AD_____

Award Number: DAMD17-01-1-0329

TITLE: Insulin-Like Growth Factor Binding Protein-1 Interacts with Integrins to Inhibit Insulin-Like Growth Factor-Induced Breast Cancer

PRINCIPAL INVESTIGATOR: Jennifer M. Gross
Doug Yee, M.D.

CONTRACTING ORGANIZATION: University of Minnesota
Minneapolis, Minnesota 55455-2070

REPORT DATE: July 2002

TYPE OF REPORT: Annual Summary

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
	July 2002	Annual Summary (1 Jul 01 - 30 Jun 02)

4. TITLE AND SUBTITLE

Insulin-like growth factor binding protein-1 interacts with integrins to inhibit insulin-like growth factor-induced breast cancer growth and migration

5. FUNDING NUMBERS

DAMD17-01-1-0329

6. AUTHOR(S)

Jennifer M. Gross
Doug Yee, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Minnesota
Minneapolis, Minnesota 55455-2070

E-Mail: Gros0228@umn.edu

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

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

The insulin-like growth (IGF) system and extracellular matrix proteins are key regulators of the malignant breast cancer phenotype. Both IGFs and extracellular matrix proteins communicate with epithelial cells by ligating cell surface receptors. Therefore ligand-receptor interactions of the two systems are relevant treatment targets in breast cancer. Studies have shown that IGFBP-1 can bind to IGF and prevent IGF from interacting with its receptor and inhibit breast cancer cell growth. IGFBP-1 has also been shown to interact with extracellular matrix protein receptors, integrins, on the cell surface through an Arg-Gly-Asp (RGD) integrin recognition sequence. This work-in-progress will test the hypothesis that IGFBP-1 interrupts ligand-receptor interactions between extracellular matrix proteins and integrins. The key research accomplishment is the cloning and expression of a mutant IGFBP-1 protein species that will not interact with integrins. The successful expression and purification of the mutant IGFBP-1 protein is needed to test whether IGFBP-1 can be used as a strategy to neutralize integrin function in an RGD-dependent mechanism. The long-term goal is to provide evidence that IGFBP-1 is a novel therapy for the treatment of breast cancer.

14. SUBJECT TERMS

breast cancer, integrins, insulin-like growth factor binding protein-1

15. NUMBER OF PAGES

8

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

Table of Contents

INTRODUCTION:

Accumulating evidence supports a role for the insulin-like growth factor (IGF) system and extracellular matrix proteins in the malignant phenotype of breast cancer. Both IGFs and extracellular matrix proteins interact with epithelial cells by ligating cell surface receptors. Our laboratory has previously shown that insulin-like growth factor binding protein-1 (IGFBP-1) can be used as a method to interrupt ligand-receptor interactions of the IGF system and inhibit the growth of breast cancer cells. IGFBP-1 contains an integrin recognition sequence and has also been shown to interact with extracellular matrix protein receptors, integrins, on the cell surface. The present proposal will test the hypothesis that IGFBP-1 inhibits the breast cancer phenotype by dual mechanisms, neutralization of IGF action and integrin function. The long-term goal is to provide evidence that IGFBP-1 is a novel therapy for the treatment of breast cancer.

BODY:

To determine whether IGFBP-1 inhibits IGF-stimulated responses through an RGD-dependent mechanism, a mutant IGFBP-1 will be expressed that does not contain an intact integrin binding sequence. The Arg in the integrin recognition site of IGFBP-1 was mutated to a Trp (R to W substitution at amino acid 221, [Trp^{221}]IGFBP-1), and has been shown to prevent binding of the protein to the $\alpha5\beta1$ fibronectin integrin receptor [1].

<u>Specific Aim 1)</u> Express wild-type IGFBP-1 protein and mutant [Trp^{221}]IGFBP-1 protein in yeast

Task 1: Month 1-6, Cloning and sequencing of wild-type IGFBP-1 cDNA and mutant [Trp^{221}]IGFBP-1 cDNA into the yeast *Pichia pastoris* vector

The *Pichia pastoris* system has been made commercially available by Invitrogen (Carlsbad, CA). *P. pastoris* is a methylotrophic yeast that has been shown to be suitable for high level expression of various heterologous proteins in secreted form. Expression of recombinant proteins in *P. pastoris* is based on the use of the alcohol oxidase-1 (AOX1) gene. In this expression system, methanol induces expression of the tightly regulated AOX1 gene which in turn drives expression and secretion of foreign proteins.

While this grant was in the process of being reviewed and funded, our laboratory was able to express and purify biologically active recombinant wild-type IGFBP-1 protein using the *Pichia pastoris* system. The yield of the wild-type IGFBP-1 protein is approximately 10mg/L yeast medium. Therefore, during the last year, I have focused my efforts on the expression of mutant [Trp^{221}]IGFBP-1 in *Pichia pastoris*.

The coding region of [Trp^{221}]IGFBP-1 was modified to incorporate an EcoR1 site at the 5'-end and a KpnI site at the 3'end using PCR. Using these restriction sites, the [Trp^{221}]IGFBP-1 gene was cloned into the yeast vector pPICZαB by fusing it to the α-factor mating secretion signal derived from *S. cerevisiae* for expression in *P. pastoris*. As described above, the α-factor mating secretion signal is under control of the AOX1 gene promoter and allows high levels of protein

secretion when induced by methanol. This vector also encodes the zeocin resistance gene *sn ble* for antibiotic selection.

The *P. pastoris* strain X-33 was transformed by electroporation with 10ug of linearized pPICZαB-[Trp^{221}]IGFBP-1, on a Bio-Rad gene pulser (1.5 KV, 25 μF, 200 Ω) for 5ms. Transformants were first selected by plating on yeast extract peptone dextrose medium (YPD) plates containing 100 ug/ml zeocin and then screened for insertion of the construct at the alcohol oxidase site by PCR using 5' AOX1 and 3'AOX primers. PCR screening confirmed the presence of [Trp^{221}]IGFBP-1 DNA in selected *P. pastoris* clones. To confirm that the clones have a methylotrophic phenotype, clones were patched onto both minimal dextrose medium plates and minimal methanol medium plates using the methylotrophic *P. pastoris* strain X-33 as a positive control and the slow methanol utilization *P. pastoris* strain KM71H as a negative control. Growth on the plates was compared to that of the positive control. One hundred percent of the transformants tested were able to utilize methanol as its carbon source, affirming their Mut+ phenotype as expected.

Task 2: Month 6-9, Expression of secreted recombinant proteins in *Pichia pastoris*

PCR-positive clones (Mut+) were initially grown in 25ml buffered glycerol complex medium (BMGY) at 30° in a shaking incubator (275rpm) to an OD600 of 2-6. Cells were then centrifuged, resuspended in buffered methanol complex medium (BMMY) and continuously incubated for 5 days. Every 24 hours 100% methanol was added to a final concentration of 0.5% to maintain induction. Aliquots of the supernatant were taken daily and examined by SDS-PAGE for expression of [Trp^{221}]IGFBP-1 protein. The figure below shows the expression profile of a selected clone before and after methanol induction.

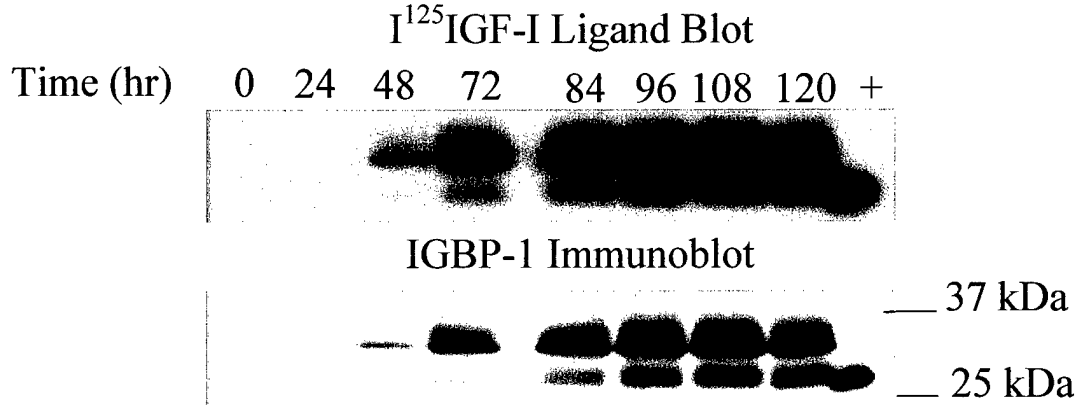

Figure 1: Time course of protein secretion into the culture medium in a *Pichia* clone. Aliquots taken from the culture medium at different time points over 5 days were analyzed by SDS-PAGE. Human recombinant IGFBP-1 produced in bacteria was used as a positive control (lane +) [2]. To determine the presence and functionality of [Trp^{221}]IGFBP-1 mutant protein secreted into the culture medium, an I^{125}IGF-I ligand blot was performed as shown in the top panel. The ligand blot was then immunoblotted with an anti-IGFBP-1 antibody as shown in the bottom panel.

The results show two immunoreactive bands that were able to bind I^{125}IGF-I. The molecular weight of mature secreted [Trp^{221}]IGFBP-1 is 33.4 kDa. Therefore, the upper band is thought to be the mature protein and the lower band is postulated to be protease-cleaved fragment of [Trp^{221}]IGFBP-1. The results demonstrate that [Trp^{221}]IGFBP-1 can be produced by the *Pichia pastoris* yeast expression system. Furthermore, the results of the ligand blot suggest that [Trp^{221}]IGFBP-1 is properly folded since it is able to bind IGF-I. These results suggest that an intact integrin recognition sequence is not required for IGF-I binding.

One problem I have encountered is expressing secreted [Trp^{221}]IGFBP-1 in *P. pastoris* at reasonable levels. Using the *P. pastoris* system, secreted protein levels have been shown to vary considerably depending on the protein (in the range of 10 mg to 5 gm per liter) [3-5]. Eight *P. pastoris* clones were screened for [Trp^{221}]IGFBP-1 secretion and all clones secreted [Trp^{221}]IGFBP-1 into the media but at less than 2 mg per liter as shown in Figure 2.

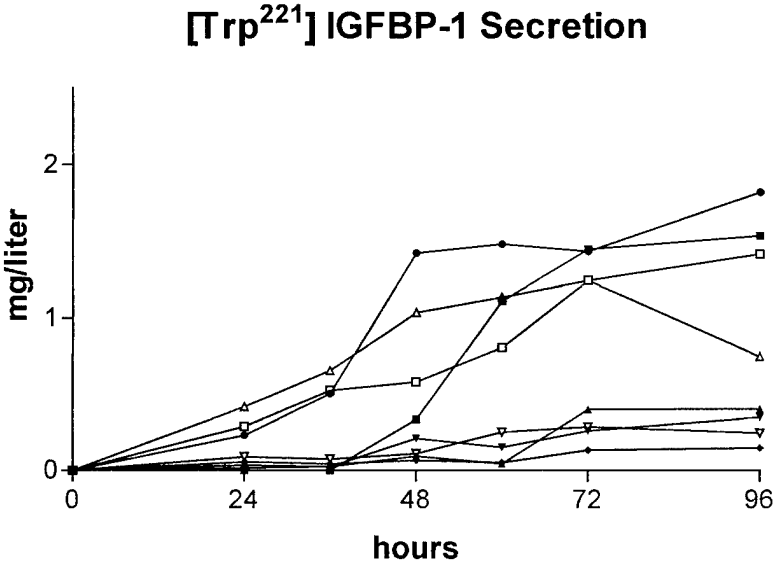

Figure 2: Time course of protein secretion into the culture media of 8 different *P. pastoris* clones. Aliquots were taken from the culture medium every 12 hours over 4 days and analyzed by an IGFBP-1 ELISA kit (Alpha Diagnostic International).

There are examples of increasing protein expression levels by using clones that have multiple copies of the gene of interest. A quick way to isolate multiple copy integrants is to plate transformants on increasing concentrations of zeocin. Therefore, electroporation was repeated and the transformants were immediately plated on yeast dextrose plates containing 500, 1000, and 2000 ug/ml of zeocin. Eight clones isolated from a 1000 ug/ml zeocin yeast dextrose plate were screened for expression and all secreted low levels of [Trp^{221}]IGFBP-1 (less than 2mg per liter). In summary, 16 clones of [Trp^{221}]IGFBP-1 were screened and none showed protein secretion levels greater than 2 mg per liter.

To address the problem of low protein expression of [Trp^{221}]IGFBP-1, I will determine whether the protein is being secreted at low levels or is being expressed at low levels. Cell pellets will be checked to determine if overall expression is low or if the protein is not being secreted properly. If the protein is expressed but is failing to be secreted, a different signal sequence could be tried.

Alternatively if the protein is expressed but secreted at low levels, I will attempt to optimize the yeast culturing condition. For example, increasing the cell density of the culture prior to induction would be expected to increase secreted protein levels.

KEY RESEARCH ACCOMPLISHMENTS

- Cloning of [Trp^{221}]IGFBP-1 into the *P. pastoris* yeast expression vector
- Electroporation of *P. pastoris* with [Trp^{221}]IGFBP-1

REPORTABLE OUTCOMES

1. Abstract: Insulin-like growth factor-1 regulates estrogen receptor-alpha transcriptional activity by multiple pathways in breast cancer. **J.M. Gross** and D. Yee. American Association for Cancer Research Meeting: Abstract No. 942, 2002.
2. Invited article: How does the estrogen receptor work? **J.M. Gross** and D. Yee. Breast Can Res Treatment 4(2): 62-64, 2002.

CONCLUSIONS

Identification of molecules that breast cancer cells require to maintain the malignant phenotype can potentially lead to new, more selective therapies. Evidence suggests that the IGF system and extracellular matrix proteins are key regulators of the malignant breast cancer phenotype. This work-in-progress will test the hypothesis that IGFBP-1 interrupts IGF action and extracellular matrix protein function. The long-term goal is to provide evidence that IGFBP-1 can be used as a novel therapy for treatment of breast cancer.

REFERENCES

1. JI Jones, A Gockerman, WH, Jr, Busby, G Wright, DR Clemmons. Insulin-like growth factor binding protein 1 stimulates cell migration and binds alpha5-beta1 integrin by means of its Arg-Gly-Asp sequence. Proc Natl Acad Sci USA 90: 10553-10557, 1993.
2. JA Figueroa, J Sharma, JG Jackson, MJ McDermott, SG Hilsenbeck, D Yee. Recombinant insulin-like growth factor binding protein-1 inhibits IGF-1, serum, and estrogen-dependent growth of MCF-7 human breast cancer cells. J Cell Phys 157: 229-236, 1993.
3. F Rajamohan, SO Doumbia, CR Engstrom, SL Pendergras, DL Maher, and FM Uckun. Expression of biologically active recombinant pokeweed antiviral protein in methylotrophic yeast *Pichia pastoris*. Protein Expres and Purif 18: 193-201, 1999.
4. K Sreekrishna, RHB Poenz, JA Cruze, WR McCombie, KA Parker, L Nelles, PK Mazzaferro, KA Holden, RG Harrison, PJ Wood, DA Phelps, CE Hubbard, and M Fuke. High level expression of heterologous proteins in methylotrophic yeast *Pichia pastoris*. J Basic Microbiol 28(4): 265-278, 1988.
5. D Mohanraj, T Olson, and S Ramakrishnan. Expression of biologically active human vascular endothelial growth factor in yeast. Growth Factors 12: 17-27, 1995.

#942 Insulin-like growth factor-I regulates estrogen receptor-α transcriptional activity by multiple pathways in breast cancer. Jennifer M. Gross and Douglas Yee. *University of Minnesota, Minneapolis, MN.*

Estrogen and insulin-like growth factor-I (IGF-I) both induce breast cancer cell proliferation. While estrogen acts through a nuclear hormone receptor to stimulate cell growth, IGF-I acts through a transmembrane tyrosine kinase receptor to affect growth. IGF-I activates transcriptional activity of estrogen receptor-α (ERα) in the absence of steroid hormone, although the mechanism is not fully understood. To determine which signaling pathways are involved in IGF-I-induced activation of the estrogen receptor, we treated the estrogen receptor-positive human breast cancer cell line MCF7 stably transfected with an estrogen-response element luciferase reporter construct (MVLN) with inhibitors of downstream signaling pathways. Treatment with IGF-I (5nM) or estrogen (1nM) resulted in a 2-3 fold increase in luciferase activity. Cotreatment with IGF-I and estrogen resulted in enhanced luciferase activity than compared to either treatment alone. Previous studies have shown that mitogen-activated extracellular regulated protein kinase (ERK) and phosphatidylinositol-3-kinase (PI3K) mediate many of the biological effects of IGF-I. Inhibition of ERK1/2 by U0126 (20 μM) or treatment with the PI3K inhibitor LY294002 (25 μM) blunted the IGF-I-induced increase in luciferase activity. U0126 but not LY294002 also blocked the estrogen-induced increase in luciferase activity. Interestingly, an inhibitor of c-jun N-terminal kinase (JNK), dicumarol (50 μM), and the p38 inhibitor SB203580 (20 μM) also suppressed IGF-I mediated activation of luciferase activity. Therefore, the ability of IGF-I to activate JNK and p38 was studied. Treatment of MCF7 cells with IGF-I (5nM) increased the level of phosphorylation of JNK but not p38, as determined by phosphospecific immunoblot analysis. These studies suggest that multiple IGF-I-activated signaling pathways contribute to transactivation of ERα. In contrast, estrogen-induced activation of ERα does not appear to require the same set of signaling pathways. While ERK signaling appears to be central to estradiol and IGF-I action, additional IGF-I-activated signaling pathways play a role in regulating ERα function.