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The insulin-like growth factor (IGF) system and extracellular matrix proteins are key regulators of the malignant breast cancer phenotype. Both IGFs and extracellular matrix proteins communicate wit epithelial cells by ligating cell surface receptors. Therefore, ligand-receptor interactions of the two systems are relevant treatment targets in breast cancer cell growth. 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 proposal will test the hypothesis that IGFBP-1 interrupts ligand-receptor interactions between extracellular matrix proteins and integrins. The key research accomplishment is the conclusion that expression of a mutant form of IGFBP-1 that cannot bind integrins at reasonable levels is not technically achievable using the *Pichia pastoris* yeast expression system. An alternative approach will be used to express mutant IGFBP-1. The successful expression and purification of 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 manner.

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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 (Figueroa, Sharma et al. 1993). 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:

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

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 *Pichia pastoris* yeast expression system at approximately 10ug/ml yeast medium. Therefore, I have focused my efforts on the expression of mutant [Trp^{221}] IGFBP-1.

During the first year of the grant, I was able to successfully clone cDNA for [Trp^{221}] IGFBP-1 into a yeast expression vector, electroporate the construct into *P. pastoris*, and screen clones for protein expression. The results demonstrated that [Trp^{221}] IGFBP-1 could be produced by the *P. pastoris* yeast expression system. However, the protein was expressed at low levels (<1ug/ml culture media). Therefore, electroporation was repeated and the transformants were immediately plated on yeast dextrose plates containing increasing concentrations of zeocin (500 to 2000 ug/ml). Because the expression vector contains a zeocin resistance gene, this method was used to isolate clones that contain multiple copies of the integrated "gene of interest" into the yeast genome. However, this approach was not successful in increasing protein yield as all clones screened expressed low levels of [Trp^{221}] IGFBP-1. In summary, 26 total clones were screened and all exhibited low expression (<1ug/ml culture media). Purification of [Trp^{221}] IGFBP-1 from the highest expressing clone is shown in Figure 1.

I tried to increase protein yield from the highest expressing clone by optimizing the culturing conditions. This included increasing the number of yeast cells in the induction culture, varying the culture media to flask volume, increasing the frequency of methanol feeding, and adding cas amino acids to the culture media to prevent protein degradation. Unfortunately, these methods did not increase protein expression or yield.

Purification of [Trp^{221}] IGFBP-1

Figure 1A)

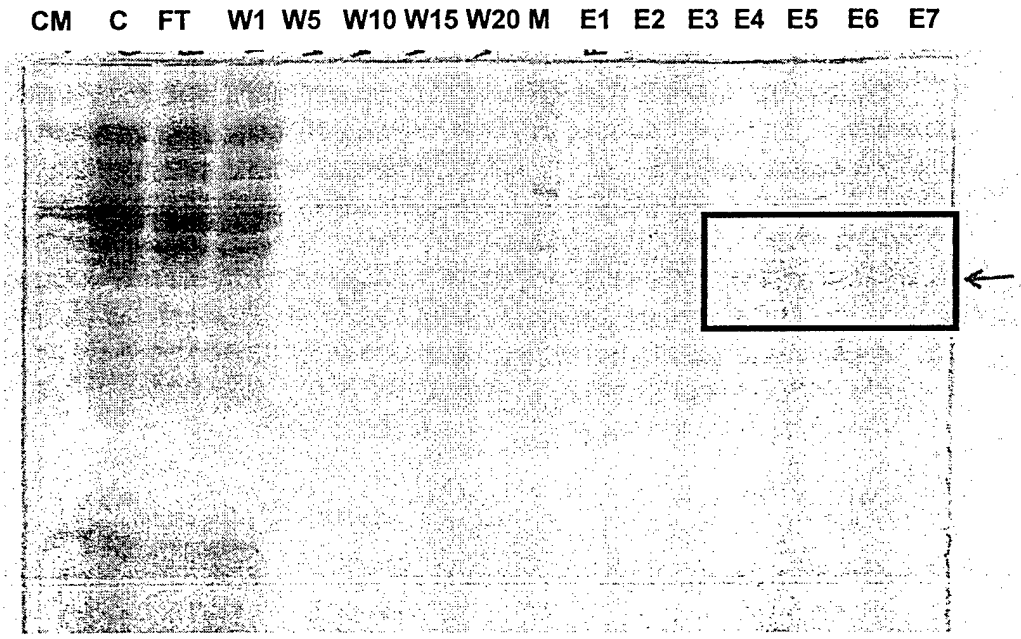

Figure 1B)

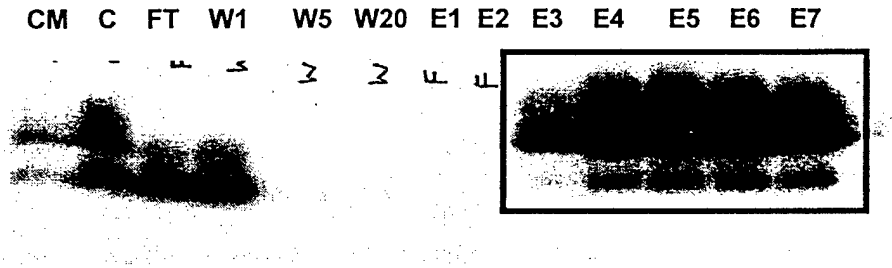

Figure 1: Purification of 6xHis-tagged [Trp^{221}] IGFBP-1 using Ni-NTA resin (Qiagen). Culture media (250ml) was concentrated by ammonium sulfate precipitation to a total volume of 10ml. Following equilibration of the concentrated sample in column loading buffer (50mM NaH_2PO_4, 300mM NaCl, 5mM imadazole, pH 8.0), the sample was loaded onto a Ni-NTA resin column at 1ml/min and subsequently washed with column loading buffer containing 10mM imidazole. [Trp^{221}] IGFBP-1 was eluted with 50mM imidazole. The elution fractions containing [Trp^{221}] IGFBP-1 are indicated by the black box in Figure 1A and 1B, respectively. These fractions were pooled, dialyzed, and concentrated. Total protein yield was 250ng/ml culture media. A) Coomassie blue-stained SDS-15% PAGE of purified [Trp^{221}] IGFBP-1. Lanes: CM: culture media, C: concentrated sample prior to column loading, FT: column flow-through, W1-W20: 10mM imidazole washes, M: protein molecular weight marker, E1-E7: 50mM imidazole elutions. B) I^{125}IGF-I ligand blot of purified [Trp^{221}] IGFBP-1 transferred from a SDS-15% PAGE. Lanes are the same as described for Figure 1A. The results of the ligand blot indicate that [Trp^{221}] IGFBP-1 is properly folded since it is able to bind IGF-I.

CONCLUSION:

I have completed all of the tasks for specific aim 1. Specifically, I have cloned [Trp^{221}] IGFBP-1 into the yeast *Pichia pastoris* vector (Task 1), expressed the construct in *P. pastoris* (Task 2), and purified/characterized [Trp^{221}] IGFBP-1 (Task 3 and 4). However, I have been unable to express [Trp^{221}] IGFBP-1 in *P. pastoris* at reasonable levels (greater than 1ug/ml). As a result, I am unable to address specific aim 2 because I do not have enough [Trp^{221}] IGFBP-1 protein to perform the biological experiments. Therefore, I have decided to use a different approach to express [Trp^{221}] IGFBP-1 protein. David R. Clemmons, University of North Carolina, Chapel Hill, NC, is the investigator who gave us the cDNA for [Trp^{221}] IGFBP-1. His laboratory used CHO-K1 cells to express [Trp^{221}] IGFBP-1 protein. The cells secreted 4ug of [Trp^{221}] IGFBP-1 per ml culture media in 48 hours (Jones, Gockerman et al. 1993). Therefore, I am in the process of making a stable CHO-K1 cell line that will express [Trp^{221}] IGFBP-1 protein. The cDNA for [Trp^{221}] IGFBP-1 and the attached 6xHis-tag will be cloned out of the yeast expression vector and put into the mammalian expression vector pcDNA3.1. CHO-K1 cells will be subsequently transfected with the construct, selected, and amplified into stable cell lines. It is expected that using CHO-K1 mammalian cells to express [Trp^{221}] IGFBP-1 will generate adequate amounts of protein (approximately 4 ug/ml culture media) to complete the experiments proposed in specific aim 2.

KEY RESEARCH ACCOMPLISHMENTS:

- Purification of soluble recombinant of [Trp^{221}] IGFBP-1 protein
- Culture conditions were optimized to increase [Trp^{221}] IGFBP-1 protein yield
- Protein yield using the yeast expression system was determined to be unreasonably low (<1ug/ml)

Since protein expression of [Trp^{221}] IGFBP-1 has been difficult and I need to advance a research project to complete my graduate training, I have also been researching another area of breast cancer. The main objective of this project is to determine the mechanisms whereby IGF-I regulates the estrogen receptor to stimulate proliferation of breast cancer cells. As my reportable outcomes indicate, I am making progress in this research project.

REPORTABLE OUTCOMES:

1. Student Volunteer to the Graduate Committee, Department of Pharmacology, University of Minnesota, 2003.

2. Review article: The type-1 IGF receptor tyrosine kinase and breast cancer: Biology and therapeutic relevance. **J.M. Gross** and D. Yee. Cancer Metastasis Rev, 22(4): 327-336, 2003.

3. Poster Presentation: Insulin-like growth factor-1 regulates estrogen receptorα transcriptional activity by multiple pathways in breast cancer. **J.M. Gross** and D. Yee. Era of Hope: Department of Defense Breast Cancer Research Program Meeting, Abstract No. P39-4, Sept. 25-28, 2002, Orlando, FL.

4. Poster Presentation: Enhanced translational efficiency mediated by insulin-like growth factor-1 augments estrogen receptorα- function in breast cancer. **J.M. Gross** and D. Yee. Gordon Conference: IGFs in Physiology and Disease, March 9-13, 2003, Ventura, CA.

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Jones, J. I., A. Gockerman, et al. (1993). "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.

Appendix

INSULIN-LIKE GROWTH FACTOR-I REGULATES ESTROGEN RECEPTOR-ALPHA TRANSCRIPTIONAL ACTIVITY BY MULTIPLE PATHWAYS IN BREAST CANCER

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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-alpha (ER-alpha) 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 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 (20μM) or treatment with the PI3K inhibitor LY294002 (25μM) blunted the IGF-I-induced increase in luciferase activity. U0126 but not LY294002 also blocked the estrogen-induced increase in luciferase activity. Interestingly, the p38 inhibitor SB203580 (20 μM) also suppressed IGF-I mediated activation of luciferase activity. However, treatment of MCF7 cells with IGF-I (5nM) did not increase the level of phosphorylation of p38, as determined by phosphospecific immunoblot analysis. These studies suggest that multiple IGF-I-activated signaling pathways contribute to transactivation of ER-alpha. In contrast, estrogen-induced activation of ER-alpha 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 ER-alpha function.

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Cancer and Metastasis Reviews 22: 327–336, 2003.

The type-1 insulin-like growth factor receptor tyrosine kinase and breast cancer: Biology and therapeutic relevance

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Key words: insulin-like growth factor receptor, tyrosine kinase, breast cancer

Summary

The development of the mammary gland requires the coordinated expression of hormones and growth factors. Likewise, some transformed breast cells continue to respond to these same extracellular signals. Thus, understanding the mechanisms that control normal development of tissues can lead to new therapeutic targets. The insulin-like growth factor (IGF) system plays an important role in the normal development and function of the mammary gland. Accumulating evidence suggests that the IGFs are also key regulators of the malignant phenotype. The IGFs stimulate proliferation, promote survival, and enhance metastatic potential of breast cancer cells. Although multiple receptors for the IGFs have been identified, the IGFs primarily exert their biologic effects through ligation of the type I IGF receptor tyrosine kinase (IGF1R). IGF binding to the IGF1R initiates an intracellular signaling cascade that leads to changes in gene expression and cell biology. This review will focus on the evidence that the IGF1R is a relevant treatment target in breast cancer.

Introduction

The mammary gland undergoes striking structural and physiological changes during puberty and pregnancy. These changes are the result of the synergistic actions of several growth factors and hormones. During puberty, the mammary gland undergoes differentiation and intense growth as epithelial cells proliferate and invade the surrounding tissue to form branching ducts [1]. Cells participating in this invasion process must be able to survive in a rapidly changing microenvironment. During pregnancy and lactation, the mammary gland undergoes additional structural changes as epithelial cells proliferate at the ends of terminal ducts to prepare for milk secretion. Once lactation is completed, gland involution occurs which is associated with massive apoptosis and removal of the cells required for milk production.

There are many parallels between the processes associated with mammary gland development and breast cancer. In breast cancer, malignant epithelial cells also proliferate, migrate, and invade the surrounding normal tissue. This invasion process is accompanied with the secretion of matrix proteases and survival factors, which causes extensive tissue remodeling in the affected area. Since normal mammary gland development requires the coordinated expression of hormones and growth factors, it seems logical that transformed breast cells could continue to respond to these same extracellular signals. Certainly, this was the logic used by Beatson who surgically removed the ovaries of a premenopausal woman with advanced breast cancer [2]. Thus, over 100 years ago the idea of a connection between normal breast biology and malignant breast disease was firmly established.

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As Beatson observed, ovarian production of a substance, later identified to be the sex steroid hormone estrogen [3], is required for normal mammary gland function. Estrogens mediate their effects in the breast by interacting with receptors (ERα and ERβ), which in turn modulate gene expression. The observation that breast cancers often express ERα coupled with the finding that receptor function could be inhibited *in vitro* lead to the development of new therapies for the treatment of breast cancer. These drugs are among the most successful in the treatment of hormone-dependent breast cancer today [4,5]. These studies highlight the importance of identifying key factors that regulate the normal development of tissues, and demonstrate how interruption of these pathways may lead to novel therapeutic treatments.

In addition to estrogen, growth factors play an important role in the development and function of the mammary gland. Several lines of experimental evidence implicate a role for the insulin-like growth factor (IGF) system in the growth and differentiation of the mammary gland. Importantly, it is also becoming apparent that the IGF system promotes phenotypes associated with malignancy. Similar to estrogen, IGFs mediate their effects by binding to a receptor. IGF binding to the type I insulin-like growth factor receptor tyrosine kinase (IGF1R) triggers an intracellular signaling cascade that leads to changes in gene expression and cell biology. This review will focus on the evidence that the IGF system promotes several phenotypes associated with cancer, including proliferation, protection from cell death, and metastasis.

The IGF system

The IGF system is composed of IGF ligands, receptors, binding proteins, and binding protein proteases [6]. Two IGF ligands exist, IGF-I and IGF-II, which share high homology to each other and to proinsulin. In contrast to other polypeptide hormones, circulating IGFs are found complexed to high affinity binding proteins (IGFBPs 1–6). IGFBPs are regulated by binding protein proteases.

The IGFs are required for normal growth and development. Mice homozygous for a deletion of the IGF-II gene are born with a reduced body size compared to their wild-type littermates, indicating the importance of IGF-II during prenatal development [7]. Since IGF-II is predominantly expressed during embryogenesis and IGF-II null mice develop into normal fertile dwarves, the role of IGF-II in the adult remains unclear. The biology of IGF-I null mice is more complex but suggests that IGF-I also plays an important role during prenatal development. Similar to IGF-II null mice, animals with a disruption of the IGF-I gene are born with a reduced body size compared to wild-type animals. However, the majority of these animals die shortly after birth due to severe respiratory muscle dystrophy and an inability to breathe [8,9]. The IGF-I null mice that reach adulthood are infertile and exhibit abnormal muscle and bone development, indicating that IGF-I is required for normal post-natal development. In accordance with this, one of the well-known physiological roles of IGF-I is to mediate the effects of growth hormone during puberty.

IGF-I can act in a paracrine or endocrine manner to affect development. The Cre/lox recombination system has been used to delete the IGF-I gene exclusively in the liver of mice [10]. In contrast to IGF-I null mice, the growth of liver-specific IGF-I null animals did not differ from that of wild-type littermate controls. Although circulating levels of IGF-I were dramatically reduced in the liver-specific IGF-I null mice, tissue mRNA levels of IGF-I were not different from wild-type littermate control animals. While coordinate regulation of liver-derived IGF-I and its major serum binding proteins may be ultimately responsible for growth of the organism, this study shows that circulating IGF-I alone is not crucial for normal development and supports the notion that paracrine and/or autocrine sources of IGF-I play an important role in mediating development.

IGF receptors

Two high affinity receptors exist for the IGF ligands; the type I IGF receptor (IGF1R) and the type II IGF receptor (IGF2R). The IGF1R can

bind IGF-I as well as IGF-II, but the preferential ligand is IGF-I. The IGF2R will only bind IGF-II. The two receptors differ completely in structure and function. The IGF1R is a tyrosine kinase receptor sharing 60% homology with the insulin receptor [11,12]. The receptor contains two identical extracellular alpha subunits and two identical transmembrane beta-subunits that are linked by disulfide bonds in a beta-alpha-alpha-beta heterotetramer. The alpha subunits make up the ligand binding domain while the beta subunits contain the tyrosine kinase activity. In contrast to other growth factor receptors, ligand binding is required for IGF1R activation. Ligand binding to the IGF1R leads to autophosphorylation of the receptor on tyrosine residues, which in turn provides docking sites for SH2-domain-containing molecules. Multiple substrates have been identified for the IGF1R including src-homology 2 [13], phosphatidyl inositol-3 kinase [14], growth factor receptor-binding protein 10 (Grb10) [15], focal adhesion kinase [16], Crk [17], CrkL [18], SHPTP2 [19], and insulin-receptor substrates 1–4 (IRS) [20–23]. However, the IRS species are considered to be the major substrate of the IGF1R. The IRS proteins contain multiple tyrosine phosphorylation sites and act as docking molecules for other signaling proteins. Our laboratory has shown that the IRS-1 is the major adaptor protein activated by IGF1R in estrogen receptor positive breast cancer cells [24]. Following ligand binding to IGF1R, IRS-1 associates with IGF1R and becomes tyrosine-phosphorylated, providing multiple docking sites for SH2-domain-containing molecules. The two most-studied signaling pathways downstream of IRS-1 in breast cancer cells are phosphatidyl inosital-3 kinase (PI3K) and mitogen-activated protein kinase (MAPK). The IGF1R is expressed by almost all cell types and plays a critical role in cell growth regulation and survival. Mice lacking the IGF1R gene have a body weight that is only 45% of their wild-type littermates and generally die after birth due to respiratory failure [9]. The results of these null mutation experiments show that the IGF1R and its ligands is required for organ development and survival.

In contrast to the IGF1R, the IGF2R does not have intrinsic tyrosine kinase activity and does not appear to signal to intracellular molecules [25]. The IGF2R is monomeric and can also bind mannose-6-phosphate containing enzymes. Thus the IGF2R is also known as the cation-independent mannose-6-phosphate receptor (IGF2/M6P receptor). Although the biologic function of the IGF2R is not clear, the receptor has been postulated to be an inhibitor of IGF-II action. Binding of IGF-II to the IGF2R results in receptor internalization and degradation, thereby reducing the interaction of IGF-II with the IGF1R. These events are thought to ultimately result in reduced IGF-II biological activity.

The IGFs have the ability to signal through two other highly related tyrosine kinase receptors, the insulin receptor (IR) and an insulin/IGF type 1 receptor hybrid (IR/IGF1R hybrid). The receptors for insulin and insulin-like growth factor-I are closely related in primary sequence and overall structure. Therefore it may not be surprising that insulin and the IGFs can bind to each other's receptor at high ligand concentrations. It has been suggested that the IGFs mediate growth responses through these receptors in malignant tissues. One group found that the IR was overexpressed in breast cancer specimens as well as cultured breast cancer cells [26], and subsequently demonstrated that activation of the IR by IGF-II lead to stimulation of cell growth [27]. The existence of a hybrid receptor between an alpha-beta heterodimer of the IGF1R and an alpha-beta heterodimer of the IR has also been reported to be involved in IGF signaling [28,29]. The IR/IGF1R hybrid receptor was found to be overexpressed in colon cancer and breast cancer [30,31]. IGF-I, rather than insulin, is considered to be the major ligand for the IR/IGF1R hybrid receptor. This is based on the finding that the hybrid receptors bind IGF-I with a higher affinity than insulin [32]. Furthermore, in contrast to insulin, IGF-I and IGF-II are produced locally in malignant tissues and thus have the potential to bind IR/IGF1R hybrid receptor. Because it is difficult to distinguish distinct signaling pathways resulting from IGF binding to the IGF1R as opposed to the IR/IGF1R hybrid, the specific physiological or pathological role for the hybrid receptor remains unclear. In spite of this, these studies emphasize the importance of considering the insulin receptor and IR/IGF1R hybrid receptor when designing therapies to block IGF action in cancer cells.

Role of the IGF system in mammary gland development

Accumulating evidence suggests that IGF-I, acting as the local mediator of growth hormone, plays a key role in mammary gland growth and differentiation [1]. Growth hormone can stimulate IGF-I mRNA production within the mammary glands of hypophysectomized rats [33]. Subsequent experiments performed upon hypophysectomized, castrated, sexually immature male rats showed that IGF-I could mimic the action of growth hormone by inducing the formation of terminal end buds, the structures through which mammary gland development occurs [34]. Evidence that IGF-I was required for mammary gland development during puberty was provided by experiments performed in IGF-I null mice. Investigators noted that female IGF-I null mice had markedly less mammary development than age-matched wild-type controls. The lack of mammary development could be directly correlated to the loss of IGF-I in these animals as IGF-I administration caused a significant increase in the number of branching ducts and terminal end buds [35]. Interestingly, treatment with estradiol was insufficient to cause mammary gland growth while IGF-I alone stimulated development. These experiments show that growth hormone induces mammary gland development by stimulating production of IGF-I.

IGF-I also may play a role in the structural changes that occur in the mammary gland during pregnancy and lactation. Transgenic mice have been created that chronically express IGF-I or des(1–3)IGF-I in the mammary gland starting from mid-pregnancy and throughout lactation [36,37]. The transgenic animals showed ductal hypertrophy and incomplete involution of the mammary gland at weaning, indicating that IGF-I can act as a mitogen and a survival factor in the mammary gland. Consistent with these observations, studies have linked IGF-I to the development of mammary tumors. Mice that have a p53 gene deletion and also overexpress des(1–3)hIGF-I in the mammary gland display a shorter period of tumor latency compared to expression of either protein alone [38].

These data are further supported by experimental evidence demonstrating a role for the IGF1R in transformation and tumorogenesis. While overexpression of the IGF1R promoted a ligand-dependent transformed phenotype in fibroblasts [39], gene deletion of the IGFIR in fibroblasts made the cells resistant to the transforming properties of several known oncogenes [40–43]. *In vivo* experiments have confirmed that the IGFs mediate tumorogenesis through the IGF1R. Tumors formed by human melanoma cells in nude mice are growth-inhibited when these cells are engineered to express antisense IGFIR [44]. Taken together, these studies implicate a role for the IGFs and the IGF1R in malignant transformation.

Expression of the IGF system in breast cancer

Investigators have measured expression of IGF system components in breast cancer cell lines and primary tumors. All components of the IGF system can be found in breast cancer specimens. Experimental studies suggest that most primary breast tumor specimens express mRNA encoding the IGF1R, the receptor that mediates the biological effects of the IGFs [45,46]. Furthermore, IGF-II has been shown to be elevated in normal stromal cells adjacent to malignant epithelial cells in the breast [45,46]. These studies have lead to the hypothesis that local production of IGF-II in the stroma provides an important paracrine growth stimulatory signal to malignant cancer cells. However, endocrine sources of IGF-I may also have a role in breast cancer. Evidence for this is provided by the observation that premenopausal women with high circulating IGF-I level are at increased risk for developing breast cancer [47,48]. These studies collectively show that the IGF1R is present on breast cancer cells and that IGF ligands are freely available to these cells from endocrine or paracrine sources. This environment within the malignant tissue would promote interaction between IGF ligands and IGF1R, allowing for IGF-mediated biological effects in breast cancer.

In contrast to IGF1R overexpression by most breast cancers, homozygous deletion of the IGF2R gene has been noted in breast cancer. Since the remaining allele has been shown to have mutated in the IGF-II binding domain [49], it has been suggested that IGF2R is a potential tumor

suppressor [50,51]. The IGF2R is thought to function as a 'sink' for IGF-II. Therefore, loss of the IGF2R gene would allow more IGF-II to interact with the IGF1R.

The association between the expression of IGF system components and cancer prognosis has been studied in breast cancer, but the results have been contradictory [52]. IGF1R expression in breast cancer specimens was positively correlated with ERα [53], and predicted a better prognosis [46,54]. The favorable prognosis provided by IGF1R is similar to that provided by the ERα in breast cancer. However, our laboratory has shown that breast tumors with an elevated expression of IRS-1, the major downstream signaling molecule of activated IGF1R, identified a subset of patients with decreased disease-free survival [55]. Given the discrepancy between the prognostic significance of IGF1R signaling pathways in primary breast cancer specimens, it seems possible that levels of receptor and key signaling molecules are not coordinately regulated.

Role of the IGFs in breast cancer growth

Multiple studies have shown that activation of the IGF1R by the IGFs promotes proliferation in some breast cancer cells. Both IGF-I and IGF-II are potent mitogens in ERα breast cancer cells [56]. Considerable crosstalk has been documented between the signaling pathways of IGFs and the steroid hormone estrogen in breast cancer [57,58]. This is of particular significance because ERα is an important prognostic marker and a valuable predictive factor for response to endocrine therapy. It is clear that only ERα tumors respond to selective estrogen modulators such as tamoxifen [59]. Estrogen and IGF-I can act synergistically with one another to stimulate proliferation of ERα+ breast cancer cells [60], so a better understanding of the ways IGF signaling can influence ERα function could have clinical relevance.

One of ways the two signaling systems appear to cooperate is through regulation of gene expression. Key signaling components of the IGF-I system are upregulated by estrogen treatment, including IRS-1 and IGF1R [61]. Furthermore, a recent study in MCF7 breast cancer cells showed that expression of ERα was directly correlated with important IGF signaling system components [62]. In this study, loss of ERα expression was associated with decreased expression of proteins in the IGF-I signaling system, diminished IGF signal transduction, and no growth response to estrogen or IGF-I. Re-expression of ERα in these cells restored expression of key molecules critical to IGF signaling as well as the proliferative responses to IGF-I and estrogen. These studies provide evidence that estrogen stimulates growth of ER+ breast cancer cells by maintaining and regulating responsiveness to the IGFs.

On the other hand, IGF-I has been shown to influence ERα biology. The selective estrogen modulator, tamoxifen, can inhibit IGF-I-mediated growth of ERα+ breast cancer cells. This suggests that IGFs stimulate growth of ERα+ breast cancer cells, at least in part, through transactivation of ERα [63]. Furthermore, IGF-I can increase the expression of the progesterone receptor, an endogenous estrogen-responsive gene, as well as estrogen-response-element-reporter constructs [64,65]. IGFBP-1, an inhibitor of IGF-I signaling, decreases IGF-I- and estrogen-induced activation of the estrogen-response-element reporter construct [65]. Taken together, these results indicate that the signaling pathways of ERα and IGF1R can potentiate the effects of one another to positively influence cancer progression.

Role of the IGF system in survival

Activation of the IGF1R has been shown to protect many different cell types from a variety proapoptotic insults. Breast cancer cells and fibroblasts treated with IGF-I are protected from radiation-induced cell death and are resistant to chemotherapeutic drugs [66–68]. Immunohistochemical analysis of primary breast tumors revealed that high levels of IGF1R were highly correlated with ipsilateral breast tumor recurrence following lumpectomy and radiation therapy [68]. Experimental studies have provided evidence that the IGF1R was responsible for mediating the effects of IGF-I on death. Downregulation of

IGF1R function by dominant-negative mutants removes the protective effect of IGFs [69]. These studies indicate that IGF1R activation may offer a survival advantage to malignant cells undergoing cancer treatment.

IGF-I-mediated protection from apoptosis occurs primarily through activation of the PI3K/Akt pathway [69]. Following IGF1R stimulation by ligand, IRS-1 associates with the receptor, leading to activation of PI3K and subsequent stimulation of AKT/PKB. The anti-apoptotic action of the PI3K/AKT pathway is thought to occur through the phosphorylation and inactivation of the death effector protein BAD. However, other anti-apoptotic signaling pathways have been described for the IGF1R [70,71].

Role of the IGF system in metastasis

Breast cancer becomes a lethal disease when tumor cells metastasize and proliferate at distant sites. Therefore, identifying the factors that contribute to metastasis is highly relevant. Several lines of evidence support a role for the growth factors in phenotypes associated with metastasis, including angiogenesis, invasion, dissemination, and extravasation. Increasing evidence suggests that the IGF system contributes to these metastatic phenotypes in breast cancer [72]. *In vitro* studies have revealed that IGF can stimulate the migration of metastatic as well as non-metastatic breast cancer cell lines [73]. These effects are mediated by IGF1R activation. Inhibition of IGF function by a dominant negative IGF1R suppressed metastasis from the mammary fat pad to distant organs of the metastatic breast cancer cell line MDA-MD-435. However, primary tumor growth was not affected [74]. Other studies have shown that IGF system components stimulate processes that enhance the invasion potential of cells. For example, both IGF-II treatment and overexpression of IGF1R in cell culture models increase the production of enzymes involved in extracellular matrix degradation [75,76]. Vascularization of the tumor also contributes to its metastatic potential and evidence suggests that IGF1R activation enhances angiogenesis [75,77]. These studies demonstrate that IGF1R activation contributes to the metastatic potential of cancer cells. Since IGF-II is synthesized by stromal cells surrounding breast neoplasms and most breast tumors express IGF1R, the potential exists for IGF-II to stimulate breast cancer cell migration and invasion *in vivo*.

Recent reports indicate that IGFs and integrins cooperate to govern biological responses elicited by a cell. Integrins are heterodimeric transmembrane receptors with two membrane-spanning domains [78]. Integrins were first recognized for their ability to mediate interaction between the cell and the extracellular matrix. However, they appear to be involved with a variety of cell processes including migration, survival, and growth. As a result, their role in mediating cancer progression is being extensively studied. Since integrins lack intrinsic kinase activity, they depend on association with other proteins to transmit signals. Several studies have reported interactions between the IGF and integrin signaling pathways. Association between the two signaling pathways was demonstrated through the physical interaction between $\alpha v\beta 3$ integrin and IRS-1, the major immediate substrate of the IGF1R [79]. Other molecules important for integrin signaling have been shown to be substrates for IGF1R or IRS-1 [16,80,81]. Furthermore, evidence exists that integrins cooperate with the IGF signaling system to positively influence the metastatic potential of breast cancer cells. In a metastatic breast cancer cell line, it has been demonstrated that $\alpha 6\beta 4$ integrin increases the invasive potential of these cells through activation of PI3K [82,83]. A subsequent recent study showed that IRS-1, one of the major downstream adaptor proteins of IGF1R, is involved in $\alpha 6\beta 4$ – induced activation of PI3K in breast cancer cells [29]. This existence of crosstalk between the IGF system and integrins is further supported by functional studies in tissue culture and *in vivo* tumor models. Inhibition of integrin function blocks IGF-stimulated cell migration [73,84]. Spontaneous metastasis of multiple tumor types required both integrin function and growth factor treatment [85]. These studies provide evidence that the IGF system is involved in the process of metastasis and provide rationale for targeting IGF-IR in the treatment of metastatic breast cancer.

Conclusion

The normal mammary gland must invade normal tissue, proliferate, and undergo cell death at the appropriate time. Thus, all of the key phenotypes of malignant breast epithelial cells have a counterpart in normal mammary gland development. Some transformed cells may require the same stimuli that were necessary during normal development. This recognition has lead to the identification of factors that are required by tumor cells to maintain the malignant phenotype. Interruption of key biological pathways responsible for each of these phenotypes may lead to novel therapeutic strategies. There is abundant experimental evidence that activation of the IGF1R contributes to breast cancer progression. The IGFs stimulate mitosis, enhance survival, and promote metastasis. Furthermore, all of the IGF system components required to initiate an intracellular signal are present in breast cancer tumors. Just as interruption of the epidermal growth factor receptor family members has proven to be a useful therapy for human cancer, it is likely that interruption of IGF1R signaling and function could have similar benefits. While many strategies have been demonstrated *in vitro*, the next challenge will be to discover a safe and effective method for disrupting tumoral IGF action without eliciting host toxicity.

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