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13. ABSTRACT *(Maximum 200 Words)*
There were two specific tasks listed in the proposal (finishing cell proliferation assays and summarizing data and manuscript preparation) for the final year of this three-year project. Since we have completed a majority of the tasks related to this project in terms of development of a PRL antagonist, hPRL-G129R. Our focus of research in the past 12 month has been on (1) to further confirm the anti-tumor effects of hPRL-G129R in vivo; (2) to develop hPRL-G129R based, breast cancer specific therapeutics; and (3) to reach a conclusion on hPRL-BP project. We have demonstrated that hPRL-G129R is able to inhibit the growth of two human breast cancer cell xenografts in nude mice (manuscript attached). In terms of designing hPRL-G129R based therapeutics, we have designed and demonstrated efficacy of dual functional protein (G129R-IL2, manuscript attached). We have also generated preliminary results regarding G129R-PE40 (abstracts attached). We further confirmed relationship between hPRL, hPRL-G129R and bcl-2 in multiple breast cancer cells (manuscript attached). On the other hand, we have exhausted our attempts in terms of producing hPRL-BP. In summary, we have demonstrated that hPRL-G129R is a true PRL receptor antagonist. It's anti-breast tumor effects were confirmed using both in vitro and in vivo assays. We are currently conducting pre-clinical studies of hPRL-G129R and hope to launch clinical phase studies.

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Introduction

Human breast cancer is the most predominant malignancy with the highest mortality rate in women from western society. Many risk factors have been identified for this disease. Several lines of evidence strongly linked human prolactin (hPRL) to breast carcinogenesis. In this proposal, two novel approaches have been designed to generate hPRL receptor specific antagonists. First approach is to adopt a site-directed mutagenesis strategy by which hGH receptor antagonist, hGH-G120R, was discovered, to produce a mutated hPRL, hPRL-G129R, and use it as hPRL receptor **blocker**. The other approach is to design and produce a soluble form of extra-cellular domain of hPRL receptor namely hPRL binding protein (hPRL-BP), and use it to **sequester** autocrine/paracrine effects of hPRL. After cloning of hPRL and hPRL-BP cDNAs, mutation will be made in hPRL cDNA to generate hPRL-G129R. Human PRL, hPRL-G129R and hPRL-BP cDNAs will be produced and purified using *E. coli* protein expression system. The purified proteins will then be used to test its bioactivities in multiple human breast cancer cell lines and two non-breast origin human cancer cell lines (as controls) for receptor binding, inhibition of phosphorylation of the STATs protein induced by hPRL (as an indicator for intracellular signaling), and inhibition of human breast cancer cell proliferation. We hope that these two novel approaches will ultimately result in generation of hPRL antagonists that could be used to improve human breast cancer therapy.

Body

There are two tasks proposed for the final year of this project.

---- finishing cell proliferation assays
---- summarizing data and manuscript preparation

Since we have completed a majority of the tasks related to this project in terms of development of a PRL antagonist, hPRL-G129R. Our focus of research in the past 12 month has been on (1) to further confirm the anti-tumor effects of hPRL-G129R *in vivo*; (2) to develop hPRL-G129R based, breast cancer specific therapeutics; and (3) to reach a conclusion on hPRL-BP project. We have demonstrated that hPRL-G129R is able to inhibit the growth of two human breast cancer cell

xenografts in nude mice (manuscript attached). In terms of designing hPRL-G129R based therapeutics, we have designed and demonstrated efficacy of dual functional protein (G129R-IL2, manuscript attached). We have also generated preliminary results regarding G129R-PE40 (abstracts attached). In summary, we have published three peer reviewed manuscripts and six abstracts (national meeting presentations) during this period.

A main negative result in this project is that we were unable to produce hPRL-BP in *E. coli* system despite many trials using different *E. coli* stains. We believe it probably has something to do with the codon usage or other unknown factors. We have considered to use eukaryotic expression system for this protein if we decided to continue this part of the project.

Key Research Accomplishment for the final Year.

We have further confirmed the antagonistic effects of hPRL-G129R using human breast cancer xenografts/nude mouse models (Chen et al., 2002). In our recent publications, we have confirmed that the inhibitory effects of hPRL-G129R is possibly through the inhibition of bcl-2 gene expression (Beck et al., 2002). We have explored possibility of creating novel fusion protein using G129R as a breast specific targeting molecule (Zhang et al., 2002).

Reportable Outcomes

Three manuscripts and four abstracts/meeting presentations (see appendices)

1. Chen NY, Li W, Cataldo L, Peirce S, and Chen WY. *In vivo* Anti-tumor Activities of a Human Prolactin Antagonist, hPRL-G129R. Int. J. Oncology 20:813-818, 2002.
2. Zhang GR, Li W, Holle H, Chen NY and Chen WY. A novel design of targeted endocrine and cytokine therapy for human breast cancer. Clin. Cancer Res., 8:1196-1205, 2002.
3. Beck MT, Peirce SK and Chen WY. Regulation of *bcl-2* gene expression in human breast cancer cells by prolactin and its antagonist, hPRL-G129R. Oncogene 21(33):5047-55, 2002.

ABSTRACTS (Presented at Endo Meeting 2002)

1. J. F. Langenheim, M.T. Beck, W.Y. Chen, From an antagonist back to an agonist: two wrongs do make a right. Endo 2002 (oral Presentation).

2. S. K. Peirce[1], M.T. Beck and W.Y. Chen. Regulation of bcl-2 expression by hPRL and its antagonist, hPRL-G129R, in human breast cancer cell lines. Endo 2002.

3. J.F. Langenheim and W.Y. Chen. Construction of human prolactin (hPRL) receptor targeting fusion toxins for breast cancer treatment using PRL antagonist and recombinant forms of pseudomonas exotoxin A. Endo 2002.

4. M.T. Beck and W.Y. Chen. Human prolactin antagonist and endostatin fusion protein for the treatment of breast cancer. Endo 2002 (Oral presentation and travel award).

Two Ph. D. students (Mike Beck and Susan Peirce) are at dissertation preparation stage of their graduate studies (partially supported through this award).

Conclusions:

In our final year of work, we have further confirmed that that hPRL-G129R acted as a true hPRL receptor antagonist in human breast cancer cells. We have also made considerable progress in terms of designing hPRL-G129R based potential therapeutics for breast cancer. This DoD idea award has brought opportunities to PI to fulfill his dreams of designing and testing novel anti-cancer drugs and actually see the ideas to become reality. The award also helped several graduate students in the lab to be able to complete their training in breast cancer research. The data generated through this award has resulted in several manuscripts and many abstracts; part of the additional data has been used to generate new proposals for future funding. The PI would like to extent his sincere thanks to DoD for this award.

Appendices

Three manuscripts and four abstracts

A Novel Design of Targeted Endocrine and Cytokine Therapy for Breast Cancer[1]

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ABSTRACT

The aim of this study is to combine endocrine therapy [human prolactin (hPRL) antagonist, G129R] and immune therapy [interleukin 2 (IL2)] in the design of a fusion protein, G129R-IL2, to treat human breast cancer. This novel approach uses the specific interaction between the G129R and hPRL receptors (PRLRs), thus directly targeting the fusion protein to the malignant breast tissues that have previously been shown to contain high levels of PRLR. The localized bifunctional fusion protein is designed to block signal transduction induced by hPRL as well as to activate T lymphocytes near the tumor site. A bacterial expression system was used to produce G129R-IL2 fusion protein that maintained both G129R and IL2 activities as demonstrated by cell-based assays such as signal transducer(s) and activator(s) of transcription (STAT)5 phosphorylation, breast cancer cell proliferation, and T-cell proliferation. The antitumor activities of G129R-IL2 were demonstrated *in vivo* using a syngeneic model system with BALB/c mice and EMT6-hPRLR breast cancer cells. After daily injection (i.p.) of G129R-IL2 (100 μg/mouse) for 18 days, the tumor growth in the G129R-IL2-treated group was only one-third the size as compared with that of the control group. The growth rate in the G129R-IL2-treated group is also significantly slower than that of the group treated with G129R alone (200 μg/mouse/day). We hope that this novel bifunctional protein will contribute significantly to human breast cancer therapy.

INTRODUCTION

One of the leading causes of cancer death in women is metastatic breast cancer. The etiology of breast cancer is complex, but its rarity among males suggests a role of female sex hormones (1, 2). In addition to estrogen, more and more evidence supports the notion that hPRL[3] is also intimately involved in breast cancer development (3–7). The following lines of evidence demonstrate the relationship between PRL and breast cancer: (a) PRL is synthesized by human breast cancer cells, which suggests its autocrine/paracrine role in the mammary gland (4); (b) PRLRs are up-regulated in the majority of malignant breast tissue (8); (c) PRL transgenic mice have high breast cancer rate (9); and (d) the inhibition of the binding of PRL to PRLR inhibits breast cancer cell growth (10).

hPRL is a single-chain, neuroendocrine, polypeptide hormone with 199 amino acids in its mature form. As a member of the GH family, PRL is primarily produced by the lactotrophs of the anterior pituitary gland in all vertebrates. The biological activities of PRL are mediated through specific membrane receptors known as PRLRs. The primary site of PRL action is 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 (11–15). The generation of PRL and *PRLR* gene knockout mice has unambiguously demonstrated that PRL and PRLR are key regulators in mammary development (12, 16).

In previous studies, Chen *et al.* (17–22) have developed a hGH antagonist by making a single amino acid substitution mutation at position 120 of the hGH molecule (hGH-G120R). The mutated hGH has been shown to block GH action both *in vitro* and *in vivo* (21) and has completed its Phase III clinical studies (23). By adopting a strategy similar to the development of the hGH antagonist, Goffin *et al.* (24) and our laboratory (10, 25–27) demonstrated that a single amino acid substitution mutation at position 129 of hPRL resulted in a hPRLR-specific antagonist (G129R). We have demonstrated that G129R is able to inhibit human breast cancer cell proliferation via the induction of apoptosis (10). G129R has also been shown to inhibit tyrosine phosphorylation of oncogene *STAT3* (26, 28) and to modulate transforming growth factor α/β levels in breast cancer cells (27). Furthermore, additive effects of hPRL-G129R and tamoxifen, which serves as an antiestrogen agent, have been

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[1] This work was supported in part by the Endowment Fund of the Greenville Hospital System and grants from the United States Army Medical Research Command (DAMD17-99-1-9129) and NIH-National Cancer Institute (1R21CA87093-01).
[2] To whom requests for reprints should be addressed, at Oncology Research Institute, Greenville Hospital System, 900 West Faris Road, Greenville, SC 29605. Phone (864) 455-1457; Fax (864) 455-1567; Email: wchen@ghs.org.

[3] The abbreviations used are: PRL, prolactin; hPRL, human PRL; CSS, charcoal-stripped serum; FBS, fetal bovine serum; GH, growth hormone; hGH, human GH; IL2, interleukin 2; hIL2, human IL2; PRLR, PRL receptor; IRMA, immunoradiometric assay; mAb, monoclonal antibody; STAT, signal transducer(s) and activator(s) of transcription; ATCC, American Type Culture Collection; RT-PCR, reverse transcription-PCR; TBS, Tris-buffered saline; MTS-PMS, (3-4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2H-tetrazolium, inner salt, phenazine methosulfate.

observed (10). Taken together, the ability of G129R to inhibit breast cancer cell proliferation, especially its additive effects with tamoxifen, makes it potentially valuable as a therapeutic agent for the treatment as well as prevention of breast cancer.

Tumor immune therapy has been of great interest for many years (29, 30). IL2 has been one of the main cytokines used for treating cancer. IL2, originally called T-cell growth factor, is a M_r 15,000 glycoprotein encoded by a single gene on chromosome 4 in humans (31). Characteristics of IL2 that make it attractive in cancer therapy include its ability to stimulate T lymphocytes as well as natural killer cells (32). However, one of the disadvantages in using IL2 is that patients systemically receiving IL2 often experience serious side effects that limit the amount of IL2 that can be administered. This limitation of dosage in turn directly affects the efficacy of treatment (33, 34).

In this study, we explore the possibility of fusing G129R with IL2 in the hope of generating a bifunctional protein that will have a dual therapeutic effect (targeted endocrine and cytokine) in the treatment of breast cancer. The targeting ability of this novel fusion protein uses the highly specific interactions between ligand (G129R) and receptor (PRLR). After G129R binds to PRLR, it not only blocks the signal transduction inducted by PRL but also localizes IL2 at the tumor site, which will play a crucial role in T lymphocyte activation, thus leading to tumor cytotoxicity. Because IL2 would be concentrated mainly in the breast cancer tissue, the severe side effects of IL2 would be greatly reduced.

MATERIALS AND METHODS

Cell Lines and Animals. To test the dual function of the fusion protein, human breast cancer cells (T-47D) and T cells (HT-2) were used for *in vitro* studies. T-47D human breast cancer cells were purchased from ATCC (Manassas, VA) and maintained in RPMI 1640 (Life Technologies, Inc. Inc., Rockville, MD) supplemented with 10% FBS and 50 μg/ml gentamicin. The HT-2 cell line, a murine T cell line requiring IL2 for growth, was also obtained from ATCC and cultured in RPMI 1640 containing 10% FBS, 200 IU of IL2 (kindly provided by Dr. Samuel Smith, Greenville Hospital System, SC), and other ATCC-recommended supplements. In addition, because of the nature of the fusion protein, a syngeneic animal tumor model (tumor cells paired with a immunocompetent host of identical genetic background) must be chosen as a study model. The EMT6 mouse mammary tumor cells, which originated from BALB/c mouse mammary carcinoma, were kindly provided by Dr. Rockwell, Yale University (New Haven, CT). After initial examination by RT-PCR, we found that the expression level of PRLR was nondetectable in these cells; therefore, a subline of EMT6 cells was generated in which full-length hPRLR cDNA was stably transfected using G418 selection as described previously (35). In a separate experiment, primers specific for mouse IL2 receptor were used to detect the IL-2 receptor expression. The results were negative (data not shown). The EMT6-hPRLR cells were maintained in DMEM (Life Technologies, Inc.) supplemented with 10% FBS (Life Technologies, Inc.) and 50 μg/ml gentamicin (Life Technologies, Inc.). All of the cell lines were grown at 37°C in a humid atmosphere in the presence of 5% CO_2.

The animals used for this study were 8–10-week-old female BALB/c mice (Jackson Laboratory; Bar Harbor, ME), which were housed in compliance with NIH guidelines.

Cloning of G129R-IL2 Fusion cDNA for *Escherichia coli* Expression. A two-step cloning procedure was used to generate a recombinant DNA encoding G129R fused to IL2. Primers corresponding to G129R (minus sequences encoding signal peptide and stop codon, and plus restriction sites of *Nde*I and *Bam*HI: 5′-*CAT ATG* TTG CCC ATC TGT CCC GGC-3′ and 5′-*GGA TCC* GCA GTT GTT GTT GTG GAT-3′) were used to amplify the G129R fragment from pCR3.1-G129R (10). Primers corresponding to hIL2 (minus sequences encoding signal peptide, and plus restriction sites of *Bam*HI and *Xho*I; 5′-*GGA TCC* GCA CCT ACT TCA AGT TCG-3′ and 5′-*CTC GAG* TTA AGT TAG TGT TGA GAT GAT-3′) were used to amplify the hIL2 fragment from hIL2 cDNA, purchased from ATCC. Both fragments were cloned into pCR2.1 TA cloning vector (Invitrogen, Inc., Carlsbad, CA) and sequenced. The fragments were reisolated by restriction digestion, purified, and ligated into the pET22b+ expression vector (Novagen, Madison, WI; Fig. 1).

Production and Purification of G129R-IL2. BL21 (DE3) cells (Novagen) were transformed with pET22b-G129R-IL2 using the calcium chloride method. An *E. coli* BL21 (DE3) seed culture (200 ml) carrying the pET22b-G129R-IL2 plasmid was grown overnight at 37°C and was used to inoculate 4 liters of L-broth (Bio 101, Carlsbad, CA) containing 100 μg/ml ampicillin (Fisher Scientific, Fair Lawn, NJ). The culture was grown at 37°C with agitation until the $A_{600\ nm}$ reached 0.9, at which time 1 mM isopropyl β-thiogalactoside (IPTG; Alexis Biochemicals, San Diego, CA) was added to induce expression of T7 RNA polymerase; the culture was incubated for an additional 3 h. The cells were then harvested by centrifugation at 6,000 × *g* for 5 min and resuspended in 0.2 M $NaPO_4$ (pH 8), 10 mM EDTA, 0.1 mg/ml lysozyme, and 0.5% Triton X-100, and incubated at 37°C for 1 h. The cells were disrupted by sonication using five 1-min pulses at 5 kHz applied with a Vibra-Cell Sonicator (Fisher Scientific). The insoluble inclusion bodies were recovered by centrifugation at 12,000 × *g* for 15 min at 4°C; resuspended in 0.2 M $NaPO_4$ (pH 7), 5 mM EDTA, 1 M urea, and 0.5% Triton X-100; recollected by centrifugation at 12,000 × *g* for 15 min; resuspended in 0.2 M $NaPO_4$ (pH 8), 8 M urea, and 1% v/v β-mercaptoethanol; and heated at 55°C for 10 min. Renaturation of the solubilized G129R-IL2 was performed by dialysis against decreasing concentrations of urea/TE buffer [20 mM Tris, 2 mM EDTA (pH 8.3)] for 4 days. The renatured protein was then filtered with 0.45 μm filters and purified using an anionic exchange column (Q-Sepharose) on a fast-performance liquid chromatography system (Amersham Pharmacia, Newark, NJ). The concentration of G129R-IL2 was determined using a hPRL IRMA kit (DPC, Inc., Los Angeles, CA), and its purity was determined via silver staining using the Silver Stain Plus kit (Bio-Rad Inc., Hercules, CA).

Verification of Fusion Protein Production via Western Analysis. Samples (200 ng) were analyzed using 4–15% SDS PAGE followed by Western blotting. After SDS-PAGE, the protein was transferred to ECL Hybond nitrocellulose (Amersham Pharmacia) at 16 W for 1.5 h. Blots were blocked with TBS containing 5% milk and 0.05% Tween 20 (blocking buffer)

for 30 min at room temperature; incubated overnight at 4°C in blocking buffer containing the appropriate antibody [IL2 antiserum, 1:200 (Santa Cruz Biotechnology Co., Santa Cruz, CA); hPRL antiserum, 1:1000 (Dr. Parlow, National Hormone & Pituitary Program, NIH, Bethesda, MD)]. The blots were then washed three times with TBS containing 0.05% Tween 20 (5 min/wash); and incubated in goat antirabbit secondary antibody (1:5000; Bio-Rad, Hercules, CA) for 1.5–2 h at room temperature with constant agitation. After secondary antibody incubation, membranes were washed three times with TBS-Tween-20 (5 min/wash); developed for 1 min using enhanced chemiluminescence reagents (Amersham Pharmacia) and captured on Kodak MR film (Fisher Scientific).

STAT5 Assay. Twenty-four h before protein extraction, T-47D cells were grown to confluency in 6-well plates containing RPMI 1640 supplemented with 10% CSS (Hyclone, Logan, UT). On the day of treatment, T-47D cells were depleted for 30 min in RPMI 1640 containing 0.5% CSS. The cells were treated for 20 min with the appropriate amount of hPRL (Dr. Parlow, National Hormone and Pituitary Program, NIH), G129R, or G129R-IL2, washed with ice-cold PBS (Life Technologies, Inc.), lysed with 200 μl of lysis buffer [50 mM Tris-HCl (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM Na_3VO_4] and incubated on an orbital rotator for 15 min. The lysate was transferred to 1.5-ml centrifuge tubes, gently passed through a 21-gauge needle five to six times to shear genomic DNA, and then placed on ice for 20 min. The lysate was centrifuged at 12,000 × *g* for 20 min at 4°C, and the supernatant was removed and stored at −20°C until ready for use.

Thirty-five μl of cell lysate (65–70 μg) was used for Western blotting analysis as described in the previous section using STAT5A + STAT5B antiserum (1:4000 dilution; UBI; Lake Placid, NY) or with anti-phospho-STAT5 antiserum (UBI) at a concentration of 1.5 μg/ml (26).

Radioreceptor Binding Assay. PRL receptor binding assays were performed on EMT6-hPRLR cells using T-47D human breast cancer cells as well EMT6 parental cells as controls, as described previously (10). Briefly, cells were grown in six-well tissue-culture plates until 90% confluent (~1×10^5 cells/well). Monolayers of cells were starved in serum-free RPMI 1640 for 0.5–1 h. The cells were then incubated at room temperature in serum-free RPMI 1640 containing 5×10^4 cpm ^{125}I- hPRL (specific activity, 30 μCi/μg; NEN Perkin-Elmer, Boston, MA) with or without 500 ng/ml hPRL. Cells were washed three times in serum-free RPMI 1640, lysed in 0.5 ml of 0.1 N NaOH/1%SDS, and the bound radioactivity was determined by scintillation counting. Total specific binding was calculated and compared.

EMT6-hPRLR Breast Cancer Cell Proliferation Assays. The assay conditions were modified from those described by Ginsburg and Vonderhaar (4). EMT6 cells were trypsinized and transferred to 96-well plates containing DMEM supplemented with 1% CSS. The optimal cell number/well for EMT6 cells was found to be 15,000 cells/well using titration assays. The cells were allowed to settle and adhere overnight (12–18 h), and various concentrations of hPRL, G129R, or G129R-IL2 were added. The cells were incubated for an additional 24 h at 37°C in a humidified 5% CO_2 incubator. After incubation, MTS-PMS solution (Cell Titer 96 Aqueous kit; Promega Corp., Madison, WI) was added to each well following the manufacturer's instructions, and the plates were read at 490 nm using a BIO-RAD benchmark microplate reader (Hercules, CA). All of the experiments were carried out in triplicate.

HT-2 Cell Proliferation Assay. Before each assay, HT-2 cells were washed three times in growth medium lacking IL2. The cells were counted, and ~5×10^3 cells were transferred to each well of a 96-well plate. Dose-response curves were obtained by varying the concentration of IL2, G129R-IL2, PRL, or G129R added to the HT-2 cells and incubating for 24 h at 37°C. Cell proliferation assays (MTS-PMS; Promega) were performed in triplicate using the same procedure described in the previous section.

***In Vivo* Studies of Antitumor Efficacy of G129R-IL2.** Two experiments were conducted to determine the antitumor efficacy of G129R-IL2 fusion protein using EMT6-hPRLR cells and a BALB/c female mouse model. In the first experiment, eight eight-week-old female BALB/c mice were inoculated s.c. with 1×10^6 EMT6-hPRLR breast cancer cells and randomized into two groups. One day after breast cancer cell inoculation, mice were injected (i.p.) with either 50 μg/mouse of G129R-IL2 or PBS every 24 h for 14 consecutive days. In the second experiment, 24 mice were given s.c. injections of 1×10^6 EMT6-hPRLR breast cancer cells and were randomized into four groups. One day after breast cancer cell inoculation, mice were given injections (i.p.) of PBS, G129R (200 μg/mouse), or G129R-IL2 (100 μg/mouse or 200 μg/mouse, respectively) for 18 consecutive days. At the end of the experiments, the tumors were dissected and weighed. It should be pointed out that the original experimental design included a group of animals that was treated with 20 μg of free IL2 mixed with 20 μg of G129R/mouse. Because of the toxicity of IL2 to the mice, the mice died and the experiment ended. The data are expressed as mean ± SE, and the Student *t* test was used to analyze the statistical difference between groups.

RESULTS

Construction of pET22b-G129R-IL2 Expression Vector. G129R-IL2 cDNA was cloned into the pET22b(+) expression vector as shown in Fig. 1. The G129R and IL2 cDNA sequences were found to be identical to those reported in GenBank, except for a single codon mutation (GGC to CGG), which resulted in Gly to Arg mutation at position 129 of hPRL (accession no. XM 033558). Two amino acids, Gly and Ser, were added at the junction of G129R and IL2 because of the addition of a *Bam*HI (GGATCC) restriction site for cloning purposes.

Production of G129R-IL2 Fusion Protein. The G129R-IL2 fusion protein was produced in the form of inclusion bodies. After refolding and ionic exchange column purification, the yield of fusion protein was ~2 mg/liter as determined by the Bradford protein assay and PRL IRMA analysis. The purified protein was analyzed by 4–15% SDS PAGE followed by silver staining (Fig. 2*A*), and the identity of the M_r 38,000 fusion protein was further confirmed by Western analysis using antiserum against hIL2 (accession no. XM 035511) or hPRL, respectively (Fig. 2*B* and 2*C*).

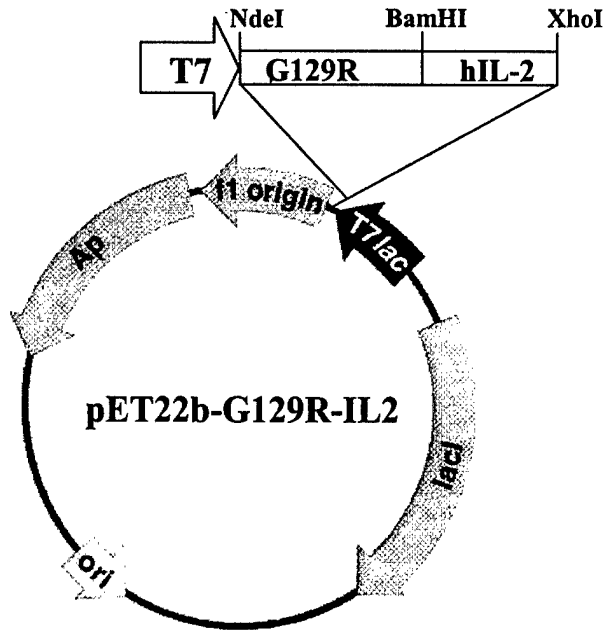

Fig. 1 Cloning and construction of the expression plasmid for G129R-IL2 production. PCR fragments amplified from G129R and hIL2 cDNAs were ligated into an *E. coli* expression vector, pET22b+, resulting in pET22b-G129R-IL2. A *Bam*HI restriction site was created between G129R and IL2 cDNAs for cloning purposes. The addition of the *Bam*HI site resulted in two extra amino acid residues (Gly and Ser).

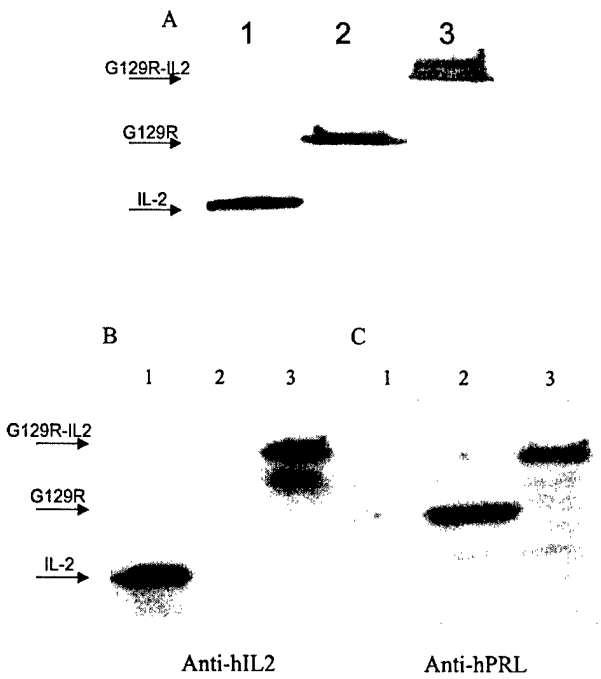

Fig. 2 Production and purification of G129R-IL2. *A*, silver staining of a SDS-PAGE gel. IL2 (*Lane 1*), G129R (*Lane 2*), or G129R-IL2 (*Lane 3*) were analyzed by 4–15% SDS-PAGE followed by silver staining (200 ng/lane). *B*, and *C*, Western blot analyses. IL2 (*Lane 1*), G129R (*Lane 2*), or G129R-IL2 (*Lane 3*) were analyzed by 4–15% SDS-PAGE (100 ng/lane) followed by Western blotting with either IL2 antiserum (*B*) or hPRL antiserum (*C*).

HT-2 Cell Proliferation Assay. An HT-2 cell proliferation assay was used to determine whether or not the IL2 portion of the fusion protein was functional. Fig. 3*A* demonstrated a dose response of IL2 in the proliferation of HT-2. The stimulatory effect of G129R-IL2 fusion protein on HT-2 cell proliferation was similar to that caused by IL2 alone (Fig. 3*B*). G129R or hPRL alone had no effect on HT-2 cell proliferation (Fig. 3*C*). The EC_{50} values for the G129R-IL2 and IL2 were ~1 ng/ml.

STAT Assay. Fig. 4*A* demonstrates a dose response of STAT5 phosphorylation in T-47D cells induced by hPRL. STAT5 phosphorylation was detected at a maximum level for 100 ng/ml hPRL (Fig. 4*A*). G129R (Fig. 4*B*) and IL2 (Fig. 4*C*), on the other hand, were inactive in this assay. To determine the antagonistic effects of G129R-IL2, T-47D cells were treated with a constant concentration of hPRL (100 ng/ml) and various concentrations of G129R or G129R-IL2 fusion protein (50 ng/ml to 1 μg/ml). It can be seen that at a 1:5 ratio (hPRL: G129R), STAT5 tyrosine phosphorylation is significantly decreased (Fig. 5*A*); and at a 1:10 ratio (hPRL:G129R), STAT5 tyrosine phosphorylation is almost completely inhibited (Fig. 5*A*). Fig. 5*B* demonstrates that G129R-IL2 fusion protein inhibits STAT5 phosphorylation induced by hPRL to nearly the same extent as G129R; therefore, the G129R portion of the fusion protein is functional.

Generation of EMT6-hPRLR Cells. The tumor cell line used for the *in vivo* studies was the EMT6 mouse mammary tumor cell line. Because this cell line has nondetectable PRLR mRNA as determined by RT-PCR (Fig. 6*A*, *Lane 2*), EMT6 cells were transfected with hPRLR cDNA to generate an EMT6-hPRLR stable cell line. Fig. 6*A* (*Lane 4*) shows the results of RT-PCR that demonstrate the expression of hPRLR mRNA in the EMT6-hPRLR cell lines. The hPRLR mRNA expression level in the EMT6-hPRLR cell line selected was still found to be much lower than that of T-47D cells (Fig. 6*A*).

The hPRL receptor status in EMT6-hPRLR cells was confirmed by using a radio receptor assay. The results of a direct comparison of the hPRL receptor-specific binding levels in the three breast cancer cell lines are shown in Fig. 6*B*. T-47D cells have the highest specific PRL receptor binding and EMT6 parental cells have close to zero binding. The EMT6-hPRLR cells demonstrate ~12% of specific binding. The results of receptor binding correlate well with the RT-PCR data.

Once the EMT6-hPRLR cell line was established, the effects of hPRL, G129R, and G129R-IL2 on cell proliferation of this cell line were investigated. When equal numbers of cells (15,000) were treated with 500 ng/ml hPRL, G129R, or G129R-IL2, the stimulatory effects were seen from only the cells treated with hPRL; whereas both G129R and G129R-IL2 demonstrated inhibitory effects on EMT6-hPRLR cell proliferation (Fig. 7*A*). More importantly, G129R or G129R-IL2 (1:10) competitively inhibited the proliferative effects induced by hPRL (Fig. 7*B*).

***In Vivo* Studies of the G129R-IL2 Fusion Protein.** Pharmacokinetic studies of G129R-IL2 were first conducted to determine the effective dose needed. Eight-week-old female BALB/c mice were given injections i.p. of either 25 μg/mouse

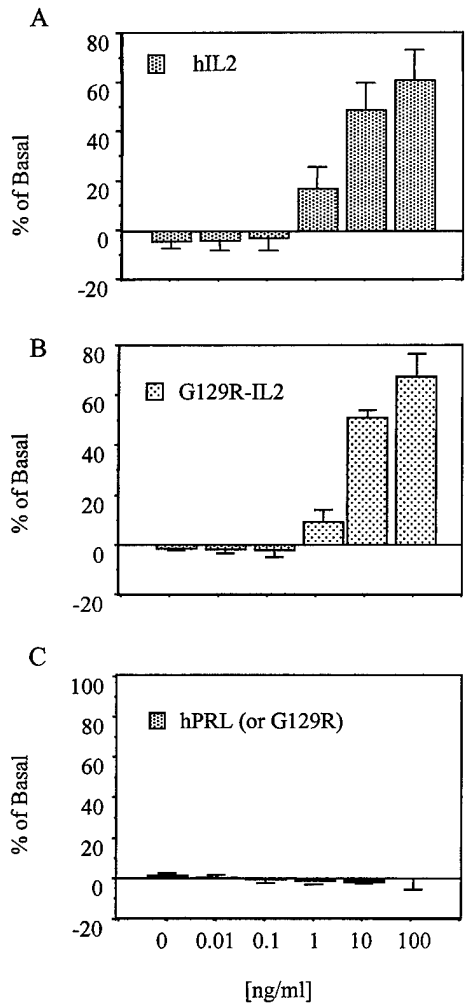

Fig. 3 HT-2 cell proliferation assay in response to IL2, G129R, or G129R-IL2. *A*, dose-response effects of HT-2 cells on hIL2. *B*, dose-response effects of HT-2 cells on G129R-IL2; *C*, HT-2 cell incubated with either hPRL or G129R.

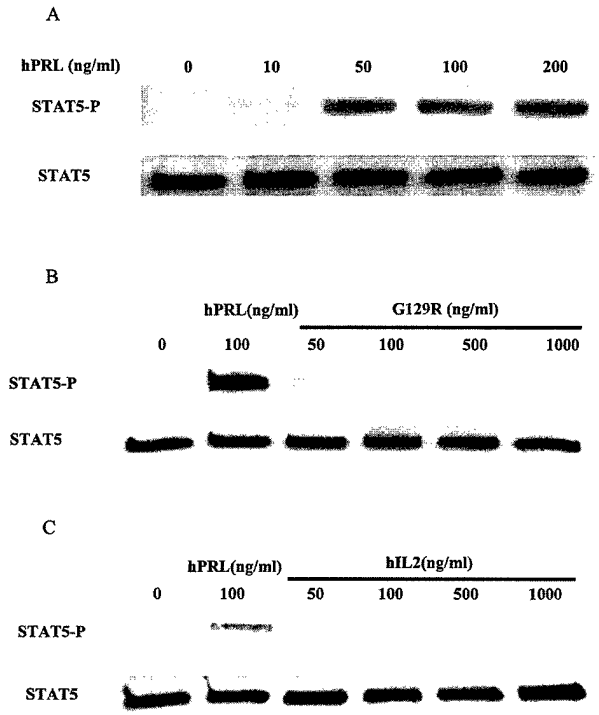

Fig. 4 Stimulation of STAT5 phosphorylation by hPRL. T-47D human breast cancer cells were treated with the indicated concentrations of hPRL, G129R, or IL2. Total protein was extracted from treated cells and analyzed via 4–15% gradient SDS-PAGE, followed by Western blotting with antiserums against either STAT5 or Phospho-STAT5 as indicated in each panel. *A*, dose-response effects of hPRL on STAT5 phosphorylation; G129R (*B*) and IL2 (*C*) are inactive in this assay.

(n = 4) or 50 μg/mouse (n = 3) of the G129R-IL2 fusion protein. After injection, serum samples were collected via tail vein bleeding at 2, 6, and 24 h. The concentration of the fusion protein was assayed by the hPRL IRMA kit (DPC, Inc.). Fig. 8 shows that the serum G129R-IL2 concentration 24 h after injection was ~20 ng/ml This finding is somewhat surprising because hPRL or G129R had a serum half-life of ~2 h and the half-life of IL2 was even shorter. We normally could not detect G129R 24 h after injection. With these encouraging results, it was decided that mice that bore mammary tumor cells would be treated every 24 h.

In our preliminary animal studies, eight female BALB/c mice were inoculated s.c. with 1×10^6 EMT6-hPRLR breast cancer cells and randomized into two groups. Each animal then received daily injections of G129R-IL2 (50 μg/mouse). We found that the serum concentration of fusion protein was maintained at ~30 ng/ml, which reduced the growth of EMT-6-PRLR in mice (115 ± 55 mm^3 *versus* 238 ± 75 mm^3 in control group). Although because of small sample numbers, no statistical difference could be found in tumor volume nor in final tumor weights between the two groups, it provided dose reference for our main animal studies.

Twenty-four female BALB/c mice were inoculated s.c. with 1×10^6 EMT6-hPRLR breast cancer cells and randomized into four groups. Fig. 10 demonstrates that the tumor growth was similar between the groups treated with G129R (241 ± 45 mm^3; 200 μg/day/mouse) and a high dose of G129R-IL2 (223 ± 41 mm^3; 200 μg/day/mouse); however, mice that were given injections of 100 μg of G129R-IL2 showed the best response, in which the average tumor volume was approximately one-third of that in the control group (125 ± 25 mm^3 *versus* 305 ± 55 mm^3).

DISCUSSION

Recent advances in the understanding of the immune system and in defining tumor antigens have motivated the development of many new strategies using immune therapy in cancer treatment (36–38). There is ample evidence that cancers express tumor-specific antigens and that hosts have T cells that can respond to these antigens (39, 40). However, it is likely that tumor cells are poor antigen-presenting cells because they do not provide second signals, which are needed for full T-cell activation (40). Therefore, the major effort in tumor immune

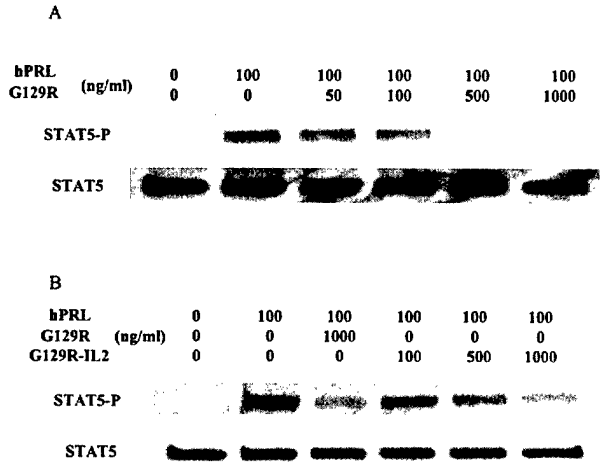

Fig. 5 Inhibition of STAT5 phosphorylation by G129R or G129R-IL2 in T-47D human breast cancer cells. T-47D cells were treated with the indicated concentrations of hPRL, G129R, and G129R-IL2 or with combination as indicated. Total protein was extracted from cells and analyzed via 4–15% gradient SDS-PAGE followed by Western blotting with antiserums against either STAT5 or Phospho-STAT5 as indicated in each panel. *A*, the competitive inhibition of STAT5 phosphorylation by G129R. *B*, the competitive inhibition of STAT5 phosphorylation by G129R-IL2.

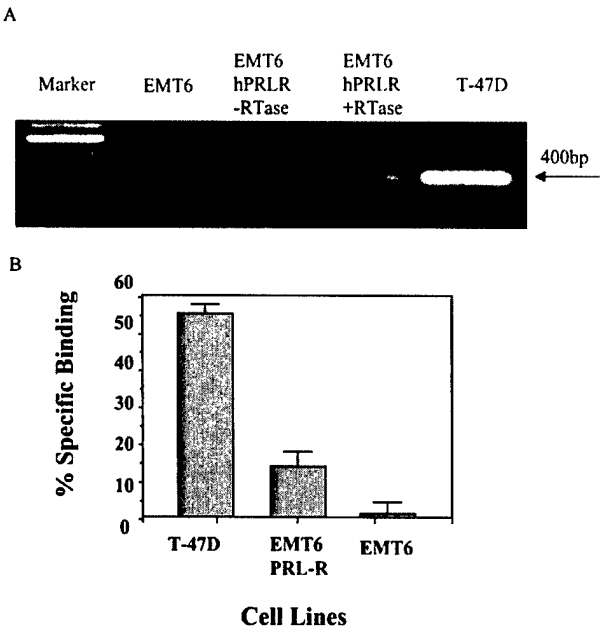

Fig. 6 Confirmation of the expression of hPRLR in EMT-6-hPRLR cells. *A*, RT-PCR analysis of hPRLR mRNA level using total RNA isolated from EMT6 or EMT6-hPRLR cells. RT-PCR products were analyzed on a 1% agarose gel as indicated. *Arrow*, a 400-bp fragment. *B*, results of radioreceptor binding assay on three breast cancer cell lines. Specific binding of PRL recepter was measured using the formula: [(cpm of total binding − cpm of nonspecific binding)/cpm of total binding] × 100.

therapy is focused on how to augment weak host immune responses to tumor antigens, such as exogenously administering cytokines to the patients. Among the many cytokines used, IL2 has been demonstrated to yield promising results (36–38).

IL2 is the principal cytokine responsible for the progression of T lymphocytes from the G_1 to S phase of the cell cycle. It is mainly produced by CD4+ T cells and in smaller quantity by CD8+ T cells (41). With the help of recombinant DNA technology, recombinant hIL2 has been used *in vivo* to treat patients with advanced renal cell carcinoma and melanoma (33, 34). The aim of such an approach is to generate tumor-reactive lymphocytes in cancer patients. However, it has been reported that cancer patients receiving systemic hIL2 often experience potentially life-threatening side effects that limit the total amount that can be administered, which in turn directly affects the efficiency of treatment (33, 34). The major efforts regarding the use of IL2 in tumor therapy, therefore, have been concentrated on how to balance the side effects and the effective dose. By increasing the specificity of administered IL2 (via the targeting of IL2 precisely to the tumor sites), it is possible to dramatically increase the therapeutic effects of hIL2 while significantly decreasing its side effects.

Recently an alternative approach for using the binding specificity of antitumor mAbs to direct cytokines to tumor sites has been introduced (40–46). This novel approach combines the unique targeting ability of mAbs with the activities of cytokines and, therefore, achieves an effective concentration of IL2 in the tumor microenvironment. The targeted IL2 therapy has been shown to be able to completely eradicate disseminated pulmonary and hepatic murine melanoma metastases in immunocompetent syngeneic mice (42, 43) and has also generated promising clinical results (47). These findings demonstrate that targeted IL2 can provide an effective tool in cancer immunotherapy and establish the missing link between T-cell-mediated cytotoxicity and objective clinical response. There are several obvious advantages of this targeted IL2 therapy. First, an mAb-IL2 fusion protein does not have to reach all of the target cells to achieve the maximum effects because it is not a direct cytotoxic reaction (46, 48). Second, it has been shown that the induction of a cellular immune response using the mAb-targeted IL2 approach facilitates the eradication of established s.c. melanoma metastases, even if the tumor displays substantial antigen heterogeneity (47). Most importantly, the therapeutic effect of targeted IL2 therapy is associated with the induction of a long-lived and transferable, protective tumor immunity. In addition, this mAb-targeted IL2 therapy is also different from, and advantageous to, the *ex vivo* transfer of cytokine genes because it concentrates IL2 in the tumor environment in a nonpersonalized way that makes this approach clinically more feasible (42–50).

In our previous studies, we have demonstrated that G129R was able to inhibit breast cancer cell proliferation via the induction of apoptosis both *in vitro* (10, 26–27) and *in vivo* (25). In this study, we used a strategy similar to that of mAb-IL2 to design a novel G129R-IL2 fusion protein that is targeted specifically to human breast cancer. The targeting ability of this novel fusion protein involves the highly specific interactions between the ligand (G129R) and receptor (PRLR), therefore, concentrating IL2 at the cancerous breast tissue in which PRLR levels have been shown to be elevated (8). We hypothesized that once the G129R-IL2 fusion protein reaches the malignant mam-

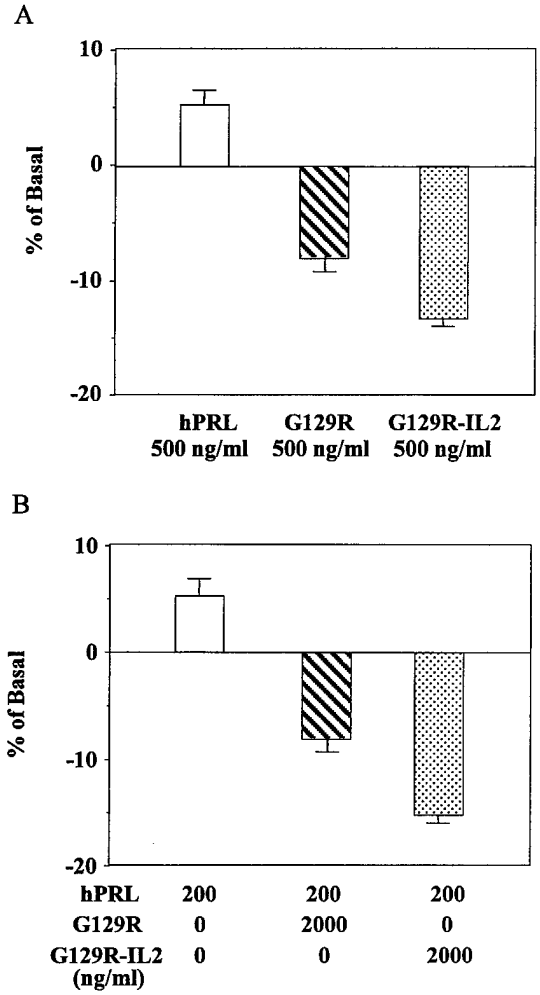

Fig. 7 Inhibition of EMT6-hPRLR cell proliferation by G129R or G129R-IL2. EMT6-hPRLR cells were treated with hPRL, G129R, G129R-IL2, or in combination as indicated. In *A*, PRL induces cell proliferation of EMT6-hPRLR cells, whereas both G129R and G129R-IL2 have inhibitory effect on the proliferation of EMT6-hPRLR cells. In *B*, G129R or G129R-IL2 is able to competitively inhibit the stimulatory effect of hPRL on EMT6-hPRLR cells. The inhibitory effect of G129R-IL2 is significantly greater than that of G129R ($P < 0.05$).

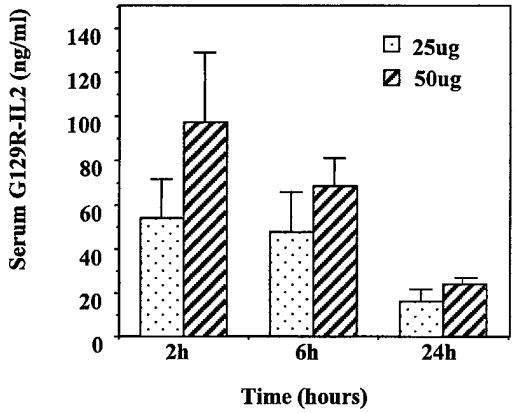

Fig. 8 Pharmacokinetic studies of G129R-IL2 in Balc/c mice. BALB/c mice were given injections (i.p.) of either 25 μg or 50 μg of G129R-IL2, and serum samples were collected via tail vein bleeding at time intervals indicated. The serum concentration of G129R-IL2 was determined via the hPRL IRMA kit.

mary tissues, it will elicit dual therapeutic effects: the G129R portion of the fusion protein will specifically block PRLR, inhibiting the autocrine/paracrine effects of endogenous PRL; and the IL2 portion of the fusion protein may elicit a T-cell-mediated antitumor cytotoxicity reaction *in situ*, as in the case of mAb-IL2 studies.

To express the G129R-IL2 fusion protein, several different cloning strategies were used. Eukaryotic expression systems were not effective and resulted in very low yields, which made it impractical for *in vivo* studies. Ultimately, the bacterial expression vector pET22b+ was used to produce relatively large quantities of the G129R-IL2 fusion proteins. although the yield was far from ideal when compared with the yield of G129R. The low yield of production may be, in part, attributable to the presence of five pairs of Cys residues (three pairs in hPRL and two pairs in hIL2) in this novel protein. Only a small portion of the protein was found to be able to refold properly and eluted from Q-Sepharose columns in low-salt fractions (0.15 mM NaCl). G129R-IL2 fusion protein in these fractions is fully active in cell-based assays. More than 60% of the fusion protein eluted from the Q-Sepharose columns in high-salt fractions (>1 M NaCl) was nonfunctional as tested by STAT5 and HT-2 assay. We believe that proteins in the high-salt fractions represent fusion protein with mismatched disulfur bonding, which results in nonfunctional conformations.

The HT-2 proliferation analysis and STAT assays indicated that properly refolded G129R-IL2 fusion protein retained its IL2-like activity, namely stimulation of T-cell proliferation, as well as G129R-like activities, namely inhibition of STAT5 phosphorylation and inhibition of breast cancer cell proliferation. Although the fusion protein was functional *in vitro*, the real challenge was to determine whether this fusion protein could function *in vivo*. Pharmacokinetic results indicated that the blood clearance of G129R-IL2 fusion protein is much slower than that of either G129R alone or IL2 alone. The serum concentration of G129R-IL2 remained at 20–30 ng/ml after daily injection (50 μg/mouse/day). These data are very significant because previous studies have shown that the half-life of G129R or hIL2 are less than 2 h because of small molecular sizes. Moreover, the serum concentration of G129R or IL2 was not detectable 24 h after injection with a dose up to 200 μg/mouse. We believe that the significantly prolonged serum half-life of G129R-IL2 could not be explained merely by the increase in size of the fusion protein. It was reported that IL2 is able to bind to α-macroglobulin in serum (51), therefore, prolonging its serum half-life. This unique feature of IL2 might help to prolong the half-life of the G129R-IL2 fusion protein.

The concentration of G129R-IL2 used in our *in vivo* studies was similar to the dose used in hGH antagonist clinical studies (5–10 mg/kg of body weight) and is also in the range of G129R used alone in our recent *in vivo* studies with human breast cancer cell xenografts in nude mice (25). It is noteworthy that the concentrations of fusion protein used in our *in vivo* studies

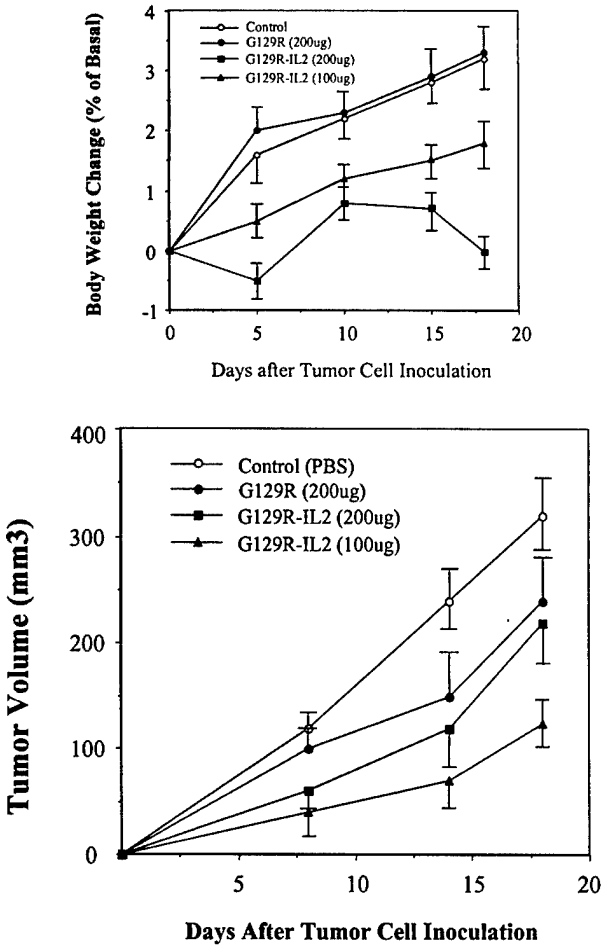

Fig. 9 Inhibition of EMT6-hPRLR cell growth *in vivo* by G129R-IL2. Twenty-four Balb/C mice were given injections of 1×10^6 EMT6-hPRLR cells. After tumor inoculation, mice were randomized into four groups and treated with PBS, G129R (200 μg/mouse/day), or G129R-IL2 (100 μg/mouse/day or 200 μg/mouse/day) for 18 consecutive days. Tumor volumes were calculated by the following equation: [(short dimension^2) × (long dimension)]/2. *Parentheses*, the average body weight of each group.

are considered highly toxic when the molar concentration of its IL2 portion is considered. In our preliminary studies, IL2 (20 μg/mouse/day) i.p. injection has proved very toxic. One animal died 3 days after i.p. injection, the remaining three were extremely ill, and the experiment was terminated. These results strongly suggest that the pharmacodistribution pattern of G129R-IL2 is different from that of free IL2 despite the prolonged serum exposure to the fusion protein. One explanation of the lower toxicity observed in G129R-IL2 fusion protein is that it binds to the PRLR and, therefore, is concentrated quickly in tissues with high levels of PRLR, thus decreasing systemic exposure of IL2. An alternative explanation is that G129R-IL2 acts differently at the receptor level (although G129R-IL2 is able to stimulate HT-2 proliferation) as compared with free IL2, because the molecular size is more than doubled. However, we noticed that the tumor-inhibitory effect of the fusion protein in the group of 100-μg/day/mouse is better than that of the 200-μg/day/mouse group. We believe that the discrepancy between these results is attributable to the toxic reaction caused by the high dose of G129R-IL2 (200 μg/day/mouse). This speculation was supported by the observation that there is a body weight loss during the treatment period in the high-dose group (Fig. 9).

We also directly compared the inhibitory effects of G129R and G129R-IL2 in cancer cell proliferation assay (Fig. 7) as well as the growth of xenografts (Fig. 9). In both cases, G129R-IL2 showed stronger inhibitory effects than G129R alone. We assume that the better *in vivo* results are attributable to the effects of targeted IL2 and prolonged serum half-life of G129R, although additional studies regarding the immune response *in vivo* after the administration of G129R-IL2 are needed. We do not have a good explanation for the difference between G129R and G129R-IL2 in the inhibition of EMT6-hPRLR cell proliferation. We speculate that G129R-IL2 is probably more stable in cultured media as compared with G129R and, therefore, results in better inhibitory effects.

In conclusion, the data presented here demonstrate that the fusion of G129R and IL2 results in a novel, bifunctional protein, G129R-IL2. This novel fusion protein is able to act as a PRLR antagonist as well as a T-cell growth factor. With a relatively long serum half-life, daily injection of G129R-IL2 at a dose of 100 μg/mouse resulted in significant inhibition of breast tumor growth *in vivo*. Additional *in vivo* studies regarding the fusion protein's biological activities using natural breast cancer cells are needed to evaluate its bifunctional properties. We believe that this targeted endocrine-immune design provides a novel and effective approach to human breast cancer treatment.

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Regulation of *bcl-2* gene expression in human breast cancer cells by prolactin and its antagonist, hPRL-G129R

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To gain insight into the molecular basis of human prolactin (hPRL) antagonist induced apoptosis, we compared the differential gene expression profile of four human breast cancer cell lines following treatment with hPRL and its antagonist (hPRL-G129R). Among the genes identified, the *bcl-2* gene was of particular interest. We found that *bcl-2* mRNA was up regulated in three of the four cell lines that were treated with hPRL. To further confirm these results, real time RT–PCR and ELISA analyses were used to detect *bcl-2* mRNA and Bcl-2 protein, respectively, in 11 different breast cancer cell lines after hPRL or hPRL-G129R treatment. Our data suggests that Bcl-2 is up-regulated in response to hPRL stimulation and is competitively inhibited by hPRL-G129R in the majority of the cell lines tested. Thus, we propose that the anti-apoptotic role of hPRL in breast cancer is mediated, at least in part, through regulation of Bcl-2.
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Keywords: prolactin; prolactin antagonist; Bcl-2; breast cancer; apoptosis

Introduction

Human PRL is a neuroendocrine polypeptide hormone primarily produced by the lactotrophs in the anterior pituitary gland of vertebrates. It is well established that hPRL is directly involved in the development and differentiation of normal mammary gland in mammalian species (Blackwell and Hammond, 1999; Clevenger *et al.*, 1995; Nagasawa *et al.*, 1985; Topper and Freeman, 1980; Vonderhaar, 1998). Controversy, however, still exists regarding the role of hPRL in human breast cancer. Emerging evidence links hPRL to human breast cancer including: (a) the detection of biologically active hPRL in human breast cancer cells, which suggests that hPRL is produced locally as an autocrine/paracrine growth factor within the mammary gland (Clevenger *et al.*, 1995; Ginsburg and Vonderhaar, 1995; Goffin *et al.*, 1996, Goffin and Kelly, 1997; Vonderhaar, 1999); (b) PRL receptor (PRLR) levels are significantly higher in human breast cancer cells than in normal breast epithelial cells (Kelly *et al.*, 1991); (c) transgenic mice over expressing hPRL have a higher breast cancer incidence (Wennbo *et al.*, 1997) and (d) the hPRL antagonist, hPRL-G129R, slows the growth rate of human breast cancer xymographs in nude mice (Chen *et al.*, 2002). These examples support hPRL's role as a mitogen in human breast cancer and suggest that its antagonist may have potential in treating human breast cancer.

Apoptosis plays a critical role in the regulation of cells that are either in a normal or cancerous state of growth. Key regulators that control apoptosis are kept highly controlled by the cells' internal machinery. One of the first and most widely studied regulators of apoptosis to be identified was Bcl-2, which is now known to be a part of a family of related proteins (Adams and Cory, 1998). *bcl-2* is a human proto-oncogene that when overexpressed, will ultimately lead to the inhibition of cell death (Korsmeyer, 1999). It suppresses apoptosis by blocking the release of cytochrome *c*, a major component of cellular respiration, from the mitochondria, thus preventing the activation of caspases, a group of proteases that carry out the process of cell death (Kumar *et al.*, 2000; Yin *et al.*, 1994). In human breast cancer cells, Bcl-2 and Bax, the inhibitor of Bcl-2, are constitutively expressed to tightly regulate apoptosis (Adams and Cory, 1998; Binder *et al.*, 1996; Kumar *et al.*, 2000; Yin *et al.*, 1994). One of many factors leading to breast malignancy is the up-regulation of *bcl-2* gene expression, ultimately resulting in the inhibition of apoptosis (Green and Beere, 1999). There are numerous molecules that can regulate Bcl-2. For example, IL-3 has been shown to increase the expression of *bcl-2* in hematopoietic cell lines (Krumenacker *et al.*, 1998). Studies using Nb2 cells, a rat lymphoma cell line, show that *bcl-2* is up-regulated in immortalized cell lines (Krumenacker *et al.*, 1998; Leff *et al.*, 1996). One of the more relevant studies involving *bcl-2* demonstrated that treatment of Nb2 cells with PRL results in *bcl-2* up-regulation and *bax* down-regulation (Krumenacker *et al.*, 1998). However, there have been no definitive studies linking hPRL to Bcl-2 activity in human breast cancer cells.

The identification of specific genes that are differentially expressed in response to exogenous treatments has been a subject of great interest to many researchers in the past. There are several methods to compare gene

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expression patterns in tissue cells, such as representational difference analysis, differential display, cDNA array hybridization and serial analysis of gene expression (DeRisi *et al*., 1996; Guiliano *et al*., 1999; Hakvoort *et al*., 1994; Oh *et al*., 1999; Zhan *et al*., 1997). All these methods are able to detect different gene expression profiles, but a newly described technique called suppression subtractive hybridization (SSH) offers additional advantages (Kuang *et al*., 1998; Yang *et al*., 2000). Briefly, SSH first uses mRNA from two populations of cells and converts them into cDNA. The cDNA from cells that contain differentially expressed genes is referred to as the 'tester' and the reference cDNA is referred to as the 'driver'. Both 'tester' and 'driver' cDNAs are first digested using a 4 base-cutter restriction enzyme to create shorter blunt-ended molecules. The ends of the tester cDNAs are modified by ligating adaptors that will serve as PCR primers. The 'tester' cDNAs are then hybridized with 'driver' cDNAs, which have no adaptors on their ends. Suppression PCR, using the adaptors as primers, is then performed to allow exponential amplification of the differentially expressed genes. SSH allows investigators to identify which genes are being turned on or off in one cell type versus another more quickly and easily than other techniques. It is also possible to compare expression profiles of the same cell line by treating a group of cells with a specific compound and using an untreated group as the control. This variation of SSH allows investigators to understand which genes are being expressed in response to a specific treatment of choice. SSH is valuable because it includes an amplification step and selection step that other methods do not, thus increasing the levels of differentially expressed genes while decreasing the levels of housekeeping genes that result in unnecessary background. The introduction of the cDNA microarray makes it possible to identify genes in a much more efficient manner. This emerging technique has proven to be an essential tool when attempting to identify which genes are responding to a certain condition (Oh *et al*., 1999; Yang *et al*., 2000). By combining these two methods it is possible to obtain and identify differentially expressed genes with precision (Beck *et al*., 2001).

In this study, we examined the profile of apoptosis related genes expressed by four human breast cancer cell lines upon treatment with either hPRL or hPRL-G129R. It was found that *bcl-2* gene expression was increased following treatment of breast cancer cells with hPRL in both estrogen receptor (ER) positive cell lines and one of two ER negative cell lines tested. To confirm the evidence linking hPRL and Bcl-2, a quantitative method of RT–PCR and a Bcl-2 ELISA were used to measure both *bcl-2* mRNA expression levels and protein levels in 11 human breast cancer cell lines after treatment with hPRL or hPRL-G129R. The data from these studies suggests that hPRL acts as an apoptosis inhibitor by increasing the expression of Bcl-2 in human breast cancer and that hPRL-G129R competitively inhibits Bcl-2 induction by hPRL.

Results

Profile of apoptosis genes in response to hPRL or hPRL-G129R in four human breast cancer cell lines

Comparisons of the relevant apoptosis related genes expressed in human breast cancer cells are shown in Figure 1. hPRL-G129R treated T-47D and MCF-7 cells shown in Figure 1a,c and hPRL treated in Figure 1b,d, respectively. It appears that in both cases the only gene that was up regulated is *bcl-2* (10-F). In Figure 1a, T-47D cells treated with hPRL-G129R exhibited a strong up-regulation of caspases, such as caspases-3 (11-A), -4 (11-F), -7 (11-I), -9 (11-L) and -10 (11-M). The Bcl-2 binding protein, $BNIP_3$, was expressed in all but the MDA-MB-468 after hPRL-G129R treatment (Table 1). In the T-47D cells, the genes related to death receptors such as serine-threonine kinase 1 (12-H), DAXX (12-I), tumor necrosis factor-related apoptosis inducing ligand (14-F) and death domain receptor 3 (15-G) were up-regulated after hPRL-G129R treatment, although the gene for caspase-8 (11-J and 11-K), which is normally associated with death receptors, has not. In MCF-7 cells the gene *BAD* (10-O in Figure 1c), an important member of the Bcl-2 family of proteins, was differentially expressed after hPRL-G129R treatment. There was no evidence of caspase expression in MCF-7 cells after hPRL-G129R treatment.

Table 1 summarizes all apoptosis related differentially expressed genes in T-47D, MCF-7, BT-549 and MDA-MB-468 cells treated with either hPRL or hPRL-G129R that were probed on an apoptosis microarray. To our knowledge, this is the first time that a list of apoptosis related differentially expressed genes has been compiled for these four breast cancer cells after treatment with hPRL and its antagonist.

Quantitative RT–PCR measurement of bcl-2 mRNA in 11 human breast cancer cell lines

To confirm that hPRL induced the expression of *bcl-2*, quantitative real time RT–PCR was used. Figure 2a represents direct real time RT–PCR output from T-47D cells treated with either hPRL or hPRL-G129R and compared to the untreated control. *bcl-2* message levels were clearly elevated in the hPRL treated samples as indicated by the amplification curves shift to the left and decrease in the hPRL-G129R treated samples as indicated by the amplification curves shift to the right, relative to untreated samples. All samples were normalized to equivalent levels of β-actin mRNA (Figure 2b). Table 2 represents the *bcl-2* levels from multiple quantitative real time RT–PCR runs relative to normalized levels of β-actin. The data is presented as levels of *bcl-2* in all 11 cell lines treated with hPRL, hPRL-G129R or a combination of hPRL and hPRL-G129R, and were compared to levels of *bcl-2* in the untreated controls. The responses are graphed as the per cent change of the experimental response to the untreated control $\pm$ s.e. and shown in Figure 3. In MCF-7 and MDA-MB-134 cells, hPRL treatment

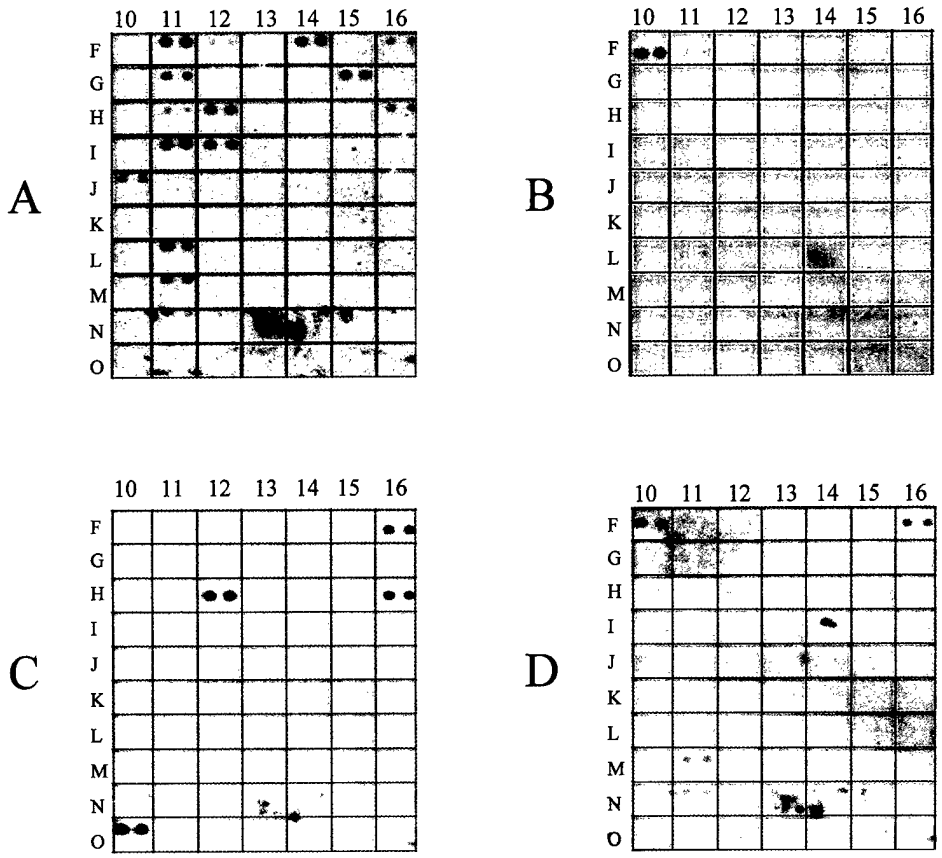

Figure 1 Representation of SSH and microarrays. Three micrograms of DIG labeled differentially expressed cDNAs were probed onto each microarray and detected by means of chemiluminescence. T-47D cells treated with hPRL-G129R (**a**) and hPRL (**b**) and MCF-7 cells treated with hPRL-G129R (**c**) and hPRL (**d**) are shown as a representative of the microarrays

resulted in a highly significant ($P<0.01$) up-regulation of *bcl-2* message, while in BT-549 and T-47D cells the per cent change was significant ($P<0.05$). In the remaining seven cell lines, *bcl-2* message levels were not significantly different from the untreated controls. Treatment with the antagonist resulted in significantly ($P<0.05$) decreased expression of *bcl-2* message in four of the cell lines (MCF-7, T-47D, BT-549 and MDA-MB-157) with no significant change in the other cell lines. A modest increase in *bcl-2* message expression was observed in four cell lines (MDA-MB-436, MDA-MB-468, MDA-MB-231, BT-483) following hPRL-G129R treatment. In seven cell lines a combination treatment of hPRL-G129R and hPRL resulted in lower levels of *bcl-2* expression than the hPRL treatment alone (MCF-7, T-47D, MDA-MB-134, MDA-MB-453, BT-474, MDA-MB-231 and BT-483). The combination treatment significantly ($P<0.05$) reduced *bcl-2* expression levels in MCF-7 and T-47D cell lines, whereas MDA-MB-134, MDA-MB-453, BT-474, MDA-MB-231 and BT-483 cells show an insignificant decrease in the level of bcl-2.

Overexpression of Bcl-2 protein in various cell lines

To further confirm that Bcl-2 was upregulated in human breast cancer cells, a Bcl-2 ELISA was performed on all 11 cell lines. Data is presented as per cent change of Bcl-2 levels in cells treated with hPRL (100 ng/ml) or in cells treated with hPRL-G129R (500 ng/ml) and the combination of hPRL (100 ng/ml) and hPRL-G129R (500 ng/ml) over cells with no treatment. Figure 4a illustrates that six of the 11 human breast cancer cell lines tested showed a highly significant increase ($P<0.01$) in Bcl-2 protein levels after treatment with hPRL. T-47D, MDA-MB-157 and MDA-MB-134 demonstrated the highest levels of Bcl-2 with an increase of approximately 175% over untreated cells. MCF-7, BT-549 and MDA-MB-483 all exhibited levels of Bcl-2 with an increase of approximately 125% over the untreated cells. MDA-MB-468, MDA-MB-453 and BT-474 demonstrated a less significant ($P<0.05$) per cent change of Bcl-2 levels after treatment with hPRL. The remaining two cell lines, MDA-MB-436 and MDA-MB-231, did not demonstrate a significant level of Bcl-2 increase after treatment with hPRL. It is clear from Figure 4b that upon treatment with hPRL-G129R (500 ng/ml), there was a highly significant ($P<0.01$) decrease in the levels of Bcl-2 in all 11 cell lines. The combination treatment of hPRL and hPRL-G129R demonstrated a highly significant ($P<0.01$) decrease of Bcl-2 protein in nine of the 11 cell lines (Figure 4c). MDA-MB-436 and BT-

Table 1 Differentially expressed gene profile for four human breast cancer cell lines

	Cell lines and their treatments							
	(PRLR+ and ER+)				*(PRLR+ and ER−)*			
	T-47D		*MCF-7*		*BT549*		*MDA-MB-468*	
Genes	*hPRL*	*G129R*	*hPRL*	*G129R*	*hPRL*	*G129R*	*hPRL*	*G129R*
Cell cycle-regulating proteins and kinases								
Cell division cycle (CDC)-like kinase 1						+		
Serine/threonine protein kinase 1	+		+					
Cyclin dependent kinase (CDK)-G2	+						+	
CDK 4 inhibitor 2D						+		
CDC10 protein homolog	+			+	+		+	
Ubiquitin-conjugating enzyme E2						+		
CDC16HS		+						
MAP kinase 3			+				+	
MAP kinase 1					+			
MAPKK 1		+				+		
MAPKK 5							+	
MAPKK 10							+	
Peptidyl-prolyl cis-transisomerase nima-interacting 1					+			
Retinoblastoma-binding protein 4					+		+	
E2F dimerization partner 1								
Bcl-2 family proteins and caspases		+						
B-cell lymphoma protein 2 (Bcl-2)	+		+		+			
Bcl-2-associated death promoter (BAD)				+		+		+
Bcl-2 binding protein ($BNIP_3$)		+		+		+		
BAK1								+
BID3		+						
Caspase-3		+				+		
Caspase-4		+						
Caspase-7		+				+		+
Caspase-8								+
Caspase-9		+						
Caspase-10		+						
Death receptors/ligands and apoptosis associated proteins								
TNF receptor 1 associated death domain protein				+		+		
TNF receptor-associated factor 6						+		
Death receptors/ligands and apoptosis associated proteins								
Receptor interacting protein (RIP)		+		+		+		+
DAXX		+						+
TNF-alpha converting enzyme		+						
TNF-related apoptosis inducing ligand		+						
Caspase Death Domain								+
Death domain receptor 3		+						
Insulin-like growth factor-binding protein 2 (IGFBP-2)	+			+				
IGFBP-4				+				
Fas-activated serine/threonine kinase (FAST)		+		+				
Nuclear kappa factor-B DNA binding subunit			+					
Glutathione peroxidase 1						+		
Glutathione S-transferase theta 1						+		

Cells were either treated with hPRL or hPRL-G129R as indicated. Genes that were differentially expressed are represented with a '+' symbol below the treatment that stimulated their expression and when not expressed the field was left blank as shown

474 cells show a less significant ($P<0.05$) per cent change of Bcl-2 levels after the combination treatment.

Discussion

Apoptosis, or programmed cell death, is a means of regulating cellular growth and differentiation without the inflammatory response generally induced by necrotic cell death (Adams and Cory, 1998). During mammary gland development, and more importantly involution, key apoptosis-inducing Bcl-2 family proteins, such as Bax, Bad and Bcl-w are up regulated (Li, 1997; Schorr *et al*., 1999a), and Bcl-2 appears to act as a regulator of Bax levels. It has been well established that decreased levels of Bax are correlated to increased levels of Bcl-2 and that this Bax/Bcl-2 ratio is also critical to normal breast development (Reed, 1998; Green, 2000; Adams and Cory, 1998). Increases in levels of Bcl-2 appear to be more important to cell survival than the down-regulation of Bax (Schorr *et al*., 1999b). The *bcl-2* oncogene has been shown to have an anti-apoptotic function and may play a role in tumorigenesis by raising the threshold for apoptosis (Adams and Cory, 1998). In our previous studies, we reported that an hPRL

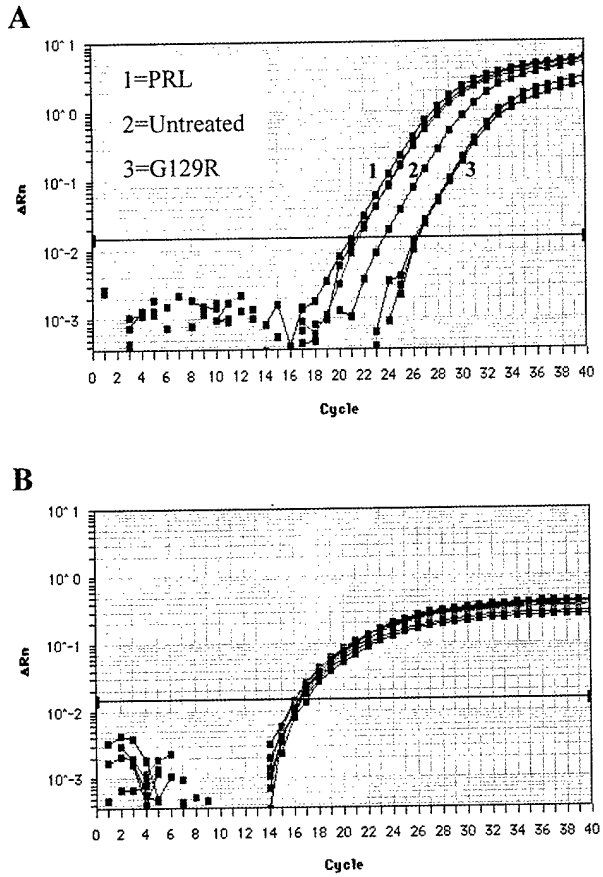

Figure 2 Real time RT–PCR spectral output measuring *bcl-2* levels in T-47D cells treated with 500 ng/ml hPRL (left-hand curve) or 500 ng/ml of hPRL-G129R (right-hand curve), relative to the untreated control (central curve) (**a**). (**b**) Represents the Real time RT–PCR spectral output measuring β-actin levels in untreated, hPRL and hPRL-G129R treated T-47D cells

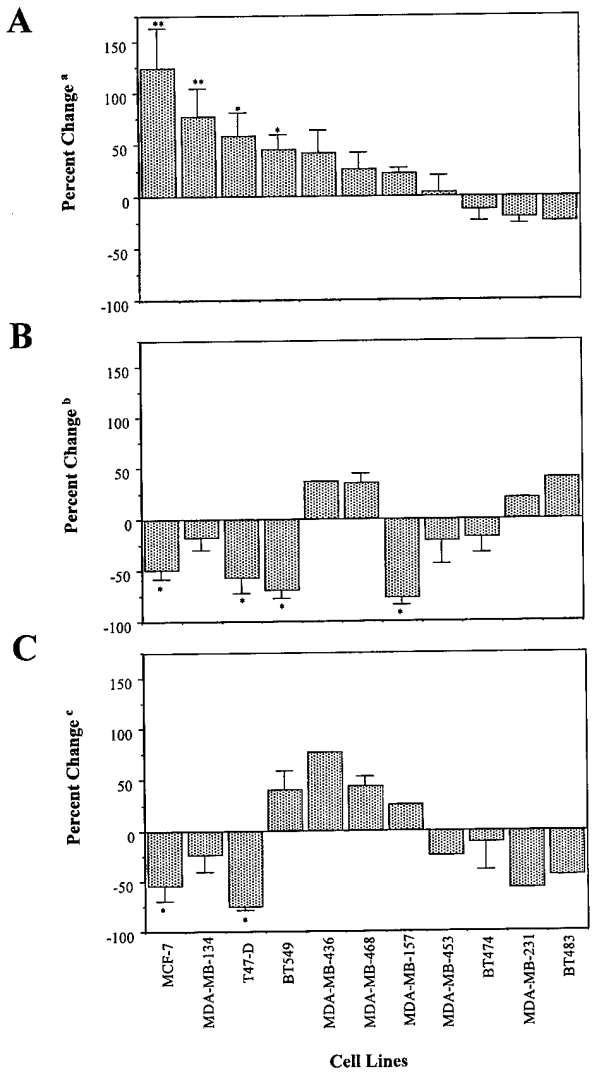

Figure 3 Real-time quantitative measurement of *bcl-2* mRNA levels in 11 breast cancer cell lines in response to 48 h treatments with hPRL (500 ng/ml; **a**), hPRL-G129R (500 ng/ml; **b**) and a combination treatment of hPRL (200 ng/ml) and hPRL-G129R (1000 ng/ml; **c**). Levels are represented as the fold change over the untreated controls, and numbers are presented as mean $\pm$ s.e. **a**, $n=2-5$; **b**, $n=2-4$; **c**, $n=2-3$. $^{**}P<0.01$ *versus* basal levels of Bcl-2; $^{*}P<0.05$ *versus* basal levels of Bcl-2

Table 2 Fold difference of *bcl-2* message of treatments over untreated cells

Cell line	*PRL*[a]	*G129R*[b]	*PRL+G129R*[c]
MCF-7	2.25±0.19	0.50±0.08	0.45±0.15
MDA-MB-134	1.78±0.27	0.82±0.12	0.76±0.17
T47-D	1.59±0.29	0.43±0.15	0.24±0.03
BT549	1.46±0.14	0.30±0.07	1.41±0.38
MDA-MB-436	1.42±0.28	1.38	2.77
MDA-MB-468	1.27±0.15	1.26±0.09	1.44±0.09
MDA-MB-157	1.22±0.05	0.33±0.07	1.26
MDA-MB-453	1.04±0.16	0.78±0.22	0.75
BT474	0.87±0.11	0.82±0.15	0.88±0.27
MDA-MB-231	0.79±0.06	1.21	0.43
BT483	0.75	1.41	0.55

Numbers represent real time RT–PCR data correlating to Figure 3 and represent *bcl-2* message levels. Cells were either treated with hPRL (500 ng/ml) or hPRL-G129R (500 ng/ml). Combination treatement (*PRL+G129R*) is as follows: 200 ng/ml PRL+1000 ng/ml G129R for 48 h. All values are represented as fold change over the untreated controls and are mean $\pm$ s.e. [a]$n=2-5$; [b]$n=2-4$; [c]$n=2-3$

antagonist, hPRL-G129R, is able to inhibit human breast cancer cell proliferation through the induction of apoptosis (Chen *et al*., 1999), suggesting that the role of hPRL in breast cancer cells may be anti-apoptotic. We have also shown that hPRL down-regulates TGFβ1 (apoptotic factor) and up-regulates TGFα (survival factor) secretion in a dose-dependent manner in human breast cancer cells (Ramamoorthy *et al*., 2001). More importantly, hPRL-G129R up-regulates TGFβ1 and down-regulates TGFα. In the same study it was also shown that caspase-3 is up regulated by hPRL-G129R. In the present study, we looked at a vast array of genes within breast cancer cells that are responding to treatment with hPRL and hPRL-G129R. We provide evidence that the potential tumorigenic effects (autocrine and paracrine) of hPRL may be mediated through the up-regulation of Bcl-2.

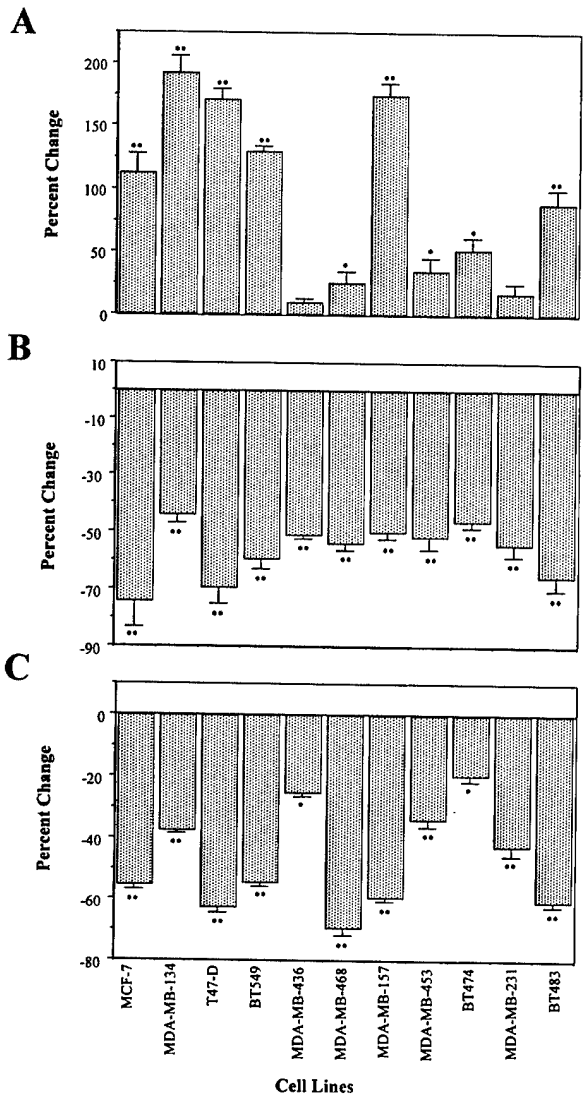

Figure 4 Expression of the Bcl-2 protein in various cell lines. Cells were treated with either hPRL (100 ng/ml; **a**), hPRL-G129R (500 ng/ml; **b**) or a combination of hPRL (100 ng/ml) and hPRL-G129R (500 ng/ml, **c**) for 48 h, lysed and ELISAs were performed to determine the relative levels of Bcl-2 present in the cells. All cells were assayed in triplicate and levels of Bcl-2 was determined using a standard curve prepared using known Bcl-2 standards from the manufacture. Fold induction and inhibition were determined using untreated cells as a control for hPRL treated cells and hPRL treated cells as a control for the combination treatment. Cell lines are arranged from left to right in order of increasing bcl-2 mRNA message level (Figure 3.). Numbers are presented mean $\pm$ s.e. of at least three independent experiments. $^{**}P<0.01$ *versus* basal levels of Bcl-2; $^{*}P<0.05$ *versus* basal levels of Bcl-2

The microarray studies using four breast cancer cell lines implied the heterogeneous nature of human breast cancer (Table 1). It is apparent in the gene expression patterns of four cell lines that the gene *bcl-2* is overexpressed in T-47D, MCF-7 and BT-549 breast cancer cells following hPRL treatment. In response to hPRL-G129R treatment, BNIP3, a 19-KD dimeric mitochondrial protein that binds to Bcl-2 and suppresses its anti-apoptotic activity (Chen *et al.*, 1997), is up-regulated in three of the four cell lines (Table 1). These data suggest an additional role for hPRL in regulation of Bcl-2 activity in breast cancer cells. To further confirm this finding, we utilized 11 breast cancer cell lines and treated them with hPRL, hPRL-G129R or a combination of the two and measured *bcl-2* mRNA as well as Bcl-2 protein levels. Increases in *bcl-2* mRNA levels in four cell lines (MCF-7, MDA-MB-134, T-47D and BT-549) are highly significant ($P<0.01$) following treatment by hPRL and are down regulated by hPRL-G129R treatment in five cell lines (MCF-7, MDA-MB-134, T-47D and BT-549 and MDA-MB-157) (Figure 3). However, the response of Bcl-2 to the treatment of hPRL or hPRL-G129R was most apparent at the Bcl-2 protein level (Figure 4). Eight of the 11 cell lines demonstrated a highly significant increase of Bcl-2 protein after hPRL treatment ($P<0.01$). The maximum increase in Bcl-2 protein after a single dose treatment with hPRL was several fold higher than the basal level in untreated controls. In contrast, the decrease of Bcl-2 levels in response to treatment by hPRL-G129R was highly significant ($P<0.01$) in all 11 cell lines (Figure 4). The cell lines (MDA-MB-436, MDA-MB-468, MDA-MB-453, BT-474, MDA-MB-231 and BT-483) demonstrated no statistically significant *bcl-2* response to hPRL treatment at the mRNA level, and showed a relatively lower response at the protein level. This would suggest that in these cell lines hPRL plays a less significant role in maintaining proliferation via the Bcl-2 protein. The discrepancy between the Bcl-2 mRNA levels and the protein levels in some cell lines after treatment may be attributed to many factors, which include the general instability of mRNA in the cells, relative to protein, where Bcl-2's half-life is greater than 10 h (Merino *et al.*, 1994).

The cell lines used in the cDNA subtraction experiments have been reported to produce PRL as an autocrine/paracrine growth factor, from the work of others (Ginsburg and Vonderhaar, 1995; Shaw-Bruha *et al.*, 1997; Yamauchi *et al.*, 2000). We do not have evidence, at this point, that hPRL-G129R contains an intrinsic ability to elicit novel signal transduction of its own. Rather, we believe that the down regulation of the *bcl-2* expression by hPRL-G129R is through the competitive inhibition of the effects induced by endogenous PRL. These findings are consistent with those of Llovera *et al.* (2000), who have found that the hPRL-G129R does not activate any specific signaling molecules in their systems.

We found no obvious correlation between the published (Ormandy *et al.*, 1997) ER status and the *bcl-2* response following hPRL and hPRL-G129R treatment. For example, both the ER negative BT-549 and the most ER positive MDA-MB-134 cell lines demonstrated high levels of *bcl-2* response, while the strongly ER positive cell line BT-483 and the ER negative MDA-MB-231 cell lines had the lowest responses. In our previous work, we determined relative PRLR mRNA expression levels in 11 breast

cancer cell lines (Peirce and Chen, 2001). In this study, the levels of *bcl-2* expression do not appear to have a linear correlation with that of PRLR mRNA. This might be attributed to the fact that there are multiple intracellular signaling mechanisms involved in regulation of *bcl-2* expression. For example, there is recent evidence of cross-talk between PRL receptor and HER2/neu through phosphorylation of Jak2 that leads to the activation of MAP kinases in breast cancer cell lines (Yamauchi *et al.*, 2000). Related to this finding, we have seen that MAP kinases are, in fact, up regulated by PRL treatment in three out of four cell lines (Table 1). Further work in this area should help to identify the specific pathways by which cellular apoptosis is regulated by hPRL and hPRL-G129R.

cDNA microarrays provide a powerful means for identifying genes differentially expressed in cells after certain alterations. However, the vast amount of information revealed after array analyses often leaves more questions than answers. In this study, we decided to focus on one gene, *bcl-2*, after analysing initial results of the cDNA subtraction and array analysis. There are clearly many questions to be addressed (Table 1). For example, we found that there was no evidence of caspase expression in MCF-7 cells following treatment by hPRL-G129R, whereas caspase expression is up regulated in T-47D, BT-549 and MDA-MB-468 cells. We previously reported that caspase-3 activity was up regulated in T-47D cells following treatment with hPRL-G129R (Ramamoorthy *et al.*, 2001). There is a lack of caspase mRNA expression and the appearance of a more direct link to Bcl-2 related apoptosis via the BAD (Bcl-2 associated death promoter) protein in MCF-7 cells (Figure 1c). During apoptosis, BAD has been shown to bind to Bcl-2 and release it from the mitochondrial membrane resulting in total cellular disruption. BAD operates upstream of the caspase pathway suggesting that MCF-7 cells activate apoptosis via a different pathway utilized by the other three breast cancer cell lines. It has been previously shown that MCF-7 cells lack caspase-3 entirely due to a 47-base pair deletion within the CASP-3 gene, although this cell line is still able to undergo apoptosis even in the absence of DNA fragmentation (Janicke *et al.*, 1998; Liang *et al.*, 2001). Thus, our data tentatively identifies one component of the caspase-3 independent signaling pathway that MCF-7 cells may use to trigger apoptosis. It is also interesting to point out that a death domain protein, the receptor-interacting protein (RIP), is differentially expressed in all four cell lines following hPRL-G129R treatment. It has been reported that over-expression of RIP induces both NF-κB activation and apoptosis (Hsu *et al.*, 1996). Our results may be of interest when investigating the death domain proteins and death domain receptors in relation to hPRL and hPRL-G129R.

In summary, a list of apoptosis related genes that are differentially expressed following treatment with either hPRL or hPRL-G129R has been compiled for four different breast cancer cell lines. These data will allow for future studies of specific genes that are involved with cellular proliferation or apoptosis in human breast cancer. By focusing on Bcl-2 mRNA or protein expression in response to hPRL and hPRL-G129R treatment in 11 cell lines, we provide further evidence that the anti-apoptotic effects of hPRL in breast cancer are likely mediated through the up regulation of Bcl-2. It is generally accepted that, for cancer therapy, one should not design an approach based solely upon increasing death signals, such as chemotherapeutics. Rather, a twofold approach combining chemotherapeutics with removal of survival factors will result in a more efficient treatment. Our data regarding hPRL-G129R further strengthens its potential therapeutic role in breast cancer therapy.

Materials and methods

Cell lines and growth conditions

Five ER positive (T-47D, MDA-MB-134, BT-474, MDA-MB-483, MCF-7) and six ER negative human breast cancer cell lines (BT-549, MDA-MB-231, MDA-MB-453, MDA-MB-468, MDA-MB-436 and MDA-MB-157) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). T-47D, BT-549, BT-474 and MDA-MB-483 cells were maintained in RPMI 1640 medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT, USA) and 100 μg/ml gentamycin (for all media used) (Hyclone). BT-459 cells were supplemented with 200 IU of Insulin (Sigma, St. Louis, MO, USA). MDA-MB-483 cells were supplemented with 0.2 mM sodium pyruvate, 2 mM HEPES buffer and 200 IU Insulin (Sigma). MCF-7 cells were maintained in DMEM (Life Technologies) supplemented with 10% FBS. MDA-MB-231, MDA-MB-134, MDA-MB-453, MDA-MB-468, MDA-MB-436 and MDA-MB-157 cells were maintained in Leibovitz L-15 (Life Technologies) media. MDA-MB-231, MDA-MB-436 and MDA-MB-453 cells were supplemented with 10% FBS with the addition of 200 IU of Insulin for MDA-MB-436. MDA-MB-468 and MDA-MB-157 cells were grown in the presence of 15% FBS and MDA-MB-134 in the presence of 20% FBS. Cell lines T-47D, BT-549, BT-474, MDA-MB-483, MCF-7 and MDA-MB-231 were grown at 37°C in a humid atmosphere in the presence of 5% CO_2. MDA-MB-134, MDA-MB-453, MDA-MB-468, MDA-MB-436 and MDA-MB-157 cells were grown at 37°C in a humid atmosphere in the absence of CO_2.

PCR-Select cDNA suppression subtraction hybridization

Before experiments, cells were split into three groups of 10 T75 flasks and grown in their specific medium supplemented with 10% charcoal-stripped fetal bovine serum (CSS) until 80% confluent. Approximately 1×10^8 cells from each group were treated with either 500 ng/ml of hPRL (hPRL was kindly supplied by Dr AF Parlow, National Hormone and Pituitary Program, NIH, USA) or 500 ng/ml of hPRL-G129R in cell specific media supplemented with 1% CSS. The untreated control cells were cultured in their respected medium supplemented with 1% CSS. All cells were treated for 48 h and immediately harvested for mRNA extraction. Polyadenylated mRNA was isolated using the Micro-Fast Track 2.0 kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. RNA yield was determined by

measuring absorbency at 260 nm. SSH was performed using the PCR-Select™ cDNA Subtraction Kit (Clontech, Palo Alto, CA, USA) as previously described (Beck *et al*., 2001). Three micrograms of purified cDNAs from the subtraction hybridizations were random primed labeled with alkali labile digoxigenin-dUTP using the DIG DNA Labeling Kit (Roche Molecular Biochemical's, Mannheim, Germany) according to the manufacturer's protocol.

cDNA microarrays

The Atlas™ human apoptosis array (Clontech) was used for all microarray analyses. The array is a nylon membrane that contains all known apoptosis related genes (205 cDNAs) spotted onto the surface in duplicate. Membranes were prehybridized with DIG Easy Hyb solution (Roche) overnight at 37°C in a hybridization incubator with gentle rotation. DIG-labeled probes were purified and resuspended appropriately in TE (pH 8.0). The probes were boiled for 10 min and placed on ice for 5 min. After prehybridization, the DIG-labeled probe was added to the microarray membrane in a total volume of 5 ml of fresh DIG Easy Hub and allowed to hybridize overnight at 68°C in a hybridization incubator with gentle rotation. Membranes were washed twice at 38°C for 5 min in 2× SSC, 1% SDS, and twice at 68°C for 15 min in 0.1× SSC, 0.5% SDS. DIG-labeled cDNAs on the hybridized Atlas™ membranes were detected by chemiluminescence using the DIG luminescent detection kit (Roche) according to the manufacturer's specifications using CSPD® as the chemiluminescent substrate. After incubation with CSPD®, membranes were placed in an autoradiography cassette and incubated at 37°C for 15 min to enhance the exposure and then exposed to Kodak Biomax™-MR film at room temperature (all hybridizations were carried out in duplicate). It was determined that using 3 μg of differentially expressed cDNAs for labeling and probing the microarrays was the correct amount for less background and optimal brightness for gene identification. Each array was exposed to the film for various periods of time to allow for the correct exposure to be captured.

Real time quantitative RT–PCR

Before treatment, 11 breast cancer cell lines were depleted of serum for 3 to 4 days in their respective medium supplemented with 1% CSS. Approximately $0.5-1\times7^7$ cells from each group were treated with 500 ng/ml of hPRL, 500 ng/ml of hPRL-G129R or a 1000 ng:250 ng ratio of hPRL-G129R to hPRL in cell specific medium supplemented with 1% CSS. The untreated control cells were cultured in cell specific medium supplemented with 1% CSS. All cells were treated for 48 h and harvested for total RNA extraction using the RNAqueous (Ambion, Austin, TX, USA) RNA isolation kit.

A one-step real time reverse transcription PCR (RT–PCR) technique was used to determine relative expression levels of *bcl-2* mRNA using the ABI Perkin Elmer Prism 7700 Sequence Detection System (Perkin-Elmer Biosystems, Foster City, CA, USA). The reaction mix included a pre-developed TaqMan® assay mixture containing both forward and reverse *bcl-2* specific primers and a 100 nm final concentration of the *bcl-2*-specific probe labeled with FAM reporter fluorescent dye (Perkin-Elmer 4319432F). A one-step reaction mixture provided in the TaqMan® Gold RT–PCR Kit (Perkin-Elmer) was used for all amplifications (5.5 mM $MgCl_2$, 50 mM KCl, 0.01 mM EDTA, 10 mM Tris-HCl pH 8.3, 300 μM deoxyATP, 300 μM deoxyCTP, 300 μM deoxyGTP, 600 μM deoxyUTP, 0.025 U/ml AmpliTaq Gold DNA polymerase, 0.25 U/ml MultiScribe Reverse Transcriptase, 0.4 U/ml RNase inhibitor). Cycle parameters for the one-step RT–PCR included a reverse transcription step at 48°C for 30 min followed by 40 cycles of 95°C denaturation and 60°C annealing/extension. Four hundred to 1500 ng of total RNA was used per reaction. The housekeeping gene β-actin was used for internal normalization. Each reaction was carried out in triplicate for each PCR run, and each run was repeated two to five times. Data are expressed as the mean ± s.e.

Bcl-2 ELISA

A Bcl-2 ELISA (Oncogene Research Products, Boston, MA, USA) analysis was performed according to manufacturer's instructions. Briefly, all 11 cell lines were treated with hPRL (100 ng/ml), hPRL-G129R (500 ng/ml) or combination of hPRL (100 ng/ml) and hPRL-G129R (500 ng/ml) for 48 h in depleted media specific for each cell line described previously. Controls for all treatments were those of untreated cells in their specific defined media. For each cell line, approximately 5×10^6 cells were resuspended in 1 ml of resuspension solution (50 mM Tris, 5 mM EDTA, 0.2 mM PMSF, 1 μg/ml pepstatin and 0.5 μg/ml leupeptin; pH adjusted to 7.4). Two hundred microliters of antigen extraction agent (Oncogene) were added to the cell suspensions and incubated on ice for 30 min. Cell debris was centrifuged and supernatant was frozen until use. Each supernatant was diluted 1:1 to obtain an optimal reading in the range of the standards. Standards were performed in duplicate. Analyses were repeated three times and data are expressed as the mean ± s.e.

Statistical analyses

All values are given as mean ± s.e. Statistical analysis was performed using the program StatsDirect version 1.9.8 (CamCode, Cambridge, UK) with one way ANOVA and a Tukey-Multiple Comparison test. *P*-values less than 0.01 were considered highly statistically significant and *P* values that were less than 0.05 were considered statistically significant.

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In vivo studies of the anti-tumor effects of a human prolactin antagonist, hPRL-G129R

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Abstract. Previously we demonstrated that a mutated human prolactin (hPRL) with a single amino acid substitution at position 129 (hPRL-G129R) was able to inhibit human breast cancer cell proliferation via the induction of apoptosis. In this study, we report the *in vivo* anti-tumor effects of hPRL-G129R in nude mice bearing human breast cancer xenografts (T-47D and MCF-7). In an effort to prolong the half-life of the proteins, hPRL or hPRL-G129R were formulated with either growth factor reduced Matrigel or into slow-releasing pellets (custom made 5 mg/5 day release). Initially, nude mice inoculated (s.c.) with T-47D human breast cancer cells were treated with either hPRL or hPRL-G129R formulated with Matrigel. At the end of the 7-week study, it was found that hPRL significantly stimulated the *in vivo* growth of T-47D xenografts (mean tumor volume, 202±62 mm^3 as compared to 124±31 mm^3 in control mice), whereas hPRL-G129R inhibited the tumor growth (mean tumor volume, 79±32 mm^3). The inhibitory effects of hPRL-G129R were further confirmed in a second experiment using nude mice bearing MCF-7 human breast cancer xenografts and treated with slow-releasing pellets containing hPRL-G129R. Based on these results, we believe that hPRL-G129R can be used to improve the outcome of human breast cancer treatment in the near future.

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Abbreviations: ER, estrogen receptor; E2, 17-ß estradiol; FBS, fetal bovine serum; FPLC, fast-performance liquid chromatography; hPRL, human prolactin; hGH, human growth hormone; IRMA, immunoradiometric assay; STAT, signal transducers and activators of transcription

Key words: prolactin antagonist, breast cancer xenografts, nude mice

Introduction

Human PRL is a neuroendocrine polypeptide hormone primarily produced by the lactotrophs of the anterior pituitary gland in all vertebrates. The biological activities of PRL are mediated by a specific membrane receptor, the PRL receptor. Although hPRL has been reported to have multiple biological activities, the best-characterized action of PRL is on the mammary gland (1). In this organ, PRL plays a decisive role in DNA synthesis, epithelial cell proliferation and milk production (2-5). It has been unambiguously demonstrated in studies using PRL or PRL receptor gene knock-out mice that PRL and PRL receptors are the key regulators in mammary tissue development (5,6).

Recently, the notion that hPRL acts as a survival growth factor in the mammary gland and is directly involved in breast cancer development has revitalized the efforts in searching for a hPRL receptor blocker. In our previous studies (7-12), we developed an hGH antagonist with a single amino acid substitution mutation from Gly→Arg at position 120 (hGH-G120R). This peptide-based therapeutic has proven to be clinically effective in blocking the hGH receptor. Human GH antagonist has completed phase III studies and will be used in patients with pathological levels of GH (13). By adopting a strategy similar to the one used in the development of the hGH antagonist, we (14-16) and others (17-20) have demonstrated that a single amino acid substitution mutation (Gly→Arg at position 129) in the hPRL molecule results in an hPRL receptor specific antagonist (hPRL-G129R). We have demonstrated that hPRL-G129R is able to inhibit human breast cancer proliferation via the induction of apoptosis. We have also demonstrated that the possible mechanism of the hPRL antagonist's inhibitory effects is mediated, at least in part, through: a) inhibition of phosphorylation of oncogene STAT3 (15); b) modulation of TGFs (up regulation of TGFß and down regulation of TGFα) (16); and c) induction of caspase 3 activities (26). The purpose of this study is to further test the anti-tumor activities of hPRL-G129R *in vivo* using nude mice inoculated with human (T-47D or MCF-7; s.c.) mammary tumor cells and treated with hPRL-G129R using two different delivery methods intent to prolong the half-life of the proteins (the Matrigel mix and slow-releasing pellets). The results from four different *in vivo* experiments demonstrated that hPRL stimulates breast cancer cell growth and more importantly hPRL-G129R significantly inhibited breast cancer cell growth *in vivo*.

Materials and methods

Cell lines and animals. The cell lines used in this study were two human breast cancer cell lines (T-47D and MCF-7) from ATCC (Manassas, VA). T-47D cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (Gibco BRL; Baltimore, MD) and ATCC recommended supplements. MCF-7 cells were grown in DMEM supplemented with 10% FBS. Six- to 8-week-old female Nuj/nude mice were obtained from the Jackson Lab (Bar Harbor, ME) and maintained in a sterile environment in compliance with NIH guidelines. Animals were allowed to adjust to the institutional animal facility for 1 week before the experiment.

Production and purification of hPRL and hPRL-G129R. Human PRL and hPRL-G129R used in this study were produced using an *E.coli* protein production system according to published protocols (15,20) with modifications. Briefly, BL21 (DE3) cells (Novagen; Madison, WI) were transformed with hPRL or hPRL-G129R expression plasmids (pET22b-hPRL or pET22b-G129R) using the calcium chloride method. The transformant was spread on an ampicillin plate, and grown overnight at 37°C. An LB seed culture was inoculated with 6-10 colonies and incubated overnight. The following day, an LB culture was generated by inoculation of 5% of the seed culture and grown for ~2.5 h at 37°C with agitation. IPTG (Fisher Scientific; Norcross, GA) was then added to the culture (1 mM final concentration) to induce expression of hPRL or hPRL-G129R and incubated for an additional 4 h. Bacteria were pelleted and resuspended in a solution containing 0.2 M $NaPO_4$ (pH 8.0), 10 mM EDTA, and 0.5% Triton X-100. The resuspended cells were lysed using a 550 Sonic Dismembrator from Fisher Scientific (Norcross, GA), and the products in the form of inclusion bodies were pelleted by centrifugation at 12,000 g for 15 min. The pellets were then resuspended in solution A [0.2 M $NaPO_4$ (pH 7.0), 5 mM EDTA, 1 M urea, 0.5% Triton X-100] and pelleted by centrifugation at 12,000 g for 15 min. These pellets were then resuspended in solution B [0.2 M $NaPO_4$ (pH 8.0), 8 M urea, 1% v/v ß-mercaptoethanol], and the refolding process was initiated. The refolding process consisted of dialyzing the protein against decreasing amounts of urea and ß-mercaptoethanol in the presence of 50 mM NH_4HCO_3 (pH 8.0) for at least 3 consecutive days. The protein product was then filtered through a 0.22 µ filter, degassed and purified using a Q-Sepharose anionic exchange column (Pharmacia; Piscataway, NJ) on the FPLC system (Pharmacia; Piscataway, NJ). The concentration of hPRL or hPRL-G129R purified from FPLC was determined using the PRL immunoradiometric assay (IRMA) kit (DPC; Los Angeles, CA). The purity of both PRL and hPRL-G129R exceeded 98% as determined by SDS-PAGE in combination with silver staining (Bio-Rad; Hercules, CA). The endotoxin level in the final products from all batches was <5 EU/mg tested by Cape Cop Inc. The recombinant proteins produced by this method has an extra Met at the N-terminus as compared to wild-type PRL. The biological function of hPRL and hPRL-G129R was confirmed by the STAT assay as described previously (15).

Radioreceptor binding assay. Human PRL receptor binding assays were performed as previously described (14). Briefly, cells were grown in 6-well tissue culture plates until 90% confluent (~$1x10^5$ cells/well). Monolayers of cells were starved in serum-free RPMI-1640 medium for 0.5-1 h. The cells were then incubated at room temperature in serum-free RPMI-1640 containing $5x10^4$ cpm ^{125}I hPRL (specific activity, 30 µCi/µg; Perkin Elmer Life Sciences; Boston, MA) with or without 500 ng/ml of hPRL. Cells were washed three times in serum-free RPMI-1640, lysed with 0.5 ml of 0.1 N NaOH/1% SDS, and the bound radioactivity was determined by a scintillation counter. Total specific binding was calculated and compared.

Delivery of hPRL and hPRL-G129R. The *in vivo* half-lives of hPRL and hPRL-G129R are less than 2 h due to their small molecular sizes. Therefore, two alternative protein delivery methods were used in this study to extend the half-lives of these proteins. Serum PRL or PRL-G129R level was determined by PRL immunoradiometric assay (IRMA) kit.

Formulation with growth factor reduced Matrigel. Matrigel (BD Biosciences; San Diego, CA) is an artificial extracellular matrix that exists in liquid form at 4°C and solidifies into a gel at room temperature. Lyophilized hPRL or hPRL-G129R proteins were first hydrated with PBS (pH 8.0) and were then mixed with growth factor reduced Matrigel at a 1:1 or 1:2 (Protein:Matrigel, v/v) ratio to a final concentration of 1 mg/ml before injection. The *in vivo* phamocokinetics of protein/PBS and protein/PBS/Matrigel formulations were compared.

Formulation with slow-releasing pellets. Purified hPRL-G129R protein was lyophilized and sent to Innovative Research of America, Inc. (Sarasota, FL) for production of slow-releasing pellets. The pellets were implanted s.c. into experimental animals. Preliminary experiments demonstrated that 5 mg/5 day slow-releasing pellets resulted in satisfactory serum concentrations and minimal wounding. This formula was employed for the remainder of the study.

Tissue distribution of hPRL-G129R. We investigated the tissue distribution pattern of hPRL-G129R after i.p. injection into nude mice. FPLC purified hPRL-G129R was iodinated using ^{125}I (Perkin Elmer Life Sciences; Boston, MA). Approximately 0.5 µCi of ^{125}I labeled hPRL-G129R was i.p. injected into each of 10 nude mice bearing either T-47D (n=6) or MCF-7 (n=4) xenografts (7 weeks after initial tumor cell inoculation). Six hours after injection, animals were sacrificed and tissues were dissected and weighed. The radioactivity in the various tissues was determined using a scintillation counter. The data were expressed as cpm/mg tissue and normalized by reference to serum cpm (% tissue CPM = cpm in tissue/mg/cpm of 100 µl serum x 100).

In vivo inhibition of tumor growth studies

Experiment one. Five million T-47D cells pre-mixed with the Matrigel were injected into the mammary fat pads of 30 Nuj/nude mice, which were then implanted s.c. with slow-releasing E2 (17-ß estradiol) pellets (0.72 mg/60 day, Innovative Research of America, Inc.) to enhance tumor growth. Three days after tumor cell inoculation, the mice were randomized into three groups. Each animal was then injected s.c. five times a week with either Matrigel alone (150 µl/mouse) or

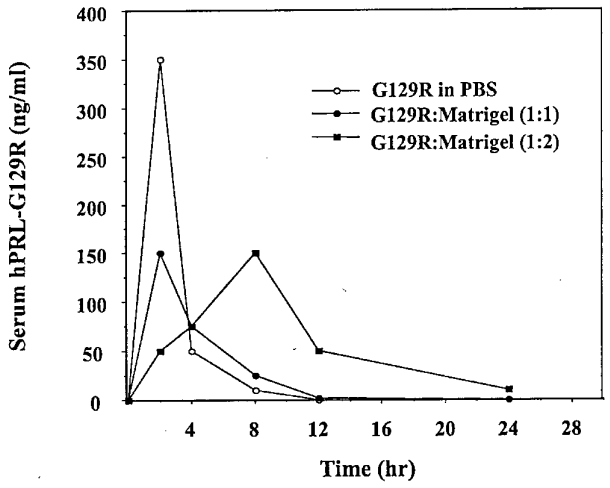


Figure 1. Pharmacokinetic studies of hPRL-G129R formulated with growth factor reduced Matrigel in mice. One hundred and fifty μg of hPRL-G129R were mixed with either Matrigel at a 1:1 or 1:2 v/v ratio (hPRL-G129R: Matrigel) or in PBS in a total volume of 150 μl and i.p. injected at time 0. Blood samples were collected at time intervals as indicated via tail vein bleeding. Serum hPRL-G129R levels were tested using an hPRL IRMA kit. Each data point represents the mean value from three animals.

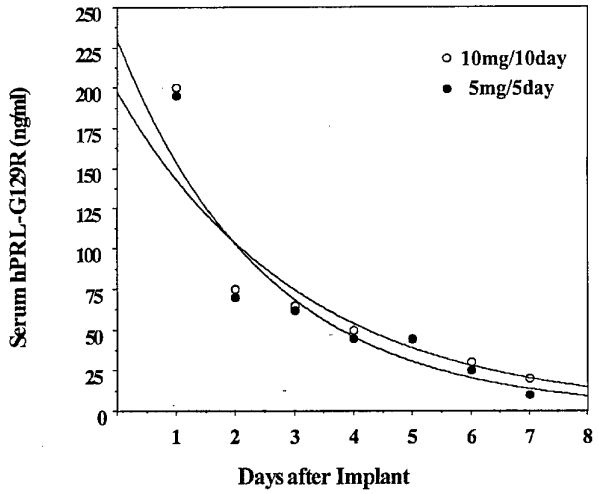


Figure 2. Pharmacokinetic studies of hPRL-G129R slow-releasing pellets. One hPRL-G129R slow-releasing pellet (5 mg/5 day or 10 mg/10 day) was s.c. implanted into each Balb/C mouse (n=3 for each group). Blood samples were collected daily via tail vein bleeding. The serum concentration of hPRL-G129R was tested using a PRL IRMA kit. Each data point represents the mean value from three animals.

Matrigel formulated (1:2 v:v ratio; 1 μg/ml) with hPRL-G129R or hPRL (150 μl/mouse) continuously for 7 weeks.

Experiment two. Since the original MCF-7 cells purchased from ATCC grow very slowly in nude mice (preliminary experiments, data not shown), secondary MCF-7 cultures were established. Briefly, a primary MCF-7 xenograft was established by injection of 10^7 MCF-7 cells into the mammary fat pad of a nude mouse in combination with an E2 slow-releasing pellet (s.c.). After the tumor was visible, it was dissected, minced and treated with trypsin. The tumor cells were then cultured and expanded. The sub-cultured MCF-7 cells were used to establish tumor xenografts in nude mice. Twelve Nuj/nude mice were inoculated with $5x10^6$ sub-cultured MCF-7 tumor cells, implanted s.c. with E2 pellets (0.72 mg/60 day) and then randomized into two groups. One group received implantation of slow-releasing hPRL-G129R pellets (5 mg/5 day), and the other group received implantation of placebo pellets. These mice received pellets once a week for 6 weeks.

Monitoring of tumor growth and statistics. Two dimensional tumor sizes were measured once a week. The tumor volume was calculated using the formula (L x W^2)/2. Tumors were dissected at the end of experiments and weighed. Assessment of statistical difference was determined by Student's t-test.

Results

Pharmacokinetics of hPRL-G129R formulated with the Matrigel or slow-releasing pellets. We compared the relative bio-availability and the duration of hPRL-G129R in serum using two different administration routes. The results demonstrated that both Matrigel formulations (1:1 or 1:2 ratio; v:v) of hPRL-G129R resulted in a more desirable serum profile than administration of hPRL-G129R/PBS. At the 1:2 ratio formulation, the peak concentration of hPRL-G129R was greatly reduced from approximately 350 ng/ml to approximately 150 ng/ml (Fig. 1). Also, the peak concentration in serum is delayed from 2 to 8 h, which resulted in much longer bio-available serum levels of hPRL-G129R (Fig. 1). Therefore, it is our belief that at a 1:2 (v:v) ratio mix, the protein:Matrigel formulation could be used as a novel protein delivery method. The 1:2 ratio is used throughout this study.

The second method of administration, implantation of slow-releasing pellets, resulted in an even more prolonged half-life of around 48 h (Fig. 2). In addition, significant serum hPRL-G129R concentrations continued to be detected 5 days after initial implantation. The average serum concentration was approximately 50 ng/ml 5 days after implantation. There was little difference in the serum profile between the two formulations (5 mg/5 day vs. 10 mg/10 day) for the slow-releasing pellets (Fig. 2). Considering the size of the pellets (directly related to the wound for each implantation), the 5 mg/5 day slow-releasing pellets were chosen for this study and were implanted once a week.

PRL receptor status in breast cancer cell lines. The results of a direct comparison of the PRL receptor specific binding levels in the three breast cancer cell lines used in this study are shown in Fig. 3. T-47D cells have the higher specific PRL receptor binding (55%), as compared to that of MCF-7 cells (25%). HeLa cells were included as a negative control. These results are consistent with findings from reverse transcriptase real time PCR assays conducted in our lab measuring expression of PRL receptor mRNA levels in these three cell lines (data not shown).

Tissue distribution. The distribution of ^{125}I hPRL-G129R 6 h after i.p. injection is represented in Fig. 4. The mammary

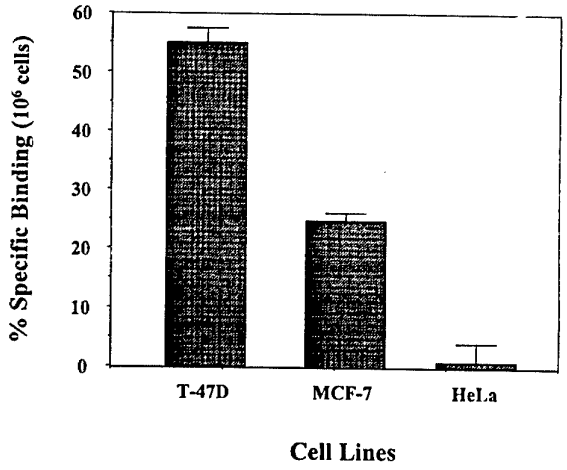


Figure 3. Radioreceptor binding assay was performed using ^{125}I labeled hPRL-G129R and three breast cancer cell lines. Specific binding of PRL receptor was measured using the formula: (cpm of total binding per 10^6 cells - cpm of non-specific binding)/cpm of total binding x 100, as described previously (14). A HeLa cell line was used as negative control.

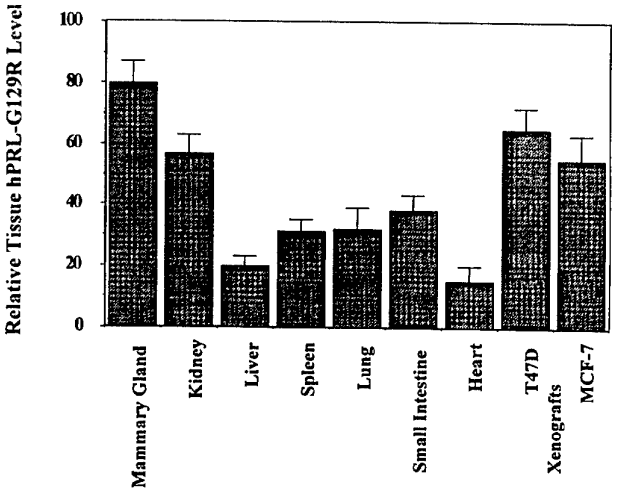
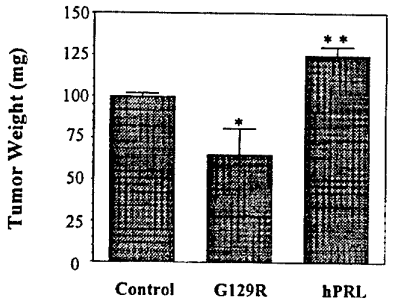


Figure 4. Tissue specific binding (pharmaco-distribution) of hPRL-G129R in nude mice bearing human breast cancer xenografts (T-47D, n=3; or MCF-7, n=3). One µCi of ^{125}I labeled hPRL-G129R was injected i.p. into experimental animals. Six hours after injection, animals were sacrificed and various tissues were dissected, weighed and the amount of radioactivity in each sample was determined by a scintillation counter. The data was normalized with reference to the cpm in 100 µl serum of each animal and expressed as relative cpm/mg tissue/cpm in 100 µl serum x 100.

gland and the breast cancer cell xenografts were the tissues containing the highest counts of ^{125}I hPRL-G129R. These findings are very important in supporting the use of an hPRL antagonist to target the mammary gland, especially the tumor cells. It is of interest that the counts in the kidney were much higher than other organs with rich blood supplies such as the liver and the lung, a finding that suggests the kidney might be one of the major organs involved in PRL metabolism. The levels of ^{125}I hPRL-G129R were lowest in the heart and the liver (Fig. 4).



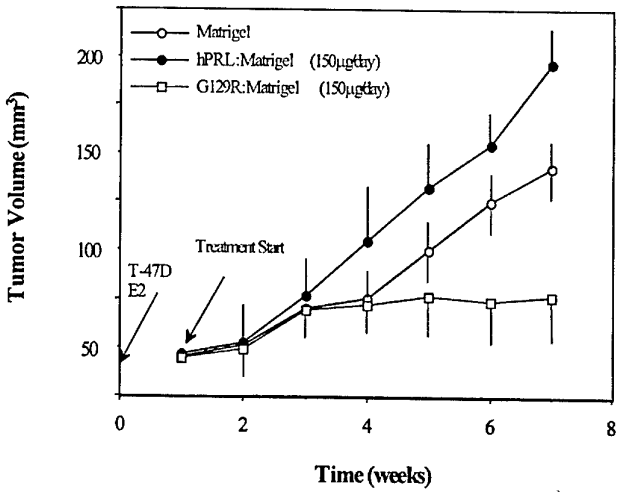


Figure 5. Effects of hPRL and hPRL-G129R on T-47D human breast cancer cell xenograft growth in nude mice. Thirty 6 to 7-week-old Nuj/nude mice were inoculated with T-47D cells and implanted s.c. with slow-releasing E2 pellets (0.72 mg/60 day). T47D cells ($5x10^6$) pre-mixed with Matrigel were injected into the mammary fat pad. One week after tumor cell inoculation, the mice were randomized into three groups and treated five times/week with either 150 µl of Matrigel (control), hPRL/Matrigel (150 µg/150 µl), or hPRL-G129R/Matrigel (150 µg/ 150 µl) for 7 consecutive weeks. The tumor volumes in each group were measured weekly. Tumors weights (mg) were taken at 7 weeks after tumor cell inoculation (upper panel). Values are expressed as mean and SE. *P<0.05; **P<0.01 vs. control.

In vivo inhibition of tumor growth

Experiment one: T-47D xenograft in nude mice treated with hPRL or hPRL-G129R/Matrigel mix. At the end of the 7-week period of treatment, Nuj/nude mice that had been implanted with T-47D cells and treated with the hPRL/Matrigel formulation exhibited enhanced tumor growth (mean tumor volume, 202±62 mm^3 vs. 124±31 mm^3 in the control mice). Those treated with the hPRL-G129R/Matrigel formulation showed inhibition of tumor growth (mean tumor volume was 79±32 mm^3 vs. 124±31 mm^3) (Fig. 5). While the tumor growth rate in the hPRL-G129R treated mice plateaued after the fifth week, tumor growth in the control and hPRL treated mice was clearly increasing beginning at around the fourth week of the experiment (Fig. 5). The final tumor weight in the three groups is also significantly different (P<0.05); (control, 100±2 mg; PRL, 121±5 mg; hPRL-G129R, 65±16 mg) (Fig. 5, upper panel).

Experiment two: MCF-7 xenograft in nude mice treated with hPRL-G129R slow-releasing pellets. Treatment with slow-

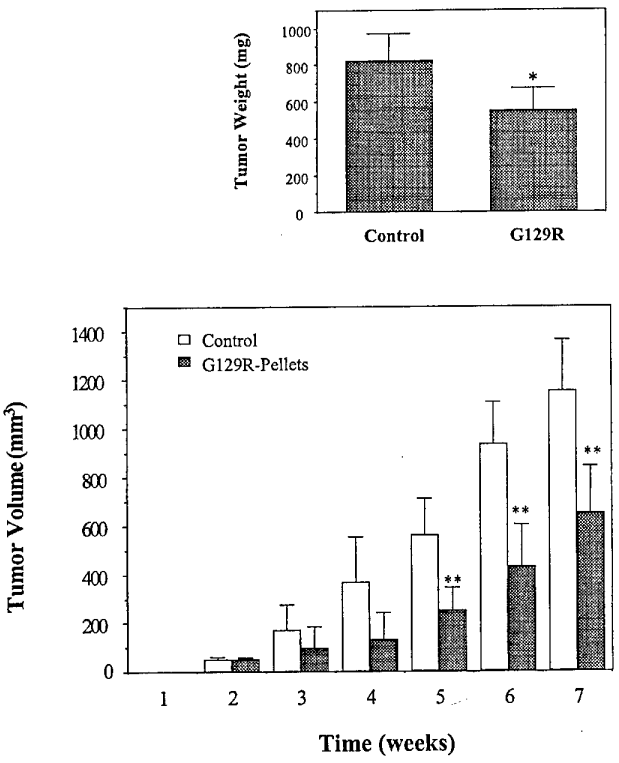


Figure 6. Effects of hPRL-G129R on MCF-7 human breast cancer cell xenograft growth in nude mice. Twelve 6- to 7-week-old Nuj/nude mice were inoculated with MCF-7 cells and implanted s.c. with slow-releasing E2 pellets (0.72 mg/60 day). MCF-7 cells ($5x10^6$) pre-mixed with Matrigel at 1:1 (v/v) ratio were injected into the mammary fat pad. Three days after tumor cell inoculation, the mice were randomized into two groups and received hPRL-G129R slow-releasing pellets or placebo (once/week) for 7 consecutive weeks. The tumor volumes in each group were measured weekly. Tumors weights (mg) were taken at 7 weeks after tumor cell inoculation (upper panel). Values are expressed as mean and SE. $^*P<0.05$; $^{**}P<0.01$ vs. control.

releasing hPRL-G129R pellets also resulted in inhibition of tumor growth in Nuj/nude mice inoculated with MCF-7 human breast cancer xenografts (Fig. 6). Since these secondary MCF-7 cells have been adapted to *in vivo* growth, they tend to grow much more aggressively as compared to original MCF-7 cells. At approximately 5 weeks after tumor inoculation, treatment with hPRL-G129R resulted in a decrease in tumor volume of about 50%. The tumor growth difference was most obvious between weeks 5 and 6. Along with tumor volume decreasing in hPRL-G129R treated nude mice, tumor weight also decreased as demonstrated in mice at 7 weeks of age bearing tumors (Fig. 6, upper panel).

Discussion

Estrogen is well known as a powerful mitogen that plays an important physiological role in human breast growth and function. The role of estrogen in breast cancer has also been well established and is supported by findings that anti-estrogen treatment has both therapeutic as well as preventive effects in the treatment of breast malignancies (21). However, the etiological role of hPRL as an autocrine/paracrine growth factor in breast cancer is still being challenged despite the fact that: a) hPRL has been shown to stimulate the proliferation of cultured breast cancer cells (14,22); b) high levels of hPRL receptor have been found in breast cancer tissues (23-25); and c) hPRL has been found to be produced locally in breast tissue (22). The controversy is largely due to the fact that there have been no convincing studies involving the use of anti-hPRL agents in an *in vivo* breast cancer model to establish the efficacy of an anti-PRL drug (26,27). In this report, we demonstrate that hPRL does indeed promote the growth of human breast cancer xenografts in nude mice (Fig. 5). More importantly, to the best of our knowledge, our data for the first time demonstrate the feasibility of using an hPRL antagonist to inhibit the growth of human breast cancer xenografts (Fig. 6).

The maintenance of relatively constant hPRL-G129R serum concentrations over a longer period of time is crucial for inducing the *in vivo* effects of the PRL antagonist. In this study, we used two alternative delivery methods to overcome the problem of a short hPRL-G129R half-life. As shown in Fig. 1, the peak serum concentration of hPRL-G129R was shifted from ~2 to 8 h after it is formulated with Matrigel, which resulted in a much longer serum half-life. Even more promising results were generated using the slow-releasing pellets of hPRL-G129R implanted once a week: in addition to a greatly extended half-life, hPRL-G129R serum concentrations were maintained within a range of 120 to 20 ng/ml for over a week (Fig. 2). We (14) and other groups (20) have, in the past, produced PRL antagonists that are highly effective in *in vitro* assays. This present study extends the therapeutic potential of hPRL-G129R protein.

The delivery methods used in this study are far from ideal from a clinical viewpoint. However, these two delivery methods provide alternatives to those used in peptide-based therapeutics. One obvious advantage of the delivery methods used in this study compared to those traditionally used to prolong the half-life of a protein (such as pegylation) is that they do not require chemical alteration of the therapeutic molecule. Therefore, functional testing is kept to a minimum before initiating *in vivo* studies.

Our tissue distribution studies provide some insight into the molecular nature of the therapeutic effects of hPRL-G129R treatment. Using ^{125}I labeled hPRL-G129R, it is clear that the human tumor xenografts contain high levels of hPRL-G129R-specific radioactivity, second only to mammary glands (Fig. 4). These findings indicate that high levels of the PRL receptor on the cancer cell surface provide the physical basis for the anti-tumor action of the PRL antagonist. We reason that the lower concentration of hPRL-G129R in tumor tissue vs. that of the mammary gland is due to the fact that the weight of the solid tumor masses dissected in these studies were as high as 900 mg (Fig. 6, upper panel); blood circulation in these solid tumors is much reduced relative to normal mammary tissue. We also note the higher levels of radioactivity in the kidney, higher even than that of the liver (Fig. 4), suggesting that the kidney may have high level of PRL receptors and is a target tissue of PRL as an osmoregulator.

The results from this study provide strong evidence suggesting that hPRL is a survival/growth factor for human breast cancer cells. By blocking the hPRL receptor with the mutated hPRL molecule (hPRL-G129R), we believe the

proliferative signaling pathways in the breast cancer cells are reversed. The exact molecular mechanism involved in this process is awaiting further elucidation. However, in our recent studies we have successfully used combination techniques of PCR-Select cDNA subtraction hybridization and cDNA microarrays to study the possible molecular mechanisms involved in the regulation of mammary gland apoptosis by hPRL (Beck MT, *et al*, 83th Annual Meeting of Endocrine Society, p199, 2001). Our preliminary results from hPRL treated T-47D cells revealed that out of the 205 apoptosis related genes only 1 gene, bcl-2, was up regulated in response to hPRL (bcl-2 is known as an apoptosis suppressor). On the other hand, many apoptosis related genes, in particular various caspases (3 and 7), Fas-activated serine/threonine (FAST) kinase, members of the tumor necrosis factor (TNF) family, and E2F were up regulated in hPRL-G129R treated T-47D cells (28). These results suggest that hPRL serves as an apoptosis inhibitor possibly through activation of bcl-2. Further studies are needed to confirm this observation.

In summary, we have successfully demonstrated that two protein delivery methods used in this study are able to maintain relatively stable concentrations of the hPRL-G129R in serum. Our results also indicate that hPRL contributes significantly to the growth of breast cancer *in vivo*. More importantly hPRL-G129R, the hPRL antagonist, was proven to be functionally active and successfully inhibited the growth of human breast cancer xenografts in nude mice. Together these results strongly indicate that the development of hPRL receptor antagonists will contribute significantly to the treatment of breast cancer.

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In vivo studies of the anti-tumor effects of a human prolactin antagonist, hPRL-G129R

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Abstract. Previously we demonstrated that a mutated human prolactin (hPRL) with a single amino acid substitution at position 129 (hPRL-G129R) was able to inhibit human breast cancer cell proliferation via the induction of apoptosis. In this study, we report the *in vivo* anti-tumor effects of hPRL-G129R in nude mice bearing human breast cancer xenografts (T-47D and MCF-7). In an effort to prolong the half-life of the proteins, hPRL or hPRL-G129R were formulated with either growth factor reduced Matrigel or into slow-releasing pellets (custom made 5 mg/5 day release). Initially, nude mice inoculated (s.c.) with T-47D human breast cancer cells were treated with either hPRL or hPRL-G129R formulated with Matrigel. At the end of the 7-week study, it was found that hPRL significantly stimulated the *in vivo* growth of T-47D xenografts (mean tumor volume, 202±62 mm^3 as compared to 124±31 mm^3 in control mice), whereas hPRL-G129R inhibited the tumor growth (mean tumor volume, 79±32 mm^3). The inhibitory effects of hPRL-G129R were further confirmed in a second experiment using nude mice bearing MCF-7 human breast cancer xenografts and treated with slow-releasing pellets containing hPRL-G129R. Based on these results, we believe that hPRL-G129R can be used to improve the outcome of human breast cancer treatment in the near future.

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Abbreviations: ER, estrogen receptor; E2, 17-ß estradiol; FBS, fetal bovine serum; FPLC, fast-performance liquid chromatography; hPRL, human prolactin; hGH, human growth hormone; IRMA, immunoradiometric assay; STAT, signal transducers and activators of transcription

Key words: prolactin antagonist, breast cancer xenografts, nude mice

Introduction

Human PRL is a neuroendocrine polypeptide hormone primarily produced by the lactotrophs of the anterior pituitary gland in all vertebrates. The biological activities of PRL are mediated by a specific membrane receptor, the PRL receptor. Although hPRL has been reported to have multiple biological activities, the best-characterized action of PRL is on the mammary gland (1). In this organ, PRL plays a decisive role in DNA synthesis, epithelial cell proliferation and milk production (2-5). It has been unambiguously demonstrated in studies using PRL or PRL receptor gene knock-out mice that PRL and PRL receptors are the key regulators in mammary tissue development (5,6).

Recently, the notion that hPRL acts as a survival growth factor in the mammary gland and is directly involved in breast cancer development has revitalized the efforts in searching for a hPRL receptor blocker. In our previous studies (7-12), we developed an hGH antagonist with a single amino acid substitution mutation from Gly→Arg at position 120 (hGH-G120R). This peptide-based therapeutic has proven to be clinically effective in blocking the hGH receptor. Human GH antagonist has completed phase III studies and will be used in patients with pathological levels of GH (13). By adopting a strategy similar to the one used in the development of the hGH antagonist, we (14-16) and others (17-20) have demonstrated that a single amino acid substitution mutation (Gly→Arg at position 129) in the hPRL molecule results in an hPRL receptor specific antagonist (hPRL-G129R). We have demonstrated that hPRL-G129R is able to inhibit human breast cancer proliferation via the induction of apoptosis. We have also demonstrated that the possible mechanism of the hPRL antagonist's inhibitory effects is mediated, at least in part, through: a) inhibition of phosphorylation of oncogene STAT3 (15); b) modulation of TGFs (up regulation of TGFß and down regulation of TGFα) (16); and c) induction of caspase 3 activities (26). The purpose of this study is to further test the anti-tumor activities of hPRL-G129R *in vivo* using nude mice inoculated with human (T-47D or MCF-7; s.c.) mammary tumor cells and treated with hPRL-G129R using two different delivery methods intent to prolong the half-life of the proteins (the Matrigel mix and slow-releasing pellets). The results from four different *in vivo* experiments demonstrated that hPRL stimulates breast cancer cell growth and more importantly hPRL-G129R significantly inhibited breast cancer cell growth *in vivo*.

Materials and methods

Cell lines and animals. The cell lines used in this study were two human breast cancer cell lines (T-47D and MCF-7) from ATCC (Manassas, VA). T-47D cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (Gibco BRL; Baltimore, MD) and ATCC recommended supplements. MCF-7 cells were grown in DMEM supplemented with 10% FBS. Six- to 8-week-old female Nuj/nude mice were obtained from the Jackson Lab (Bar Harbor, ME) and maintained in a sterile environment in compliance with NIH guidelines. Animals were allowed to adjust to the institutional animal facility for 1 week before the experiment.

Production and purification of hPRL and hPRL-G129R. Human PRL and hPRL-G129R used in this study were produced using an *E.coli* protein production system according to published protocols (15,20) with modifications. Briefly, BL21 (DE3) cells (Novagen; Madison, WI) were transformed with hPRL or hPRL-G129R expression plasmids (pET22b-hPRL or pET22b-G129R) using the calcium chloride method. The transformant was spread on an ampicillin plate, and grown overnight at 37°C. An LB seed culture was inoculated with 6-10 colonies and incubated overnight. The following day, an LB culture was generated by inoculation of 5% of the seed culture and grown for ~2.5 h at 37°C with agitation. IPTG (Fisher Scientific; Norcross, GA) was then added to the culture (1 mM final concentration) to induce expression of hPRL or hPRL-G129R and incubated for an additional 4 h. Bacteria were pelleted and resuspended in a solution containing 0.2 M $NaPO_4$ (pH 8.0), 10 mM EDTA, and 0.5% Triton X-100. The resuspended cells were lysed using a 550 Sonic Dismembrator from Fisher Scientific (Norcross, GA), and the products in the form of inclusion bodies were pelleted by centrifugation at 12,000 g for 15 min. The pellets were then resuspended in solution A [0.2 M $NaPO_4$ (pH 7.0), 5 mM EDTA, 1 M urea, 0.5% Triton X-100] and pelleted by centrifugation at 12,000 g for 15 min. These pellets were then resuspended in solution B [0.2 M $NaPO_4$ (pH 8.0), 8 M urea, 1% v/v ß-mercaptoethanol], and the refolding process was initiated. The refolding process consisted of dialyzing the protein against decreasing amounts of urea and ß-mercaptoethanol in the presence of 50 mM NH_4HCO_3 (pH 8.0) for at least 3 consecutive days. The protein product was then filtered through a 0.22 µ filter, degassed and purified using a Q-Sepharose anionic exchange column (Pharmacia; Piscataway, NJ) on the FPLC system (Pharmacia; Piscataway, NJ). The concentration of hPRL or hPRL-G129R purified from FPLC was determined using the PRL immunoradiometric assay (IRMA) kit (DPC; Los Angeles, CA). The purity of both PRL and hPRL-G129R exceeded 98% as determined by SDS-PAGE in combination with silver staining (Bio-Rad; Hercules, CA). The endotoxin level in the final products from all batches was <5 EU/mg tested by Cape Cop Inc. The recombinant proteins produced by this method has an extra Met at the N-terminus as compared to wild-type PRL. The biological function of hPRL and hPRL-G129R was confirmed by the STAT assay as described previously (15).

Radioreceptor binding assay. Human PRL receptor binding assays were performed as previously described (14). Briefly, cells were grown in 6-well tissue culture plates until 90% confluent (~$1x10^5$ cells/well). Monolayers of cells were starved in serum-free RPMI-1640 medium for 0.5-1 h. The cells were then incubated at room temperature in serum-free RPMI-1640 containing $5x10^4$ cpm ^{125}I hPRL (specific activity, 30 µCi/µg; Perkin Elmer Life Sciences; Boston, MA) with or without 500 ng/ml of hPRL. Cells were washed three times in serum-free RPMI-1640, lysed with 0.5 ml of 0.1 N NaOH/1% SDS, and the bound radioactivity was determined by a scintillation counter. Total specific binding was calculated and compared.

Delivery of hPRL and hPRL-G129R. The *in vivo* half-lives of hPRL and hPRL-G129R are less than 2 h due to their small molecular sizes. Therefore, two alternative protein delivery methods were used in this study to extend the half-lives of these proteins. Serum PRL or PRL-G129R level was determined by PRL immunoradiometric assay (IRMA) kit.

Formulation with growth factor reduced Matrigel. Matrigel (BD Biosciences; San Diego, CA) is an artificial extracellular matrix that exists in liquid form at 4°C and solidifies into a gel at room temperature. Lyophilized hPRL or hPRL-G129R proteins were first hydrated with PBS (pH 8.0) and were then mixed with growth factor reduced Matrigel at a 1:1 or 1:2 (Protein:Matrigel, v/v) ratio to a final concentration of 1 mg/ml before injection. The *in vivo* phamocokinetics of protein/PBS and protein/PBS/Matrigel formulations were compared.

Formulation with slow-releasing pellets. Purified hPRL-G129R protein was lyophilized and sent to Innovative Research of America, Inc. (Sarasota, FL) for production of slow-releasing pellets. The pellets were implanted s.c. into experimental animals. Preliminary experiments demonstrated that 5 mg/5 day slow-releasing pellets resulted in satisfactory serum concentrations and minimal wounding. This formula was employed for the remainder of the study.

Tissue distribution of hPRL-G129R. We investigated the tissue distribution pattern of hPRL-G129R after i.p. injection into nude mice. FPLC purified hPRL-G129R was iodinated using ^{125}I (Perkin Elmer Life Sciences; Boston, MA). Approximately 0.5 µCi of ^{125}I labeled hPRL-G129R was i.p. injected into each of 10 nude mice bearing either T-47D (n=6) or MCF-7 (n=4) xenografts (7 weeks after initial tumor cell inoculation). Six hours after injection, animals were sacrificed and tissues were dissected and weighed. The radioactivity in the various tissues was determined using a scintillation counter. The data were expressed as cpm/mg tissue and normalized by reference to serum cpm (% tissue CPM = cpm in tissue/mg/cpm of 100 µl serum x 100).

In vivo inhibition of tumor growth studies

Experiment one. Five million T-47D cells pre-mixed with the Matrigel were injected into the mammary fat pads of 30 Nuj/nude mice, which were then implanted s.c. with slow-releasing E2 (17-ß estradiol) pellets (0.72 mg/60 day, Innovative Research of America, Inc.) to enhance tumor growth. Three days after tumor cell inoculation, the mice were randomized into three groups. Each animal was then injected s.c. five times a week with either Matrigel alone (150 µl/mouse) or

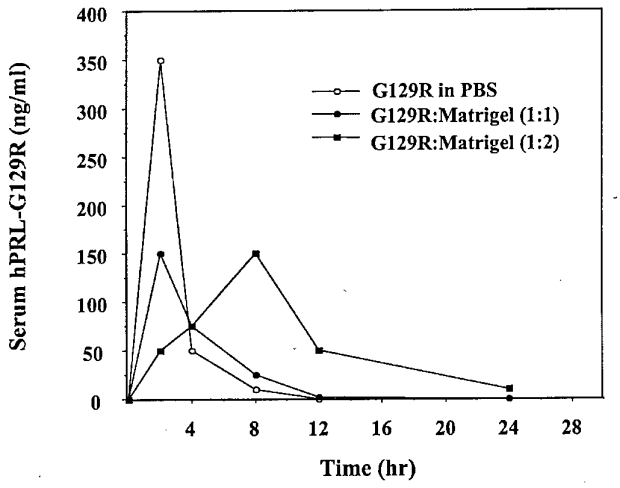


Figure 1. Pharmacokinetic studies of hPRL-G129R formulated with growth factor reduced Matrigel in mice. One hundred and fifty μg of hPRL-G129R were mixed with either Matrigel at a 1:1 or 1:2 v/v ratio (hPRL-G129R: Matrigel) or in PBS in a total volume of 150 μl and i.p. injected at time 0. Blood samples were collected at time intervals as indicated via tail vein bleeding. Serum hPRL-G129R levels were tested using an hPRL IRMA kit. Each data point represents the mean value from three animals.

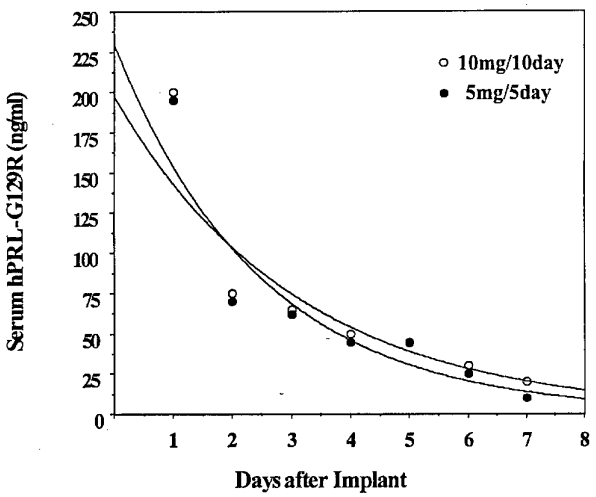


Figure 2. Pharmacokinetic studies of hPRL-G129R slow-releasing pellets. One hPRL-G129R slow-releasing pellet (5 mg/5 day or 10 mg/10 day) was s.c. implanted into each Balb/C mouse (n=3 for each group). Blood samples were collected daily via tail vein bleeding. The serum concentration of hPRL-G129R was tested using a PRL IRMA kit. Each data point represents the mean value from three animals.

Matrigel formulated (1:2 v:v ratio; 1 μg/ml) with hPRL-G129R or hPRL (150 μl/mouse) continuously for 7 weeks.

Experiment two. Since the original MCF-7 cells purchased from ATCC grow very slowly in nude mice (preliminary experiments, data not shown), secondary MCF-7 cultures were established. Briefly, a primary MCF-7 xenograft was established by injection of 10^7 MCF-7 cells into the mammary fat pad of a nude mouse in combination with an E2 slow-releasing pellet (s.c.). After the tumor was visible, it was dissected, minced and treated with trypsin. The tumor cells were then cultured and expanded. The sub-cultured MCF-7 cells were used to establish tumor xenografts in nude mice. Twelve Nuj/nude mice were inoculated with $5x10^6$ sub-cultured MCF-7 tumor cells, implanted s.c. with E2 pellets (0.72 mg/60 day) and then randomized into two groups. One group received implantation of slow-releasing hPRL-G129R pellets (5 mg/5 day), and the other group received implantation of placebo pellets. These mice received pellets once a week for 6 weeks.

Monitoring of tumor growth and statistics. Two dimensional tumor sizes were measured once a week. The tumor volume was calculated using the formula (L x W^2)/2. Tumors were dissected at the end of experiments and weighed. Assessment of statistical difference was determined by Student's t-test.

Results

Pharmacokinetics of hPRL-G129R formulated with the Matrigel or slow-releasing pellets. We compared the relative bio-availability and the duration of hPRL-G129R in serum using two different administration routes. The results demonstrated that both Matrigel formulations (1:1 or 1:2 ratio; v:v) of hPRL-G129R resulted in a more desirable serum profile than administration of hPRL-G129R/PBS. At the 1:2 ratio formulation, the peak concentration of hPRL-G129R was greatly reduced from approximately 350 ng/ml to approximately 150 ng/ml (Fig. 1). Also, the peak concentration in serum is delayed from 2 to 8 h, which resulted in much longer bio-available serum levels of hPRL-G129R (Fig. 1). Therefore, it is our belief that at a 1:2 (v:v) ratio mix, the protein:Matrigel formulation could be used as a novel protein delivery method. The 1:2 ratio is used throughout this study.

The second method of administration, implantation of slow-releasing pellets, resulted in an even more prolonged half-life of around 48 h (Fig. 2). In addition, significant serum hPRL-G129R concentrations continued to be detected 5 days after initial implantation. The average serum concentration was approximately 50 ng/ml 5 days after implantation. There was little difference in the serum profile between the two formulations (5 mg/5 day vs. 10 mg/10 day) for the slow-releasing pellets (Fig. 2). Considering the size of the pellets (directly related to the wound for each implantation), the 5 mg/5 day slow-releasing pellets were chosen for this study and were implanted once a week.

PRL receptor status in breast cancer cell lines. The results of a direct comparison of the PRL receptor specific binding levels in the three breast cancer cell lines used in this study are shown in Fig. 3. T-47D cells have the higher specific PRL receptor binding (55%), as compared to that of MCF-7 cells (25%). HeLa cells were included as a negative control. These results are consistent with findings from reverse transcriptase real time PCR assays conducted in our lab measuring expression of PRL receptor mRNA levels in these three cell lines (data not shown).

Tissue distribution. The distribution of ^{125}I hPRL-G129R 6 h after i.p. injection is represented in Fig. 4. The mammary

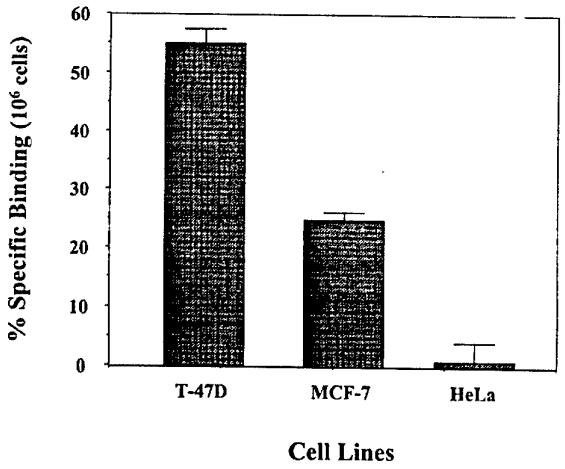


Figure 3. Radioreceptor binding assay was performed using ^{125}I labeled hPRL-G129R and three breast cancer cell lines. Specific binding of PRL receptor was measured using the formula: (cpm of total binding per 10^6 cells - cpm of non-specific binding)/cpm of total binding x 100, as described previously (14). A HeLa cell line was used as negative control.

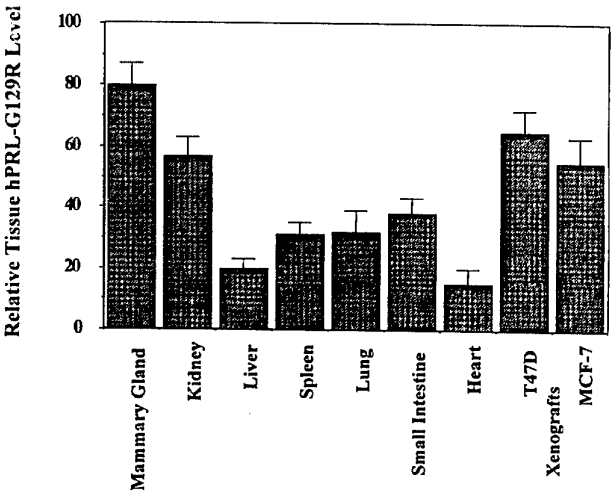
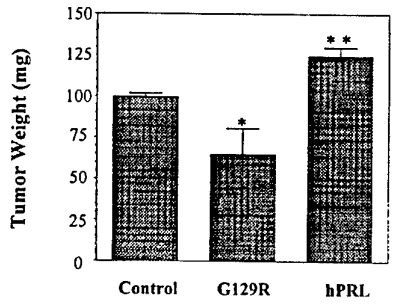


Figure 4. Tissue specific binding (pharmaco-distribution) of hPRL-G129R in nude mice bearing human breast cancer xenografts (T-47D, n=3; or MCF-7, n=3). One μCi of ^{125}I labeled hPRL-G129R was injected i.p. into experimental animals. Six hours after injection, animals were sacrificed and various tissues were dissected, weighed and the amount of radioactivity in each sample was determined by a scintillation counter. The data was normalized with reference to the cpm in 100 μl serum of each animal and expressed as relative cpm/mg tissue/cpm in 100 μl serum x 100.

gland and the breast cancer cell xenografts were the tissues containing the highest counts of ^{125}I hPRL-G129R. These findings are very important in supporting the use of an hPRL antagonist to target the mammary gland, especially the tumor cells. It is of interest that the counts in the kidney were much higher than other organs with rich blood supplies such as the liver and the lung, a finding that suggests the kidney might be one of the major organs involved in PRL metabolism. The levels of ^{125}I hPRL-G129R were lowest in the heart and the liver (Fig. 4).



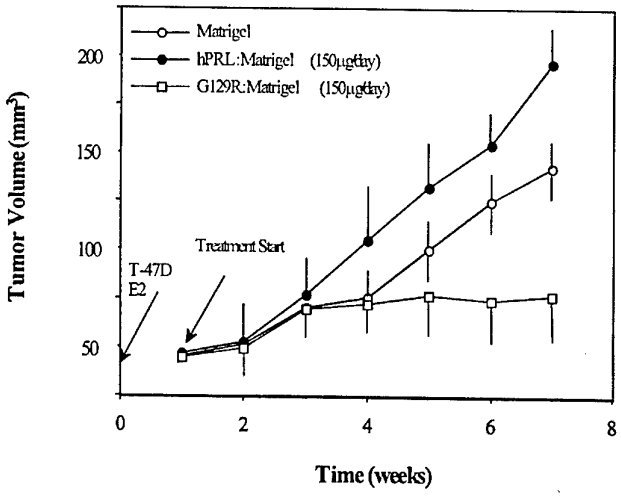


Figure 5. Effects of hPRL and hPRL-G129R on T-47D human breast cancer cell xenograft growth in nude mice. Thirty 6 to 7-week-old Nuj/nude mice were inoculated with T-47D cells and implanted s.c. with slow-releasing E2 pellets (0.72 mg/60 day). T47D cells ($5x10^6$) pre-mixed with Matrigel were injected into the mammary fat pad. One week after tumor cell inoculation, the mice were randomized into three groups and treated five times/week with either 150 μl of Matrigel (control), hPRL/Matrigel (150 μg/150 μl), or hPRL-G129R/Matrigel (150 μg/ 150 μl) for 7 consecutive weeks. The tumor volumes in each group were measured weekly. Tumors weights (mg) were taken at 7 weeks after tumor cell inoculation (upper panel). Values are expressed as mean and SE. *P<0.05; **P<0.01 vs. control.

In vivo inhibition of tumor growth

Experiment one: T-47D xenograft in nude mice treated with hPRL or hPRL-G129R/Matrigel mix. At the end of the 7-week period of treatment, Nuj/nude mice that had been implanted with T-47D cells and treated with the hPRL/Matrigel formulation exhibited enhanced tumor growth (mean tumor volume, 202±62 mm^3 vs. 124±31 mm^3 in the control mice). Those treated with the hPRL-G129R/Matrigel formulation showed inhibition of tumor growth (mean tumor volume was 79±32 mm^3 vs. 124±31 mm^3) (Fig. 5). While the tumor growth rate in the hPRL-G129R treated mice plateaued after the fifth week, tumor growth in the control and hPRL treated mice was clearly increasing beginning at around the fourth week of the experiment (Fig. 5). The final tumor weight in the three groups is also significantly different (P<0.05); (control, 100±2 mg; PRL, 121±5 mg; hPRL-G129R, 65±16 mg) (Fig. 5, upper panel).

Experiment two: MCF-7 xenograft in nude mice treated with hPRL-G129R slow-releasing pellets. Treatment with slow-

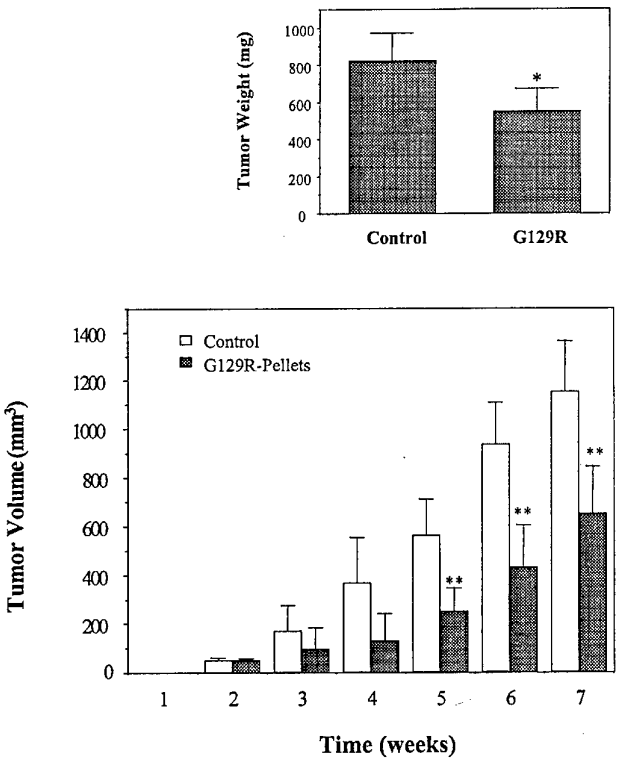


Figure 6. Effects of hPRL-G129R on MCF-7 human breast cancer cell xenograft growth in nude mice. Twelve 6- to 7-week-old Nuj/nude mice were inoculated with MCF-7 cells and implanted s.c. with slow-releasing E2 pellets (0.72 mg/60 day). MCF-7 cells ($5x10^6$) pre-mixed with Matrigel at 1:1 (v/v) ratio were injected into the mammary fat pad. Three days after tumor cell inoculation, the mice were randomized into two groups and received hPRL-G129R slow-releasing pellets or placebo (once/week) for 7 consecutive weeks. The tumor volumes in each group were measured weekly. Tumors weights (mg) were taken at 7 weeks after tumor cell inoculation (upper panel). Values are expressed as mean and SE. *P<0.05; **P<0.01 vs. control.

releasing hPRL-G129R pellets also resulted in inhibition of tumor growth in Nuj/nude mice inoculated with MCF-7 human breast cancer xenografts (Fig. 6). Since these secondary MCF-7 cells have been adapted to *in vivo* growth, they tend to grow much more aggressively as compared to original MCF-7 cells. At approximately 5 weeks after tumor inoculation, treatment with hPRL-G129R resulted in a decrease in tumor volume of about 50%. The tumor growth difference was most obvious between weeks 5 and 6. Along with tumor volume decreasing in hPRL-G129R treated nude mice, tumor weight also decreased as demonstrated in mice at 7 weeks of age bearing tumors (Fig. 6, upper panel).

Discussion

Estrogen is well known as a powerful mitogen that plays an important physiological role in human breast growth and function. The role of estrogen in breast cancer has also been well established and is supported by findings that anti-estrogen treatment has both therapeutic as well as preventive effects in the treatment of breast malignancies (21). However, the etiological role of hPRL as an autocrine/paracrine growth factor in breast cancer is still being challenged despite the fact that: a) hPRL has been shown to stimulate the proliferation of cultured breast cancer cells (14,22); b) high levels of hPRL receptor have been found in breast cancer tissues (23-25); and c) hPRL has been found to be produced locally in breast tissue (22). The controversy is largely due to the fact that there have been no convincing studies involving the use of anti-hPRL agents in an *in vivo* breast cancer model to establish the efficacy of an anti-PRL drug (26,27). In this report, we demonstrate that hPRL does indeed promote the growth of human breast cancer xenografts in nude mice (Fig. 5). More importantly, to the best of our knowledge, our data for the first time demonstrate the feasibility of using an hPRL antagonist to inhibit the growth of human breast cancer xenografts (Fig. 6).

The maintenance of relatively constant hPRL-G129R serum concentrations over a longer period of time is crucial for inducing the *in vivo* effects of the PRL antagonist. In this study, we used two alternative delivery methods to overcome the problem of a short hPRL-G129R half-life. As shown in Fig. 1, the peak serum concentration of hPRL-G129R was shifted from ~2 to 8 h after it is formulated with Matrigel, which resulted in a much longer serum half-life. Even more promising results were generated using the slow-releasing pellets of hPRL-G129R implanted once a week: in addition to a greatly extended half-life, hPRL-G129R serum concentrations were maintained within a range of 120 to 20 ng/ml for over a week (Fig. 2). We (14) and other groups (20) have, in the past, produced PRL antagonists that are highly effective in *in vitro* assays. This present study extends the therapeutic potential of hPRL-G129R protein.

The delivery methods used in this study are far from ideal from a clinical viewpoint. However, these two delivery methods provide alternatives to those used in peptide-based therapeutics. One obvious advantage of the delivery methods used in this study compared to those traditionally used to prolong the half-life of a protein (such as pegylation) is that they do not require chemical alteration of the therapeutic molecule. Therefore, functional testing is kept to a minimum before initiating *in vivo* studies.

Our tissue distribution studies provide some insight into the molecular nature of the therapeutic effects of hPRL-G129R treatment. Using ^{125}I labeled hPRL-G129R, it is clear that the human tumor xenografts contain high levels of hPRL-G129R-specific radioactivity, second only to mammary glands (Fig. 4). These findings indicate that high levels of the PRL receptor on the cancer cell surface provide the physical basis for the anti-tumor action of the PRL antagonist. We reason that the lower concentration of hPRL-G129R in tumor tissue vs. that of the mammary gland is due to the fact that the weight of the solid tumor masses dissected in these studies were as high as 900 mg (Fig. 6, upper panel); blood circulation in these solid tumors is much reduced relative to normal mammary tissue. We also note the higher levels of radioactivity in the kidney, higher even than that of the liver (Fig. 4), suggesting that the kidney may have high level of PRL receptors and is a target tissue of PRL as an osmoregulator.

The results from this study provide strong evidence suggesting that hPRL is a survival/growth factor for human breast cancer cells. By blocking the hPRL receptor with the mutated hPRL molecule (hPRL-G129R), we believe the

proliferative signaling pathways in the breast cancer cells are reversed. The exact molecular mechanism involved in this process is awaiting further elucidation. However, in our recent studies we have successfully used combination techniques of PCR-Select cDNA subtraction hybridization and cDNA microarrays to study the possible molecular mechanisms involved in the regulation of mammary gland apoptosis by hPRL (Beck MT, *et al*, 83th Annual Meeting of Endocrine Society, p199, 2001). Our preliminary results from hPRL treated T-47D cells revealed that out of the 205 apoptosis related genes only 1 gene, bcl-2, was up regulated in response to hPRL (bcl-2 is known as an apoptosis suppressor). On the other hand, many apoptosis related genes, in particular various caspases (3 and 7), Fas-activated serine/threonine (FAST) kinase, members of the tumor necrosis factor (TNF) family, and E2F were up regulated in hPRL-G129R treated T-47D cells (28). These results suggest that hPRL serves as an apoptosis inhibitor possibly through activation of bcl-2. Further studies are needed to confirm this observation.

In summary, we have successfully demonstrated that two protein delivery methods used in this study are able to maintain relatively stable concentrations of the hPRL-G129R in serum. Our results also indicate that hPRL contributes significantly to the growth of breast cancer *in vivo*. More importantly hPRL-G129R, the hPRL antagonist, was proven to be functionally active and successfully inhibited the growth of human breast cancer xenografts in nude mice. Together these results strongly indicate that the development of hPRL receptor antagonists will contribute significantly to the treatment of breast cancer.

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FROM AN ANTAGONIST BACK TO AN AGONIST: TWO WRONGS DO MAKE A RIGHT

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It is generally accepted that the initial step of signal transduction for human growth hormone (hGH) as well as human prolactin (hPRL) is to bind to their respective receptors. The binding process is reported to be sequential: one ligand binds to the first receptor through its binding site one with high affinity and then finds its second receptor through its binding site two with lower affinity resulting in a one ligand/two receptor complex. This ligand induced dimerization of the receptors is essential for hGH and hPRL signal transduction. Amino acid substitution mutation in binding site two of either hGH (hGH-G120R) or hPRL (hPRL-G129R) results in mutants with antagonistic effects both *in vitro* and *in vivo* demonstrated by many labs including ours. In our recent attempts to generate a more potent hPRL antagonist with a longer serum half-life, we produced a G129R-G129R homo-dimer using an *E. coli* expression vector, pET22b. The protein was purified using Q-sepharose anion exchange chromatography and a FPLC system. To our astonishment, the G129R-G129R homo-dimer acts in every aspect as an agonist assayed by STAT5 phosphorylation in human breast cancer cells. We found that the G129R-G129R homo-dimer is able to induce STAT5 phosphorylation in a concentration-dependent manner at a dose range similar to that of wild type hPRL. The induction of STAT5 phosphorylation is not only dose-dependent but also show self-antagonism in high concentration as seen in the case of hPRL. It is interesting to point out that the activation of STAT5 phosphorylation by G129R-G129R homo-dimer can be inhibited by G129R monomer. Our results suggest that as long as there are two binding sites (site 1 plus site 2 in wild type hPRL or site 1 plus another site 1 in G129R-G129R homo-dimer) in one molecule, the ligand serves as an agonist. Our data also suggests that the overall size of the ligand is not a crucial factor (23kd monomer or 46kd dimer) to induce PRL signal transduction. The potential use of homo-dimers of antagonists as longer half-life agonists needs further testing.

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HUMAN PROLACTIN ANTAGONIST AND ENDOSTATIN FUSION PROTEIN FOR THE TREATMENT OF BREAST CANCER.

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In recent studies, we have demonstrated that human prolactin (hPRL) with a single amino acid substitution mutation at position 129 (G129R) was able to bind to hPRL receptor (PRLR) with high affinity and block PRL induced signal transduction. More importantly, we have shown that G129R inhibits breast cancer cell proliferation both *in vitro* and *in vivo*. Another promising anticancer protein, endostatin, has also been shown to have great promise as a cancer therapeutic by inhibiting tumor angiogenesis. In this study we have created a novel fusion protein (G129R-Endo), which combines the PRLR specific recognition ability of G129R with the anti-angiogenic activity of endostatin. G129R-Endo, along with G129R and endostatin alone, was produced in the form of inclusion bodies using the expression vector pET22b(+) in *E. coli*. Proteins were purified either using HPLC or gel filtration then tested for their activities using cell-based assays. We have demonstrated that the novel fusion protein was able to inhibit STAT-5 phosphorylation induced by hPRL in the human breast cancer cell line, T47-D, indicating that the G129R portion of this fusion protein was active. The potency of inhibition of STAT-5 phosphorylation by the fusion protein was similar to that of G129R alone. It was also shown that G129R-Endo inhibited human umbilical vein endothelial cell (HUVEC) proliferation, thus demonstrating that the endostatin portion of the fusion protein was active. The anti-angiogenesis activity of the endostatin portion of the fusion protein was equivalent to that of endostatin alone. Therefore, we have demonstrated that the G129R-Endo fusion protein is a bi-functional protein that might be used as a breast cancer specific angiogenesis inhibitor. *In vivo* characterization of the novel protein using breast cancer xymographs in nude mice is currently ongoing.

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CONSTRUCTION OF HUMAN PROLACTIN (HPRL) RECEPTOR TARGETING FUSION TOXINS FOR BREAST CANCER TREATMENT USING PRL ANTAGONIST AND RECOMBINANT FORMS OF PSEUDOMONAS EXOTOXIN A

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Human prolactin (hPRL) promotes growth, differentiation, and proliferation of normal and cancerous mammary cells and may play a role in tumor development. The high affinity receptor for prolactin (PRLR) has been found to be up regulated in many breast cancers making it a target for endocrine therapy. Human PRL antagonist, hPRL-G129R, has been shown to have an antiproliferative effect through the induction of breast cancer cell apoptosis. Based on these findings a variety of PRL antagonist fusion proteins have been constructed attempting to create targeted therapeutics for breast cancer cells. Here we report the construction of two novel fusion proteins using hPRL-G129R and *Pseudomonas* exotoxin A (PE) that are intended to specifically kill PRLR positive breast cancer cells. G129R-PE_{40}KDEL was constructed by replacing the cell recognition domain of PE with G129R and the natural endoplasmic reticulum (ER) retention signal with the amino acids KDEL. PE_{37}-G129RKDEL was constructed by fusing G129R near the C-terminus of the active fragment of PE (aa 280-609) followed by the ER retention signal KDEL. Both fusion proteins were produced in *E.coli* BL21(DE3) using the expression vector pET22b, purified from inclusion bodies, and refolded. The PRL and PE portions of the constructs were confirmed to be present by immunoradiometric assay and Western blots, respectively. In vitro as well as in vivo assays for these novel fusion proteins are ongoing.

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REGULATION OF BCL-2 EXPRESSION BY HPRL AND ITS ANTAGONIST, HPRL-G129R, IN HUMAN BREAST CANCER CELL LINES.

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In previous studies, we have shown that the human prolactin antagonist hPRL-G129R is able to induce apoptosis in four human breast cancer cell lines. In the current study, a combination of PCR subtraction and cDNA microarray methodologies was used to examine the gene expression profiles of two estrogen receptor (ER) positive, and two ER negative human breast cancer cell lines following treatment with hPRL or hPRL-G129R. Among the many genes that were found to be differentially expressed, *bcl-2* was strongly induced following hPRL treatment in three of the four cell lines tested. To confirm the evidence linking hPRL and *bcl-2* expression, a quantitative method of real time RT-PCR and a Bcl-2 ELISA were used to measure *bcl-2* mRNA expression levels and Bcl-2 protein levels, respectively. Eleven human breast cancer cell lines were assayed following treatment with hPRL, hPRL-G129R, or a combination of these proteins. We found that hPRL induced, while hPRL-G129R inhibited, *bcl-2* mRNA expression in a majority of the cell lines tested, and that the induction of *bcl-2* by hPRL was competitively inhibited by hPRL-G129R in most of these cell lines. The pattern of mRNA expression following these treatments correlated to the Bcl-2 protein levels. In particular, MCF-7, T47D and BT549 cells all demonstrated a three to four fold difference in *bcl-2* expression levels between hPRL and hPRL-G129R treatments. In contrast, BT474, MDA-MB-231 and BT483 cells showed minimal *bcl-2* response to hPRL or hPRL-G129R treatments. There was no correlation between estrogen receptor (ER) status and *bcl-2* response to hPRL or hPRL-G129R treatment. The data from these studies suggest that hPRL increases the expression of *bcl-2* message and Bcl-2 protein in some human breast cancer cell lines, and hPRL-G129R competitively inhibits *bcl-2* expression induced by hPRL in these cell lines.

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