AD_____

Award Number: DAMD17-99-1-9344

TITLE: Leptin Regulation of Mammary Cell Growth

PRINCIPAL INVESTIGATOR: Gina M. Pighetti, Ph.D.

CONTRACTING ORGANIZATION: The University of Tennessee
Knoxville, Tennessee 37901-1071

REPORT DATE: October 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
	October 2002	Annual Summary (1 Oct 01 - 30 Sep 02)

4. TITLE AND SUBTITLE
Leptin Regulation of Mammary Cell Growth

5. FUNDING NUMBERS
DAMD17-99-1-9344

6. AUTHOR(S) :
Gina M. Pighetti, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Tennessee
Knoxville, Tennessee 37901-1071

E-Mail: Pighetti@utk.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)* *(abstract should contain no proprietary or confidential information)* After menopause, breast cancer risk rises with obesity. The expanding adipocyte (ie. fat cell) population may contribute directly to breast cancer by providing excess factor(s) that maintain normal growth. *To test the hypothesis that leptin regulates mammary epithelial cell growth*, obesity (ob/ob) mice that do not synthesize were leptin were evaluated for mammary gland development before and after treatment with estrogen and progesterone. Initial analysis revealed very little ductal growth in the ob/ob mutant phenotype, similar to lean littermates. Following 3 wk of treatment, the obese phenotype had limited ductal outgrowth, smaller terminal end buds, and no alveolar development when compared to lean littermates. Current efforts are more objectively analyzing development by calculating the number and area of terminal end buds, as well as ductal branch points in relation to mice with similar levels of mammary gland development. The results of these and subsequent studies will contribute directly to the knowledge of mammary gland development and possibly tumor development by providing new information regarding the signaling pathways between the adipocyte-rich stroma and mammary epithelial cells. Understanding these pathways in relation to both normal and pathologic mammary cell growth is imperative because of the greater risk of breast cancer that occurs with obesity.

14. SUBJECT TERMS:	15. NUMBER OF PAGES
breast cancer, obesity, leptin, mammary development	29
	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

The risk of developing breast cancer rises with obesity, however the cause of this phenomenon is unknown. The growing adipocyte population during obesity may contribute directly to breast cancer by providing an excess of growth factor(s) that are required for normal mammary epithelial cell proliferation. An intriguing candidate is leptin, a protein produced almost exclusively by adipocytes for the regulation of energy metabolism. Leptin also influences reproductive development. As lactation is an extension of reproduction, the premise is established for leptin to participate in mammary gland development. Preliminary evidence in our lab indicates that mammary epithelial cells express leptin receptors and may be capable of responding to leptin released by the surrounding adipocytes. **The studies of this proposal test the hypothesis that the interaction of leptin with its receptor regulates normal and pathologic mammary epithelial cell proliferation and/or differentiation.** Aim 1 will examine leptin's role in mammary gland development by assessing the mRNA and protein expression of leptin and its receptor in the mammary glands of virgin, pregnant, and lactating mice. Archival human breast tissue samples collected from healthy and breast cancer patients also will be examined to determine if leptin or leptin receptor expression changes with tumor development. Biochemical, immunohistochemical, molecular techniques will be used to qualify mRNA and protein expression. Aim 2 will evaluate if the interaction of leptin with its receptor induces proliferation and/or differentiation of normal and tumor-derived mammary epithelial cells. The first objective of this aim will judge the proliferative effects induced by leptin-containing pellets implanted in the mammary glands of leptin-deficient (*ob/ob*) mice. The second objective will complement the *in vivo* studies by examining the direct effects of leptin administration on the proliferation and differentiation of normal and tumor derived mammary epithelial cells. The final objective will examine the biochemical signaling mechanisms by which leptin activates epithelial cell responses by determining the activation of specific signal transducers and activators of transcription (STAT) proteins through electrophoretic mobility shift and supershift assays. Understanding the functional role of adipocyte-derived factors on mammary cell growth is imperative, especially because of the increased risk of breast cancer that occurs with obesity. The results of these studies will contribute directly to the knowledge of mammary gland development and possibly tumor development by providing new information regarding the signaling pathways between the adipocyte-rich stroma and mammary epithelial cells.

Body

As indicated in the cover letter, the process of transferring the award from The Pennsylvania State University to The University of Tennessee resulted in a delay of one year. The award was reinitiated January 2002 with a completion date of October 2003. In an effort to minimize confusion, the October 2003 date will be considered the final 36 months that was indicated in the original statement of work. All other dates will be adjusted accordingly. Moreover, an extension of one year will be requested to allow completion of the budgeted projects.

Technical Objective 1. Expression of leptin and leptin receptor isoforms varies with mammary gland development or tumor formation (1-24: 24th month = September 2002).

A. Expression of leptin and leptin receptor mRNA and protein during mammary gland development.

Since the last report, all tissues have been collected for analysis of leptin and leptin receptor mRNA and protein expression at 4 and 10 weeks of age; 9, 14, and 20 days of pregnancy; 4 days of involution and 4 days of lactation; with 4 replicate samples at each time point. Samples were collected and immediately placed in RNAlater solution and stored at -20°C. Thus far no samples have been analyzed for leptin or leptin receptor mRNA expression. Immunohistochemical analysis of leptin receptor expression has been initiated. Unfortunately, difficulties have been encountered in routine analysis within the laboratory relating to poor intensity of positive staining – regardless of antibody specificity or source. Current efforts are aimed at troubleshooting this problem and are focusing primarily on the paraffin-embedding and antigen retrieval process. It is expected that this problem will be corrected in the next 3 months.

B. Expression of leptin and leptin receptor mRNA and protein in primary human tumors.

Samples are continuing to be collected by Dr. Stan Lightfoot at the University of Oklahoma Health Sciences Center. Once the immunohistochemical analysis is functional in the lab, the human samples will be initiated. The original plan of work indicated that these studies would be completed within the 30th month of this award, which would correlate to March 2003. However, due to the difficulties encountered with the immunohistochemical analysis, additional time will be requested.

Technical Objective 2. Determine if leptin interaction with its receptor regulates mammary epithelial cell proliferation and/or differentiation (months 18-36: March 2002 – Oct 2003).

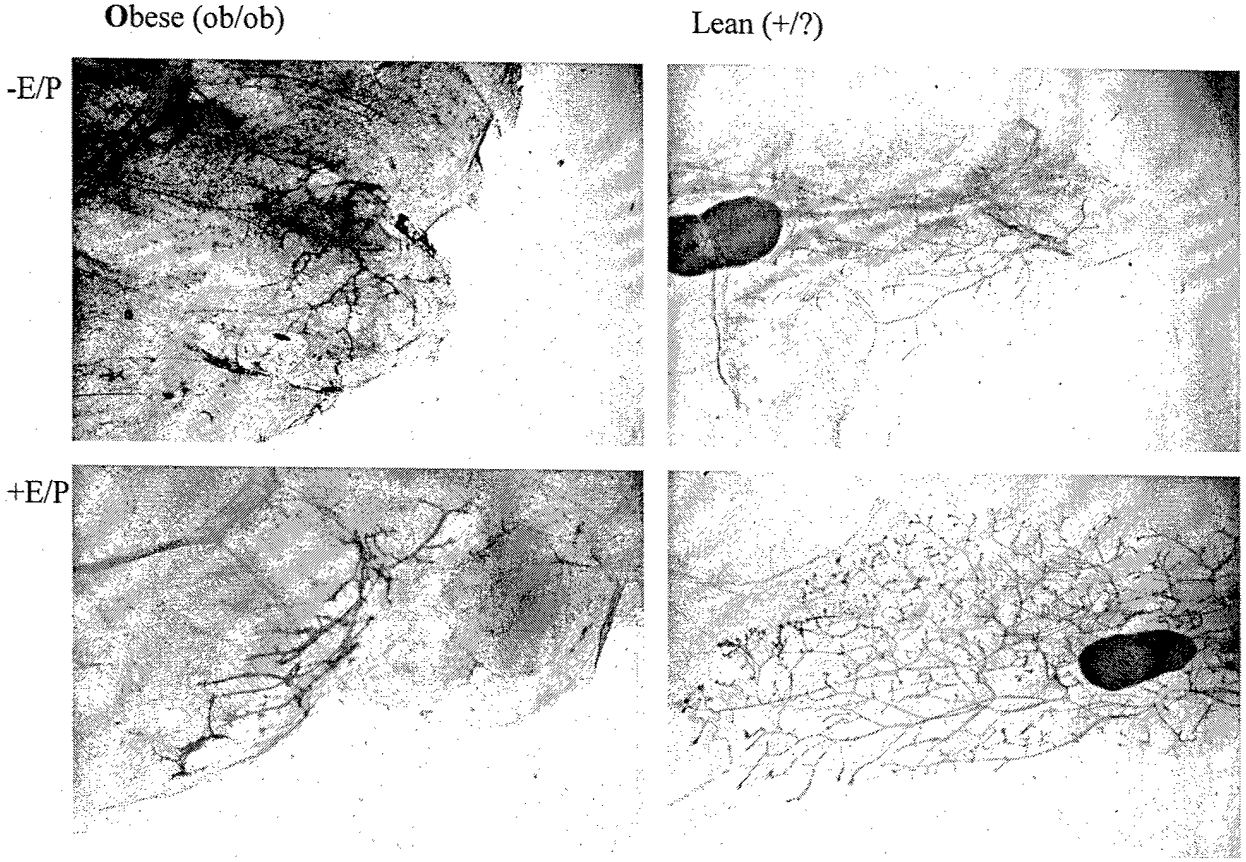
A. Analysis of leptin function in vivo (months 18-22).

1. Samples to evaluate the morphological development of mammary glands from obesity (ob/ob) mice and their lean littermates at 4 and 10 weeks have been collected. The initial analysis of morphological development revealed that there is no ductal or alveolar

growth in the ob/ob mutant phenotype, regardless of age. This contrasts with lean littermates that have normal ductal and alveolar development. Current efforts are more objectively analyzing development by calculating the number and area of terminal end buds, as well as ductal branch points.

One potential explanation for the lack of ductal and alveolar development in ob/ob mice is the lack of estrogen and progesterone, as these hormones are not expressed because these mice do not undergo puberty. To test this hypothesis, estrogen and progesterone injections were given to ovariectomized ob/ob and lean (ob+/ob?) mice for 0, 1, 2, and 3 weeks. Development was assessed in mammary gland whole mounts and revealed limited ductal outgrowth, smaller terminal end buds, and no alveolar development (figure 1). Current efforts are more objectively analyzing development by calculating the number and area of terminal end buds, as well as ductal branch points in relation to mice of similar to 3 weeks of mammary gland development.

Figure 1. Representative whole mammary gland mounts of obese (ob/ob) or lean (+/?) mice given three weeks of daily injections with 1 μg estrogen and 1 mg progesterone. Magnification 10x.



Methods

Animals. C57Bl/6J mice were raised utilizing standard husbandry practices; whereas ob/ob mice and their lean littermates were purchased as needed (Jackson Laboratories). To establish dates of conception, female mice were examined visually 12-24 hours after copulation for plug formation. At either 4 and 10 weeks of age; 9, 14, and 20 days of pregnancy; 4 days of involution or 4 days of lactation mice were euthanized, all mammary glands collected, placed in RNAlater solution (Ambion), and frozen at -20°C for later mRNA and immunohistochemical analysis.

Estrogen and progesterone injections. The ob/ob mice and their lean littermates were ovariectomized one week after arrival, when the mice were approximately 5 weeks of age. Mice were allowed to rest for one week after surgery to allow recovery and removal of endogenous estrogen and progesterone sources. Mice were given daily am injections of water-soluble estrogen (1 μg; Sigma) and progesterone (1 mg; Sigma) for 1, 2, or 3 weeks. Mice then were euthanized, the inguinal mammary glands collected for whole mount analysis and the thoracic glands collected in RNAlater for subsequent RNA analysis.

Whole mount fixation. Whole mounts were processed as outlined by Rasmussen, et al. Briefly, the number 4 inguinal glands were removed from the right and left sides and placed on a Superfrost Plus slides. The tissues were fixed with Carnoy's fixative 2 (10% glacial acetic acid: 30% chloroform: 60% absolute ethanol) overnight. The glands were rehydrated through decreasing concentrations of alchohol washes.

B. Analysis of leptin function in vitro (months 20-26).

As indicated in the previous report, an in vitro system was being developed that would enhance leptin receptor expression in vitro without transfecting in exogenous DNA. Essentially, culture of HC11 cells on collagen induced the expression of the long isoform of leptin receptor mRNA; whereas, the addition of insulin and epidermal growth factor induced the short isoform of leptin receptor mRNA. Current efforts are directed towards verifying leptin receptor protein expression to authenticate this as a viable system for studying the effects of leptin on mammary epithelial cell function without resorting to more artificial means such as transfection.

C. Evaluate leptin activation of specific STAT proteins in mammary epithelial cells (months 27-36).

Not initiated.

Key research accomplishments

- ▸ Mammary gland development requires some level of leptin
- ▸ In vitro culture system to stimulate leptin receptor expression without transfection

Reportable outcomes

Manuscripts, Abstracts, and Presentations relevant to this award:

Abstracts

Pighetti, GM, 2001. Impact of leptin on in vitro cytokine production during early and mid lactation. J Dairy Sci 84 supplement 1: 151.

Pighetti, GM, 2002. Leptin regulation of mammary cell growth. Department of Defense Breast Cancer Research Meeting Proceedings volume.

Presentations

Impact of leptin on in vitro cytokine production during early and mid lactation. Annual American Dairy Science Association Joint Meeting. Indianapolis, IN, July 2001.

Does obesity cause breast cancer risk? The University of Tennessee Nutrition Departmental Seminar Series. Knoxville, TN, March 2002. *(Invited)*

Pighetti, GM, 2002. Leptin regulation of mammary cell growth. Era of Hope Meeting, Department of Defense Breast Cancer Program, Orlando, FL, September 2002.

Employment Opportunities Obtained

Assistant Professor of Animal Science (tenure-track), Department of Animal Science, The University of Tennessee, Knoxville started February 1, 2001.

Concluding comments.

Overall, I am disappointed with the lack of progress that has been made with respect to this grant proposal, and I'm certain the reviewers are as well. The following comments are not offered as excuses, but as factors contributing to the lack of progress. The primary reason can be attributed to the process of moving twice during the tenure of this award. In each case, it was necessary to set up a laboratory to conduct my research, taking at least 6 months each time. I also was reluctant to continue this research upon arrival at my current position because of limited funds available within the department. Hence, little research and reestablishment of specific assays was not conducted until the award was transferred in January 2002. Thus, the past 10 months have been spent collecting samples and re-developing the assays to a new location. A second contributing factor would be the growing pains associated with being a new faculty

member. Although I recognized considerable time would need to be devoted to areas other than research, I did not appreciate the extent to which this occurred. After having completed a full year, I believe I'm better prepared at managing my research, teaching, and service commitments. Moreover, the people within my laboratory have more experience, and will require less-individualized attention, allowing me to focus on this work more intensively. Accordingly work has moved forward steadily over the past several months and I fully expect this to continue. A request will be submitted to extend the statement of work, in recognition that the delays in progress have been recognized and solved. I respectfully request that these considerations be taken into account upon review of this award.

References

Aoki, N., M. Kawamura, et al. (1999). "Lactation-dependent down regulation of leptin production in mouse mammary gland." Biochim Biophys Acta 1427: 298-306.

Chomczynski, P. and N. Sacchi (1987). "Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction." Anal Biochem 162: 156-159.

Laud, K., I. Gourdou, et al. (1999). "Detection and regulation of leptin receptor mRNA in ovine mammary epithelial cells during pregnancy and lactation." FEBS Lett 463: 194-198.

O'Brien, S. N., B. H. Welter, et al. (1999). "Presence of leptin in breast cell lines and breast tumors." Biochem Biophys Res Commun(259): 695-698.

Appendices

A. Curriculum vitae.

B. Abstracts.

Pighetti, GM, 2001. Impact of leptin on in vitro cytokine production during early and mid lactation. J Dairy Sci 84 supplement 1: 151.

Pighetti, GM, 2002. Leptin regulation of mammary cell growth. Department of Defense Breast Cancer Research Meeting Proceedings volume.

C. Presentations.

Impact of leptin on in vitro cytokine production during early and mid lactation. Annual American Dairy Science Association Joint Meeting. Indianapolis, IN, July 2001.

Does obesity cause breast cancer risk? The University of Tennessee Nutrition Departmental Seminar Series. Knoxville, TN, March 2002. *(Invited)*

Pighetti, GM, 2002. Leptin regulation of mammary cell growth. Era of Hope Meeting, Department of Defense Breast Cancer Program, Orlando, FL, September 2002.

D. Current statement of work.

E. Revised statement of work to be submitted.

Gina M. Pighetti

• The University of Tennessee • Department of Animal Science •
• 2640 Morgan Circle, 114 McCord Hall • Knoxville, TN 37996 •
• PH (865) 974-7225 • FAX (865) 974-3394 • EMAIL pighetti@utk.edu•

EDUCATION

PhD	1998	Pathobiology, The Pennsylvania State University Major emphasis: Immunology Thesis: Vitamin E and selenium deficiency impairs transferrin receptor internalization, but not interleukin-2, interleukin-2 receptor, or transferrin receptor expression
MS	1994	Pathobiology, The Pennsylvania State University
BS	1991	Dairy Production/Business Minor, The Pennsylvania State University

POSITIONS HELD

2001-present	Assistant Professor, Department of Animal Science, The University of Tennessee
1999- 2001	DOD Post-doctoral Fellow, Department of Dairy and Animal Science, The Pennsylvania State University
1998 -1999	NIH-NRSA Post-doctoral Fellow, Department of Surgery, University of Oklahoma Health Sciences Center
1992 - 1998	Graduate Research Assistant, Department of Veterinary Science, The Pennsylvania State University

GRANT AWARDS

Department of Defense Breast Cancer Program Postdoctoral Training Award (DAMD17-99-1-9344) entitled "Leptin regulation of Mammary Cell Growth" $125,962, 1999-2003

The University of Tennessee Food Safety Center of Excellence entitled "The interaction of interleukin-8 receptor expression with neutrophil function and disease resistance" $36,550, 2001-2002

The University of Tennessee Food Safety Center of Excellence entitled "Identifying genes differentially expressed by mastitis resistant and susceptible dairy cows" $39,446, 2002-2003

The University of Tennessee Food Safety Center of Excellence entitled "Food Safety Doctoral Student Training" $9,500, 2002-2003

FELLOWSHIP and ACADEMIC AWARDS

- NIH NRSA Post-doctoral Institutional Training Grant (1998-99)
- NASA Space Grant Fellowship (1994-96)
- Grier Scholarship for Outstanding Pathobiology Graduate Student (1995)

- American Dairy Science Association Award for Outstanding Research Paper Presentation (1994)

MEMBERSHIPS

- American Association for the Advancement of Science
- American Dairy Science Association
- National Mastitis Council
- National Dairy Shrine
- Honor Society of Phi Kappa Phi
- Honor Society of Gamma Sigma Delta

PUBLICATIONS

1. **Pighetti, GM** and LM Sordillo. 1994. Regulation of mammary gland macrophage tumor necrosis factor production with interferon-gamma. Res Vet Sci. 56: 252.

2. Sordillo, LM, CR Hicks, and **GM Pighetti**. 1994. Altered interleukin-2 production by lymphocyte populations from bovine leukemia virus-infected cattle. Proc Soc Exp Biol Med. 207: 268.

3. **Pighetti, GM** and LM Sordillo. 1994. Enhanced antigen-specific responses in bovine mammary glands following administration of interleukin-2. J Dairy Sci. 78: 528.
4. Sordillo, LM, **GM Pighetti**, and MR Davis. 1995. Enhanced production of bovine tumor necrosis factor-α during the periparturient period. Vet Immunol Immunopathol. 49: 263.

5. **Pighetti, GM** and LM Sordillo. 1996. Specific immune responses of dairy cattle after primary inoculation with recombinant bovine interferon-γ as an adjuvant when vaccinating against mastitis. Am J Vet Res. 57: 819.

6. Shafer-Weaver, KA, **GM Pighetti**, and LM Sordillo. 1996. Diminished mammary gland lymphocyte functions parallel shifts in trafficking patterns during the postpartum period. Proc Soc Exp Biol Med. 212: 271.

7. **Pighetti, GM**, ML Eskew, CC Reddy, and LM Sordillo. 1998. Selenium and vitamin E deficiency impairs transferrin receptor internalization but not interleukin 2, interleukin 2 receptor, or transferrin receptor expression. J Leuk Biol. 63: 131.

8. Gimble, JM, **GM Pighetti**, MR Lerner, X Wu, SA Lightfoot, DJ Brackett, K Darcy, and AB Hollingsworth. 1998. Expression of peroxisome proliferator activated receptor mRNA in normal and tumorigenic rodent mammary glands. Biochem Biophys Res Commun. 253: 813-817.

9. **GM Pighetti**, DC Hitt, and JM Gimble. 1999. Leptin its role in hematopoiesis and bone formation. J Clin Ligand Assay. 22/2: 239-241.

10. **Pighetti, GM**, W Novosad, C Nicholson, DC Hitt, C Hansens, AB Hollingsworth, ML Lerner, D Brackett, SA Lightfoot, JM Gimble. 2001. Therapeutic treatment of DMBA-induced mammary tumors with PPAR ligands. Anticancer Res 21(2A): 825-829.

11. **Pighetti, GM**, C Nicholson, X Chen, K Davis, W Novosad, DC Hitt, C Hansens, AB Hollingsworth, ML Lerner, D Brackett, SA Lightfoot, JM Gimble. Omega-3 and omega-6 fatty acids modify therapeutic treatment of DMBA-induced mammary tumors with PPAR ligands. In preparation.

Book Chapters and Non-Refereed Articles

1. **Pighetti, GM** and LM Sordillo. 1994. Feasibility of using interleukin-2 as an adjuvant with mastitis vaccines. in Proceedings of the 33rd Annual Meeting of the National Mastitis Council. Orlando, FL. pp. 328-330.

2. **Pighetti, GM** and LM Sordillo. 1994. Using cytokines to improve mastitis vaccines. in The Center for Mastitis Research Newsletter, College of Agricultural Sciences, The Pennsylvania State University, p. 1.

3. **Pighetti, GM** and LM Sordillo. 1995. Role of cytokines in coliform mastitis. in The Center for Mastitis Research Newsletter, College of Agricultural Sciences, The Pennsylvania State University, p. 2.

4. **Pighetti, GM** and LM Sordillo. 1997. Mechanisms of bovine leukosis virus infection in dairy cattle. in Herd Health Memo, Cooperative Extension, College of Agricultural Sciences, The Pennsylvania State University, p. 3-4.

5. Gimble, JM, S Chen, **GM Pighetti**, and DC Hitt. 2000. Adipocyte biology of the bone. in (ed), Biomedical and Health Research Vol 37: Adipocyte Biology and Hormone Signaling, IOS Press. Washington, DC. 231-238.

6. Oliver SP and **Pighetti GM**. 2002. Mastitis pathogens (b) environmental pathogens. In Encyclopedia of Dairy Science, H Roginski, PF Fox, and JW Fuquay, Eds., Academic Press, London pp 1728-1734.

Abstracts

1. **Pighetti, GM** and LM Sordillo. 1992. Effects of interferon-gamma on *in vitro* tumor necrosis factor production by lipopolysaccharide stimulated mammary gland macrophages. Proceedings of the Conference of Research Workers in Animal Diseases. Chicago, IL. p. 63.

2. Sordillo, LM and **GM Pighetti**. 1993. Production of tumor necrosis factor by mammary gland macrophages following *in vitro* exposure to interferon-□. Proceedings of the 32nd Annual Meeting of the National Mastitis Council, Kansas City, MI. p. 215.

3. Sordillo, LM and **GM Pighetti**. 1993. Regulation of tumor necrosis factor production by mammary gland macrophages with recombinant bovine interferon-gamma. J. Dairy Sci. 76 (Supplement 1):261.

4. **Pighetti GM** and LM Sordillo. 1993. Specific immune response of dairy cattle to mastitis immunization protocols using recombinant interleukin-2. Proceedings of the Conference of Research Workers in Animal Diseases. Chicago, IL. p. 20.

5. Sordillo, LM, C Hicks, and **GM Pighetti**. 1993. Phenotypic and functional characteristics of peripheral blood leukocytes obtained from dairy cattle with bovine leukosis virus. Proceedings of the Conference of Research Workers in Animal Diseases. Chicago, IL. p. 70.

6. **Pighetti, GM** and LM Sordillo. 1994. Feasibility of using interleukin-2 as an adjuvant to enhance mastitis vaccines. J. Dairy Sci. 77 (Supplement 1): 67.

7. **Pighetti, GM** and LM Sordillo. 1994. Enhancing specific mammary gland immunity with interleukin-2. Proceedings of the Conference of Research Workers in Animal Diseases. Chicago, IL. abstract 91.

8. **Pighetti, GM** and LM Sordillo. 1994. Mastitis immunization protocols using interferon-γ as an adjuvant. Proceedings of the Conference of Research Workers in Animal Diseases. Chicago, IL. abstract P46.

9. Davis, MR, **GM Pighetti**, and LM Sordillo. 1994. Enhanced production of bovine tumor necrosis factor-α production during the periparturient period. Proceedings of the Conference of Research Workers in Animal Diseases. Chicago, IL. abstract P43.

10. **Pighetti, GM**, KA Shafer-Weaver, and LM Sordillo. 1995. Alteration of bovine lymphoid phenotype and function during the periparturient period. J. Dairy Sci. 78 (Supplement 1): 166.

11. Shafer-Weaver, KA, **GM Pighetti**, and LM Sordillo. 1995. Enhancing bactericidal mechanisms of bovine lymphoid cells. J. Dairy Sci. 78 (Supplement 1): 288.

12. **Pighetti, GM**, CC Reddy, and LM Sordillo. 1996. Down regulation of transferrin and interleukin-2 receptor expression in lymphocytes during vitamin E deficiency. FASEB J. 10(6): 1097.

13. **Pighetti, GM**, CC Reddy, and LM Sordillo. 1996. Dimished lymphocyte interleukin 2 and transferrin receptor expression during vitamin E deficiency. Proceedings of the Conference of Research Workers in Animal Diseases. Chicago, IL. abstract 126.

14. Eskew, ML, LM Sordillo, **G Pighetti**, and CC Reddy. 1997. Effect of deficiency of vitamin E and selenium on production of nitric oxide in zymosan-stimulated rat peritoneal and pulmonary macrophages. FASEB J.

15. Weaver, JA, **GM Pighetti**, CC Reddy, and LM Sordillo. 1998. Vitamin E and Se deficiency impair iron internalization by disrupting lymphocyte transferrin receptor expression and internalization. FASEB J.

16. Brackett, DJ, MR Lerner, **GM Pighetti**, DE Branam, JS. Hanas, ER Jupe, and RG Postier. 1999. Evaluation of changes in gene expression during endotoxemia. Fourth International Shock Congress.

17. **Pighetti, GM**, W Novosad, C Nicholson, DC Hitt, CJ Hansens, ML Lerner, D Brackett, SA Lightfoot, X Wu, K Darcy, AB Hollingsworth, JM Gimble. 2000. Peroxisome proliferator activated receptors in rodent mammary gland carcinogenesis. Department of Defense Breast Cancer Research Meeting Proceedings volume II: 515.

18. **Pighetti, GM**, 2001. Impact of leptin on in vitro cytokine production during early and mid lactation. J Dairy Sci 84 supplement 1: 151.

19. Pighetti, GM, 2002. Leptin regulation of mammary cell growth. Department of Defense Breast Cancer Research Meeting Proceedings.

20. Youngerman, S.M. J.L. Edwards, F.N. Schrick, S. van Amstel, H. H. Dowlen, S.P. Oliver, and **G.M. Pighetti**. 2002. Leukocyte Phenotypes Expressed by a Calf Cloned from a Mastitis Susceptible Dairy Cow. Proceedings of the Conference of Research Workers in Animal Diseases. Chicago, IL. Abstract

21. Rambeaud, M. **G.M. Pighetti**, S.P. Oliver. 2002. Dynamics of leukocytes and cytokines during experimentally-induced *Streptococcus uberis* mastitis. Proceedings of the Conference of Research Workers in Animal Diseases. Chicago, IL. Abstract

22. **Pighetti, G.M.**, S.M. Youngerman, and S.P. Oliver. 2002. A novel and promising candidate gene for mastitis resistance. Proceedings of the Conference of Research Workers in Animal Diseases. Chicago, IL. Abstract

23. Waller, J.C., F.N. Schrick, F.M. Hopkins, M. Davis, B. Pitts, G. E. Bates, **G.M. Pighetti**, J.L. Edwards, J.W. Oliver, K.D. Gwinn, M.A. Mueller, and B.A. Sims. 2002. Pastures of tall fescue cultivars with different endophytes and/or clover for intact beef males and steers on the Highland Rim. SERAIEG-8 Tall Fescue Toxicosis/Endophyte Workshop. Wildersville, TN.

SELECT PRESENTATIONS

1. Poster presentation entitled "Effects of interferon-gamma on *in vitro* tumor necrosis factor production by lipopolysaccharide stimulated mammary gland macrophages" at the Conference of Research Workers in Animal Diseases, Chicago, IL, November 1992.

2. Poster presentation entitled "Regulation of tumor necrosis factor production by mammary gland macrophages with recombinant bovine interferon-gamma" at the Annual American Dairy Science Association Meeting, College Park, MD, June 1993.

3. Oral presentation entitled "Specific immune response of dairy cattle to mastitis immunization protocols using recombinant interleukin-2" at the Conference of Research Workers in Animal Diseases, Chicago, IL, November 1993.

4. Oral presentation entitled "Feasibility of using interleukin-2 as an adjuvant to mastitis vaccines" at the Annual American Dairy Science Association Meeting, Minneapolis, MN, July 1994.

5. Oral presentation entitled "Enhancing specific mammary gland immunity with interleukin-2" at the Conference of Research Workers in Animal Diseases. Chicago, IL, November 1994.

6. Poster presentation entitled "Mastitis immunization protocols using interferon-□ as an adjuvant" at the Conference of Research Workers in Animal Diseases. Chicago, IL, November 1994.

7. Oral presentation entitled "Alteration of bovine lymphoid phenotype and function during the periparturient period" at the Annual American Dairy Science Association Meeting, Ithaca, NY, June 1995.

8. Poster presentation entitled "Downregulation of transferrin and interleukin-2 receptor expression in lymphocytes during vitamin E deficiency" at the Federation of American Societies for Experimental Biology joint meeting for ASBMB/ASIP/AAI, New Orleans, LA, June 1996.

9. Oral presentation entitled "Dimished lymphocyte interleukin 2 and transferrin receptor expression during vitamin E deficiency" at the Conference of Research Workers in Animal Diseases. Chicago, IL, November 1996.

10. Poster presentation entitled "Mechanisms of lymphocyte immunosuppression at the Molecular Biology and Pathology of Neoplasia Workshop, American Association for Cancer Research. Keystone, CO, July 1997.

11. Oral presentation entitled "Leptin regulation of mammary cell growth" at the Wilderness Conference XVII, mini-Gordon Conference. Cedar Run, PA, September 1999.

12. Poster presentation entitled "Peroxisome proliferator activated receptors in rodent mammary carcinogenesis " at the Era of Hope Meeting, Department of Defense Breast Cancer Program, Atlanta, GA, June 2000.

13. Oral presentation entitled "Possible mechanisms of leptin impact upon the mammary gland" at the Wilderness Conference XVIII, mini-Gordon Conference. Cedar Run, PA, October 2000.

14. Oral presentation entitled “Mastitis and acute phase reactants produced in the mammary gland” at Pfizer Global Research and Development mini-symposium. Terre Haute, IN, May 2001.

15. Oral presentation entitled “Impact of leptin on in vitro cytokine production during early and mid lactation” at the American Dairy Science Association Joint Meeting. Indianapolis, IN, July 2001.

16. Oral presentation entitled "Improving dairy cattle resistance to mastitis" at the Mastitis Research Workers Conference. Chicago, IL, November 2001.

17. Does obesity cause breast cancer risk? The University of Tennessee Nutrition Departmental Seminar Series. Knoxville, TN, March 2002. **(Invited)**

18. Searching for the two billion dollar gene. The University of Tennessee Science Forum. Knoxville, TN, April 2002. **(Invited)**

19. Leptin regulation of mammary cell growth at the Era of Hope Meeting, Department of Defense Breast Cancer Program, Orlando, FL, Sept 2002.

20. Searching for the two billion dollar gene. The University ofTennessee Institute of Agriculture Development Board, Knoxville, TN, October 2002. **(Invited)**

LEPTIN REGULATION OF MAMMARY CELL GROWTH

Gina M. Pighetti

The University of Tennessee

pighetti@utk.edu

The risk of developing breast cancer after menopause rises with obesity, although the cause is unknown. The expanding adipocyte (ie. fat cell) population during obesity may contribute directly to breast cancer by providing excess factor(s) that maintain normal growth. An intriguing candidate is leptin, a protein produced almost exclusively by adipocytes. To test the hypothesis that leptin regulates mammary epithelial cell growth, a mouse mammary epithelial cell line, HC11, was incubated with increasing doses of leptin (0-100 ng/ml) either in the presence or absence of insulin (50 ng/ml) and epidermal growth factor (EGF; 10 ng/ml). Leptin alone had no effect on mammary epithelial cell growth, whereas leptin prevented cellular proliferation in the presence of insulin and EGF. Only the lowest concentration of leptin (1 ng/ml) reduced DNA synthesis. These results suggest that leptin may be a potential inhibitor of mammary epithelial cell proliferation when leptin concentrations are low, but not when concentrations are high as occurs with obesity. Although changes were detectable, they may have been limited by low leptin receptor expression due to the culture of cells on plastic. In an effort to increase receptor expression and maximize responses, HC11 cells were cultured in a collagen matrix, thereby representing a more natural environment. This three-dimensional system generated differential mRNA expression of both long and short receptor isoforms. With collagen alone, the long leptin receptor isoform was prevalent. In contrast, addition of insulin and EGF to the medium altered expression so that the short receptor isoform was more prevalent. The ability to induce differential expression of these receptors is critical as these receptors vary depending upon stage of mammary gland development. The results of these and subsequent studies will contribute directly to the knowledge of mammary gland development and possibly tumor development by providing new information regarding the signaling pathways between the adipocyte-rich stroma and mammary epithelial cells. Understanding these pathways in relation to both normal and pathologic mammary cell growth is imperative because of the greater risk of breast cancer that occurs with obesity.

The U.S. Army Medical Research Materiel Command under DAMD17-99-1-9344 supported this work.

Impact of leptin on *in vitro* cytokine production during early and mid lactation. Gina M. Pighetti, Department of Animal Science, University of Tennessee, Knoxville, TN

The ability of leptin to regulate energy stores within the body has allowed it to evolve and help regulate other energy-dense processes such as reproduction and immunity. However, very little if any information exists regarding the consequences of leptin on bovine immune function. Therefore, the objective of the current study is to compare the *in vitro* responses of peripheral blood mononuclear cells isolated from both mid-to-late (ML) and periparturient (PP; within 3 days after calving) dairy cows to leptin. Increasing doses of concanavalin A (0-2 ug/ml) and/or recombinant human leptin (0-50 ng/ml) were administered to the cells 12 hours prior to collection for RNA. Interferon (IFN)-γ and interleukin (IL)-4 mRNA were measured as indicators of cellular and humoral immunity, respectively. Preliminary evidence indicates that cells isolated from PP and ML cows respond in a similar fashion to leptin, but vary as to which concentrations they respond to. Leptin approximately doubled the relative expression of IFNγ mRNA in cells from ML cows, regardless of dose. In contrast, cells from PP cows only responded to the lowest leptin dose (1 ng/ml). Little, to no IL-4 mRNA was produced in stimulated cells collected from ML lactation cows. However, costimulation with a minimum of 5 ng/ml leptin increased IL-4 mRNA to levels comparable to IFN-γ. In contrast, it required 50 ng/ml to achieve the same effect in cells from PP dairy cows. These preliminary results indicate that immune cell populations are responsive to leptin and that this response can vary with the stage of lactation. Moreover, with the fluctuations in leptin that can occur with body condition and pregnancy, it is imperative to further investigate this link between leptin, energy metabolism/storage, and immune function in order to promote better animal health.

Impact of Leptin on *in vitro* Cytokine Production during Early and Mid Lactation

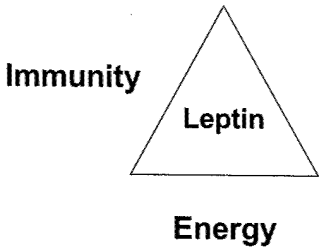
Gina M. Pighetti
Department of Animal Science
University of Tennessee

Greater incidence of metabolic and infectious diseases during the periparturient period.

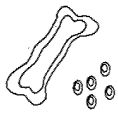
Immunosuppression during the Periparturient Period

- Glucocorticoids
- Leukocyte trafficking
- Cytokine production
- Negative energy balance

Is there a common factor that influences both immunity and energy regulation?



Leptin Participates in Immune Function



Hematopoiesis

<u>T-cell mediated immunity</u>
↑ IFN-γ, IL-2
↓ IL-4

<u>Acute inflammation</u>
Macrophage &
neutrophil killing
↑ TNF, GM-CSF

Why would leptin be relevant during the periparturient period?

Leptin and Pregnancy

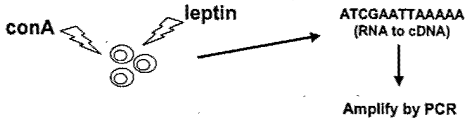
- Sera leptin increases 5-10 fold 2nd/3rd trimester
- Drop immediately after birth
- Provide energy for fetus? Lactation?

Hypothesis

Immune cells isolated from periparturient dairy cows have lower sensitivity to leptin modification of cytokine production.

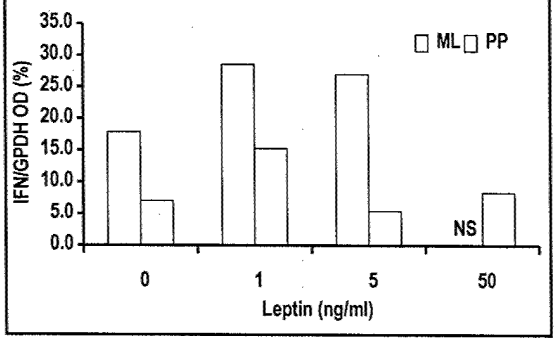
Methods

- Isolate peripheral blood mononuclear cells
- Stimulate for 12 hr w/ concanavalin A ✂ leptin
- Isolate RNA
- Determine relative cytokine mRNA expression by RT-PCR

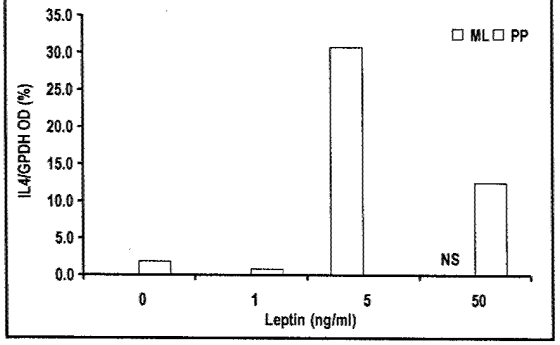


Preliminary Results

Does leptin influence IFN-γ RNA levels?



Does leptin influence IL-4 RNA levels?



Preliminary Results Indicate

- Bovine immune cells responsive to leptin
- Cytokine response may vary w/ stage of lactation

Current and Future Research

- Expand current research
- Evaluate association w/ *in vitro* leptin levels
- Signal transduction
  - Reduced STAT/MAPK signaling?
  - Increased SOCS expression/activity?

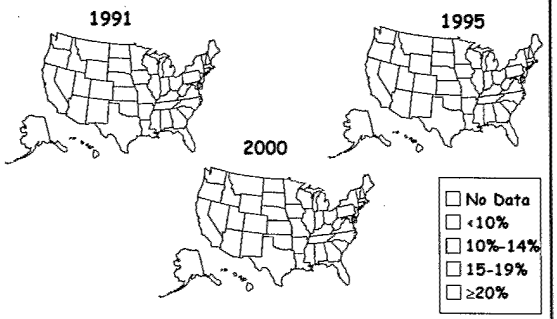
The End

Does Obesity Contribute to Breast Cancer?

Gina M. Pighetti
Department of Animal Science
University of Tennessee

Obesity: A Public Health Epidemic

BRFSS, 1991, 1995 and 2000



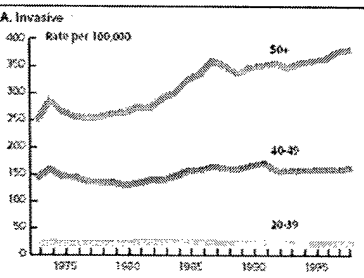
Source: Mokdad A H, et al. *J Am Med Assoc* 1999;282:16, 2001;286:10.

Obesity: A Public Health Epidemic

- High blood pressure & cholesterol
- Coronary heart disease & stroke
- Type-2 diabetes
- Insulin resistance
- CANCER
  - colon, prostate, endometrial, & BREAST

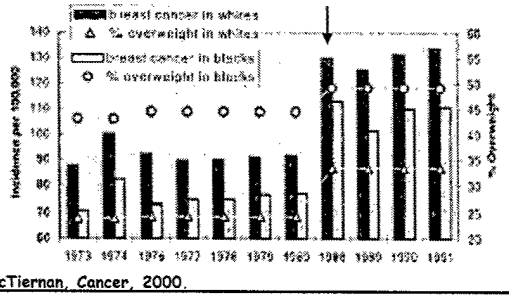
NIH, 1988

Female Breast Cancer Incidence Rates SEER 1994-1998



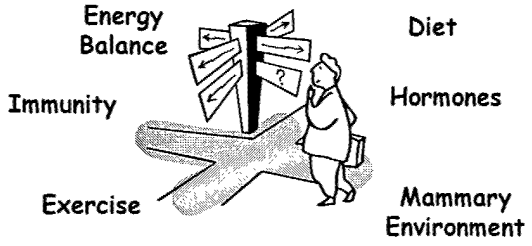
Breast Cancer Facts & Figures 2001, American Cancer Society

Female Breast Cancer Incidence Rates (SEER) & Overweight Prevalence (NHANES)

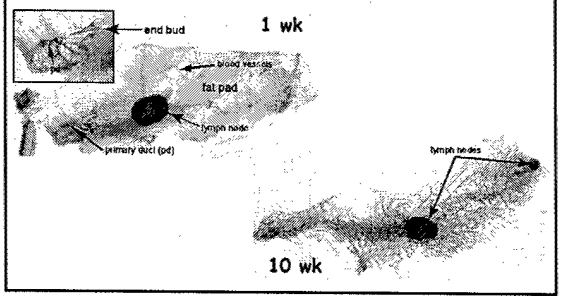


McTiernan, Cancer, 2000.

No surprise that obesity can contribute to breast cancer...



No surprise that obesity can contribute to breast cancer...



Adipocyte-derived factors that influence mammary growth...

- Estrogen
- Insulin-like growth factor
- Leptin??

What is leptin?

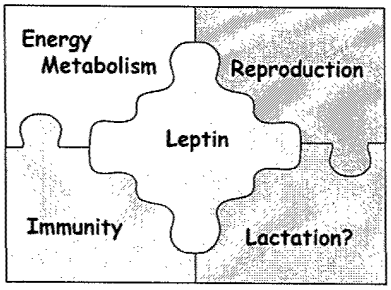
"Adipostat"

FAT → Brain, pancreas, fat, etc.

- Feed intake
- Energy metabolism
- Insulin levels
- Lipid stores

As such, it can "control" high energy functions

Leptin



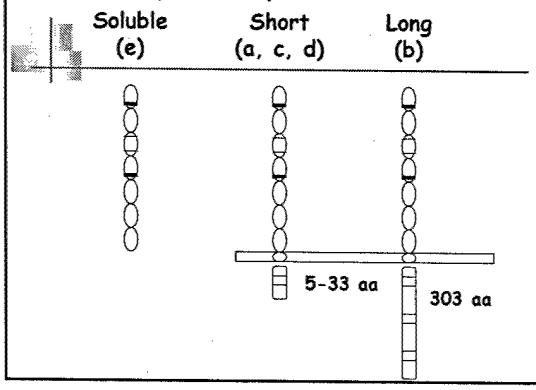
Leptin

- Adipocytes, placenta, muscle, brain, mammary epithelial cells
- 16 kDa
- 2-10 ng/ml plasma
- normally bound by binding proteins in blood

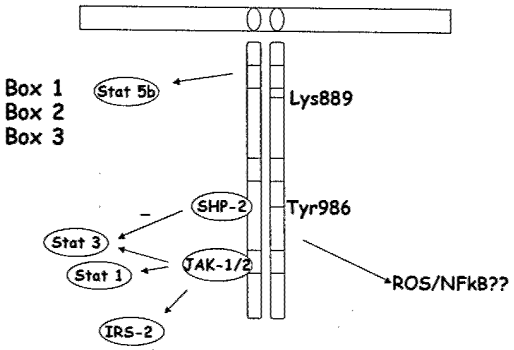
Leptin Receptor

- Cytokine receptor family (gp-130)
- Highly conserved
- High affinity binding 200-700 pM
- Homodimerization
- Alternative splicing for multiple isoforms

Leptin Receptor Isoforms



Leptin Receptor Signaling



Leptin...

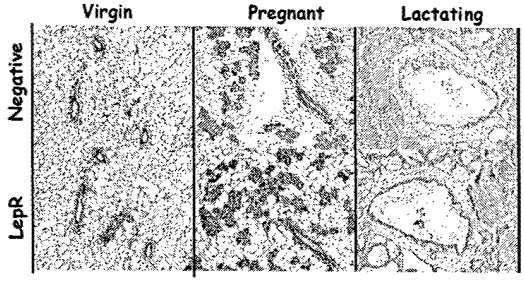
excellent candidate for an adipocyte-derived mammary gland growth factor.

- Fat necessary for mammary growth
- Leptin produced by fat cells
- Leptin regulates hi energy processes

1st Question...

Are mammary epithelial cells capable of responding to leptin ???

Do mammary epithelial cells express leptin receptors?



Does leptin receptor expression change during mammary development?

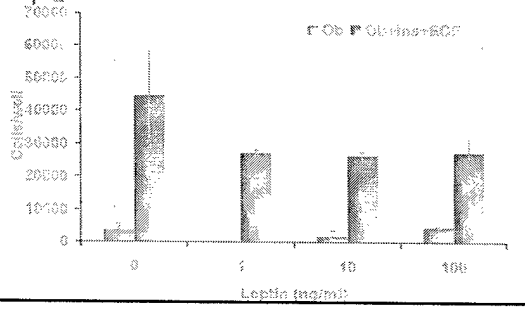
		Pregnancy			
		Virgin	Mid	Late	Lactation
Short	ObRa	−	+	+	+
	ObRc	+	+	−	+
Long	ObRb	+	−	−	−
Soluble	ObRe	+	+	+	+

2nd Question...

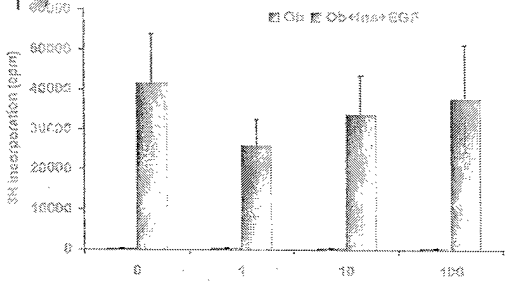
What effect does leptin have on mammary epithelial cells?

In vitro models...

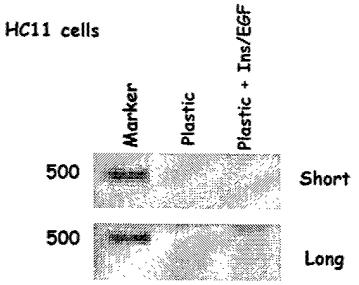
Leptin inhibits cell proliferation induced by insulin and epidermal growth factor



Only low leptin levels reduce DNA synthesis

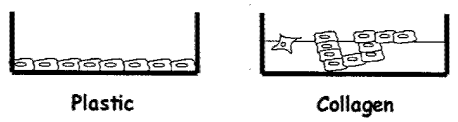


In vitro Leptin Receptor mRNA



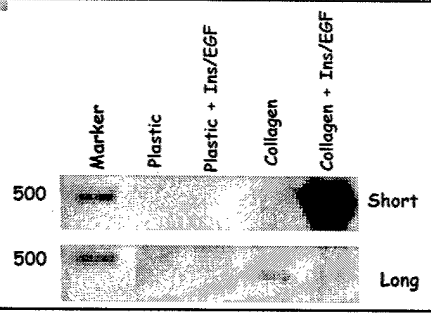
Develop an in vitro environment that more closely mimics mammary gland environment...

+/- Ins & EGF



3-dimensional culture system

HC11 Leptin Receptor mRNA

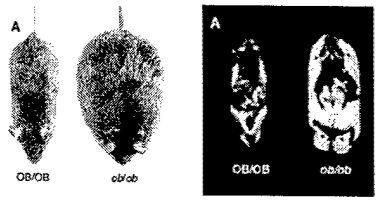


2nd Question...

What effect does leptin have on mammary epithelial cells?

In vivo models...

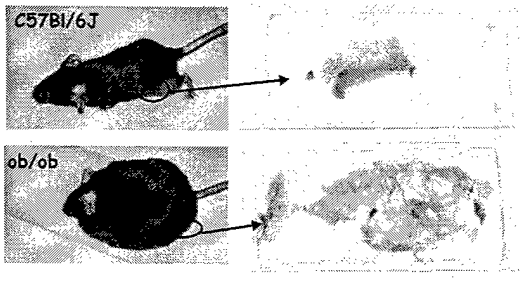
Obesity (ob) Mouse: Leptin deficient



Hyperglycemia, Type II diabetes, hypertriglyceridemia, decreased metabolism, sterile

Pictures taken from: *J.C. Erickson, G. Hollopeter, and R.D. Palmiter. 1996 Science 274: 1704-1707.*

Mammary gland whole mounts: Nine mo virgin mice



Current Experiment ...

Whole Mount Analysis

4 wk ovx

E/P

2 wk

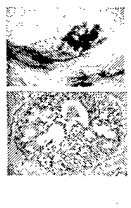
- No growth → Growth Factor
- Normal growth → Unnecessary
- Excessive growth → 'Braking' Factor

Potential leptin implications for breast cancer...

Obesity

Growth factor → Excess leptin

Growth inhibitor → Impaired leptin signal



Does Obesity Contribute to Breast Cancer?

Leptin Regulation of Mammary Cell Growth

Gina M. Pighetti, The University of Tennessee

Abstract

The risk of developing breast cancer after menopause rises with obesity, although the cause is unknown. The expanding adipocyte (ie. fat cell) population during obesity may contribute directly to breast cancer by providing excess factor(s) that maintain normal growth. An intriguing candidate is leptin, a protein produced almost exclusively by adipocytes. *To test the hypothesis that leptin regulates mammary epithelial cell growth*, a mouse mammary epithelial cell line, HC11, was incubated with increasing doses of leptin (0-100 ng/ml) either in the presence or absence of insulin (50 ng/ml) and epidermal growth factor (EGF; 10 ng/ml). Leptin alone had no effect on mammary epithelial cell growth, whereas leptin prevented cellular proliferation in the presence of insulin and EGF. Only the lowest concentration of leptin (1 ng/ml) reduced DNA synthesis. These results suggest that leptin may be a potential inhibitor of mammary epithelial cell proliferation when leptin concentrations are low, but not when concentrations are high as occurs with obesity. Although changes were detectable, they may have been limited by low leptin receptor expression due to the culture of cells on plastic. In an effort to increase receptor expression and maximize responses, HC11 cells were cultured in a collagen matrix, thereby representing a more natural environment. This three-dimensional system generated differential mRNA expression of both long and short receptor isoforms. With collagen alone, the long leptin receptor isoform was prevalent. In contrast, addition of insulin and EGF to the medium altered expression so that the short receptor isoform was more prevalent. The ability to induce differential expression of these receptors is critical as these receptors vary depending upon stage of mammary gland development. The results of these and subsequent studies will contribute directly to the knowledge of mammary gland development and possibly tumor development by providing new information regarding the signaling pathways between the adipocyte-rich stroma and mammary epithelial cells. Understanding these pathways in relation to both normal and pathologic mammary cell growth is imperative because of the greater risk of breast cancer that occurs with obesity.

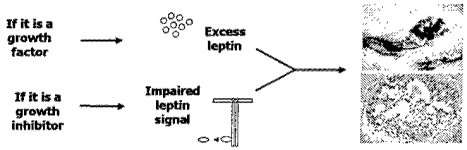
Introduction

- Almost 9 out of 10 Americans are overweight!
- Obesity increases breast cancer risk two-fold after menopause

As such, the number of women with breast cancer will continue to increase unless we better understand the mechanisms by which obesity contributes to breast cancer.

- Fat produces a protein, LEPTIN (ob), that signals the body that adequate energy stores are present.
  - Leptin overproduced during OBESITY
  - Interferes with leptin signaling through its receptor
- Leptin receptors are present on mammary epithelial cells, BUT WHAT DO THEY DO?
  - Proliferation??
  - Differentiation??

How can excess leptin produced by fat cells (adipocytes) during obesity influence breast cancer?

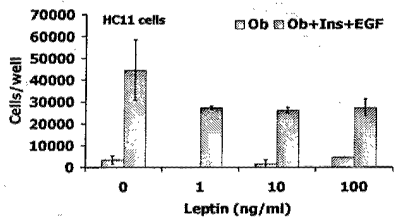


Hypothesis & Objectives

To test the hypothesis that a fat-cell derived product, leptin, regulates mammary cell growth, the following objectives are being conducted:

- Assess mammary epithelial cell proliferation following incubation with leptin in the presence or absence of growth factors.
- Assess mammary epithelial cell leptin receptor expression under different culture conditions

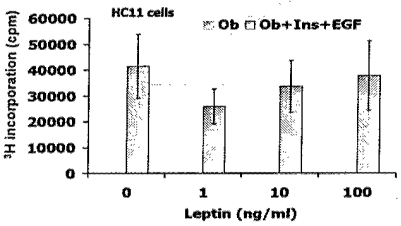
Figure 1. Does leptin promote mammary epithelial cell proliferation in the absence or presence of growth factors (insulin & epidermal growth factor)?



NO, it doesn't promote...

- Leptin alone has no effect, but
- INHIBITS proliferation in the presence of growth factors.

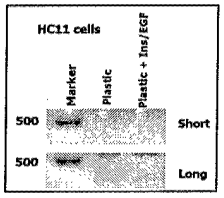
Figure 2. Does reduced cell proliferation coincide with decreased DNA synthesis?



Yes, depending upon the concentration...

- Decreased synthesis with 1 ng/ml
- Less effect at higher concentrations

Figure 3. Do mammary epithelial cells express the short and/or long forms of the leptin receptor?



The mRNA for leptin receptor long and short forms was dectected using reverse-transcription polymerase chain reaction (RT-PCR).

NO...

- mRNA was not detectable
- Common problem for this RNA when using cultured cells
- Wanted to enhance leptin receptor expression without transfecting in specific DNA to force expression

Can providing a more 'natural' environment where mammary epithelial cells are cultured in a 3-dimensional matrix and are able to form ducts and tubules promote leptin receptor expression?

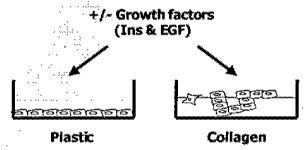
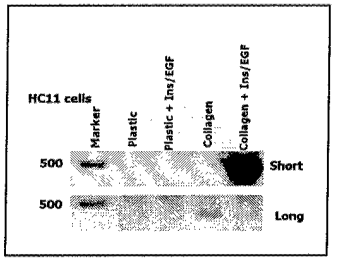


Figure 5. Does culturing mammary epithelial cells on either plastic or collagen influence leptin receptor expression?



YES...

- Leptin receptor expression mRNA non-detectable when cells grown on plastic.
- LONG form mRNA predominates when grown on collagen WITHOUT growth factors
- Short form mRNA predominates when grown on collagen WITH growth factors.

Conclusions

- Do high leptin concentrations shift DNA synthesis from proliferation towards differentiation?
  - → All leptin concentrations (1-100 ng/ml) inhibit cell proliferation BUT only the lowest concentration inhibited DNA synthesis
- Culturing cells in a 3-dimensional matrix of collage provides a BETTER MODEL: Increases our ability to understand how leptin impacts mammary epithelial cells and how this may contribute to the increased risk of breast cancer with obesity.
  - → Addition of growth factors (Ins & EGF) shifts expression of long & short leptin receptor forms
  - → More closely resembles the situation in the animal where the long but not the short receptor is expressed in mammary glands from virgin mice and the opposite occurs during pregnancy and lactation.

Research Fundedby: The U.S. Army Medical Research and Materiel Command under DAMD17-99-1-9344.

D. Current Statement of Work

Task 1.Determine if epithelial cell expression of Lep or LepR isoforms varies with mammary gland development or tumor formation. (months 18-30)

A. Expression of Lep and LepR mRNA and protein during mammary gland development
- Collect and process mammary glands from C57Bl mice during various stages of development (n=4 replicate experiments; if time permits)
- Immunohistochemical analysis of Lep and LepR protein expression (if time permits)
- RT-PCR and *in situ* hybridization analysis of Lep and LepRa, b, c, and e mRNA expression (if time permits)

B. Expression of Lep and LepR mRNA and protein in primary human tumors
- Collect paraffin-embedded and frozen tissue sections taken from breast cancer and reduction mammoplasty patients (months 18-30)
- Immunohistochemical analysis of Lep and LepR protein expression (months 18-30)
- RT-PCR and *in situ* hybridization analysis of Lep and LepRa, b, c, and e mRNA expression (months 18-30)

Task 2. Determine if Lep interaction with its receptor regulates mammary epithelial cell proliferation and/or differentiation (months 18-36)

A. Analysis of Lep function *in vivo*
- Dose response of *in vivo* leptin administration on mammary epithelial cell proliferation (n=4 replicate experiments; months 18-21)
- Whole mount analysis of *in vivo* proliferation by end bud number (months 18-22)
- Immunohistochemical analysis of bromodeoxyuridine expression (months 18-22)

B. Analysis of Lep function *in vitro*
- Characterize the LepR protein (Western) and mRNA expression (RT-PCR) by mammary epithelial cell lines (months 20-22)
- Evaluate the proliferation and differentiation of mammary epithelial cell lines to leptin administration *in vitro*, dose and time course (months 21-24)
- Western and Northern analysis of WDNM1, β-casein, and α-lactalbumin expression by HC11 cells (months 23-26)

C. Evaluate Lep activation specific STAT proteins in mammary epithelial cells
- Electromobility shift analysis of STAT 1, STAT 3, and STAT 5a (months 27-36)
- Gel supershift analysis STAT 1, STAT3, and STAT 5 (months 27-36)

E. Revised Statement of Work - Proposed ending date OCT 2004, a 1 year extension

Task 1. Determine if epithelial cell expression of leptin or leptin receptor isoforms varies with mammary gland development or tumor formation.

A. Expression of leptin and leptin receptor mRNA and protein during mammary gland development. (JAN 2003 – DEC 2003)
- Collect and process mammary glands from C57Bl mice during various stages of development. *DONE*
- Immunohistochemical analysis of leptin and leptin receptor protein expression
- RT-PCR analysis of leptin and leptin receptor isoforms
- In situ hybridisation of leptin and leptin receptor isoforms – if time permits

B. Expression of leptin and leptin receptor mRNA and protein in primary human tumors. (until AUG 2004)
- Collect paraffin-embedded tissue sections taken from breast cancer and reduction mammoplasty patients (in progress)
- Immunohistochemical analysis of leptin and leptin receptor protein expression (JAN 2004 – AUG 2004)
- RT-PCR analysis of leptin and leptin receptor isoforms (JAN 2004 – AUG 2004)
- In situ hybrization of leptin and leptin receptor isoforms – if time permits

Task 2. Determine if leptin interaction with its receptor regulates mammary epithelial cell proliferation and/or differentiation.

A. Analysis of leptin function in vivo. (JAN 2003 – JUNE 2003)
- Impact absence leptin on mammary gland development while in presence estrogen and progesterone... *DONE*
- Whole mount analysis of in vivo proliferation by end bud number (JAN 2003–APRIL 2003)
- Immunohistochemical analysis of proliferation (JAN 2003 – JUNE 2003)

B. Analysis of leptin function in vitro. (JAN 2003 – DEC 2003)
- Characterize leptin receptor protein (western) and mRNA (RT-PCR) expression by mammary epithelial cell lines
- Evaluate proliferation and differentiation of mammary epithelial cell lines to leptin administration in vitro, dose and time course
- Western and Northern analysis of WDNM1, β-casein, and α-lactalbumin expression by HC11 cells

C. Evaluate leptin activation of specific STAT proteins in mammary epithelial cells (JAN 2004 – AUG 2004)
- Electromobility shift analysis of STAT1, STAT3, and STAT5a
- Gel supershift analysis STAT1, STAT3, and STAT5