Expression of Insulin-like Growth Factor I, Its Binding Proteins, and Its Receptor in Ovarian Cancer

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ABSTRACT

Insulin-like growth factor (IGF) I is a polypeptide hormone important in normal growth and development. Although IGF-I is a mitogen for many cancer cell lines, previous work has suggested that autocrine production of IGF-I is uncommon in cancers of epithelial origin. In this study, expression of IGF-I, its binding proteins, and its receptor were examined in ovarian cancer cell lines and tissues. Of 10 ovarian cancer cell lines, 3 (OVCAR-3, OVCAR-7, and PEO4) expressed IGF-I mRNA. RNase protection assays using probes derived from IGF-IA, IGF-B, and alternate exon 1 IGF-I complementary DNAs demonstrated that these cells contained a predominant IGF-I transcript with an alternate first exon. RNA extracted from primary and metastatic ovarian cancer tissues also expressed IGF-I mRNAs (7/7) with the alternate first exon. IGF-I protein was detected in OVCAR-3-conditioned media; this activity was secreted in conjunction with several IGF-binding proteins (IGFBPs). IGFBP-2, IGFBP-3, and an M, 24,000 species, and an M, 30,000 species could also be demonstrated in OVCAR-3. Type I IGF receptor mRNA was found in all 10 of the ovarian cancer cell lines and all 7 of the primary or metastatic ovarian cancer tissues. IGF-I was a mitogen for OVCAR-3, demonstrating the presence of a functional type I IGF receptor. These data show that all the necessary components for an IGF-I-mediated autocrine loop are expressed by ovarian cancer cells.

INTRODUCTION

Polypeptide growth factors are mitogens for many types of cancer cells in vitro. This observation has suggested that endocrine, paracrine, or autocrine growth factor-mediated stimulation of tumors may account, in part, for the unlimited proliferative capacity of human cancer. Production of growth factors by some tumors of epithelial origin has been well characterized, suggesting that autocrine stimulation may be important (1). Recent evidence has suggested that ovarian cancers may produce autocrine growth factors (2-6), and these factors may be necessary for optimal in vivo growth of the tumor (7).

We have previously shown that IGF1 is not commonly expressed by tumor cell lines of epithelial origin. However, neuroectodermal tumors and ovarian cancers were found to contain IGF-I transcripts (8-9). Furthermore, the IGF-I mRNA transcript produced by the ovarian cancer cell line OVCAR-3 is alternately spliced when compared to the major transcript produced by normal human liver (9). However, this transcript contains essentially all of the coding exons of the IGF-I gene, and it is possible that ovarian cancer cells produce IGF-I peptide. We undertook this study to examine the possibility that the IGF system could be important in regulating the autocrine growth of ovarian cancer cells. We were also interested in studying the production of IGF-I mRNA by ovarian cancer tissue specimens. We report that ovarian cancer cell lines and tissues express IGF-I, the type I IGF receptor, and IGF-binding proteins, which suggests that IGF-I may be an autocrine growth factor for ovarian cancer.

MATERIALS AND METHODS

Materials. The IGF-1A, type I IGF receptor, and IGF-binding protein cDNA probes were kindly supplied by Ken Gabbay (Baylor College of Medicine, Houston, TX), Axel Ullrich (Max Planck Institute, Martinsried, Germany), and David Powell (Baylor College of Medicine, Houston, TX), respectively. The IGF-B, alternate exon 1 IGF-C cDNA and genomic IGF-B exon 4 and 5 probes were a gift from Peter Rotwein (Washington University School of Medicine, St. Louis, MO). The cell lines OVCAR-2 to -10 were originally characterized in one of our laboratories (T. C. H.). SK-OV-3, CaOV-4, and T47-D were obtained from the American Type Tissue Culture Collection (Rockville, MD). PEO4 was provided by John F. Smyth (Imperial Cancer Research Fund, Edinburgh, United Kingdom) (10). MCF-7 was originally obtained from Dr. Marvin Rich of the Michigan Cancer Foundation. CHF-100 was provided by Children's Hospital of Pennsylvania. The ovarian cancer tissue specimens were obtained from a frozen bank of excess tumor tissue which had been sent for in vitro drug sensitivity testing in the San Antonio Human Tumor Cloning Laboratory. Tissue specimens were obtained from primary and metastatic sites.

RNase Protection Assay and Northern Blot Assay. RNA was extracted from cell lines and tissues using the technique of Chomczynski and Sacchi (11). The cRNA probes we used to detect IGF-1 mRNA were transcribed from a 514-base pair BamHI-ECORI IGF-1A, a 685-base pair EcoRI-PstI IGF-1B cDNA, and a 221-base pair HindIII-HindIII alternate exon 1 IGF cDNA (9) and are depicted in Fig. 1. Two fragments of the type I IGF receptor cDNA were used to detect the type I IGF receptor mRNA: a 164-base pair Stul-SmaI and a 293-base pair Aval-Aval fragment (12). RNase protection assays were performed as previously described (13).

To detect IGFBP mRNA expression, we examined total cellular RNA by Northern blot analysis. The probes we used were a 1.2-kilobase pair EcoRI-EcoRI rodent IGFBP-2 cDNA and a 535-base pair PstI-EcoRI human IGFBP-3 cDNA labeled by random priming. Gels and blots were performed as described previously (13).

Cell Culture and IGF-1 Mitogenesis. Cells were maintained in IMEM (Gibco/BRL, Bethesda, MD) with 10% fetal calf serum (Innovar, Gaithersburg, MD) in a humidified 5% CO2 atmosphere. The mitogenic effects of IGF-1 (Amgen, Thousand Oaks, CA) were determined in serum-free conditions. Cells (25,000) were plated in triplicate 12-well plates with RPMI 1640 plus 10% fetal calf serum (Day 0). Later (24 h, day 1), the cells were washed three times with phosphate-buffered saline and placed in serum-free media alone (IMEM plus 292 mg/liter glutamine, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, 2 mg/liter fibronectin, 2 mg/liter transferrin, and trace elements), serum-free IMEM plus 5 nM IGF-1, or IMEM plus 10% fetal calf serum. The media was exchanged on day 3. The cells were detached using trypsin and counted by hemocytometer on days 3 and 6. Three separate IGF-I mitogenesis experiments yielded similar results.

IGF-1 Radioimmunoassay. OVCAR-3 cells were plated in IMEM plus 10% fetal calf serum until subconfluent. They were then rinsed three times with phosphate-buffered saline and refed with serum-free...
media. After 24 h, this media was discarded, and the cells were placed in fresh serum-free IMEM. After 48 h, the medium was collected in the presence of protease inhibitors (aprotinin, phenylmethysulfonyl fluoride, pepstatin A, leupeptin, and EDTA) and then concentrated 100-fold in an ultrafiltration apparatus (Amicon Corp., Danvers, MA) using an M, 5000 cutoff filter (YM5). To remove IGF-binding proteins, 100 µl of concentrated conditioned media was mixed with 900 µl of 1 mM acetic acid and 5% bovine serum albumin and loaded on a SepPak C18 column (Waters, Milford, MA). The column was washed with 10 ml of 4% acetic acid, and IGF-I was eluted in 1 ml 50% acetonitrile and 4% acetic acid. The sample was lyophilized and resuspended directly in 100 µl of radioimmunoassay buffer. SepPak filtration has been reported to be an efficient method of separating IGF-binding proteins from IGF-I (14). IGF-I radioimmunoassay was performed using a rabbit anti-serum and a second antibody bound to magnetic beads. The assays were performed according to the instructions of the manufacturer (Amersham, Arlington Heights, IL). Charcoal IGF-binding protein assays were performed as previously described (15).

**Western Ligand Blot.** Protein concentrations of concentrated conditioned media were determined using the Bio-Rad (Richmond, CA) protein assay kit. Protein (15 µg) was separated by electrophoresis on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel along with molecular weight protein standards (Bio-Rad). The proteins were transferred to nitrocellulose and hybridized with radiolabeled IGF-I as described before (16). The blot was exposed to X-ray film for 24 h at -70°C.

**RESULTS**

**IGF-I mRNA Expression in Cell Lines and Tissues.** Alternate splicing of the IGF-I gene at its 3' end accounts for the generation of two well-characterized IGF-I transcripts found in human liver, IGF-IA and IGF-IB. Fig. 1 schematically demonstrates the structure of the IGF-I cDNAs. In this figure, the coding sequences are represented by boxes, and the untranslated regions are thin lines. The 3’ ends of IGF-I transcripts are derived from exon 5 of the IGF-I gene, and IGF-IB mRNA contains exon 4 sequences (17). We used probes derived from IGF-IA and IGF-IB cDNAs (Fig. 1) in RNase protection assays.

Of the 10 ovarian cancer cell lines we examined, 3 (OVCA-R-3, OVCAR-7, and PEO4) expressed IGF-I mRNA (Fig. 2A, and data not shown; OVCAR-2, OVCAR-4, OVCAR-5, OVCAR-8, OVCAR-10, Ca-OV-4, and SK-OV-3 were negative). Fig. 2A shows that a 514-base pair IGF-IA probe was fully protected by human liver; however, OVCAR-3 and PEO4 RNAs protected fragments that were approximately 100 base pairs smaller. This pattern of protection suggests that the ovarian cancer cell line transcripts are altered within 100 base pairs of either the 5’ or 3’ ends. In contrast, a neuroectodermal tumor cell line (CHP-100) expressed a full-length IGF-IA transcript, similar to that seen in human liver.

Since the 3’ end of the IGF-IA probe we used contains 101 base pairs that are not contained in IGF-IB transcript, it was possible that this alteration in protected fragment size could be due to a preponderance of IGF-IB transcripts produced by ovarian cancer cell lines. Therefore, we examined these RNAs with a 685-base pair IGF-IB probe by RNase protection assay (Fig. 2B). This probe differs from the IGF-IA probe in that it contains a larger portion of sequences derived from exon 1 (Fig. 1). OVCAR-3 and OVCAR-7 RNAs protected faint bands at 685 and 585 base pairs that corresponded to normal IGF-IB and IGF-IA transcripts, respectively. However, two other predominant fragments of 439 and 339 base pairs were also seen.

RNA extracted from a metastatic ovarian cancer tissue sample (OCM-1) protected a pattern of transcripts identical with those seen in OVCAR-3. In contrast, human liver protected a full 685-base pair IGF-IB fragment as well as the other fragments.

Since the predominant IGF-I RNA transcripts produced by ovarian cancer cells could not protect either a full-length IGF-IA or IGF-IB probe, this suggested that the 5’ sequences were altered. The 685-base pair IGF-IB probe contained 246 base pairs of exon 1 and it was possible that the 439 and 339 base pair-protected fragments resulted from transcripts with an alternate exon 1 while the splicing at the 3’ ends remained intact (exon 4- and exon 5-containing transcripts). Results of genomic exon 4 and exon 5 probes supported this model; OVCAR-3 RNAs fully protected both exon 4 and 5 probes (data not shown).

Taken together these results suggested that the ovarian cancer transcripts contained an altered 5’ sequence. We recently confirmed this suggestion by cloning a cDNA with an alternate 5’ end from a breast cancer xenograft tumor (9). This 226-base pair cDNA contains 165 base pairs derived from the alternate first exon with ovarian cancer cell lines. Therefore, we examined these RNAs due to a preponderance of IGF-IA transcripts produced by human liver.

We performed RNase protection assays using a probe derived from a cDNA containing the alternate first exon with ovarian cancer RNAs. Fig. 3 shows that RNA derived from cell lines, primary tumors (labeled OCP), and tumors metastatic within the peritoneal cavity (labeled OCM) protected full-length fragments of the alternate exon 1 IGF-I cDNA probe. The pattern of protection was complex. All RNAs protected a full-length 221-base pair fragment which corresponds to the full-length probe; however a 165-base pair fragment and a smaller series of protected fragments from 145 to 110 base pairs were identified; these smaller fragments probably represent diverse IGF-I mRNAs generated from within alternate exon 1 (9).

Since any tumor specimen contains normal and malignant tissues, it was possible that some of these transcripts could be derived from normal cells. Certainly, the primary ovarian cancer specimens were likely to contain normal ovary, and normal ovary is a source of IGF-I mRNA (18). However, since several of the metastatic implants contained IGF-I mRNA, it seems likely that the malignant epithelial cells in these samples were producing IGF-I mRNA. Furthermore, we have not...
normal tissues in which the alternate first exon transcripts predominate (9).

Fig. 1 summarizes the structure of the IGF-I transcripts detected in ovarian cancer. Two 5' portions were identified, one corresponding to exon 1 and the other derived from alternate exon 1. Exons 2 and 3 contain all of the coding sequences of the mature hormone, and these are intact in the ovarian cancer RNAs. Sequences derived from exons 4 and 5 are also expressed by ovarian cancer cells. Thus, the IGF-I mRNA transcripts produced by these cells are complex.

IGF-I and IGF-binding Protein Expression. Although the ovarian cancer cells appear to predominantly express an alternate IGF-I mRNA transcript, this transcript contains all of the coding exons of mature, circulating IGF-I. We next examined OVCAR-3-conditioned media for the presence of IGF-I and IGF-binding proteins. Unextracted conditioned media contain a significant amount of binding protein when examined by charcoal-binding assay, and extraction with SepPak C₁₈ columns can remove >97% of this activity (data not shown). After SepPak removal of the IGFBPs, the extracted conditioned media displaced authentic IGF-I in radioimmunoassay (Fig. 4), demonstrating that the alternate IGF-I transcripts produced by ovarian cancer cell lines were translated. However, since OVCAR-3 also contained IGF-IA and IGF-IB transcripts, it is possible that the protein we detected was derived from those mRNAs. IGF-II, insulin, and concentrated serum-free media

Fig. 2. IGF-I RNase protection assays of ovarian cancer cell lines. A, a 514-base pair IGF-IA probe was used in RNase protection assay. Normal human liver and the neuroepithelioma cell line CHP-100 mRNAs protected full-length fragments. OVCAR-3 and PEO4 predominantly protected species were 100 base pairs smaller. Exposure time was 48 h. B, IGF-IB RNase protection assay of ovarian cancers. A 685-base pair IGF-IB cDNA was used. OCM-1 is an RNA sample isolated from a tissue specimen of metastatic ovarian cancer. Liver protected a full-length fragment corresponding to IGF-IB mRNAs. A faint band at 685 base pairs was seen during prolonged exposure in all the ovarian cancer cells. The 685-base pair fragment seen in liver, OVCAR-3, and OCM-1 represent IGF-IA transcripts. Smaller bands at 439 and 339 base pairs were the predominant species seen in ovarian cancer cells. The 585-base pair fragment seen in liver is the product of a prematurely terminated probe species. A 24-h exposure is shown.

Ordinates, base pairs.

Fig. 3. Alternate exon 1 IGF-I RNase protection assay. Ovarian cancers metastatic within the peritoneum (OCM), ovarian cancer primary tumors (OCP), and ovarian cancer cell lines (OVCAR-3 and -7) were studied using the 221-base pair alternate exon 1 IGF-I probe. Multiple protected fragments are seen. Prolonged exposure demonstrated a 221-base pair full-length fragment in all RNAs, while smaller fragments from 165 to 100 base pairs are also demonstrated. These smaller fragments probably represent multiple IGF-I mRNAs generated within the alternate exon 1. Exposure is for 24 h. Ordinate, base pairs.
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Fig. 4. IGF-I radioimmunoassay of OVCAR-3-conditioned media. Binding proteins were removed from conditioned media by SepPak C18 chromatography, and the increasing volumes of the eluate (OVCAR3 CM) were examined for immunoreactive IGF-I. Increasing amounts of conditioned media displaced authentic IGF-I in radioimmunoassay. Insulin, IGF-II, and 100-fold concentrated serum-free media (IMEM) did not appreciably displace IGF-I.

Since the conditioned media also contained a significant amount of IGF-binding activity, we next examined ovarian cancer cells for the production of these binding proteins using Northern blots and Western ligand blots. Northern blot analysis showed that IGFBP-2 mRNA was found in the cell lines OVCAR-3 and CaOV-4 (Fig. 5, bottom), while SK-OV-3 did not produce this binding protein mRNA. However, all three cell lines produced IGFBP-3 mRNA (Fig. 5, top). None of the ovarian cancer cell lines we examined produced IGFBP-1 (data not shown).

To confirm that these IGFBPs could be found in the conditioned media, we examined OVCAR-3-conditioned media by Western ligand blotting (Fig. 6). Several IGFBP species could be found in OVCAR-3-conditioned media: an Mr 38,000–41,000 doublet, an Mr 34,000, an Mr 32,000, and an Mr 24,000 species. The Mr 38,000–41,000 doublet is compatible with the sizes reported for IGFBP-3, and since OVCAR-3 expressed this mRNA, it seems likely that this is indeed IGFBP-3 (19). The 34-kDa band is compatible with the molecular mass reported for IGFBP-2 (20). This band comigrated with the predominant binding protein species produced by the neuroectodermal tumor cell line CHP-100. We have previously shown that this IGFBP produced by CHP-100 is IGFBP-2 (15). Since OVCAR-3 also produced IGFBP-2 mRNA, it seems likely that this Mr 34,000 band is IGFBP-2. Additionally, OVCAR-3 produced an Mr 24,000 binding protein which is also found in the breast cancer cell lines T47-D and MCF-7; its size is compatible with that reported for IGFBP-4 (21, 22). An additional binding protein species migrating at approximately Mr 30,000–32,000 is also seen in OVCAR-3 and in the breast cancer cell lines; this binding protein species has not yet been fully characterized. These data show that, in addition to producing IGF-I, OVCAR-3 cells produced IGFBP-2, IGFBP-3, and at least one other IGFBP species.

Type I IGF Receptor mRNA Expression. Type I IGF receptor mRNA expression was examined in RNase protection analysis using two different probes obtained from the type I IGF receptor gene (Fig. 7). All of the ovarian cancer cell lines and tissues expressed type I IGF receptor mRNA. This finding is common to most cancer cell lines we have examined (23); type I IGF receptor mRNA is ubiquitously expressed.

IGF-I-induced Mitogenesis in OVCAR-3. Since all the components of an autocrine system are present in the cell line OVCAR-3, we examined the mitogenic effects of exogenous IGF-I. In short-term culture experiments, 5 nM IGF-I in serum-free media caused similar growth stimulation to media containing 10% fetal calf serum (Fig. 8). This experiment suggests that the OVCAR-3 cells contain functional type I IGF receptors, and under the conditions we used, the endogenously produced binding proteins did not inhibit IGF action. Therefore, endogenously produced IGF-I could potentially function as an autocrine growth factor for these tumors.

DISCUSSION

It is apparent that complex interactions between the IGFs, their binding proteins, and their receptors are important in determining IGF action in normal growth and development (24, 25). In cancer cells, similar complex interactions are likely to exist. Although other investigators have suggested that IGF-I may be an autocrine growth factor for several types of cancer, our previous work has suggested that IGF-I mRNA expression by malignant epithelial cells is not a common event (8). This discrepancy may be due to the different techniques used; we examined cancer cells with the sensitive and specific RNase protection assay, while other investigators did not characterize the mRNA in the cells producing immunoreactive IGF-I. We

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found that many cancer cells produce IGF-binding proteins, and these proteins can give false-positive results in IGF-I radioimmunoassay (14). In addition to interfering with the detection of IGF-I, these binding proteins probably play a critical role in modulating the interaction between IGF-I and the type I IGF receptor (26-28).

In this study, we characterized the expression of IGF-I, binding proteins, and type I IGF receptor mRNA in ovarian cancer cell lines and tissues. We found that some ovarian cancer cell lines express IGF-I mRNA with an alternately spliced transcript. In the cell line, OVCAR-3, immunoreactive IGF-I can be found in the conditioned media, suggesting that this alternate transcript may be efficiently translated. However, since OVCAR-3 also expresses smaller amounts of IGF-IA and IGF-IB mRNA, it is possible that the peptide we detected is translated from these IGF-I transcripts. IGF-I expression by normal human ovaries is well documented (18, 29, 30). In the normal ovary, it appears that granulosa cells are the source of IGF-I. The ovarian cancer specimens we examined are derived from the surface epithelium of the ovary; we did not examine granulosa cell tumors. It seems unlikely that the IGF-I mRNA we found originated from normal ovarian tissue since most of our tumor specimens were taken from metastatic implants. Since samples taken from ovarian cancer tissues and tumor implants also contain elements from normal stromal cells, in situ hybridization will be necessary to absolutely prove that the ovarian cancer cells are the in vivo source of the IGF-I message. However, we found that the alternate transcript is not found in abundance in most normal tissues, suggesting that the IGF-I mRNA we measured originated from the ovarian cancer cells.

We also found that ovarian cancer cells, like many other cell lines (31-33), express IGF-I-binding proteins. In OVCAR-3, an M, 24,000 binding protein (possibly IGFBP-4), IGFBP-2, and IGFBP-3 are expressed. The physiological function for these proteins is not known. It has been suggested that IGFBP-3, can either augment or inhibit IGF-I action, depending on the experimental conditions used (28). However, this observation does not address the role for these proteins in an IGF-I-producing cell, nor does it address the role of multiple species of IGF-binding proteins. OVCAR-3 represents a good model system for the study of these relationships.

Although we have shown that all the components necessary for an autocrine loop are present in ovarian cancer cells, we have not proven that such a loop is operative. We, and others, have used a strategy of antibody (aIR3) receptor blockade in order to demonstrate a functional autocrine loop (15, 34, 35). Although this antibody can block IGF-I-binding to the type I IGF receptor, Steele-Perkins et al. (36) showed that this is accompanied by receptor phosphorylation, suggesting that aIR3 is not physiologically inert. Therefore, it is difficult to
interpret the results of these blockade experiments. It is also conceivable that the coexpression of IGF-I and IGF-binding proteins by a cell may result in high-affinity binding, making it more difficult for the antibody to block the ligand/receptor interaction. Finally, IGF-I ligand/receptor interactions could occur internally, which would functionally bypass blockade of the external receptors. We are currently investigating the potential autocrine loop in ovarian cancer cells by examining more direct ways to inhibit gene expression of IGF-I and its receptor.

REFERENCES


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