruses containing the GnRH receptors. Two days after infection, GH<sub>4</sub> cells were selected with G418 for 2 wk, and selected clones were expanded. GH<sub>4</sub> cell lines expressing rat GnRHR or bfGnRHR-3 with the highest IP fold induction were chosen and maintained in DMEM containing 10% FBS until use.

Generation of recombinant adenoavirus

Ad-h2S-LSS, an E1-deleted adenoavirus expressing bFGnRHR-2-SDY mutant, was generated by Neurogenex (Seoul, Korea). Briefly, DNA encoding bFGnRHR-2-SDY (30) was inserted into the pHShuttle-cytomegalovirus vector (Clontech). The pHShuttle-cytomegalovirus with bFGnRHR-2-SDY was linearized with Pmel and cotransformed into BJ 5183 Escherichia coli along with the pAdEasy-1 (Clontech) adenoaviral vector. Transformed cells were overlaid on kanamycin-containing agarose plates, and individual colonies were checked for the presence of the proper recombinant. After sequence confirmation, recombinant adenoaviral stocks were expanded by infection of HEK-293A cells, followed by extraction and CsCl gradient purification. Viral titers were determined using plaque assays and reported as multiplicity of infection (MOI), in which one MOI represents one plaque-forming unit/plate. Viruses expressing enhanced green fluorescent protein (Ad-EGFP) were used as controls.

Cell binding assay

GnRH-II and GnRH-Ia were iodinated with the chloramine-T (Sigma) method and purified by chromatography on a Sephadex G-25 (Sigma) column in 0.01% acetic acid and 0.1% BSA. Cells (3 × 10<sup>6</sup> cells/well) were grown in 12-well plates for 24 h before assay. The cells were washed with warm PBS and incubated at 4°C for 60 min with the iodinated ligand (60,000–80,000 cpm/well) in DMEM containing 0.1% BSA in the absence or presence of cold GnRH (10 μM). The cells were then washed twice
with PBS. After solubilizing the cells in 1% sodium dodecyl sulfate (SDS) and 0.2 mM NaOH, radioactivity was determined.

**RT-PCR analysis**

Total RNA was isolated from ALVA-41, DU-145, PPC-1, HEP-293, and HeLa cells using Tri-reagent (Sigma). Five micrograms of total RNA were reverse transcribed with Superscript II Ribonuclease H- reverse transcriptase (Life Technologies, Inc., Rockville, MD), according to a provided protocol. The first-strand cDNA was amplified by PCR using the following primers: hGnRHR1-R (5'-CTGAGGCTGTGTTCCGG-3'); hGnRHR1-F (5'-AAAGCTGAGGTCCTGTGATGA-3'; 426–445); hGnRHR2e1-R (5'-ATGTGCTCAGCGCCACGCT-3'; 380–396); hGnRHR2e2-R (5'-CTGGGCAACGACAGCAATCT-3'; 865–888); hGnRHR2e2-F (5'-CTGTTTCTCTTCACAAGCAGGAC-3'; 880–907); hGnRHR2e2-R (5'-CATGCGTCCCCTCTTCCT-3'; 1083–1099); F-β-actin (5'-CTGAAATCACCATTGAA-CATGGC-3').

**Terminal deoxynucleotidyltransferase-mediated deoxyuridine 5-triphosphate nick-end labeling (TUNEL)**

To analyze DNA fragmentation histologically, TUNEL was performed using an in situ cell death detection kit, POD (Roche Diagnostics Corp., Indianapolis, IN). Cells were seeded onto poly-t-lysine-coated glass cover slips in 6-well plates at a density of 1×10⁶ cells/well. After 24 h, the cells were treated with GnRH antagonists for 72 h. The cells were then rinsed with PBS at 25°C and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. After rinsing and permeabilization with 0.1% Triton X-100 and 0.1% sodium citrate for 15 min at room temperature. After rinsing and permeabilization with 0.1% Triton X-100 and 0.1% sodium citrate for 15 min at room temperature. After rinsing and permeabilization with 0.1% Triton X-100 and 0.1% sodium citrate for 15 min at room temperature.

**Phoaffinity labeling**

[Azidobenzoyl-L-Lys]-GnRH-I or 125I-[Azidobenzoyl-L-Lys]-GnRH-II were iodinated using the chloramine-T method and purified on a Sephadex G-25 (Sigma) column with 0.01% acetic acid under a minimal light source. Prostate cancer cells were grown to maximum confluence in 100-mm dishes. The cells were washed twice with PBS and incubated in 2 ml buffered solution containing 0.1% EDTA, 0.5% Triton X-100 and 0.1% sodium citrate for 2 min at 37°C. The cells were then rinsed with 2 ml PBS and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. The cells were then permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate for 2 min at 37°C.

**Photofinity labeling**

[Azidobenzoyl-L-Lys]-GnRH-I and [azidobenzoyl-L-Lys]-GnRH-II were iodinated using the chloramine-T method and purified on a Sephadex G-25 (Sigma) column with 0.01% acetic acid under a minimal light source. Prostate cancer cells were grown to maximum confluence in 100-mm dishes. The cells were washed twice with PBS and incubated in 2 ml buffered solution containing 0.1% EDTA, 0.5% Triton X-100 and 0.1% sodium citrate for 2 min at 37°C.

**Photoaffinity labeling**

[Azidobenzoyl-L-Lys]-GnRH-I and [azidobenzoyl-L-Lys]-GnRH-II were iodinated using the chloramine-T method and purified on a Sephadex G-25 (Sigma) column with 0.01% acetic acid under a minimal light source. Prostate cancer cells were grown to maximum confluence in 100-mm dishes. The cells were washed twice with PBS and incubated in 2 ml buffered solution containing 0.1% EDTA, 0.5% Triton X-100 and 0.1% sodium citrate for 2 min at 37°C.
gels (4, 10, and 16.5% polyacrylamide) using a tricine buffer system. The gels were fixed and subjected to autoradiography.

Data analysis

Results were expressed as mean ± SEM. Plots were produced using PRISM2 software (GraphPad, San Diego, CA).

Results

Androgen-independent prostate cancer cells bind to GnRH-II with a higher affinity than GnRH-I

To examine whether prostate cancer cells bind to GnRH-I or GnRH-II, we employed three different androgen-independent prostate cancer cell lines, DU-145, ALVA-41, and PPC1, a bladder carcinoma cell line TSU-Pr1 (36), and an androgen-sensitive cell line LNCaP. Cancer cells were incubated with 125I-GnRH-Ia or 125I-GnRH-II in the presence or absence of cold GnRMs. 125I-GnRH-Ia efficiently bound to GH3 cells expressing the rat GnRHR and the binding was inhibited by excess cold GnRH-I and GnRH-II. DU-145 cells did not bind 125I-GnRH-Ia (Fig. 1A), indicating that DU-145 cells do not or only marginally express the conventional mammalian type 1 GnRH receptor. In contrast, DU-145 cells bound 125I-GnRH-II as efficiently as GH3 cells stably expressing bFGnRHR-3. Both cold GnRH-I and GnRH-II were able to inhibit the binding of 125I-GnRH-II to GH3 cells expressing bFGnRHR-3, whereas only cold GnRH-II efficiently decreased binding of 125I-GnRH-II to DU-145 cells (Fig. 1B). The binding affinity of ALVA-41, PPC-1, and TSU-Pr1 cells for GnRH-I and GnRH-II was examined. All three cell types showed a higher affinity for GnRH-II than GnRH-I as observed in DU-145 cells (Fig. 1C). The binding affinity of LNCaP cells for GnRH-I and GnRH-II was examined. LNCaP cells did not specifically bind to 125I-GnRH-I. For GnRH-II, however, the LNCaP cells have a low-affinity binding because a GnRH-II analog [Tyr5]GnRH-II significantly inhibited the binding, although native and other GnRH-II analogs did not (Fig. 1D). To further define the binding specificity of DU-145 cells, GnRH-II analogs, Tyr5-GnRH-II, Leu4-Ala6-GnRH-II, d-Ala6-Leu5-GnRH-II, and d-Ala6-Tyr7-Trp8-GnRH-II were employed. Except for Tyr5-GnRH-II, GnRH-II analogs, having considerable hydrophobicity, failed to displace 125I-GnRH-II binding (Fig. 1E), suggesting specific binding of 125I-GnRH-II. Binding of DU-145 cells to 125I-GnRH-II was inhibited by unlabeled GnRH-II and salmon GnRH in a dose-dependent manner (Fig. 1F). These results indicate that GnRH-II specifically binds to prostate cancer cells, although it has a relatively low affinity.

RT-PCR analysis of GnRH receptors in prostate cancer cells

Because the binding assay indicates the presence of a receptor with a higher affinity to GnRH-II than GnRH-I, the presence of mRNAs for already known human mammalian type I and type 2 GnRH receptors was sought using RT-PCR. It should be noted that 35 rounds of PCR amplification brought about very faint signals for each GnRH receptor, whereas 40 rounds of PCR amplification produced intense signals for all receptors (Fig. 2). ALVA-41 and DU-145 cells expressed the mRNA for type 1 GnRH receptor. Interestingly, the mRNAs for individual exon 1 or exon 2 of the human type 2 GnRH receptor were amplified in all cell lines. However, we failed to obtain PCR products consisting of exon 1 and exon 2 together (Fig. 2). This result is consistent with a previous observation that mRNA specific for human type 2 GnRH receptor is expressed ubiquitously, but the full-length mRNA may be truncated due to inappropriate posttranscriptional processing (25). This result indicated that high-affinity binding of GnRH-II to prostate cancer cells is not due to expression of the conventional type 2 GnRH receptor.

GnRHs failed to induce IP production in prostate cancer cells

It is well known that both mammalian type 1 and type 2 GnRH receptors are coupled to Gq/11, producing IP through PLC activation. To examine the presence of functional conventional GnRH receptors, we determined IP production in response to GnRH stimulation. Treatment of prostate cancer cells with GnRMs failed to increase IP formation (Fig. 3). One may suggest that the GnRH receptor in prostate cancer cells does not couple with Gq/11 or that prostate cancer cells lack the Gq/11/PLC machinery. To test these possibilities, we infected prostate cancer cells with adenovirus containing a mutant bFGnRHR-2 (bFGnRHR-2-SDY) that has a high affinity for GnRH-II (30). The cells infected with the adenovirus containing bFGnRHR-2-SDY significantly induced IP production in response to GnRH (Fig. 3). However, the cells infected with adenovirus with EGFp did not respond to GnRH stimulation. Similar results were observed with MCF-7, a breast cancer cell line and CV-1, a monkey kidney cell line (Fig. 3). These results indicate that prostate cancer cells contain the functional Gq/11 and PLC system and thus may express an uncharacterized GnRH receptor that does not link to the Gq/11/PLC system. Because GnRH did not induce IP production in prostate cancer cells, we examined other signaling systems, such as CAMP: GnRH did not alter basal or forskolin-induced CAMP levels (data not shown).

GnRHs induce calcium mobilization in prostate cancer cells

Although GnRH did not influence IP or cAMP production, both GnRH-I and GnRH-II provoked an increase in intracellular Ca2+ concentration ([Ca2+]i) in prostate cancer cells (Fig. 4). All androgen-independent prostate cancer cells, ALVA-41, DU-145, and PPC-1, responded to both GnRH-I and GnRH-II. TSU-Pr1 cells responded to only GnRH-II. Concurrently androgen-sensitive LNCaP cells and nonprostate MCF-7 and HEK-293 cells did not respond to either GnRH-I or GnRH-II (Fig. 4). BAPTA-AM is known to effectively chelate Ca2+ release from internal stores but is not as effective at chelating Ca2+ entering through plasma membrane channels (37). To determine the Ca2+ source responsible for GnRH-induced [Ca2+]i increase, GnRMs were applied to DU-145 cells in the absence of extracellular Ca2+ or under a BAPTA-AM-pretreated condition. Removal of Ca2+ in the incubation medium abrogated the [Ca2+]i response to GnRH-I but did not affect the response to GnRH-II (Fig. 5A). Replacement of Ca2+-free with Ca2+-containing medium restored the [Ca2+]i response to GnRH-I (data not shown). In contrast, in BAPTA-AM-pretreated cells, GnRH-I but not
GnRH-II induced an increase in \([Ca^{2+}]_i\), (Fig. 5B). These observations indicate that GnRH-II causes \([Ca^{2+}]_i\) increase via mobilization of intracellular stores, whereas GnRH-I provokes \([Ca^{2+}]_i\) influx probably through activation of a certain \([Ca^{2+}]_i\) channel. Furthermore, graded concentrations of GnRH-I and GnRH-II were applied to DU-145 cells. In \([Ca^{2+}]_i\)-free medium, the log EC\(_{50}\) value of GnRH-II was \(-8.21 \pm 0.08\), whereas the log EC\(_{50}\) value of GnRH-I was very high \((-5.25 \pm 0.10\), such that GnRH-II appeared to be 1000 times more potent than GnRH-I in this condition (Fig. 5C). In the BAPTA-AM-pretreated cells, however, GnRH-I (log EC\(_{50}\) \(-7.96 \pm 0.10\) was more potent than GnRH-II (log EC\(_{50}\) \(-8.21 \pm 0.08\).
Thus, it is likely that GnRH-I may activate a membrane Ca$^{2+}$ channel, causing Ca$^{2+}$ entry into the cells, whereas GnRH-II provokes Ca$^{2+}$ mobilization from internal stores. It is also noteworthy that the GnRH-II-evoked [Ca$^{2+}$]$_i$ increase rapidly declined in Ca$^{2+}$-free medium but induced a sustained response in the presence of extracellular Ca$^{2+}$, indicating a capacitative Ca$^{2+}$ entry.

**GnRH-II activates the ryanodine receptor to release Ca$^{2+}$**

The release of Ca$^{2+}$ from internal stores may be mediated by either the IP$_3$ receptor or ryanodine receptor. To determine which mechanism was responsible for GnRH-II-induced Ca$^{2+}$ mobilization, cells were treated with U73122, a PLC inhibitor, or dantrolene, a ryanodine receptor inhibitor. In Ca$^{2+}$-free medium, GnRH-I induced an increase of [Ca$^{2+}$]$_i$ in GH$_3$ cells stably expressing rat GnRHR. This increase was inhibited by U73122 but not by dantrolene, indicating that the [Ca$^{2+}$]$_i$ increase induced by rat GnRHR can be accounted for by Ca$^{2+}$ mobilization from IP$_3$-sensitive intracellular sources (Fig. 6A). In contrast, in DU-145 cells, the GnRH-II-induced [Ca$^{2+}$]$_i$ increase was completely blocked by dantrolene but not U73122 (Fig. 6B). Involvement of G$_i$ coupling or receptor tyrosine kinase in GnRH-induced Ca$^{2+}$ mobilization was determined using pertussis toxin, an inhibitor of G$_i$ and genistein, an inhibitor of tyrosine protein kinase. Neither of these inhibitors affected Ca$^{2+}$ mobilization by GnRH-I and GnRH-II in DU-145 cell (data not shown). These data, together with the observation that neither GnRH-I nor GnRH-II-induced IP$_3$ production in prostate cancer cells, strongly suggest that the GnRH-II-induced [Ca$^{2+}$]$_i$ increase is mediated by the ryanodine receptor rather than the IP$_3$ receptor.

**Effect of GnRH antagonists on [Ca$^{2+}$]$_i$**

Two types of GnRH antagonists were used to study the pharmacological characteristics of GnRH-induced [Ca$^{2+}$]$_i$ transients: cetrorelix, a potent GnRH-I antagonist, and trp-torelix-1 and trp-torelix-2, potent GnRH-II antagonists (22, 29). It should be noted that none of these antagonists induced an increase in [Ca$^{2+}$]$_i$, in GH$_3$ cells expressing conventional GnRH receptors, such as rat GnRHR, bFGnRHR-2, or monkey type 2 GnRH receptor, and that these antagonists efficiently blocked the GnRH-induced [Ca$^{2+}$]$_i$ increase in these cells (data not shown). In prostate cancer cells, however, all three antagonists induced a bell-shaped [Ca$^{2+}$]$_i$ response in the presence of extracellular Ca$^{2+}$: low concentrations of antag-
onists induced an increase in \([\text{Ca}^{2+}]_i\), whereas high concentrations of antagonists did not (Fig. 7A). Trporelix-1 was the most potent analog because it could induce a massive increase in \([\text{Ca}^{2+}]_i\) in DU-145 cells at a concentration as low as 10 pm. In the absence of extracellular \([\text{Ca}^{2+}]_i\), neither of the antagonists induced a \([\text{Ca}^{2+}]_i\) increase (data not shown). Pretreatment of DU-145 cells with cetrorelix blocked the \([\text{Ca}^{2+}]_i\) increase induced by low doses of GnRH-I (1 and 10 nm) but did not block the GnRH-II-induced \([\text{Ca}^{2+}]_i\) increase. Thus, trporelix-1 can inhibit both GnRH-I- and GnRH-II-induced \([\text{Ca}^{2+}]_i\) increase (Fig. 7B).

A GnRH-II antagonist induces prostate cancer cell death

Because GnRHs are known to influence prostate cancer cell proliferation, we examined the effects of GnRHs and their antagonists on thymidine incorporation in DU-145 and ALVA-41 cells. GnRH-II and cetrorelix did not affect thymidine incorporation, whereas trporelix-1 reduced thymidine incorporation in a dose-dependent manner (Fig. 8). Despite the failure of \([\text{Ca}^{2+}]_i\) induction in LNCaP cells by GnRH-I and GnRH-II, LNCaP cells may have a low-affinity GnRH-II binding protein (Fig. 1D). In addition, trporelix-1 has a growth-inhibitory effect on LNCaP cells (data not shown). This observation suggests that trporelix-1 could be useful for the treatment of prostate cancer cells that retain some sensitivity to androgens as well as androgen-independent cancer cells. To determine whether the trporelix-1-induced decrease in thymidine incorporation was due to a decreased proliferation rate or an increased cell death, cellular morphology and DNA fragmentation were examined in cetrorelix- or trporelix-1-treated DU-145 and ALVA-41 cells. Marked changes in cellular morphology were seen in trporelix-1- but not cetrorelix-treated cells. When cells were stained with DAPI, fragmented nuclei and cells lacking nuclei were observed in trporelix-1-treated cells (Fig. 9A). To further confirm that trporelix-1 induced apoptosis of pros-

![Fig. 5. Identification of the source of \([\text{Ca}^{2+}]_i\) mobilized by GnRH. Changes in \([\text{Ca}^{2+}]_i\) by GnRHs were examined in \([\text{Ca}^{2+}]_i\)-free medium (A and C) or BAPTA-AM-pretreated DU-145 cells (B and D). The effects of graded concentrations of GnRH-I and GnRH-II on the amplitude of the \([\text{Ca}^{2+}]_i\) responses were examined in calcium-free medium (C) or BAPTA-AM-pretreated cells (D).](image_url)

![Fig. 6. Ryanodine receptor-mediated \([\text{Ca}^{2+}]_i\) mobilization by GnRH-II. The \([\text{Ca}^{2+}]_i\) responses to GnRH-II were determined in \([\text{Ca}^{2+}]_i\)-free medium to exclude the possible contribution of external \([\text{Ca}^{2+}]_i\) sources. A, GH3 cells expressing rat GnRHR (GH3-rat-GnRHR) were exposed to GnRH-I (1 \(\mu M\)) in the presence of 1 \(\mu M\) U73122, 100 \(\mu M\) dantrolene, or vehicle. B, DU-145 cells were exposed to GnRH-II (1 \(\mu M\)) in the presence of 1 \(\mu M\) U73122, 100 \(\mu M\) dantrolene, or vehicle. Each point is the mean \(\pm\) SEM from six individual cells.](image_url)
tate cancer cells, DNA fragmentation was visualized by electrophoresis and TUNEL. DNA laddering, and TUNEL-positive cells were observed only in the trptorelix-1-treated group (Fig. 9, B and C).

GnRH-II antagonist induces a decrease in Ca\(^{2+}\) concentration of internal stores

It is well known that depletion of internal Ca\(^{2+}\) pools induces apoptosis of cells (38, 39). To elucidate the possible mechanism for trptorelix-1-induced cell death, we examined Ca\(^{2+}\) concentration in internal stores of cells treated with 10 \(\mu\)M trptorelix-1 or cetrorelix for 3 d in DU-145 and ALVA-41 cells. Ca\(^{2+}\) concentration in internal stores was measured by treating Tg, a sarcoendoplasmic Ca\(^{2+}\)-ATPase inhibitor that generally results in emptying Ca\(^{2+}\) from the internal Ca\(^{2+}\) pool (40). Tg-releasable Ca\(^{2+}\) was greatly reduced in trptorelix-1-treated cells, compared with control and the cetrorelix-treated group (Fig. 10). This finding indicates that trptorelix-1 may affect Ca\(^{2+}\) homeostasis in prostate cancer cells.

Photoaffinity labeling of GnRH-II binding protein in prostate cancer cells

The presence of a novel GnRH-II binding protein in prostate cancer cells was investigated by photoaffinity labeling using an azidobenzoyl-conjugated GnRH-II. \(^{125}\)I-[azidobenzoyl-d-Lys\(^{6}\)]-GnRH-II cross-linked with an approximately 80-kDa protein in all prostate cancer cells but not in HEK-293 and CV-1 cells (Fig. 11A). This band was abolished in the presence of an excess of unlabeled [azidobenzoyl-d-Lys\(^{6}\)]-GnRH-II. \(^{125}\)I-[azidobenzoyl-d-Lys\(^{6}\)]-GnRH-I exhibited a different binding pattern from that of [azidobenzoyl-d-Lys\(^{6}\)]-GnRH-II. It bound to an approximately 35- to 40-kDa protein in ALVA-41 and DU-145 cells. The intensity of the 80-kDa band that bound to [azidobenzoyl-d-Lys\(^{6}\)]-GnRH-II was dose-dependently decreased by cold natural GnRH-II but not GnRH-I (Fig. 11C). It should be noted that there were many other bands whose intensities were decreased by cold azidobenzoyl-d-Lys\(^{6}\)-GnRH-II, but the 80-kDa band was the only one whose intensity was decreased by cold native GnRH-II.

Discussion

The present study has demonstrated differential effects of GnRH-I and GnRH-II on signaling and proliferation of androgen-independent prostate cancer cells (Table 1). Although these cells express transcripts for either conventional mammalian type 1 and/or type 2 GnRH receptors, however, the amount of protein translated from the transcript for the type 1 receptor must be very low because we did not observe specific binding of GnRH-Ia to these cells. Protein translated from the type 2 receptor transcript may be truncated or abnormal because we failed to obtain the transcript consisting of exon 1 and exon 2 together. Furthermore, the responses to GnRhs in prostate cancer cells were completely different from those found in cells expressing conventional mammalian type 1 or type 2 GnRH receptors.

Conventional GnRH receptors are known to induce [Ca\(^{2+}\)], transients through an increased formation of IP\(_3\) lead-
ing to mobilization of IP₃-sensitive intracellular Ca²⁺ stores (16). In contrast, GnRHs failed to induce IP₃ formation in prostate cancer cell. This failure of GnRH to activate polyphosphoinositide turnover is not due to a defect of the signaling machinery, such as a lack of Gq/11 or PLC, in prostate cancer cells because we observed GnRH-induced IP₃ production when a conventional GnRH receptor was introduced into these cells. Interestingly, the GnRH-II-induced increase in [Ca²⁺]ᵢ was completely blocked by a ryanodine receptor antagonist but not by a PLC inhibitor, indicating that GnRH-II in prostate cancer cells causes Ca²⁺ mobilization through activation of the ryanodine receptor. However, the signaling cascade that is responsible for activation of the ryanodine receptor remains to be elucidated.

The effect of GnRH antagonists on [Ca²⁺]ᵢ in prostate cancer cells was also different from that observed in cells expressing conventional GnRH receptors. Neither trptorelix-1 nor cetrorelix induced a [Ca²⁺]ᵢ increase in cells expressing conventional GnRH receptors, yet low concentrations of both antagonists did induce a [Ca²⁺]ᵢ increase in prostate cancer cells incubated in Ca²⁺-containing medium. It is of interest to note that the effects of GnRH antagonists on [Ca²⁺]ᵢ increase is comparable with that of ryanodine. Ryanodine stimulates Ca²⁺ release from the endoplasmic vesicle at nanomolar to micromolar concentrations, whereas it inhibits Ca²⁺ release at higher concentrations (41, 42). GnRH-II antagonists, however, may not directly affect the ryanodine receptor activation because trptorelix-1 and cetrorelix failed to induce the [Ca²⁺]ᵢ in the absence of Ca²⁺ source. Thus, it is likely that, in prostate cancer cells, the GnRH antagonists can act as agonists for the GnRH-II receptor under a certain condition. In support of this notion, we found that TSU-Pr1 cells, which responded to only GnRH-II but not GnRH-I, were not affected by trptorelix-1 and cetrorelix. Furthermore, the molecular mass of the protein that binds to GnRH-II in prostate cancer cells is approximately 80 kDa, whereas that of the conventional GnRH receptors is 40–50 kDa (15, 35, 43). Altogether these results strongly suggest that GnRH-II exerts its action through a novel GnRH-II binding protein in androgen-independent prostate cancer cells. However, we do not exclude the possibility that GnRH-II could act through the mammalian GnRH-I receptor because the ligand selec-
activity and intracellular responses of a single receptor can be vastly altered by receptor conformation and the cellular context.

Several lines of evidence also support the idea of a functional receptor for GnRH-II in humans. In human T cells, either GnRH-I or GnRH-II can induce laminin receptor gene expression and cell migration. Interestingly, the effect of GnRH-I is completely blocked by cetrorelix, whereas the effect of GnRH-II is not (12). In human ovarian cancer cells, cetrorelix also exerts an antiproliferative effect in very much the same as GnRH-I agonists, indicating that the distinction between GnRH-I agonists and antagonists may not apply to the GnRH-I system (17). When GnRH-I receptors are depleted in human ovarian cancer cells, the antiproliferative effect of a GnRH-I agonist is abolished, whereas the effects of a GnRH-I antagonist and GnRH-II are still observed (44), indicating that the effect of GnRH-I antagonists and GnRH-II in these cells are not mediated by the conventional GnRH-I receptor.

Prostate cancer develops from an androgen-dependent stage to an androgen-independent stage. It is likely that during the androgen-dependent stage, the main effect of GnRH analogs as prostate cancer therapy is to block or to desensitize the pituitary type 1 GnRH receptor, thereby reducing serum androgen levels (3–5), whereas during the androgen-independent stage, GnRH-I and GnRH-II may act directly on prostate cancer cells. According to our findings, GnRH-I and GnRH-II themselves are not likely to affect proliferation of prostate cancer cells as neither peptide altered thymidine incorporation. However, the $[\text{Ca}^{2+}]_i$ increase induced by GnRHs indicates a possible role of GnRHs in cancer cell activity. Indeed, cytosolic $\text{Ca}^{2+}$ is known to control numerous cell functions, including contraction, proliferation, differentiation, and migration (40, 45, 46). Although the exact regulatory role of GnRHs in prostate cancer cells is currently unknown, it is conceivable that GnRH-I and/or GnRH-II may affect gene expression and/or cell migration in androgen-independent prostate cancer cells. In particular, ryanodine receptor activation by GnRH-II in prostate cancer cells is likely important because activation of these receptors is involved in the control of cell migration (46).

To elucidate the role of GnRHs in prostate cancer cell regulation, it is also of interest to determine the sources of endogenous GnRHs. The GnRH-I and/or GnRH-II genes are expressed in a number of peripheral tissues such as placenta (8), T cells (12), breast (10), endometrium (9), and ovarian surface cells (47). However, the GnRH peptides produced

TABLE 1. Differential effects of GnRH-I and GnRH-II on DU-145 cells

<table>
<thead>
<tr>
<th>Item</th>
<th>GnRH-I</th>
<th>GnRH-II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding assay</td>
<td>Very low expression of GnRH-I binding protein</td>
<td>Expression of GnRH-II binding protein with a low affinity</td>
</tr>
<tr>
<td>IP$_3$/cAMP assay</td>
<td>No alteration</td>
<td>No alteration</td>
</tr>
<tr>
<td>$\text{Ca}^{2+}$ mobilization</td>
<td>Induction of $[\text{Ca}^{2+}]_i$, probably via $\text{Ca}^{2+}$ influx from extracellular source</td>
<td>Induction of $[\text{Ca}^{2+}]_i$ mobilization from internal stores. Inhibition of the $[\text{Ca}^{2+}]_i$, increase by a ryanodine receptor antagonist but not by PLC inhibitor.</td>
</tr>
<tr>
<td>Proliferation and death</td>
<td>Marginal effect of cetrorelix and trptorelix-1</td>
<td>Trptorelix-1 has a growth-inhibitory effect and induces cell death</td>
</tr>
<tr>
<td>UV cross-linking</td>
<td>Binding to 35- to 40-kDa proteins</td>
<td>Binding to an 80-kDa protein</td>
</tr>
</tbody>
</table>
from these tissues are generally thought to play an autocrine or paracrine role rather than endocrine activities. Considering the extremely short half-life of GnRHs, an endocrine action of the peptides would not be expected. Because the GnRH genes are expressed in prostate cancer cells (6, 48), it seems likely that GnRHs can act as paracrine or autocrine factors in prostate cancer development. Therefore, regulation of the GnRH-I and GnRH-II genes during prostate cancer differentiation, and biosynthesis of GnRH from these transcripts deserve further investigation.

An interesting finding in this study is that trptorelix-1 induced prostate cancer cell death, probably through apoptosis. Although there are numerous reports indicating that GnRH analogs decrease proliferation of a variety of reproductive tissue cancer cells (14, 17, 43, 44, 49–51), few of them have shown that a GnRH analog can induce cancer cell death. The mechanism involved in trptorelix-1-induced apoptosis of prostate cancer cells is currently a matter of speculation. A possible explanation is that trptorelix-1 may disturb Ca2+ homeostasis because high concentrations of trptorelix-1 completely blocked either GnRH-I- or GnRH-II-induced [Ca2+]i increase. It is well established that depletion of internal Ca2+ pools induces apoptosis of cells (38, 39). Moreover, Ca2+ mobilization via the ryanodine receptor is known to play a role in apoptosis of androgen-dependent prostate cancer cells (52). Indeed, we observed that trptorelix-1, but not cetorelix, decreased Ca2+ concentration in internal store. Alternatively, it is possible that trptorelix-1 can induce apoptosis through a yet-unknown mechanism.

In conclusion, GnRH-I agonists and antagonists are already widely used in various reproductive tissue cancer therapies. However, the direct effects of GnRH-II agonists and antagonists have been barely examined in these cancer cells, although many of them are responsive to GnRH-II (38). Whether these cancer cells express novel GnRH-II receptors, as observed in prostate cancer cells, and whether GnRH-II antagonists can induce their death needs to be elucidated. This study provides evidence that androgen-independent prostate cancer cells possess novel receptors for GnRH-I and GnRH-II. Further information on these receptors will facilitate development of new drugs for the treatment of reproductive tissue cancers.

Acknowledgments

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