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THE INSULIN-LIKE GROWTH FACTOR/INSULIN SYSTEM IN EPITHELIAL OVARIAN CANCER

Kimberly R. Kalli and Cheryl A. Conover

Endocrine Research Unit, Mayo Clinic and Foundation, Rochester, MN

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1. ABSTRACT

Ovarian cancer is the most lethal of the gynecologic malignancies, in part because the lack of striking symptoms in many women often results in diagnosis only after the disease has spread. In order to develop rational biologic or pharmacologic therapies, it is important to understand the biology of both normal and malignant ovarian epithelial cells. This review will summarize current knowledge of the expression and function of proteins in the insulin-like growth factor system in ovarian epithelium, including the peptide ligands, receptors, binding proteins, and binding protein proteases. All components of this complex regulatory system are present in the ovarian epithelium and may play important roles in regulating the normal biology of ovarian surface epithelial cells or the transformed phenotype of ovarian carcinoma cells.

2. INTRODUCTION

Epithelial ovarian cancer arises from a single layer of ovarian surface epithelial cells (OSE) (1) and is the most lethal of the gynecological malignancies (2). The initiation of carcinogenesis is not fully understood, but has been proposed to be a consequence of altered hormone levels after menopause or repeated growth stimulation following ovulation as OSE proliferate, migrate, and differentiate to heal the ovulation-associated wound (3). Beyond its role in wound repair responses, the insulin-like growth factor (IGF) system plays several important roles controlling cell proliferation, survival under stress, and maintenance of the transformed phenotype.

The insulin-like growth factors (IGFs) are ubiquitously expressed ligands for a family of receptor proteins. IGF-I, IGF-II, and high (M x 10^{-6}) doses of insulin mediate mitogenic effects through the IGF-I receptor (also known as the type I IGF receptor). Insulin and IGF-II in nM concentrations also mediate mitogenic effects through isoform A of the insulin receptor, a protein lacking the alternatively spliced exon 11 that encodes 12 amino acids present in the metabolically active isoform B. In addition, IGF-II levels are locally controlled by the IGF-II receptor (also known as the type II IGF receptor or the mannose 6-phosphate receptor), a non-signaling protein that targets IGF-II for internalization and degradation.

IGF and insulin receptors are ubiquitous, and the bioavailability of the IGF peptides is controlled by association with IGF binding proteins (IGFBP). There are six high affinity binding proteins that can sequester IGFs, enhance their action, or mediate IGF-independent functions. An additional layer of control is added by proteases specific for IGFBPs. These proteases, including the recently identified pregnancy-associated plasma protein-A (PAPP-A), can be regulated in both expression and activity. This complex regulatory network poses interesting new questions that may impact our understanding of the role of the IGF system in the biology of both normal and malignant ovarian epithelium.

Table 1. Loss of imprinting of the IGF-II gene in malignant epithelial ovarian tumors

Cases with LOI	Informative Tumors	Reference
0	11	14
2	8	15
5	20	16
2	15	17
Total: 9 (~ 17%)	Total: 54	

This table summarizes studies detailing loss of imprinting (LOI) of the IGF-II gene in epithelial ovarian cancers. Note that the cited references may have included benign or non-epithelial ovarian tumors that were not included here.

3. EXPRESSION OF IGF SYSTEM PROTEINS IN OVARIAN CARCINOMA CELL LINES

Several reports in the early 1990's showed that the IGF system was expressed in ovarian carcinoma cell lines as well as in normal ovary. Yee *et al.* (4) reported that 3 of 10 ovarian carcinoma cell lines expressed mRNA for IGF-I and all cell lines expressed IGF-I receptor mRNA. OVCAR-3 cells expressed a subset of binding proteins and responded to IGF-I with increased proliferation. This cell line and CaOv-4 cells used IGF-based autocrine growth loops, as demonstrated by the ability of antisense oligonucleotides targeting the IGF-I receptor to inhibit IGF-I-stimulated growth and growth in serum-free medium (5). Our laboratory characterized cell lines derived from ovarian tumors and showed that 6 of 6 cell lines express the IGF-I receptor (6), and most responded in a proliferative manner to exogenous IGF-I or 100 nM insulin. All cell lines expressed various IGF binding proteins, IGF-I or IGF-II. Ovarian carcinoma cell lines may become dependent on the expression of IGF peptides, as demonstrated by the ability of antisense oligonucleotides targeting IGF-II mRNA to induce apoptosis of the AO ovarian carcinoma cell line (7).

Baldwin *et al.* suggest that estrogen responsiveness of the BG-1 ovarian carcinoma cell line correlates with IGF-I receptor expression (8). OV266, another estrogen-responsive (Kalli and Conover, unpublished data) ovarian carcinoma line (6), expresses IGF-I receptors, but did not have elevated ^{125}I-IGF binding in comparison to the five ER negative cell lines. It has been suggested that there is an interplay between steroid hormone receptors and IGF-I receptor signaling in ovarian epithelial cells because the response of the BL ovarian carcinoma cell line to sub-optimal levels of IGF is augmented by treatment with estradiol (9).

4. EXPRESSION OF IGF LIGANDS *IN VIVO*

4.1. IGF-I

RNase protection analyses were used to show that 7 of 7 ovarian carcinoma samples expressed mRNA encoding IGF-I (4). Similarly, IGF-I mRNA was detectable by non-quantitative PCR in 100% of 31 samples comprised of ovarian cancer biopsies, benign ovarian tumors, normal ovaries, cancer cell lines and xenografts (10). Because IGF-I protein detected in tissues may be bound to binding proteins and therefore unavailable to receptors, the functional significance of IGF-I expression in tissues is generally addressed by assessing the presence and activity of IGF-I receptors as well as IGF-I itself.

4.2. IGF-II

Regulation of IGF-II expression is potentially important in a number of malignancies. The two splice forms of the IGF-II gene were first and second in rank order of fold-induction of genes overexpressed in colorectal malignancies as compared to normal colonic epithelium (11). The IGF-II gene is maternally imprinted (12), meaning that normal cells express IGF-II only through the paternal allele. In addition, it is located on chromosome 11, a site of common chromosomal rearrangement in ovarian malignancies (13). Despite these interesting gene characteristics, no IGF-II gene amplifications were reported in 9 of 9 ovarian tumors with chromosome 11 alterations (13). IGF-II mRNA was detected in 27 ovarian tumors and 10 normal ovaries (14). In addition, loss of imprinting (LOI), a condition potentially increasing IGF-II levels by allowing transcription from both alleles, has been reported in approximately 17% of malignant ovarian epithelial tumors (table 1). Interestingly, Kim *et al.* also reported LOI in 2 of 3 benign mucinous adenomas in the referenced study (15), and 4 of 5 of the LOI-positive samples in the Chen analysis (16) were of low clinical stage. Semi-quantitative RT-PCR showed overexpression of the IGF-II transcript in all 7 informative tumors showing IGF-II LOI (16, 17), as well as in 5 of 18 uninformative ovarian tumors (17). There is much to be learned about the regulation of this important gene and gene product.

4.3. IGF in serum

Sera from patients with ovarian cancer have reduced levels of total IGF-I when compared to women with benign ovarian disease or healthy controls (18, 19). Monitoring IGF-I levels in serum gave no benefit over monitoring CA125 alone in patients undergoing chemotherapy for ovarian cancer (20). It is not apparent whether reduced serum IGF-I indicates reduced production, increased localization to tumors, or alterations in binding proteins that result in aberrant systemic distribution of IGF-I. Data regarding serum IGF-II levels in women with ovarian cancer are not available.

5. RECEPTORS FOR IGF

Many careful reviews of the role of the IGF-I receptor in cancer are available (21-23). In brief, IGF-I receptors play a role in proliferation, protection from stress-induced apoptosis, and maintenance of the transformed phenotype. These functions obviously could serve as targets for therapies designated to impede the growth of cancer cells. Interestingly, however, the other receptors responsive to IGFs may also play a role in malignancy as detailed below.

5.1 IGF-I receptor

Studies using molecular techniques to detect mRNA for IGF-I also found IGF-I receptor message in 100% of the samples analyzed (4, 10). Specific radioimmunoassays and/or ^{125}I-IGF autoradiography demonstrated that receptors for both IGF-I (24, 25) and

insulin (25) were expressed in the majority of ovarian samples analyzed, again comprising a mixture of epithelial and non-epithelial ovarian malignancies and benign/normal ovaries. Finally, flow cytometry (26) and immunohistochemistry (24) using alpha IR-3, a monoclonal antibody specific for the IGF-I receptor, demonstrated IGF-I receptor expression on normal and malignant ovarian cells. While relative quantitation of receptors in some of these early reports is complex due to the different epithelial content of whole ovaries compared to tumors, it is clear that IGFR is expressed in the majority of ovarian tumors. The presence of both ligand and receptor that could participate in autocrine growth regulation has obvious implications for ovarian cancer biology and are potential targets for growth-inhibiting strategies.

There is vast literature on the inhibition of growth and tumorigenicity by downregulated IGF-I receptor expression in a variety of cell types. It is likely that these concepts will hold true for ovarian carcinoma cells as well. Engineered expression of the IGF-I receptor in rabbit ovarian mesothelial cells increased their growth in soft agar and tumorigenicity in nude mice (27). Introduction of antisense oligonucleotides targeting the IGF-I receptor reduced the growth of both unstimulated and IGF-treated OVCAR-3 cells (28). Interestingly, even expression of a construct encoding 112 amino acids of the carboxy terminus of the IGF-I receptor attached to a membrane-localizing myristylation sequence was sufficient to decrease the growth of Ca-OV-3 cells in soft agar, presumably by favoring the association of signaling intermediates with a cytoplasmic domain that is unresponsive to extracellular ligand. This construct induced apoptosis of Ca-OV-3 cells in vivo and prevented the growth of the transfected cells in nude mice (29). Strategies targeting IGF-I receptor signaling capabilities are valid approaches to the control of ovarian cancer growth, but will require thorough testing to determine whether the optimal control point rests at the level of intracellular signaling, receptor binding, or ligand availability.

5.2. IGF-II Receptor

The IGF-II receptor, also identified as the cation-independent mannose 6-phosphate receptor that plays a role in lysosomal trafficking of mannose 6-phosphate-containing proteins, internalizes IGF-II and targets it for degradation rather than initiating mitogenic signaling pathways. It also can activate the proform of the antiproliferative cytokine transforming growth factor-beta (30)? another function that would tend to decrease local proliferative responses. Decreased levels of IGF-II receptor can effectively raise local levels of bioavailable IGF-II (31, 32). The importance of limiting IGF-II bioavailability was demonstrated by the large size of IGF-II receptor knockout mice as compared to their wild-type littermates (33).

The IGF-II receptor gene is paternally imprinted in rodents, but often biallelically expressed in humans (34, 35). It contains repeating mononucleotide sequences susceptible to rearrangement in cells with microsatellite instability due to mismatch repair defects. In addition, it is located at chromosome 6q26 near the end of the FRA6E common fragile site (36), a region of frequent chromosomal breakage under certain tissue culture conditions. As might be expected given these molecular characteristics, expression of the IGF-II receptor is lost in some breast, hepatocellular, and lung carcinomas (37 – 40). In these cancers, loss of heterozygosity of the IGF-II receptor is observed accompanied by mutations in the remaining receptor allele, leading to its proposed classification as a tumor suppressor gene (41, 42).

Loss of heterozygosity in chromosome 6q26-27 is seen frequently in ovarian tumors (43, 44). While loss of heterozygosity of the 6q27-qter region was observed in 5 of 8 ovarian tumors (45), the IGF-II receptor coding sequence was perfectly conserved, unlike observations in other malignancies showing loss of IGF-II receptor expression or mutations, some of which were shown to negatively affect ligand binding (46). Microsatellite instability was observed in 0 of 39 (47) and 1 of 33 (48) of ovarian carcinomas and only 1 of 8 (49) ovarian carcinoma cell lines, indicating that this mechanism of IGF-II receptor gene rearrangement does not appear to be common in ovarian cancer, unlike hepatocellular (37), endometrial, stomach, and colorectal (47) cancer. Recent semi-quantitative RT-PCR analyses show that 0 of 7 malignant ovarian epithelial cell lines and 0 of 14 ovarian tumor samples had a loss of IGF-II receptor expression when compared to normal ovarian surface epithelium (36). These results, taken at surface value, may suggest that loss of a functional IGF-II receptor protein may play a less significant role in ovarian carcinoma than in other malignancies. However, it has been proposed that overexpression of pro-cathepsins, intracellular IGF-II receptor ligands that can be overexpressed in tumors, or overexpression of IGF-II itself in ovarian tumors (17) could lead to IGF-II receptor saturation and consequent increased availability of IGF-II (45). Alterations in the secretion of lysosomal enzymes are observed in breast tumors (see 45 for discussion), providing indirect evidence for altered function of the IGF-II receptor. The importance of the IGF-II receptor as a tumor suppressor in various malignancies is undoubtedly worthy of further investigation.

5.3. Insulin receptor

Work in the late 80's showed that two forms of the insulin receptor exist as a result of alternative splicing (50). Isoform B is the classic metabolic insulin receptor prevalent on metabolic target tissues such as hepatocytes, adipocytes, and muscle. Isoform A differs from isoform B by lacking the 36 nucleotides of exon 11 that encode 12 amino acids in the membrane-proximal region of the extracellular alpha chain. This version of the insulin receptor is expressed in fetal and cancer cells (51) and mediates mitogenic effects, although the mechanisms of differential signaling are not well understood. Importantly, the absence of the amino acids encoded by exon 11 allows isoform A of the insulin receptor to bind IGF-II as well as insulin with affinities in the nM range, while isoform B binds only insulin.

Insulin receptors are expressed in normal ovary and malignant ovarian tumors (52, 25). Engineered overexpression of insulin receptors can induce ligand-

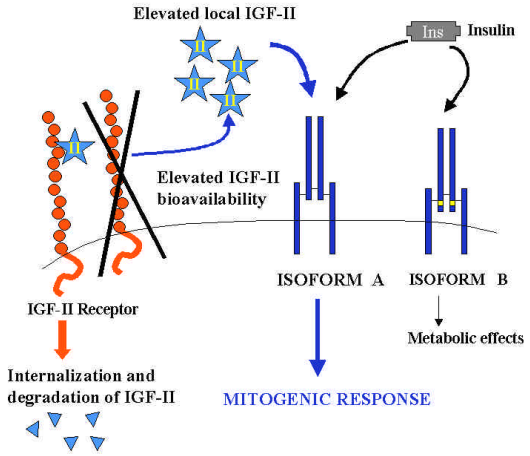

Figure 1. Model of the potential effect of IGF-II receptor aberrations in tumors expressing mitogenic insulin receptor isoform A. Alterations in normal IGF-II receptor function, whether from gene deletion, mutation, or saturation with aberrantly expressed ligands, may result in increased local levels of IGF-II. The presence of insulin receptor isoform A in the same local area could initiate mitogenic signals through this isoform of the insulin receptor as well as the IGF-I receptor.

dependent transformation (53, 54). While hyperinsulinemia and diabetes mellitus have weak, if any, association with increased risk of ovarian cancer (55), the presence of isoform A would allow responses to IGF-II as well as to insulin. Higher levels of insulin receptor isoform A are expressed in malignant cells than normal cells in breast and thyroid carcinomas (56, 57). We have investigated the presence and function of insulin receptors in epithelial ovarian carcinoma cell lines (58). Five of six cell lines expressed more ^{125}I-insulin binding ability than normal ovarian surface epithelial cell lines. Mitogenesis is induced in these lines by nM concentrations of insulin. In addition, IGF-II can displace ^{125}I-insulin binding to the insulin receptor and was shown to bind to the insulin receptor by cross-linking and immunoprecipitation analyses. These results indicate that ovarian carcinoma cells are likely to express insulin receptor isoform A, which was confirmed by 5' nuclease assays showing preferential expression of isoform A over isoform B in six of six ovarian carcinoma cell lines tested (58).

As discussed above, the loss or saturation of the IGF-II receptor in tumors may allow elevated IGF-II expression that could interact with insulin receptor isoform A (figure 1). Besides mitogenesis, IGF-II signaling through insulin receptor isoform A or the IGF-I receptor could influence cell behavior (migration, differentiation) and survival. The presence of an IGF-II/insulin receptor isoform A growth loop could make strategies designed to obstruct IGF-I receptor signaling in tumor cells be less effective than hoped. Further studies are required to determine the role of the insulin receptor in ovarian cancer.

6. INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS

6.1. IGFBPs *in vitro*

Ovarian carcinoma cells in culture have been shown to express five of the six known IGFBPs (IGFBP-2 through –6), although the pattern of expression appears to be cell- and condition-specific, similar to what has been described for other cell types (59). The established ovarian cancer cell lines, OVCAR-3, CaOV-4 and SKOV-3 express IGFBP-3 mRNA, and OVCAR-3 and CaOV-4 express IGFBP-2 mRNA as assessed by Northern blot (4). IGFBP-2 through –6 were detected by RNase protection assay in PE04, an estrogen receptor-positive ovarian cancer cell line (60). Hofmann et al. (61) derived four primary cultures of ovarian cancer cells from various tumor ascites and metastases. They concluded that ovarian cancer cells frequently (4 of 4 lines) express IGFBP-3, -4, and –6 and to a lesser extent (3 of 4) IGFBP-2, rarely (1 of 4) express IGFBP-5, and do not express IGFBP-1. Our data from cell lines derived from primary epithelial ovarian tumors also indicated that IGFBP-2, -3, -4 and -6 mRNA and protein were expressed in various combinations by all of the cell lines (6). Only one of the six cell lines expressed IGFBP-5 and none expressed IGFBP-1. Interestingly, two of the ovarian carcinoma cell lines exhibited cell surface–associated IGFBP detected by binding of ^{125}I-IGF to washed cells followed by cross-linking, gel electrophoresis, and autoradiography. In one of the lines this clearly represented cell-associated IGFBP-3, since this was the only IGFBP expressed by that cell (6). Cell-associated IGFBP-3 has been shown to potentiate IGF action in other systems (62, 63). However, the functional significance of any secreted or cell-associated IGFBP in ovarian cancer cells is currently unknown.

6.2. IGFBPs *in vivo*

Ovarian epithelial tumors have been reported to express mRNA for IGFBP-2 through –5 (60). IGFBP-2 may be of particular relevance in ovarian cancer as well as other malignancies (64). IGFBP-2 was identified as one of the up-regulated genes in ovarian cancer by serial analysis of gene expression (65). Kanety *et al.* (66) detected IGFBP-2 mRNA in all ovarian specimens tested (14 malignant epithelial ovarian tumors, 6 benign ovarian neoplasms). However, levels of IGFBP-2 mRNA were elevated 2-to 30-fold in malignant compared to benign tumors. Levels of IGFBP-2 expression correlated with tumor aggressiveness. Furthermore, IGFBP-2 was found elevated in serum and cyst fluid from women with ovarian cancer (67). Levels of IGFBP-2 were higher in cyst fluid than the corresponding sera implying local production. Serum IGFBP-2 levels correlated with CA125, a clinically useful marker of ovarian cancer. On the other hand, serum and cyst fluid levels of IGFBP-3 were decreased in women with ovarian cancer (68). Low IGFBP-3 correlated with unfavorable prognostic features (69). The lower levels of IGFBP-3 in cyst fluid were associated with the presence of proteolytic products (68). There was also evidence of IGFBP-2 proteolytic fragments in cyst fluid (67). The presence of these IGFBP fragments suggests a role for

proteolytic modification as a means of IGFBP regulation in the ovary.

7. IGFBP PROTEASES

It is known that certain invasive ovarian cancers produce proteases that can use IGFBPs as substrates. Cathepsin D expressed by human breast and prostate cancer cells has been shown to be an acid-activated IGFBP-3 protease (70). In breast cancer cells, this enzyme is regulated by estrogen. Our ovarian carcinoma cell lines also secrete cathepsin D, but it is not regulated by estrogen in an estrogen receptor-positive cell line (unpublished data). An IGFBP-2 protease has been reported in cultured neuroblastoma cells (71, 72), but there are no data to suggest whether or not this enzyme is related to the protease responsible for IGFBP-2 fragments in cyst fluid.

Recently, two IGFBP proteases have been identified as down-regulated genes in ovarian cancer: HtrA and pregnancy-associated plasma protein-A (PAPP-A). HtrA is a serine protease in the stress response pathway that selectively cleaves IGFBP-5 of the six IGFBPs (73). HtrA was downregulated in ovarian tumors as compared to normal OSE in four subtraction suppression hybridization libraries (listed as PRSS11 in Reference 74). HtrA is up-regulated in osteoarthritis (75), however its function in ovarian cancer has only begun to be addressed (76).

PAPP-A is an IGF-dependent IGFBP-4 protease expressed by normal human fibroblasts, osteoblasts, and granulosa cells in culture (77, 78). In situ localization of PAPP-A mRNA in human and murine ovaries shows significant expression of PAPP-A in granulosa cells as follicle maturation occurs (79, 80). In addition, human OSE in culture secrete abundant IGFBP-4 proteolytic activity in conditioned media (81). Differential display PCR comparing ovarian carcinoma cell lines to normal OSE cultures showed decreased expression of PAPP-A mRNA in the carcinoma cell lines (82). Whether regulated proteolysis of IGFBP-4 in normal ovarian epithelium or ovarian malignancies plays a role in the biology of ovarian cancer is under current investigation.

8. SUMMARY

IGF peptides, receptors, binding proteins, and binding protein proteases are all expressed in normal and malignant ovarian epithelial cells. Because of the importance of the IGF-I receptor in maintenance of the transformed phenotype, strategies to undermine the ability of this receptor to function in malignant cells may be therapeutically useful. However, it may be simplistic to think of the IGF system as merely the balance of IGF-I availability and IGF-I receptor expression. IGF-II gene expression may be dysregulated and protein expression altered if IGF-II receptor functions are changed with development of malignancy. In addition, IGF-II could initiate mitogenic signaling through insulin receptor isoform A in malignant ovarian epithelial cells. Finally, the contribution of binding proteins and binding protein proteases to the regulation of local IGF levels is a new area of study that should add to our understanding of the role of the IGF system in ovarian cancer.

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Send correspondence to: Kimberly R. Kalli, Ph.D. Endocrine Research Unit, Joseph 5-194, 200 1st St SW, Mayo Clinic and Foundation, Rochester, MN 55905; Tel: 507-255-4467, Fax 507-255-4828, E-mail: kalli.kimberly@mayo.edu