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Award Number: DAMD17-99-1-9129

TITLE: Rational Design of Human Prolactin Receptor Antagonists for Breast Cancer Therapy

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REPORT DATE: October 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2000	Annual (15 Sep 99 - 14 Sep 00)

4. TITLE AND SUBTITLE
Rational Design of Human Prolactin Receptor Antagonists for Breast Cancer Therapy

5. FUNDING NUMBERS
DAMD17-99-1-9129

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
This report contains colored photos

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

There were three tasks proposed for the first year of this project. All three tasks have been accomplished. We have cloned and confirmed by sequencing the cDNAs encoding hPRL, hPRL-G129R and hPRL-BP (task 1). All three cDNAs have been transfected into mouse L cells and stable mouse L cells have been established (task 2). We have also maintaining 12 human cancer cell lines purchased from ATCC(task 3). In addition, we have demonstrated that hPRL-G129R is able to inhibit breast cancer cell proliferation via induction of apoptosis (Clinical Cancer Research, 5:3583-3593, 1999). We are confident that the project will go on as proposed in years two and three.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
24

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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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 then be used to establish stably transfected mouse L cells. The proteins produced from stably transfected mouse L cells will be purified and used to test its bioactivities in ten different human breast cancer cell lines and two non-breast origin human cancer cell lines (as controls) for receptor binding, inhibition of phosphorylation of the STAT 5 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 three tasks proposed for the first year of this project. All three tasks have been accomplished. We have cloned and sequence confirmed cDNAs encoding hPRL, hPRL-G129R and hPRL-BP (task 1). All three cDNAs have been transfected into mouse L cells and stable mouse L cells have been established (task 2). We have also purchased all twelve human cancer cell lines and maintaining them in the lab (task 3).

In addition, we have made significant progress regarding hPRL antagonist (hPRL-G129R). Proteins (hPRL and hPRL-G129R)

produced from stable L-cell conditioned media have been partially purified and used for *in vitro* studies. We have demonstrated in our resent publications that hPRL-G129R is truly a hPRL receptor antagonist (Clinical Cancer Research, 5:3583-3593, 1999; appendix A). We have also demonstrated in our preliminary studies (two abstracts; appendix A) that the mechanism of the apoptosis induced by the hPRL-G129R is probably through the modulation of transforming growth factors (TGF β and α) and inhibition of STAT3.

We have encountered an unexpected problem regarding established stable L cells expressing hPRL-BP. We have found that the expression levels of the hPRL-BP in these cell lines is much less than that of PRL or PRL-G129R producing stable cell lines. This is probably due to the intrinsic properties of the hPRL-BP. Out of approximately 20+ cell lines we have established, the mean expression level is <50ng/ml as compared to >500ng/ml in the case of hPRL or hPRL-G129R stables. We are in the process of collecting enough protein to start proposed assays. In the meantime, we have also started a second round of transfection and stable cell line selection. In addition, we have chosen an alternative protein expression method (pET22b system from Novegene, Inc.) in the hope of produce enough protein for in vitro studies. In this regard, we have completed the sub-cloning process (Fig.1).

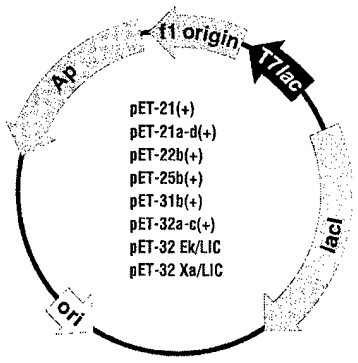

Figure. 1. Schematic representation of cloning and construction of the expression plasmid of pET22b-hPRL-BP cDNA. A PCR fragment amplified from hPRL-BP cDNA (deletion of signal peptide) will be inserted into the multiple cloning site of an E. coli expression vector (pET22b+). The hPRL-BP produced by this method has an extra Met at the N-terminus.

Key Research Accomplishment for the First Year.

We have cloned all three cDNAs and have established stable L-cell lines as proposed. In addition, we have demonstrated that hPRL-G129R acts as a PRLR antagonist in human breast cancer cells. It inhibits human breast cancer cell proliferation through induction of apoptosis shown in multiple human breast cancer cell lines. We have also demonstrated that the PRL antagonist has an additive effect when applied together with the anti-estrogen agent tamoxifen.

Reportable Outcomes

One manuscript, two abstracts and meeting presentations (see appendix A):

A Human Prolactin Antagonist Modulates Transforming Growth Factors Beta-1 and Alpha in Human Breast Cancer Cells. Ramamoorthy, P., Wagner, T.E. and Chen,W.Y

American Association for Cancer Research Annual Meeting, April 1-4, 2000, San Francisco, CA

Inhibition of the Oncogene STAT3 by a Human Prolactin (PRL) Antagonist is a PRL Receptor Specific Event Cataldo L, Chen, N.Y. Yuan, Q. Li, W, Wagner, T.E. and Chen,W.Y

82nd Annual Endocrine Society Meeting, June 21-24, 2000, Toronto, Canada

One Ph. D. student was graduated (partially supported by this award)
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Two proposals submitted based upon the work supported by this award (see appendix B, for abstracts only)

One post-doctoral fellow was employed by this award (Dr. Wei Li)

Conclusions:

In our first year of work, we have demonstrated that hPRL-G129R acted as a PRL antagonist in human breast cancer cell lines. We have shown that hPRL-G129R is able to inhibit breast cancer cell proliferation via induction of apoptosis. We have also made considerable progress elucidating the mechanisms involved in hPRL-G129R induced apoptosis. Although we have established a number of stable L cell lines expressing hPRL-BP, we would like to optimize levels of expression for the production of hPRL-BP for *in vitro* studies. To this end, we have also completed the sub-cloning necessary for developing an alternative expression system using the pET22b vector.

Appendix A

A Human Prolactin Antagonist, hPRL-G129R, Inhibits Breast Cancer Cell Proliferation through Induction of Apoptosis[1]

Wen Y. Chen,[2] Preveen Ramamoorthy, Nian-yi Chen, Robert Sticca, and Thomas E. Wagner

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

ABSTRACT

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

Received 3/18/99; revised 7/19/99; accepted 8/17/99.

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[1] This work was supported in part by the Endowment Fund of the Greenville Hospital System and the Greenville Hospital System/Clemson University Biomedical Cooperative and a Grant BC980253 from the United States Army Medical Research Command.

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INTRODUCTION

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

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

Several lines of evidence strongly link hPRL to breast cancer development: (*a*) it has been reported that female hGH transgenic mice have a high incidence of breast cancer in contrast to sporadic cases found in bovine GH transgenics (6). The high incidence of breast cancer in hGH transgenic mice is believed to be attributable to the lactogenic activity of hGH, which is a unique feature of primate GHs. A recent report of breast cancer development in hPRL transgenic mice further confirmed the role of hPRL in the stimulation of breast cancer (7); and (*b*) the finding of hPRL mRNA in mammary tissues (8–10) and the detection of biologically active hPRL in human breast cancer cells (11) suggest that hPRL is produced locally as

[3] The abbreviations used are: hPRL, human prolactin; PRLR, PRL receptor; GH, growth hormone; hGH, human GH; bGH, bovine GH; RT-PCR, reverse transcription-PCR; ER, estrogen receptor; FBS, fetal bovine serum; IRMA, immunoradiometric assay; E2, estradiol; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

an autocrine/paracrine growth factor within the mammary glands. This extrapituitary production of hPRL might not cause detectable systemic change of hPRL in serum yet could exert significant local stimulatory effects (12). In support of this concept, it has also been reported that the expression levels of PRLRs are significantly higher in human breast cancer cells or in surgically removed breast cancer tissues than in normal breast epithelial tissues (13–15). The high levels of PRLRs in malignant breast tissue make these cells highly sensitive to stimulation by hPRL (15).

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

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

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

		129
Human	PRL	IEEQTKRLLR **G** MELIVS-QVHP
Rat	PRL	IEEQNKRLLE **G** IEKIIG-QAYP
Mouse	PRL	IEEQNKQLLE **G** VEKIIS-QAYP
Hamster	PRL	IGEQNKRLLE **G** IEKILG-QAYP
Fin whale	PRL	EEEENKRLLE **G** MEKIVG-QVHP
Mink	PRL	IEEENRRLLE **G** MEKIVG-QVHP
Cattle	PRL	IEEQNKRLIE **G** MEMIFG-QVIP
Sheep	PRL	EEEENKRLLE **G** MENIFG-QVIP
Pig	PRL	IEEQNKRLLE **G** MEKIVG-QVHP
Camel	PRL	IEEQNKRLLE **G** MEKIVG-QVHP
Horse	PRL	EIEQNRRLLE **G** MEKIVG-QVQP
Elephant	PRL	VKEENQRLLE **G** IEKIVD-QVHP
Ancestral mammal	PRL	IEEENKRLLE **G** MEKIVG-QVHP
Chicken	PRL	IEEQNKRLLE **G** MEKIVG-RVHS
Turkey	PRL	IEEQDKRLLE **G** MEKIVG-RIHS
Sea turtle	PRL	IEEQNKRLLE **G** MEKIVG-QVHP
Crocodile	PRL	IEEQNKRLLE **G** MEKIIG-RVQP
Alligator	PRL	IEEQNKRLLE **G** MEKVIG-RVQP
Ancestral amniote	PRL	IEEQNKRLLE **G** MEKIVG-QVHP
Xenopus	PRL	VEEQNKRLLE **G** MEKIVG-RIHP
Bullfrog	PRL	VEEQTKRLLE **G** MERIIG-RIQP
Lungfish	PRL	VEDQTKQLIE **G** MEKILS-RMHP
Tilapia	PRL	MQQYSKSLKD **G** LD-VLSSKMGS
Tilapia	PRL	MQEHSKDLKD **G** LD-ILSSKMGP
Common carp	PRL	LQENINSLGA **G** LEHVF-NKMDS
Bighead carp	PRL	LQDNINSLGA **G** LERVV-HKMGS
Silver carp	PRL	LQDNINSLVP **G** LEHVV-HKMGS
Chum salmon	PRL	LQDYSKSLGD **G** LD-IMVNKMGP
Chinook salmon	PRL	LQDYSKSLGD **G** LD-IMVNKMGP
Trout	PRL	LQDYSKSLGD **G** LD-IMVNKMGP
		120
Human	GH	VYDLLKDLEE **G** IQTLMRELEDG
Bovine	GH	VYEKLKDLEE **G** ILALMRELEDG

[a] Two GH sequences are also included. Gly 129 of hPRL is in bold.

MATERIALS AND METHODS

RT-PCR

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

Rational Design of hPRL-G129R

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

Oligonucleotide-directed Mutagenesis

hPRL-G129R cDNA was generated using a PCR mutagenesis protocol. Oligonucleotides containing the desired mutation (5′-CTTCTAGA**GCGC**ATGGAGCTCATA-3′ and 5′-CCCT-CTAGACTCGAGCGGCCGCC-3′) were synthesized by National Biosciences, Inc. (Plymouth, MN). The codon for 129 Arg is in boldface, and the restriction site *Xba*I is underlined.

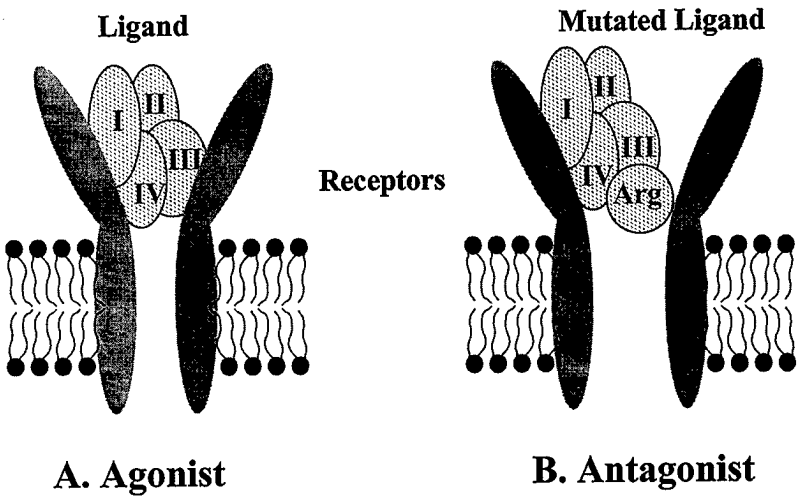

Fig. 1 Schematic illustration of the mechanism of GH or hPRL (ligand) antagonist. Four helical regions in the ligand (*dotted ovals*) are labeled as *I, II, III,* and *IV*. Two membrane bound receptors (*shaded dark ovals*) are also shown in the figure. *Arg,* substitution mutation in the third α-helix, resulting in hindering a second receptor to form a functional complex (from *A* to *B*).

The PCR product was digested with *Xba*I and ligated back into the vector described previously. The mutation was then confirmed by DNA nucleotide sequencing.

Human Breast Cancer Cell Lines

The human breast cancer cell lines used in this study are MDA-MB-134, T-47D, BT-474, and MCF-7 from the American Type Culture Collection. These human breast cancer cell lines have been characterized as ER-positive and PRLR-positive cell lines (28). T-47D and BT-474 cells were grown in RPMI 1640 (phenol red free to avoid its potential estrogen-like activities) supplemented with 10% FBS (Life Technologies, Inc.) and American Type Culture Collection recommended supplements. MCF-7 cells were grown in DMEM (phenol red free), supplemented with 10% FBS. The cells were grown at 37°C in a humid atmosphere in the presence of 5% CO_2. The MDA-MB-134 cells were grown in Leibovitz's L-15 medium supplemented with 20% FBS and grown in a CO_2-free atmosphere.

Expression and Production of hPRL and hPRL-G129R Proteins

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

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

Radioreceptor Binding Assay

hPRLR binding assays were performed as described previously (19, 31). Briefly, T-47D cells were grown in six-well tissue culture plates until 90% confluent (~10^5 cells/well). Monolayers of cells were starved in serum-free RPMI 1640 medium for 2 h. The cells were then incubated at room temperature in serum-free RPMI 1640 containing 8×10^4 cpm ^{125}I-labeled hPRL (specific activity, 30 μCi/μg; NEN DuPont, Boston, MA) with or without various concentrations of hPRL (from NIH as standard) and hPRL-G129R. Cells were then washed three times in serum-free RPMI 1640 and solubilized in 0.5 ml of 0.1 N NaOH/1% SDS, and the bound radioactivity was determined by a gamma counter (model 4/600plus; ICN Biomedical, Costa Mesa, CA). EC_{50}s of hPRL and hPRL-G129R were then determined and expressed as mean ± SD. Comparison was made by Student's *t* test.

Human Breast Cancer Cell Proliferation Assays

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

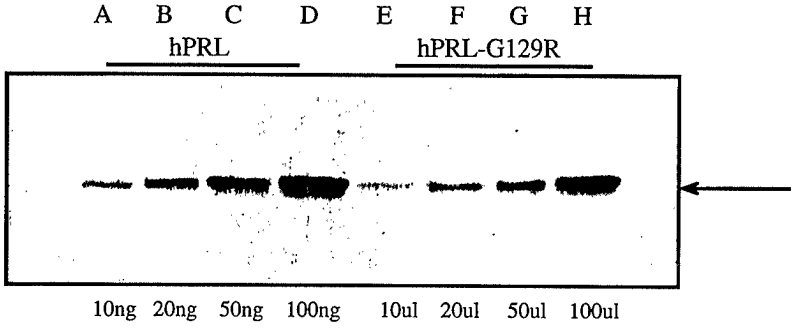

Fig. 2 Immunoblot analysis of the hPRL-G129R gene expression by mouse L cells. A polyclonal rabbit anti-hPRL (1:500; Biodesign International, Kennebunk, ME) was used as primary antibody, and a goat anti-rabbit IgG horseradish peroxidase conjugate (1:500; Boehringer Mannheim) was used as secondary antibody. *Lanes A–D*, samples containing purified hPRL (from NIH) as standards. *Lanes E–H*, culture media from stably transfected mouse L cells.

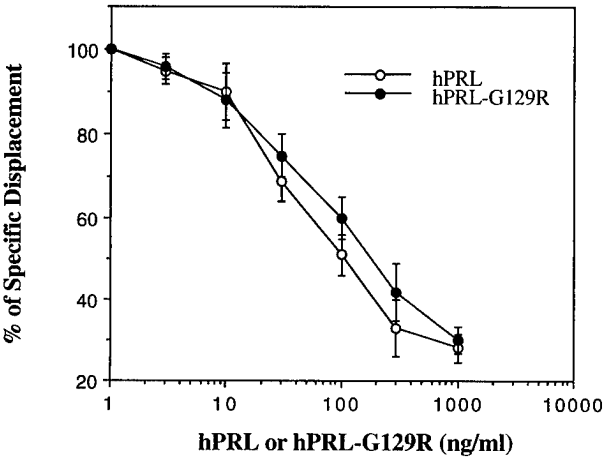

Fig. 3 Competitive radioreceptor binding assays. The data from triplicate determinations of three separate experiments are presented as the means; *bars*, SD. *Ordinate*, hPRL or hPRL-G129R concentrations. *Abscissa*, percentage of displacement of the total binding. EC_{50}s were determined and compared using Student's *t* test. There was no significant difference between the two EC_{50}s ($P > 0.05$).

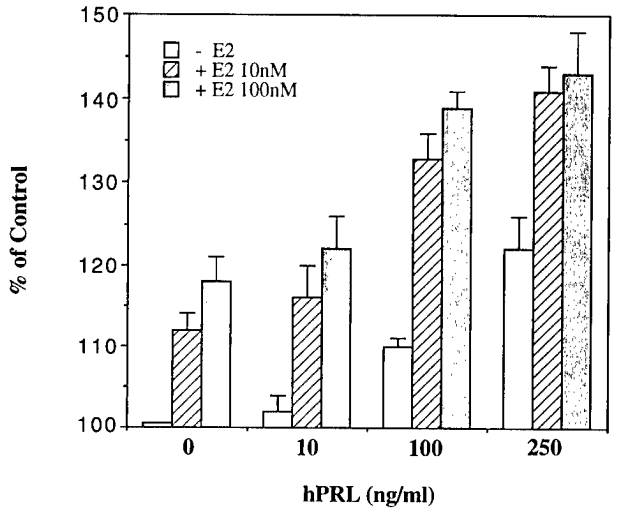

Fig. 4 Dose-response effects of hPRL and its additive effects with E2 in T-47D human breast cancer cell proliferation assay. *X axis*, the hPRL concentration either in the absence (□) or presence of E2. Each data point represents a mean of at least three independent experiments with triplicate wells; *bars*, SD.

The cells were allowed to settle and adhere overnight (12–18 h), and subsequently various concentrations of either hPRL, hRPL-G129R, E2, or 4-OH-tamoxifen in a total volume of 100 μl of culture media were added. Purified hPRL (kindly provided by Dr. Parlow, National Hormone and Pituitary Program, NIH, Bethesda, MD) was used as a positive control for hPRL produced from stable L cells. Cells were incubated for an additional 96 h at 37°C in a humidified 5% CO_2 incubator. After incubation, MTS-PMS solution (Cell Titer 96 Aqueous kit; Promega Corp.) was added to each well, following the manufacturer's instructions. Plates were read at 490 nm using a Bio-Rad benchmark microplate reader. The experiments were carried out in triplicates and repeated three to six times for each cell line.

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

TUNEL Assay

This assay (Fluorescein Apoptosis detection system; Promega Corp.) works by labeling the nicks of the fragmented DNA at the 3-OH ends. The fluorescein-labeled dUTP is incorporated at the 3-OH ends by terminal deoxynucleotidyl transferase. Four human breast cancer cell lines were used in this study. Before the assay, the breast cancer cells were switched to 10% charcoal/dextran-treated FBS (CCS) for a week. Subsequently, the cells were plated onto an eight-chambered slide

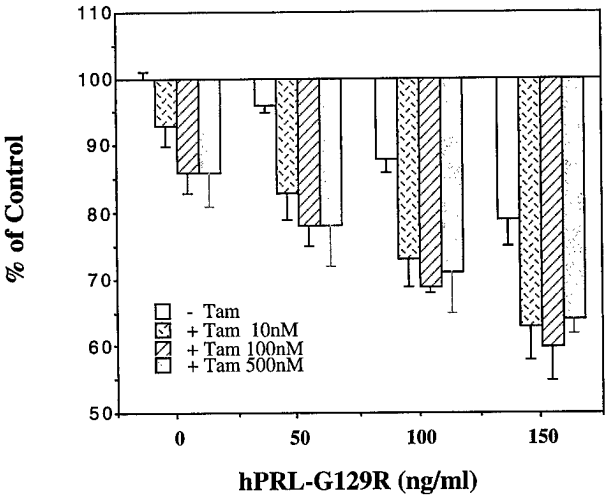

Fig. 5 Dose-response inhibitory effects of hPRL-G129R and its additive effects with 4-OH-Tamoxifen in T-47D human breast cancer cell proliferation assay. *X axis,* the hPRL-G129R concentration either in the absence (□) or presence of 4-OH-Tamoxifen. Each data point represents a mean of at least three independent experiments with triplicate wells; *bars,* SD.

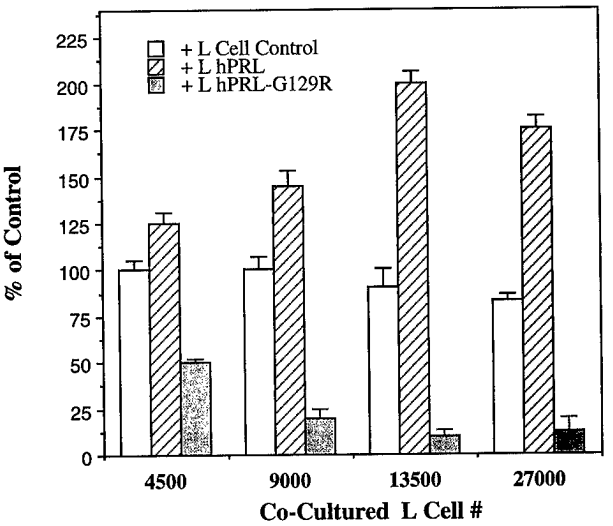

Fig. 7 Dose-response inhibitory effects of hPRl-G129R in T-47D human breast cancer cells using the coculture method. *X axis,* the cocultured L cell (control, L-PRL, or L-hPRL-G129R) numbers. Each data point represents a mean of at least three independent experiments with triplicate wells; *bars,* SD.

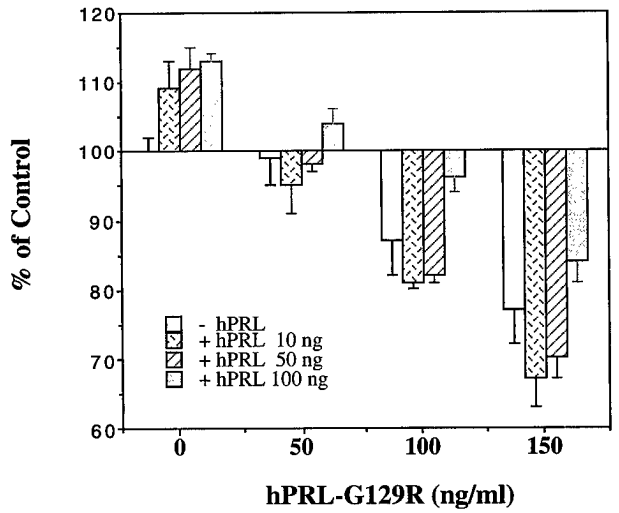

Fig. 6 Dose-response inhibitory effects of hPRL-G129R on hPRL-induced T-47D cell proliferation. *X axis,* concentration of hPRL-G129R either in the absence of hPRL (□) and the presence of hPRL. Each data point represents a mean of at least three independent experiments with triplicate wells; *bars,* SD.

system (Lab TekII) at a confluence of 60–70% per chamber. The next day, the breast cancer cells were treated with various concentrations of hPRL-G129R in conditioned medium (0.5% CCS) or 4-OH-Tamoxifen (in 0.5% CCS containing growth medium). To demonstrate the specificity of the antagonist, hPRL-G129R was also either mixed with PRL or with polyclonal anti-hPRL antibodies (kindly provided by Dr. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases) before being applied to breast cancer cells. In the case of anti-hPRL antibody experiments, 125 ng/ml of hPRL-G129R were preincubated with anti-hPRL antibodies for 6 h at 4°C before adding to the cells. After the assigned period of treatment, the chambers were dismantled, and the assay was performed as per the manufacturer's instructions. The slides were examined under a FITC filter using an Olympus IX 70 microscope system.

RESULTS

Cloning and Mutagenesis of hPRL

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

Expression of hPRL and hPRL-G129R

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

Radioreceptor Binding Assay

The assay was carried out in a homologous system using ^{125}I-labeled hPRL in the presence or absence of various con-

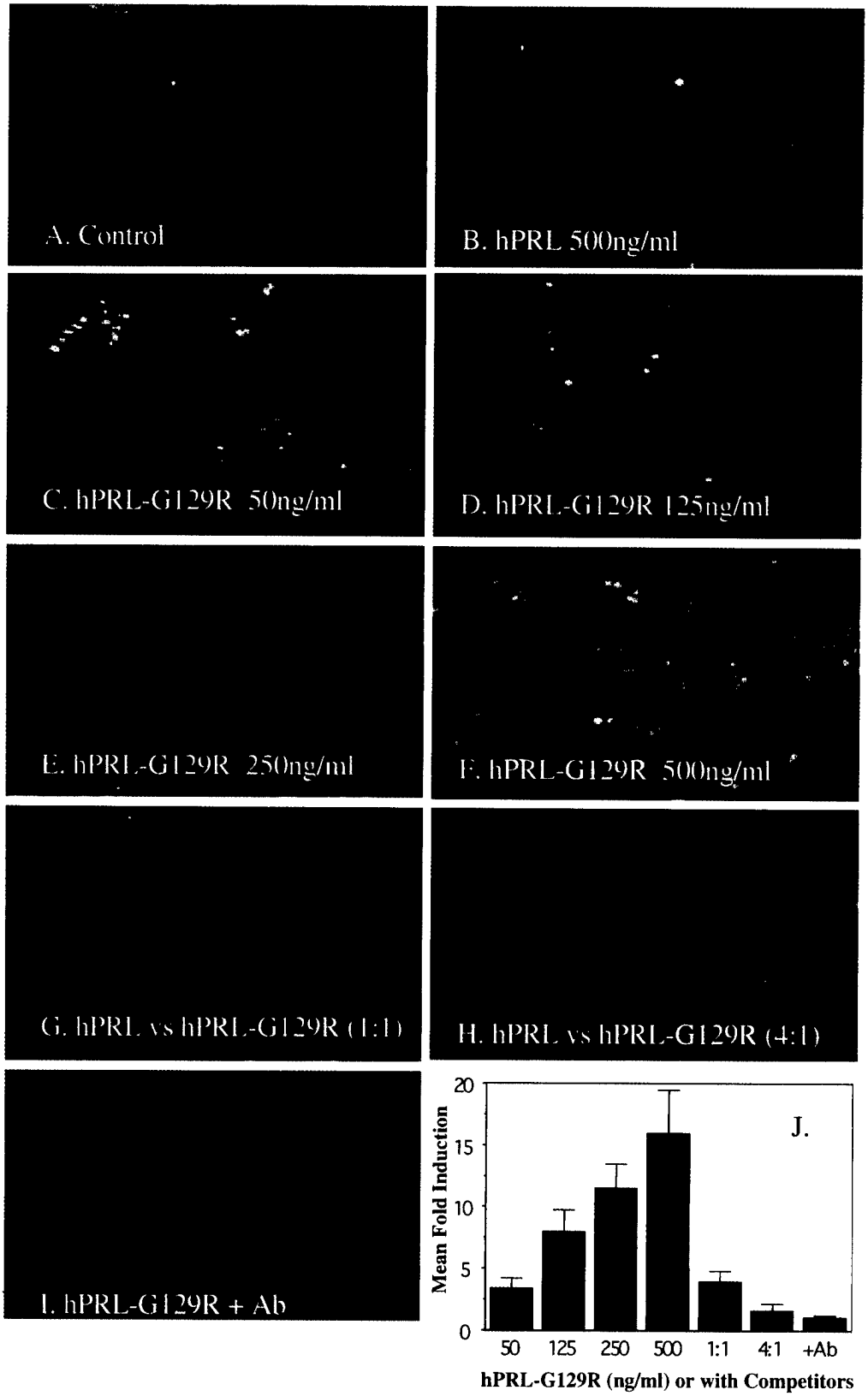

Fig. 8 Dose-response of T-47D human breast cancer cells to hPRL-G129R after 24 h of treatment using the TUNEL assay (*A–F*). *G* and *H*, results of competition between hPRL and hPRL-G129R at 1:1 ratio (125 ng/ml of each; *G*) and 4:1 ratio (500 ng/ml hPRL + 125 ng/ml hPRL-G129R; *H*). *I*, result of anti-hPRL antibody pretreatment (125 ng/ml of hPRL-G129R in 100-μl volume + 100-μl antiserum). *J*, quantification of the same experiment (fold induction of apoptotic cells/field over control; average of three measurements).

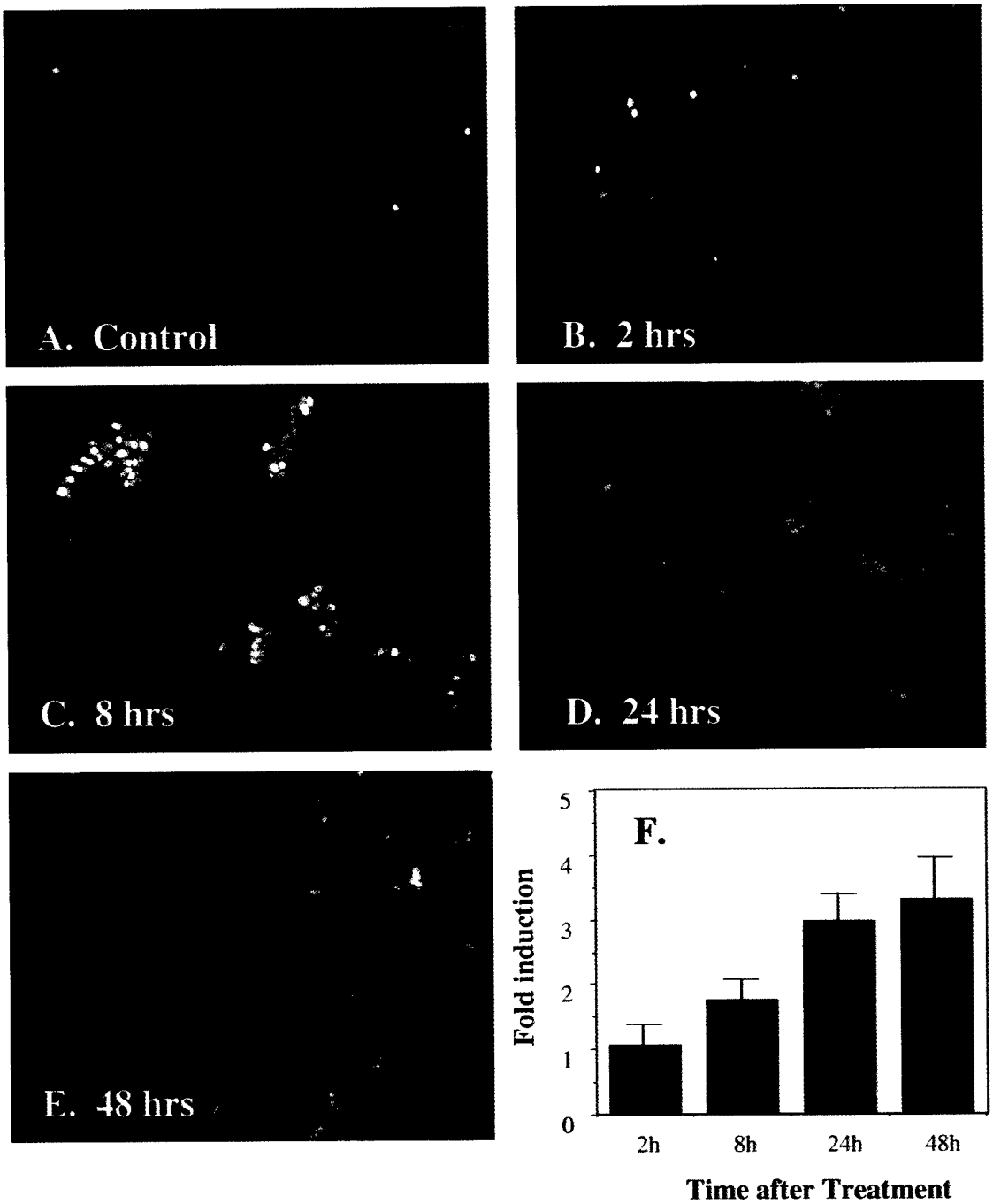

Fig. 9 Time course of T-47D human breast cancer cells responding to hPRL-G129R treatment (50 ng/ml) using the TUNEL assay (*A–E*). *F*, quantification of the same experiment (fold induction of apoptotic cells/field over control; average of three measurements).

centrations of unlabeled hPRL or hPRL-G129R and T-47D cells. The results demonstrated that there was no significant change in EC_{50}s ($P > 0.05$) of hPRL-G129R (3.01 nM ± 0.24 nM) as compared with hPRL (1.89 ± 0.18 nM; Fig. 3). These results were similar to our previous studies regarding bGH antagonist (bGH-G119R; Ref. 16) and hGH antagonist (hGH-G120R; Ref. 19).

Human Breast Cancer Cell Proliferation Assays

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

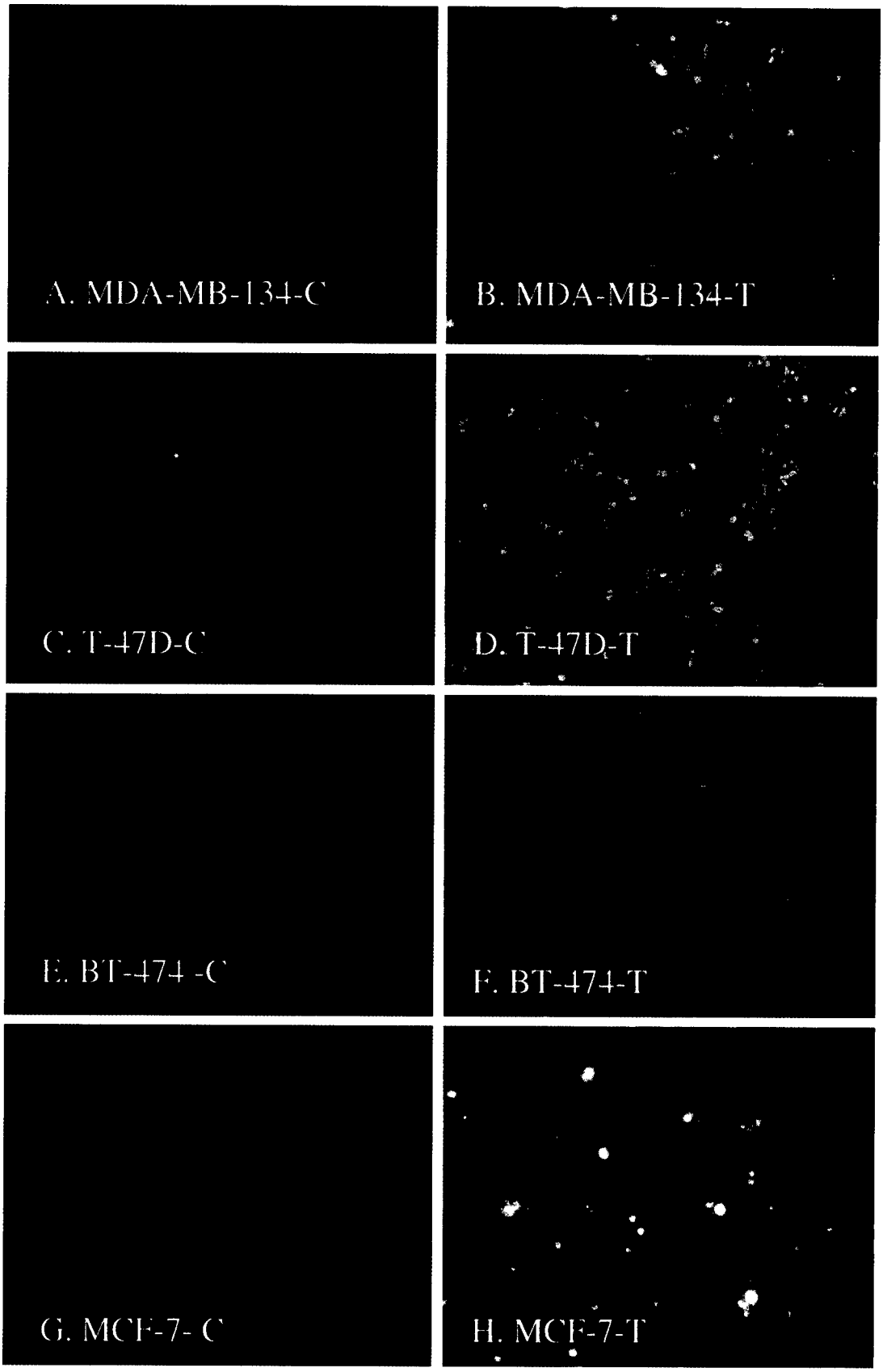

Fig. 10 Response of multiple breast cancer cells (as labeled) to treatment with 250 ng/ml hPRL-G129R for 24 h using the TUNEL assay. *-C*, control cells; *-T*, treated cells.

basal levels after a single dose/4-day incubation. However, when hPRL and E2 were applied simultaneously, an additive effect was observed. The maximum response of hPRL (100 ng/ml) in the presence of 10 nM of E2 was more than tripled as compared with hPRL alone (Fig. 4).

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

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

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

TUNEL Assay

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

DISCUSSION

Human breast cancer is known to be a heterogeneous mixture of cell clones characterized by different biological features. The primary target of endocrine therapy for breast cancer has been E2, by either surgical or pharmacological methods of estrogen deprivation (1, 2). Among the pharmacological methods, the most notable has been the development of tamoxifen. Recently, the National Surgical adjuvant Breast and Bowel Project has reported the results of the Breast Cancer Prevention Trial demonstrating a 49% decrease in the incidence of invasive breast cancer in a large cohort of high risk women as a result of the use of tamoxifen (32, 33). Despite these encouraging results, a fraction of ER-positive tumors escape first- or second-line endocrine treatment because of the initial presence of estrogen-negative clones or the development of drug resistance. It is this complexity that partly explains why tamoxifen is not universally effective, even in ER-rich tumors (2). In addition, any progress in the development of better antiestrogen therapy for breast cancer is unlikely to impact on the treatment of ER-negative tumors. For these reasons, it is our belief that the scope of the search for drugs to treat breast cancer should be expanded to effectively control tumor growth and/or recurrence in all tumors.

Recently, several lines of evidence strongly suggest that hPRL acts as an autocrine/paracrine growth factor contributing to breast cancer development (11, 34, 35). More importantly, it has recently been reported that sex steroid hormones and PRL interact synergistically to control cancerous growth within the mammary gland (28). ER and PRLR were found being coexpressed and cross-regulated in mammary tumor cell lines as well as in primary breast cancers (28). These findings further suggest that the use of antiestrogen therapy in breast cancer may be attacking only half of the synergistic equation, which leaves an opportunity for further improvement of the ultimate therapeutic approach to breast cancer (28). In support to this notion, a combined regimen using an antiestrogen (Tamoxifen), an anti-GH secretion drug (octreotide), and an anti-PRL secretion drug (CV 205-502) has been reported to have significantly better clinical results in metastatic breast cancer patients as compared with tamoxifen therapy alone (36). Although this regimen does not block the autocrine/paracrine action of PRL on breast cancer, inhibition of circulating PRL from the pituitary did seem to have an additive benefit in the treatment of advanced breast cancer. This raises exciting prospects for even better results with complete PRL blockade with an antagonist that acts at the receptor level.

In this study, we report the design and production a hPRL antagonist, hPRL-G129R. We first demonstrated that hPRL and E2 exhibited an additive stimulatory effect in human breast cancer cell proliferation (Fig. 4). We believe that the synergistic effects between hPRL and estrogen reflect the real physiological status because the breast tissue is constantly exposed to both newly synthesized estrogen and hPRL. These results also indicate the possibility of developing new therapeutic regimens, targeting possible tumor stimuli other than the ER. The potential

for additive and therefore improved benefits is significant. We further demonstrated that hPRL-G129R possessed an inhibitory effect on T-47D cell proliferation (Fig. 5). More importantly, when anti-estrogen (4-OH-Tamoxifen) and anti-PRL (hPRL-G129R) agents were applied simultaneously, as we had anticipated, an additive effect was observed. The inhibitory effect on cell proliferation was more than doubled (Fig. 5). We reason that the direct inhibitory effects of hPRL-G129R on T-47D cell proliferation are by competitive inhibition of the hPRL produced by T-47D cells (11). The hPRLR-specific antagonistic effects of hPRL-G129R were further substantiated by an assay that uses combinations of hPRL and hPRL-G129R. It is encouraging to note that even at the ratio of 1:1, hPRL-G129R could stop the T-47D cell proliferation induced by hPRL (Fig. 6).

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

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

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

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

ACKNOWLEDGMENTS

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

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A Human Prolactin Antagonist Modulates Transforming Growth Factors Beta-1 and Alpha in Human Breast Cancer Cells Ramamoorthy, P., Wagner, T.E. and Chen,W.Y. *Oncology Research Institute, Cancer Center, Greenville Hospital System, Greenville SC 29650; and Department of Microbiology and Molecular Medicine, Clemson University, Clemson, SC 29634*

Human prolactin (hPRL) has been shown to be one of the important survival/growth factors that promote the proliferation of breast cancer cells in an autocrine/paracrine manner. In our recent studies, we have developed a hPRL antagonist by a single amino acid substitution mutation in hPRL molecule (hPRL-G129R). We reported that hPRL-G129R was able to inhibit breast cancer cell proliferation via induction of apoptosis (Chen et al., Clinical Cancer Res. Nov.1, 1999, in press). It is also believed that the mammary epithelium is subject to influences by mitogenic growth factors such as Estrogen (E2), PRL, as well as Transforming Growth Factor (TGF) alpha. hPRL has also been found to be able to inhibit the production of TGF-beta that is believed to be an apoptotic agent. In the present study, we investigate the relationships among hPRL/hPRL-G129R and TGFs. We hypothesized that the hPRL antagonist, hPRL-G129R, would down regulate TGF alpha (survival factor) and up-regulate TGF beta-1 (apoptotic factor) in breast cancer cells. Our preliminary data using two human breast cancer cell lines (T-47D and MCF-7) demonstrated that hPRL down regulates TGF beta secretion and up regulates TGF alpha in a dose dependent manner measured by ELISA. On the other hand, the hPRL antagonist, hPRL-G129R, up-regulates TGF beta-1 and down-regulates TGF alpha secretion also in a dose dependent manner. More importantly, when hPRL-G129R was applied together with hPRL, it specifically blocked the effects of hPRL. We believe that the hPRL antagonist, by blocking the hPRL receptor, serves as an apoptotic factor through down-regulating TGF alpha and up-regulating TGF-beta-l, and therefore may be valuable as a therapeutic agent in breast cancer treatment.

82nd Annual Endocrine Society Meeting, June 21-24, 2000, Toronto, Canada

Inhibition of the Oncogene STAT3 by a Human Prolactin (PRL) Antagonist is a PRL Receptor Specific Event Cataldo L, Chen, N.Y. Yuan, Q. Li, W, Wagner, T.E. and Chen,W.Y. *Oncology Research Institute, Cancer Center, Greenville Hospital System, Greenville SC 29650; and Department of Microbiology and Molecular Medicine, Clemson University, Clemson, SC 29634*

STAT (signal transducers and activators of transcription) proteins are latent transcription factors that mediate growth hormone (GH) and prolactin (PRL) induced transcription, as well as transcription induced by other cytokines and growth factors. Recently, it has been reported that STAT3 is persistently activated in may human cancers and transformed cell lines suggesting that STAT3 represents a novel target for cancer therapy. In our previous studies, we have developed a hPRL antagonist by a single amino acid substitution mutation in the hPRL molecule (hPRL-G129R). We have demonstrated that hPRL-G129R was able to inhibit breast cancer cell proliferation via induction of apoptosis (Chen et al., Clinical Cancer Res. 5: 3583-93, 1999). In the present study, we set out to test the hypothesis that the inhibitory effects of hPRL-G129R is specifically mediated through the inhibition of STAT3 phosphorylation. The cell lines used in this study are T47D human breast cancer cells (containing both hPRLR and hGHR) and mouse L cells expressing hGH receptors (L-hGHR cells). The hGHR cDNA was cloned from human pituitary mRNA (Clontech Inc.) and stably transfected into mouse L-cells using a pcDNA 3.1 vector (Invitrogen Inc.). Cells were treated with either hPRL, hGH, hPRL-G129R, or a hGH antagonist (hGH-G120R) alone, or in combinations (agonists i.e. hPRL or hGH in the presence of antagonists i.e. hPRL-G129R or hGH-G120R, respectively) and harvested. Cell lysates were then analyzed by western blotting with antisera against either phosphorylated STAT3 or phosphorylated STAT5. In T47D cells, the results demonstrated that STAT5 and STAT3 could be activated by either agonists (hGH or hPRL). However, only STAT5, not STAT3, was activated in L-hGHR cells by hGH. These data strongly suggest that STAT3 activation is mediated through the PRLR. It is also interesting to point out that the levels of STAT3 phosphorylation induced by hGH (presumably through hGH binding to hPRLR) in T47D cells is much less as compared to that of hPRL suggesting that the efficacy of hPRL/hPRLR signaling through STAT3 is much better than that of hGH/hPRLR. More importantly, we were able to demonstrate that STAT3 phosphorylation can only be inhibited by hPRL-G129R (not hGH-G120R) in T47D cells. In conclusion, we believe that hPRL-G129R would be a useful therapeutic agent especially in breast cancer

Appendix B

Department of Health and Human Services
Public Health Service

Grant Application

Follow instructions carefully.

Do not exceed character length restrictions indicated on sample.

OMB No. 0925-000

LEAVE BLANK-FOR PHS USE ONLY.

Type	Activity	Number
Review Group	Formerly	
Council/Board *(Month, Year)*		Date Received

1. TITLE OF PROJECT *(Do not exceed 56 characters, including spaces and punctuation.)*

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

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT ☒ NO ☐ YES *(If "Yes," state number and title*

Number: Title: Academic Research Enhancement Award

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR New Investigator ☐ YES

3a. NAME *(Last, first, middle)*: Chen, Wen

3b. DEGREE(S): Ph.D

3c. SOCIAL SECURITY NO. *Provide on Form Page RR.*

3d. POSITION TITLE: Asst. Professor

3e. MAILING ADDRESS *(Street, city, state, zip code)*

Dept. of Microbiology and Molecular Medicine
124 Long Hall
Clemson University
Clemson, SC 29634-0327

3f. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT: Dept. of Micro and Molecular Medicine

3g. MAJOR SUBDIVISION: College of Ag. Forestry & Life Sciences

3h. TELEPHONE AND FAX *(Area code, number and extension)*

TEL: 864-656-3057
FAX: 864-656-1127

E-MAIL ADDRESS: wenc@clemson.edu

4. HUMAN SUBJECTS
☒ No
☐ Yes
4a. If "Yes," Exemption no. or IRB approval date
☐ Full IRB or ☐ Expedited Review
4b. Assurance of compliance No.

5. VERTEBRATE ANIMALS
☐ No
☒ Yes
5a. If "Yes," IACUC approval date: Pending
5b. Animal welfare assurance no.: A3737-01

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year-MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)
07/01/01	06/30/05	150,000.	210,000.	750,000.	1,050,000.

9. APPLICANT ORGANIZATION

Name: Clemson University
Address: Office for Sponsored Programs
300 Brackett Hall
Clemson, SC 29634

10. TYPE OF ORGANIZATION
Public: → ☐ Federal ☒ State ☐ Local
Private: → ☐ Private Nonprofit
Forprofit: → ☐ General ☐ Small Business

11. ORGANIZATIONAL COMPONENT CODE: 20

12. ENTITY IDENTIFICATION NUMBER: 57 6000254
DUNS NO. *(if available)*

Congressional District: 3

13. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE

Name: Chris Thurston
Title: Grants Administrator
Address: Office for Sponsored Programs
300 Brackett Hall
Clemson University
Clemson, SC 29634
Telephone: 864-656-5032
FAX: 864-656-0881
E-Mail: irisour@clemson.edu

14. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION

Name: Doris Helms
Title: Interim Vice-Pres of Academic Affairs/ Vice Provost
Address: Office for Sponsored Programs
300 Brackett Hall
Clemson University
Clemson, SC 29634
Phone: 864-656-2424
FAX: 864-656-0881
E-Mail: Biol110@clemson.edu

15. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE:
I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PI/PD NAMED IN 3a. *(In ink. "Per" signature not acceptable.)*: Wen Chen

DATE: 10/02/00

16. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE:
I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 14. *(In ink. "Per" signature not acceptable.)*: Doris R Helms

DATE: 10/02/00

DESCRIPTION: State the application's broad, long-term objective and specific aims, making reference to the health relatedness of th project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and th use of the first person. This description is meant to serve as a succinct and accurate description of the proposed work when separate from the application. If the application is funded, this description, as is, will become public information. Therefore, do not includ proprietary/confidential information. DO NOT EXCEED THE SPACE PROVIDED.

Human prolactin (hPRL) has been demonstrated to be an autocrine/paracrine growth factor in human breast cancer development. In our recent studies, we have demonstrated that a hPRL antagonist (hPRLA) with a single amino acid substitution mutation (hPRL-G129R) is able to inhibit breast cancer cell proliferation via induction of apoptosis. We have also demonstrated that the possible mechanisms of hPRLA's inhibitory effects include: (1) the inhibition of phosphorylation of oncogene STAT3, (2) modulation of transforming growth factors (TGFs) (i.e. up regulation of TGFβ and down regulation of TGFα); and (3) induction of caspase 3 activities. Our preliminary data in nude mice further demonstrated the antitumor potential of hPRLA. Recently, targeted tumor immunotherapy using specific monoclonal antibody (mAb) and interleukin 2 (mAb-IL2) fusion proteins has been demonstrated to hold therapeutic promise in animal tumor models as well as in clinical trials. It has been reported that mAb-IL2 fusion protein dramatically increases the effectiveness of IL2 and at the same time decreases side effects induced by systemic use of IL2. In this proposal, we propose to combine the essence of endocrine therapy (anti-hPRL) and immunotherapy in the design of a targeted endocrine/cytokine (hPRLA-IL2) fusion protein specific for human breast cancer treatment. This novel approach has several advantages: (1) It utilizes the highly specific interaction between hPRLA and PRL receptors (PRLR), thus targeting the fusion protein specifically to the malignant breast tissues which have been shown to contain higher levels of PRLR; (2) The antagonistic effect of hPRLA of the fusion protein is designed to block the signal transduction induced by hPRL as endocrine therapy; (3) Localized IL2 can then play a crucial role in T lymphocyte activation leading to tumor cytotoxicity; (4) Targeted IL2 can be used at concentrations much less than free IL2, thus decreasing its side effects. There are three specific aims of this project. **Aim one** is to produce and purify >100 mg of hPRLA/IL2 fusion protein using the pET E coli expression system and FPLC purification system. After the fusion protein is produced, we will use four different *in vitro* assays (**aim two**) to confirm its bi-functional biological activities. For its hPRL antagonistic activities, we are going to use human breast cancer cells for (a) receptor binding assay; (b) inhibition of STAT3 phosphorylation assay; and (c) induction of apoptosis assay; and for its IL2-like activity, an IL2 dependent HT2 cells proliferation assay (d) will be used. The fusion protein will be further tested for its anti-breast tumor effects in syngeneic mice (**aim three**). We hope that this targeted drug design will provide a novel and effective approach to human breast cancer.

PERFORMANCE SITE(S) (Organization, city, state)

700 W. Faris Road
Oncology Research Institute, Greenville Hospital System,
and Dept. of Microbiology and Molecular Medicine, Clemson University
Greenville, SC 29605

KEY PERSONNEL See instruction on Page 11. *Use continuation pages as needed* to provide the required information in the format shown below.

Name	Organization	Role on Project
Chen, Wen Yuan	Greenville Hospital System and Clemson University	PI