A Human Prolactin Antagonist, hPRL-G129R, Inhibits Breast Cancer Cell Proliferation through Induction of Apoptosis

Wen Y. Chen, Preveen Ramamoorthy, Nian-yi Chen, Robert Sticca, and Thomas E. Wagner

Oncology Research Institute, Cancer Center, Greenville Hospital System [W. Y. C., N-y. C., R. S., T. E. W.]; and Department of Microbiology and Molecular Medicine, Clemson University [W. Y. C., P. R., T. E. W.], Greenville, South Carolina 29605

ABSTRACT

Human breast cancer is the predominant malignancy and the leading cause of cancer death in women from Western societies. The cause of breast cancer is still unknown. Recently, the association between human prolactin (hPRL) activity and breast cancer has been reemphasized. Biologically active hPRL has been found to be produced locally by breast cancer cells that contain high levels of PRL receptor. A high incidence of mammary tumor growth has also been found in transgenic mice overexpressing lactogenic hormones. More importantly, it has been demonstrated that the receptors for sex steroids and PRL are coexpressed and cross-regulated. In this study, we report that we have designed and produced a hPRL antagonist, hPRL-G129R. By using cell proliferation assays, we have demonstrated that: (a) hPRL and E2 exhibited an additive stimulatory effect on human breast cancer cell (T-47D) proliferation; (b) hPRL-G129R possessed an inhibitory effect on T-47D cell proliferation; and (c) when antiestrogen (4-OH-tamoxifen) and anti-PRL (hPRL-G129R) agents were added together, an inhibitory effect was observed. We further investigated the mechanism of the inhibitory effects of hPRL-G129R in four hPRLR positive breast cancer cell lines. We report that hPRL-G129R is able to induce apoptosis in all four cell lines in a dose-dependent manner as determined by the Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay. The apoptosis is induced within 2 h of treatment at a dose as low as 50 ng/ml. We hope that the hPRL antagonist could be used to improve the outcome of human breast cancer therapy in the near future.

INTRODUCTION

Human breast cancer is the predominant malignancy and leading cause of cancer death in women from Western societies (1, 2). According to a recent estimation by the American Cancer Society, one in every eight women from the United States will develop breast cancer, and the disease will kill 43,500 women in 1998. The cause of breast cancer is still unknown, but its great rarity among males indicates an etiological role for the female sex hormones, whereas varying geographic distribution also points to the importance of environmental factors (2). Although generally slow growing, breast cancer develops invasive properties early in its pathogenic progression. By the time it has become clinically apparent, it is likely to have already metastasized to distant sites. It is this pattern that accounts for the failure of purely local treatment to control the disease. For decades, the primary therapy for women with breast cancer has been surgery or radiation or a combination of both (1, 2).

hPRL is a neuroendocrine polypeptide hormone discovered nearly 60 years ago. It is primarily produced by the lactotrophs in the anterior pituitary gland of all vertebrates. The biological activities of PRL are mediated by specific membrane receptors, i.e., PRLRs (3). On the basis of several conserved features (a single transmembrane domain and conserved amino acid sequences in the extracellular domain), PRLRs together with GH receptor, have been categorized into the cytokine receptor superfamily (3). The best-characterized action of PRL is on the mammary gland. In this organ, PRL plays a decisive role in the stimulation of DNA synthesis, epithelial cell proliferation, and the promotion of milk production (4). The generation of PRL (4) and PRLR (5) gene knock-out mice have unambiguously demonstrated that PRL and PRLR are the key regulators in mammary development.

Several lines of evidence strongly link hPRL to breast cancer development: (a) it has been reported that female hGH transgenic mice have a high incidence of breast cancer in contrast to sporadic cases found in bovine GH transgenics (6). The high incidence of breast cancer in hGH transgenic mice is believed to be attributable to the lactogenic activity of hGH, which is a unique feature of primate GHs. A recent report of breast cancer development in hPRL transgenic mice further confirmed the role of hPRL in the stimulation of breast cancer (7); and (b) the finding of hPRL mRNA in mammary tissues (8–10) and the detection of biologically active hPRL in human breast cancer cells (11) suggest that hPRL is produced locally as a lactogenic factor in breast cancer cells.
an autocrine/paracrine growth factor within the mammary glands. This extrapituitary production of hPRL might not cause detectable systemic change of hPRL in serum yet could exert significant local stimulatory effects (12). In support of this concept, it has also been reported that the expression levels of PRLRs are significantly higher in human breast cancer cells or in surgically removed breast cancer tissues than in normal breast epithelial tissues (13–15). The high levels of PRLRs in malignant breast tissue make these cells highly sensitive to stimulation by hPRL (15).

In our previous studies, we demonstrated that the third α-helix of GH is important for its growth-promoting activities (16–21). We further demonstrated that Gly 119 of bGH (18) or Gly 120 of hGH (19) plays a critical role in the action of GH in stimulating growth enhancement. The mechanism of these GH antagonists was further studied by other groups (22, 23). It is generally accepted that GH transduces its signal via a sequential receptor binding mechanism to form a one hormone-two receptor complex (22, 23). Receptor dimerization is thought to be a key step for GH signal transduction. Any amino acid substitution (other than Ala), especially one with a bulky side chain such as Arg at position 120 of hGH, will prevent receptor dimerization, resulting in a GH antagonist (16–21). As a member of the GH family, hPRL is believed to share a signal transduction mechanism similar to GH (24–27). It is, therefore, reasonable to predict that if a key amino acid within the third α-helix of hPRL is substituted, it may be possible to produce a hPRL-specific antagonist in much the same manner that GH antagonists have been produced.

In this paper, we report that by adopting a strategy similar to that which we used in designing the GH antagonist, we have developed a hPRL antagonist in which a Gly residue at position 129 was substituted with Arg (hPRL-G129R). We have demonstrated the following three hPRL-related findings: (a) single amino acid substitution mutation at position 129 of hPRL (hPRL-G129R) resulted in a hPRL antagonist, confirmed by cell proliferation assays; (b) when hPRL-G129R was applied together with 4-OH-tamoxifen, an additive inhibitory effect was observed; and (c) the inhibitory effect of hPRL-G129R on human breast cancer cells is through the induction of apoptosis. We believe that development of the hPRL-G129R, a hPRL antagonist, might open a new avenue in the design of adjuvant therapy to improve the treatment of breast cancer.

MATERIALS AND METHODS

RT-PCR

The RT-PCR technique was used to clone hPRL cDNA. Human pituitary mRNA was purchased from Clontech Laboratory, Inc. (Palo Alto, CA). A RT-PCR kit was from Perkin-Elmer, Inc. (Norwalk, CT). The hPRL antisense primer (for the reverse transcriptase reaction) was designed 2 bases from the stop codon (shown in boldface) of hPRL cDNA (5’-GCCTAAGCAGTGTGGTGTGTG-3’), and the sense primer was designed from the translational start codon ATG (5’-ATGAAACATCAAAGGAT-3’). The RT-PCR reaction was carried out following the manufacturer’s recommendation. The PCR product was then cloned into an expression vector pCDNA3.1 from Invitrogen Corp. (Carlsbad, CA). The expression of hPRL cDNA was controlled by the human immediate-early cytomegalo-virus enhancer/promoter and a polyadenylation signal and transcription termination sequence from the bGH gene. This vector also contains a neomycin gene that allows for selection of neomycin-resistant mammalian cells.

Rational Design of hPRL-G129R

We have compared the amino acid sequences of all known PRLs in the third α-helical region and aligned them with GH sequences (Table 1). It is clear that Gly 129 of hPRL is invariant among PRLs and corresponds to hGH 120, suggesting a potentially important role in its function. We, therefore, decided to make a single amino acid substitution mutation at Gly 129 of hPRL (hPRL-G129R). We have used a similar approach to that which we have used successfully previously in the discovery of GH antagonists in the hope of producing a hPRLR-specific antagonist (Fig. 1).

Oligonucleotide-directed Mutagenesis

hPRL-G129R cDNA was generated using a PCR mutagenesis protocol. Oligonucleotides containing the desired mutation (5’-CTTCTAGAGCGCATGACTGATA-3’ and 5’-CCCTCTAGACTCGAGCCGC-3’) were synthesized by National Biosciences, Inc. (Plymouth, MN). The codon for 129 Arg is in boldface, and the restriction site XbaI is underlined.

### Table 1 Comparison of amino acid sequences within the third α-helical region among PRLs (42)

<table>
<thead>
<tr>
<th>Species</th>
<th>PRL</th>
<th>Mutation Site</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>PRL</td>
<td>129</td>
<td>Arg</td>
</tr>
<tr>
<td>Rat</td>
<td>PRL</td>
<td>129</td>
<td>Arg</td>
</tr>
<tr>
<td>Mouse</td>
<td>PRL</td>
<td>129</td>
<td>Arg</td>
</tr>
<tr>
<td>Hamster</td>
<td>PRL</td>
<td>129</td>
<td>Arg</td>
</tr>
<tr>
<td>Fin whale</td>
<td>PRL</td>
<td>129</td>
<td>Arg</td>
</tr>
<tr>
<td>Mink</td>
<td>PRL</td>
<td>129</td>
<td>Arg</td>
</tr>
<tr>
<td>Cattle</td>
<td>PRL</td>
<td>129</td>
<td>Arg</td>
</tr>
<tr>
<td>Sheep</td>
<td>PRL</td>
<td>129</td>
<td>Arg</td>
</tr>
<tr>
<td>Pig</td>
<td>PRL</td>
<td>129</td>
<td>Arg</td>
</tr>
<tr>
<td>Camel</td>
<td>PRL</td>
<td>129</td>
<td>Arg</td>
</tr>
<tr>
<td>Horse</td>
<td>PRL</td>
<td>129</td>
<td>Arg</td>
</tr>
<tr>
<td>Elephant</td>
<td>PRL</td>
<td>129</td>
<td>Arg</td>
</tr>
<tr>
<td>Ancestral mammal</td>
<td>PRL</td>
<td>129</td>
<td>Arg</td>
</tr>
<tr>
<td>Chicken</td>
<td>PRL</td>
<td>129</td>
<td>Arg</td>
</tr>
<tr>
<td>Turkey</td>
<td>PRL</td>
<td>129</td>
<td>Arg</td>
</tr>
<tr>
<td>Sea turtle</td>
<td>PRL</td>
<td>129</td>
<td>Arg</td>
</tr>
<tr>
<td>Crocodile</td>
<td>PRL</td>
<td>129</td>
<td>Arg</td>
</tr>
<tr>
<td>Alligator</td>
<td>PRL</td>
<td>129</td>
<td>Arg</td>
</tr>
<tr>
<td>Ancestral amniote</td>
<td>PRL</td>
<td>129</td>
<td>Arg</td>
</tr>
<tr>
<td>Xenopus</td>
<td>PRL</td>
<td>129</td>
<td>Arg</td>
</tr>
<tr>
<td>Bullfrog</td>
<td>PRL</td>
<td>129</td>
<td>Arg</td>
</tr>
<tr>
<td>Lungfish</td>
<td>PRL</td>
<td>129</td>
<td>Arg</td>
</tr>
<tr>
<td>Tilapia</td>
<td>PRL</td>
<td>129</td>
<td>Arg</td>
</tr>
<tr>
<td>Common carp</td>
<td>PRL</td>
<td>129</td>
<td>Arg</td>
</tr>
<tr>
<td>Bighhead carp</td>
<td>PRL</td>
<td>129</td>
<td>Arg</td>
</tr>
<tr>
<td>Silver carp</td>
<td>PRL</td>
<td>129</td>
<td>Arg</td>
</tr>
<tr>
<td>Chum salmon</td>
<td>PRL</td>
<td>129</td>
<td>Arg</td>
</tr>
<tr>
<td>Chinook salmon</td>
<td>PRL</td>
<td>129</td>
<td>Arg</td>
</tr>
<tr>
<td>Trout</td>
<td>PRL</td>
<td>129</td>
<td>Arg</td>
</tr>
<tr>
<td>Human</td>
<td>GH</td>
<td>120</td>
<td>Arg</td>
</tr>
<tr>
<td>Bovine</td>
<td>GH</td>
<td>120</td>
<td>Arg</td>
</tr>
</tbody>
</table>

*Two GH sequences are also included. Gly 129 of hPRL is in bold.*
The PCR product was digested with XbaI and ligated back into the vector described previously. The mutation was then confirmed by DNA nucleotide sequencing.

**Human Breast Cancer Cell Lines**

The human breast cancer cell lines used in this study are MDA-MB-134, T-47D, BT-474, and MCF-7 from the American Type Culture Collection. These human breast cancer cell lines have been characterized as ER-positive and PRLR-positive cell lines (28). T-47D and BT-474 cells were grown in RPMI 1640 (phenol red free to avoid its potential estrogen-like activities) supplemented with 10% FBS (Life Technologies, Inc.) and American Type Culture Collection recommended supplements. MCF-7 cells were grown in DMEM (phenol red free), supplemented with 10% FBS. The cells were grown at 37°C in a humid atmosphere in the presence of 5% CO2. The MDA-MB-134 cells were grown in Leibovitz’s l-15 medium supplemented with 20% FBS and grown in a CO2-free atmosphere.

**Expression and Production of hPRL and hPRL-G129R Proteins**

Mouse L-cell transfection and stable cell selection were performed as described previously with minor modification (29). Briefly, cells were plated in a six-well plate and cultured until the culture was 50% confluent. On the day of transfection, cells were washed once with serum-free medium and cultured in 1 ml of serum-free medium containing 1 µg of pcDNA3-hPRL or pcDNA3-hPRL-G129R and 10 µl of LipofectAmine (Life Technologies, Inc.) for 5 h. Two ml of growth medium were added to the DNA/LipofectAmine solution, and incubation continued. After 18–24 h of incubation, fresh growth medium was used to replace the medium containing DNA/LipofectAmine mixture. At 72 h after transfection, cells were diluted 1:10 and passed into the selective medium (400 µg/ml G418) to select for neo gene expression. Individual colonies were isolated and expanded. The expression levels of the individual cell lines were determined by using an IRMA kit from Diagnostic Products Corp. (Los Angeles, CA). The cell lines with high expression levels were expanded.

Conditioned medium containing hPRL and hPRL-G129R was prepared as follows. Stable cells were plated in T-150 culture flasks at 85–90% confluence. The growth medium were then replaced with 50 ml of RPMI 1640 containing 0.5% dextran-coated charcoal-FBS and collected every other day for three times. The collected media were then pooled and filtered through a 0.22 µm filter units to remove cell debris and stored at –20°C until use. The concentration of hPRL or hPRL-G129R was determined by hPRL IRMA. Each batch product was further verified using a Western blot analysis protocol (30). We have used this protocol in hGH analogue studies, including hGH antagonists, for *in vitro* studies (19).

**Radioreceptor Binding Assay**

hPRLR binding assays were performed as described previously (19, 31). Briefly, T-47D cells were grown in six-well tissue culture plates until 90% confluent (~10^5 cells/well). Monolayers of cells were starved in serum-free RPMI 1640 medium for 2 h. The cells were then incubated at room temperature in serum-free RPMI 1640 containing 8 × 10^4 cpm 125I-labeled hPRL (specific activity, 30 µCi/µg; NEN DuPont, Boston, MA) with or without various concentrations of hPRL (from NIH as standard) and hPRL-G129R. Cells were then washed three times in serum-free RPMI 1640 and solubilized in 0.5 ml of 0.1 N NaOH/1% SDS, and the bound radioactivity was determined by a gamma counter (model 4/600plus; ICN Biomedical, Costa Mesa, CA). EC50s of hPRL and hPRL-G129R were then determined and expressed as mean ± SD. Comparison was made by Student’s *t* test.

**Human Breast Cancer Cell Proliferation Assays**

**hPRL-G129R Conditioned Media.** The assay conditions were modified from that described by Ginsburg and Vonderharr (11). T-47D cells were trypsinized and passed into 96-well plates in RPMI 1640 containing 0.5% charcoal/dextran-treated FBS (Hyclone, Logan, UT) in a volume of 100 µl/well. The optimal cell number/well for each cell line was predetermined after titration assay. We have found that 15,000 cell/well are optimal for T-47D cells.

---

*Fig. 1* Schematic illustration of the mechanism of GH or hPRL (ligand) antagonist. Four helical regions in the ligand (dotted ovals) are labeled as I, II, III, and IV. Two membrane bound receptors (shaded dark ovals) are also shown in the figure. Arg substitution mutation in the third α-helix, resulting in hindering a second receptor to form a functional complex (from A to B).
The cells were allowed to settle and adhere overnight (12–18 h), and subsequently various concentrations of either hPRL, hPRL-G129R, E2, or 4-OH-tamoxifen in a total volume of 100 μl of culture media were added. Purified hPRL (kindly provided by Dr. Parlow, National Hormone and Pituitary Program, NIH, Bethesda, MD) was used as a positive control for hPRL produced from stable L cells. Cells were incubated for an additional 96 h at 37°C in a humidified 5% CO2 incubator. After incubation, MTS-PMS solution (Cell Titer 96 Aqueous kit; Promega Corp.) was added to each well, following the manufacturer’s instructions. Plates were read at 490 nm using a Bio-Rad benchmark microplate reader. The experiments were carried out in triplicates and repeated three to six times for each cell line.

Coculture Experiments. This design of the cell proliferation assay is to take advantage of stable mouse L cell lines we have established that produce hPRL and hPRL-G129R. Increasing numbers of L cells (or L-hPRL or L-hPRL-G129R cells) in a range of 4,500 –27,000 cells/well were cocultured with fixed number of T-47D (9,000/well) in 96-well plates. At the same time, a correspondent set of L cells (or L-hPRL or L-hPRL-G129R cells) was cultured in the same plate (without coculture with T-47D) as background controls. The total volume of the coculture was 200 μl. The concentrations of hPRL or hPRL-G129R at the end of 72-h coculture were measured at 20–200 ng/ml, which is within the physiological range and is similar to that of the conditioned media experiments. After incubation, MTS-PMS solution was added to each well at 24, 48, or 72 h (best response was observed at 72 h and reported in this paper). Plates were then read at 490 nm using a Bio-Rad benchmark microplate reader. The absorbance (A) of T-47D cells was calculated as total A (A of T-47D plus L, L-hPRL or L-hPRL-G129R cells, respectively) minus the background As (L, L-hPRL, or L-hPRL-G129R cells alone).

TUNEL Assay
This assay (Fluorescein Apoptosis detection system; Promega Corp.) works by labeling the nicks of the fragmented DNA at the 3-OH ends. The fluorescein-labeled dUTP is incorporated at the 3-OH ends by terminal deoxynucleotidyl transferase. Four human breast cancer cell lines were used in this study. Before the assay, the breast cancer cells were switched to 10% charcoal/dextran-treated FBS (CCS) for a week. Subsequently, the cells were plated onto an eight-chambered slide.
system (Lab TekII) at a confluence of 60–70% per chamber. The next day, the breast cancer cells were treated with various concentrations of hPRL-G129R in conditioned medium (0.5% CCS) or 4-OH-Tamoxifen (in 0.5% CCS containing growth medium). To demonstrate the specificity of the antagonist, hPRL-G129R was also either mixed with PRL or with polyclonal anti-hPRL antibodies (kindly provided by Dr. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases) before being applied to breast cancer cells. In the case of anti-hPRL antibody experiments, 125 ng/ml of hPRL-G129R were preincubated with anti-hPRL antibodies for 6 h at 4°C before adding to the cells. After the assigned period of treatment, the chambers were dismantled, and the assay was performed as per the manufacturer’s instructions. The slides were examined under a FITC filter using an Olympus IX 70 microscope system.

RESULTS

Cloning and Mutagenesis of hPRL

hPRL cDNA was cloned from human pituitary mRNA using the RT-PCR technique. The size of the corresponding PCR product was 663 bp in length (data not shown), and it was cloned into the pcDNA 3.1 expression vector. The nucleotide sequence of hPRL was determined by the dideoxy chain-termination method using an automatic sequencer (PE Applied Biosystems, Foster City, CA). The hPRL cDNA sequence was found identical to that reported in GenBank, except for one base difference that results in a silent mutation at codon 21 (CTG → CTC). hPRL-G129R cDNA was also generated by PCR and sequenced.

Expression of hPRL and hPRL-G129R

Mouse L cell were stably transfected with either hPRL or hPRL-G129R cDNAs, and neo-resistant clones were selected and expanded. Conditioned media were collected and tested for expression by use of an IRMA kit. We have generated hPRL and hPRL-G129R stable mouse L-cell lines that produced hPRL and hPRL-G129R in a quantity of ~1 mg/l every 24 h/million cells (Fig. 2).

Radioreceptor Binding Assay

The assay was carried out in a homologous system using $^{125}$I-labeled hPRL in the presence or absence of various con-
Fig. 8  Dose-response of T-47D human breast cancer cells to hPRL-G129R after 24 h of treatment using the TUNEL assay (A–F). G and H, results of competition between hPRL and hPRL-G129R at 1:1 ratio (125 ng/ml of each; G) and 4:1 ratio (500 ng/ml hPRL + 125 ng/ml hPRL-G129R; H). I, result of anti-hPRL antibody pretreatment (125 ng/ml of hPRL-G129R in 100-μl volume + 100-μl antiserum). J, quantification of the same experiment (fold induction of apoptotic cells/field over control; average of three measurements).
centrations of unlabeled hPRL or hPRL-G129R and T-47D cells. The results demonstrated that there was no significant change in EC_{50} (P > 0.05) of hPRL-G129R (3.01 nM ± 0.24 nM) as compared with hPRL (1.89 ± 0.18 nM; Fig. 3). These results were similar to our previous studies regarding bGH antagonist (bGH-G119R; Ref. 16) and hGH antagonist (hGH-G120R; Ref. 19).

### Human Breast Cancer Cell Proliferation Assays

**Conditioned Media.** Human PRL and hPRL-G129R were tested further for its ability to stimulate/inhibit breast cancer cell proliferation in cell culture. Ninety-six-well cell proliferation assay results are shown in Figs. 4–6. hPRL stimulated T-47D proliferation in a dose-dependent manner. The maximum stimulation of hPRL (250 ng/ml) was ~20% over

---

Fig. 9 Time course of T-47D human breast cancer cells responding to hPRL-G129R treatment (50 ng/ml) using the TUNEL assay (A–E). F, quantification of the same experiment (fold induction of apoptotic cells/field over control; average of three measurements).
Fig. 10  Response of multiple breast cancer cells (as labeled) to treatment with 250 ng/ml hPRL-G129R for 24 h using the TUNEL assay. -C, control cells; -T, treated cells.
basal levels after a single dose/4-day incubation. However, when hPRL and E2 were applied simultaneously, an additive effect was observed. The maximum response of hPRL (100 ng/ml) in the presence of 10 nm of E2 was more than tripled as compared with hPRL alone (Fig. 4).

hPRL-G129R, on the other hand, exhibited dose-dependent inhibitory effects on cell proliferation (Fig. 5, □). It is noteworthy to point out that the inhibitory effect of hPRL-G129R (150 ng/ml) was more potent than the maximal 500 nm dose of 4-OH-Tamoxifen in our assay system (Fig. 5). The maximum inhibition of a single dose of 4-OH-Tamoxifen (500 nm) is ~85% of control, whereas the maximum inhibition by a single dose of hPRL-G129R resulted in 75% of control. More importantly, when hPRL-G129R was applied together with 4-OH-Tamoxifen, the inhibitory effects were doubled as compared with either the maximum dose of hPRL-G129R or 4-OH-Tamoxifen (Fig. 5). For example, 100 nm of 4-OH-Tamoxifen resulted in an 85% inhibition; yet in the presence of 150 ng/ml of hPRL-G129R, the inhibitory effect resulted in ~58% of control. hPRL-G129R was also able to competitively inhibit hPRL-induced cell proliferation. At a 1:1 molar ratio, hPRL-G129R was able to stop the stimulatory effect of hPRL, and at 2:1 molar ratio, it inhibits cell proliferation (Fig. 6).

Coculture Experiments. We found that stable mouse L-cell lines grow at a similar rate as do regular L cells, regardless of producing either hPRL or hPRL-G129R (data not shown) because of the fact that mouse L cells possess nondetectable levels of PRLR (20). We believe that the coculture experimental set-up sustained the presence of biologically active hPRL-G129R, resulting in a maximal response in these breast tumor cells.

T-47D cells, after coculture with L-PRL or hPRL-G129R cells, demonstrated dose-dependent growth stimulation (with L-PRL) or inhibition (with L-PRL-G129R; Fig. 7). The responses were rather dramatic as compared with conditioned media experiments. We nearly achieved complete inhibition of cell proliferation.

TUNEL Assay

In this report, we have presented data to demonstrate that the hPRLR antagonist, hPRL-G129R, is able to induce apoptosis by DNA fragmentation in multiple human breast cancer cell lines. The hPRL-G129R induces apoptosis in a dose-dependent manner after 24-h treatment (Fig. 8, A–F), and the apoptosis is obvious, even at physiological concentration (50 ng/ml; Fig. 8C). To demonstrate the specificity of hPRL-G129R to the hPRLR, hPRL or an anti-hPRL antiserum to reverse the apoptosis process and hPRL-G129R were simultaneously used to treat the cells (Fig. 8, G–I). As shown in Fig. 8H, hPRL is able to competitively reverse the DNA fragmentation induced by hPRL-G129R at a ratio of 4:1 (500 ng/ml of hPRL versus 125 ng/ml of hPRL-G129R). The same results were obtained using BT-474 cells (data not shown). The DNA fragmentation in breast cancer cells is apparent even after 2 h of exposure to hPRL-G129R at a concentration of 50 ng/ml (Fig. 9, A–D). We also confirmed that hPRL-G129R could induce apoptosis by DNA fragmentation in four hPRLR-positive breast cancer cell lines after 24 h of treatment. (Fig. 10). To demonstrate the specificity of hPRL-G129R, an anti-hPRL antibody titration experiment was also included (Fig. 8f). It was shown that the anti-hPRL antibody could completely block the apoptotic effects of hPRL-G129R in T-47D cells after 6 h of preincubation.

DISCUSSION

Human breast cancer is known to be a heterogeneous mixture of cell clones characterized by different biological features. The primary target of endocrine therapy for breast cancer has been E2, by either surgical or pharmacological methods of estrogen deprivation (1, 2). Among the pharmacological methods, the most notable has been the development of tamoxifen. Recently, the National Surgical adjuvant Breast and Bowel Project has reported the results of the Breast Cancer Prevention Trial demonstrating a 49% decrease in the incidence of invasive breast cancer in a large cohort of high risk women as a result of the use of tamoxifen (32, 33). Despite these encouraging results, a fraction of ER-positive tumors escape first- or second-line endocrine treatment because of the initial presence of estrogen-negative clones or the development of drug resistance. It is this complexity that partly explains why tamoxifen is not universally effective, even in ER-rich tumors (2). In addition, any progress in the development of better antiestrogen therapy for breast cancer is unlikely to impact on the treatment of ER-negative tumors. For these reasons, it is our belief that the scope of the search for drugs to treat breast cancer should be expanded to effectively control tumor growth and/or recurrence in all tumors. Recently, several lines of evidence strongly suggest that hPRL acts as an autocrine/paracrine growth factor contributing to breast cancer development (11, 34, 35). More importantly, it has recently been reported that sex steroid hormones and PRL interact synergistically to control cancerous growth within the mammary gland (28). ER and PRLR were found being coexpressed and cross-regulated in mammary tumor cell lines as well as in primary breast cancers (28). These findings further suggest that the use of antiestrogen therapy in breast cancer may be attacking only half of the synergistic equation, which leaves an opportunity for further improvement of the ultimate therapeutic approach to breast cancer (28). In support to this notion, a combined regimen using an antiestrogen (Tamoxifen), an anti-GH secretion drug (octreotide), and an anti-PRL secretion drug (CV 205-502) has been reported to have significantly better clinical results in metastatic breast cancer patients as compared with tamoxifen therapy alone (36). Although this regimen does not block the autocrine/paracrine action of PRL on breast cancer, inhibition of circulating PRL from the pituitary did seem to have an additive benefit in the treatment of advanced breast cancer. This raises exciting prospects for even better results with complete PRL blockade with an antagonist that acts at the receptor level.

In this study, we report the design and production of a hPRL antagonist, hPRL-G129R. We first demonstrated that hPRL and E2 exhibited an additive stimulatory effect in human breast cancer cell proliferation (Fig. 4). We believe that the synergistic effects between hPRL and estrogen reflect the real physiological status because the breast tissue is constantly exposed to both newly synthesized estrogen and hPRL. These results also indicate the possibility of developing new therapeutic regimens, targeting possible tumor stimuli other than the ER. The potential
for additive and therefore improved benefits is significant. We further demonstrated that hPRL-G129R possessed an inhibitory effect on T-47D cell proliferation (Fig. 5). More importantly, when anti-estrogen (4-OH-Tamoxifen) and anti-PRL (hPRL-G129R) agents were applied simultaneously, as we had anticipated, an additive effect was observed. The inhibitory effect on cell proliferation was more than doubled (Fig. 5). We reason that the direct inhibitory effects of hPRL-G129R on T-47D cell proliferation are by competitive inhibition of the hPRL produced by T-47D cells (11). The hPRL-specific antagonistic effects of hPRL-G129R were further substantiated by an assay that uses combinations of hPRL and hPRL-G129R. It is encouraging to note that even at the ratio of 1:1, hPRL-G129R could stop the T-47D cell proliferation induced by hPRL (Fig. 6).

We speculated that if we could sustain the effects of hPRL-G129R by providing a continuous fresh supply of antagonist, we might obtain even better results than by a single application and prolonged incubation. To address this question, we designed the coculture experiments. When stable L cells that produce hPRL-G129R were cocultured with T-47D cells, much more dramatic inhibitory effects were observed (Fig. 7). The actual concentration of hPRL-G129R at the end of the experiment is approximately the same as the beginning high dose in the conditioned media experiment; yet apparently because these antagonists are produced continuously, the effects are more dramatic.

Apoptosis (programmed cell death) is one of the central physiological mechanisms that regulates the timely and orderly death of cells (37). The biochemical hallmark of apoptosis is internucleosomal DNA cleavage (38 – 40), and it can be detected by the TUNEL assay or by conventional gel electrophoresis (41). In this report, we have presented data to demonstrate that the hPRLR antagonist, hPRL-G129R, is able to induce apoptosis by DNA fragmentation in multiple human breast cancer cell lines. The hPRL-G129R induces apoptosis in a dose-dependent manner after 24-h treatment (Fig. 8). The DNA fragmentation in breast cancer cells is apparent even after 2 h of exposure to hPRL-G129R at a concentration of 50 ng/ml (Fig. 9). We further demonstrated the specificity of hPRL-G129R by using either hPRL or an anti-hPRL antiserum to reverse the apoptosis process (Fig. 8). The mitogen rescue effect of hPRL is yet another indication that hPRL-G129R induces apoptosis (39). To our surprise, 4-OH-Tamoxifen did not induce apoptosis in the cell lines we tested at concentrations as high as 1 µM, as assayed by the same protocol (data not shown), suggesting that a different mechanism might be involved. It also explains the additive inhibitory effects on cell proliferation when two agents (hPRL-G129R and 4-OH-Tamoxifen) were applied together (Fig. 5).

The mechanism of induction of apoptosis by this hPRLR antagonist needs further experimental elucidation. The mammary gland is one of the few organs that undergoes most of its development in the mature organism. More importantly, the mammary gland undergoes sequential waves of apoptosis during development and involution beginning with each pregnancy and ending with each weaning. We speculate that PRL might serve as one of the major controlling factors that decides whether the breast cells should go into proliferation/differentiation (by producing more PRL) or apoptosis (deprived of PRL) under physiological conditions. In the case of breast cancer, the cancer cells are adapted to using PRL as a major growth factor by producing PRL on their own (as an autocrine/paracrine growth factor), therefore maintaining their proliferative status. Hence, it is conceivable that when we effectively deprived the mitogenic signal of PRL in breast cancer cells by competitive binding of hPRL-G129R to the hPRLR, apoptosis is induced. Whatever the mechanism of hPRL-G129R-induced apoptosis of breast cancer cells, it is clear that the hPRLR antagonist hPRL-G129R has a strong potential to be used as another line of endocrine therapy along with Tamoxifen or by itself in the treatment of breast cancer.

In summary, the appalling death rate from breast cancer is still a major health care problem in the United States. History and biology have taught us that instead of finding a single magic “bullet” for breast cancer or for any tumor, we are more likely to improve the outcome of patients with oncogenic disease if we consider the heterogeneity of the disease and explore alternative and/or combination treatment regimens. We have reported in this paper a new agent to inhibit breast cancer development, hPRL-G129R, which acts as a hPRL antagonist. These results provided strong evidence of the involvement of hPRL in human breast cancer cell proliferation and also offer a novel approach for the treatment of breast cancer. It is our belief that the development of the hPRL antagonist will have a significant impact on effective human breast cancer therapy.

ACKNOWLEDGMENTS

We thank Jeremy Tzeng, Long Yan, Yanzhang Wei, and George Huang for excellent technical assistance. Special thanks also go to Drs. Michael Kilgore, Ross Wilkinson, and Lyndon Larcon for valuable discussions. Purified hPRL and polyclonal rabbit anti-hPRL antiserum were kindly supplied by Dr. Parlow, National Hormone & Pituitary Program, NIH. We are grateful for the excellent clerical assistance of Diann Tinsley, June Huff, and Lakendra Workman.

REFERENCES


Clinical Cancer Research

A Human Prolactin Antagonist, hPRL-G129R, Inhibits Breast Cancer Cell Proliferation through Induction of Apoptosis


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/5/11/3583

Cited articles
This article cites 37 articles, 11 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/5/11/3583.full#ref-list-1

Citing articles
This article has been cited by 15 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/5/11/3583.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.