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Regulation of Insulin-like Growth Factor Binding Proteins in Ovarian Cancer Cells by Oestrogen

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Insulin-like growth factor-I (IGF-I), its receptor and its binding proteins are expressed by ovarian cancer cells. In this study, we examined oestradiol (E_2) regulation of IGF-I and IGF binding proteins (IGFBP) in an oestrogen-responsive ovarian cancer cell line, PE04. In serum-free conditions, PE04 cell monolayer growth was increased 1.64-fold by 3 nmol/l E_2 compared with controls, although IGF-I mRNA levels were not increased. In contrast to IGF-I mRNA, IGFBP mRNA was regulated by E_2. E_2 caused a marked decrease in IGFBP-3 RNA, but IGFBP-2, -4 and -6 levels were only minimally depressed. IGFBP-5 mRNA levels were increased by E_2. Tamoxifen had less effect on IGFBP mRNA regulation. Ligand blotting showed that E_2 reduced IGFBP levels in conditioned media. IGFBP RNA was also detected in human ovarian tissue samples. Thus, IGFBP expression can be regulated in oestrogen-responsive ovarian cancer by E_2.
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INTRODUCTION

IT HAS LONG been known that gonadotropins play a central role in the regulation of ovarian follicle growth and differentiation. Locally produced intra-ovarian peptide hormones may also modulate normal follicular development. For example, it has been suggested that insulin-like growth factor-I (IGF-I) has autocrine and paracrine actions on the ovary. IGF-I production by granulosa cells has been clearly demonstrated and it has been found that IGF-I may act synergistically with the gonadotropins in the normal ovary [1].

In addition to the regulation of normal ovarian function, IGF-I may play a role in the growth regulation of ovarian cancer. We have previously shown that ovarian cancer cells, which are derived from the surface epithelium of the ovary, also express IGF-I and its receptor [2]. However, the potential interactions in the IGF system are complex. In addition to the two well characterised specific cell surface receptors [3, 4], the IGFs are associated with high affinity binding proteins (BP) in all extracellular fluids. To date, six IGFBPs have been cloned from human tissues designated IGFBP-1 to -6 [5]. All the binding proteins are derived from separate genes, vary in size, yet have highly conserved cysteine residues suggesting some shared functions. The physiological function(s) of the IGFBP are not well characterised; evidence supports their ability to both inhibit and enhance IGF-I receptor/ligand interactions [6].

If ovarian cancer growth is regulated by IGF-I, then modulation of either IGFBP or IGF-I expression may affect cell growth. We used an oestrogen receptor (ER)-positive ovarian cancer cell line, PE04, as a model system to study the relationship between oestrogen-stimulated growth and expression of the components of the IGF system. We found that oestrogen did not regulate IGF-I mRNA, however, levels of IGFBP mRNA and protein were affected. Our data suggest that IGFBP regulation could play a role in the growth regulation of ovarian cancer.

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MATERIALS AND METHODS

Materials

The IGF-IA cDNA was provided by Ken Gabbay (Baylor College of Medicine, Houston, Texas, U.S.A.) and the IGF-IB cDNA was provided by Peter Rotwein (Washington University School of Medicine, St Louis, Missouri, U.S.A.). IGFBP-1 and -3 cDNA probes were supplied by David Powell (Baylor College of Medicine). The other IGFBP cDNA were cloned in one of our laboratories. The 36B4 probe was provided by Professor Chambon (INSERM, Strasbourg, France). All probes used in this study were derived from human sources. The ER ovarian cancer cell line, PE04, was provided by John F. Smyth (Imperial Cancer Research Fund, Edinburgh, U.K.) [7]. The ovarian cancer tissue specimens and malignant pleural effusions were obtained from a frozen bank of excess ovarian tumours which had been sent for *in vitro* drug sensitivity testing in the San Antonio Human Tumor Cloning Laboratory. Tissue specimens were obtained from metastatic sites. All chemicals were purchased from Sigma (St Louis, Missouri, U.S.A.) unless otherwise noted.

Cell culture and growth curve

PE04 cells were maintained in RPMI 1640 (Gibco/BRL, Bethesda, Maryland, U.S.A.) with 10% fetal calf serum (Innover, Gaithersburg, Maryland, U.S.A.), and insulin (Lilly, Indianapolis, Indiana, U.S.A.) in humidified 5% CO_2 atmosphere at 37°C. For monolayer cell growth experiments, PE04 cells were grown in RPMI 1640 media as described above and harvested with trypsin, plated at a density of 50 000 cells/well in 24-well dishes (Nunc), and incubated at 37°C in humidified 5% CO_2 atmosphere with RPMI 1640 media. After 48 h, the adherent cells were washed with phosphate-buffered saline and medium was changed to phenol red-free improved minimal essential medium (IMEM, Gibco/BRL) with 5% fetal calf serum stripped of oestrogen by sulphatase and dextran-coated charcoal

treatment (DCFCS) with or without 3×10^{-9} mol/l 17β-oestradiol (E_2). Cells were harvested from triplicate wells and counted by haemocytometer at days 0, 3 and 6. Experimental medium was changed at 3-day intervals. Three separate experiments were performed with similar results; a representative experiment is shown.

Similar conditions were used for harvesting RNA and serum-free conditioned media. For RNA extraction, PE04 cells were grown to subconfluence in T-150 flasks then washed three times with phosphate buffered saline. The medium was changed to IMEM plus 5% DCFCS for 48 h. After this time period, medium was again exchanged to IMEM plus DCFCS with or without 3×10^{-9} mol/l E_2 or 1×10^{-6} mol/l tamoxifen (TAM).

Serum-free media were collected in a similar manner. After 48 h of oestrogen depletion, medium was changed to phenol red-free IMEM plus 2 mg/l fibronectin, 2 mg/l transferrin, 20 m mol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, 292 mg/l glutamine, and trace elements (Biofluids). After 24 h this serum-free medium was discarded, and was replaced with media containing E_2 or tamoxifen. Media were harvested after 48 h of treatment. Cells in each flask were counted by haemocytometer to measure differences in cell growth over the experimental time period.

Ribonuclease (RNase) protection assay

RNA was extracted from the PE04 cell line and ovarian cancer tissue specimens using the technique of Chomczynski and Sacchi [8]. The cRNA probes used to detect IGF-I mRNA were transcribed from a 519 bp *Bam*HI-*Eco*RI IGF-IA and a 685 bp *Eco*RI-*Pst*I IGF-IB cDNA [2]. The cRNA probes used to detect IGFBP mRNA were transcribed from different portions of each cDNA; probes for IGFBP-1 and IGFBP-3 have previously been described [9]. Other cDNA used for templates were: a 382 bp *Sty*I-*Eco*RV IGFBP-2 fragment [10], a 505 bp *Eco*RI-*Hind*III IGFBP-4 fragment [11], a 317 bp *Sac*II-*Sac*I IGFBP-5 fragment [12], and a 267 bp *Pst*I-*Pst*I IGFBP-6 [13]. 36B4, a non-oestrogen-regulated cDNA, was hybridised simultaneously with each probe [14]. RNase protection assays were performed as described previously [2]. pBR322 fragments digested with *Msp*I were end-labelled and used as size markers. The autoradiograms were exposed to X-ray film for 12–96 h at −70°C. The levels of IGFBP mRNA were also quantified by directly scanning of the gel with the Ambis radioanalytic system (San Diego, California, U.S.A.). For regulation experiments, three separate RNA preparations were prepared and similar results were obtained. A representative experiment is shown.

Western ligand blot

Proteins obtained from PE04 serum-free medium after 48 h of hormonal treatment were concentrated 20-fold using Centricon 3 (Amicon, Danvers, Massachusetts, U.S.A.). Aliquots of PE04 were separated by electrophoresis on a 12% SDS–PAGE gel along with molecular weight protein standards (Amersham, Illinois, U.S.A.). The amount of PE04 conditioned media loaded on the gel was corrected for cell count at the time of harvest. Three separate harvests were performed with similar results. A representative experiment is shown. The proteins were transferred to nitrocellulose and hybridised with radiolabelled IGF-I (Amersham) as described [15]. The blot was exposed to X-ray film for 12–72 h at −70°C. The blot was also directly scanned using the Ambis radioanalytic scanner.

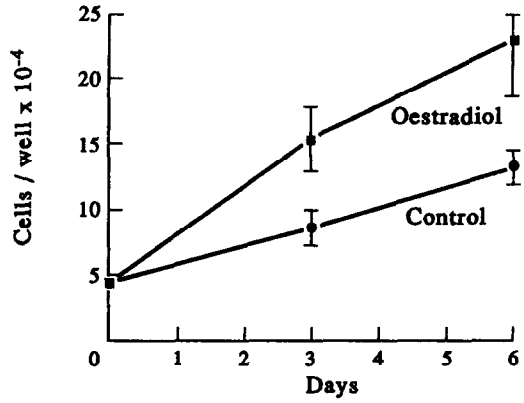

Fig. 1. Estradiol (E_2) effects on monolayer cell growth of ovarian cancer cell line PE04. 50 000 cells/well were plated in 24-well dishes and grown in oestrogen-free conditions for 48 h prior to exposure to 3×10^{-9} mol/l E_2. Points represent the mean total cell count from triplicate wells. A representative experiment is shown.

RESULTS

PE04 growth curve and IGF-I expression

Monolayer growth of PE04 cells grown was compared in the presence and absence of E_2 (Fig. 1). On day 6 of culture, the growth rate of the cell line treated with E_2 was 1.64 times that of untreated cells. These results were similar to those previously obtained by other investigators [7]. To determine whether increased expression of IGF-I could account for E_2-induced growth, IGF-I mRNA was evaluated by RNase protection from PE04 cells treated with or without E_2. As reported previously, the most abundant IGF-I transcripts produced by PE04 cells are produced by alternate splicing of the IGF-I gene [2]. The levels of these transcripts were not increased after 24 or 48 h (Fig. 2 and data not shown) of E_2 exposure. Thus, increased cell growth after E_2 treatment was not accompanied by induction of IGF-I.

Detection of PE04 IGFBP mRNA

Since E_2 did not alter expression of IGF-I mRNA, we next determined whether IGFBP RNA levels were regulated by E_2 or

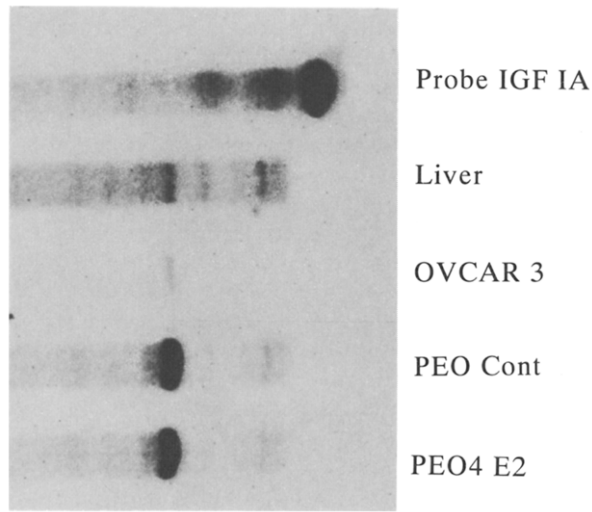

Fig. 2. IGF-IA ribonuclease protection assay of liver, ovarian cancer cell line OVCAR-3, and ovarian cancer cell line PE04 in the presence or absence of 1×10^{-9} mol/l E_2. Liver and PE04 cells express a full-length IGF-IA cDNA transcript seen as the largest band. The ovarian cancer cell lines also express an alternately spliced transcript detected as a smaller band. This band is a result of alternate splicing of the 5′ end of the IGF-I gene [2]. A 48-h exposure is shown.

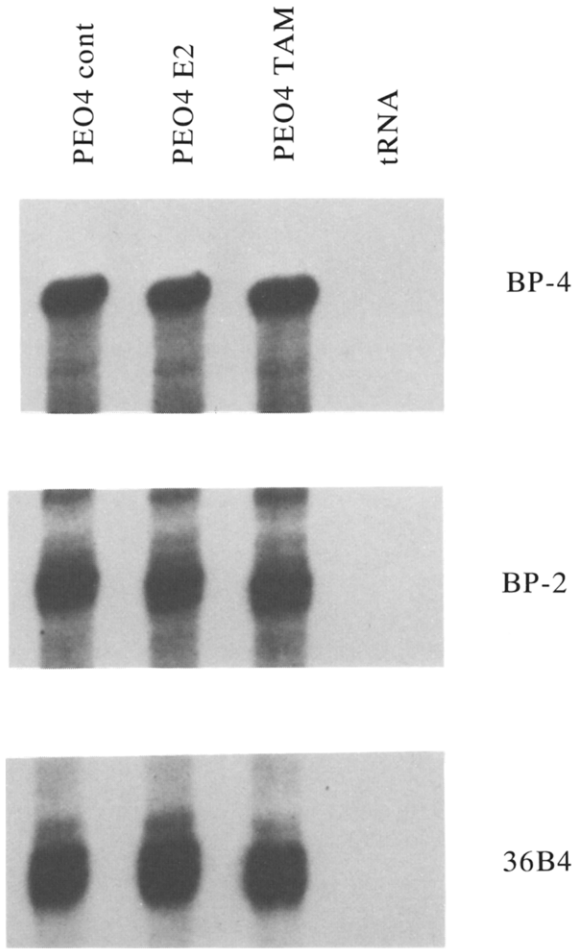

Fig. 3. IGFBP-2 and -4 ribonuclease protection assay of PE04 under three conditions: control (PE04 cont), 1×10^{-9} mol/l oestradiol (PE04 E_2) and 1×10^{-6} mol/l tamoxifen (PE04 TAM). Each RNA sample was hybridised simultaneously with each IGFBP probe and 36B4 to control for equal loading. A 96-h exposure is shown.

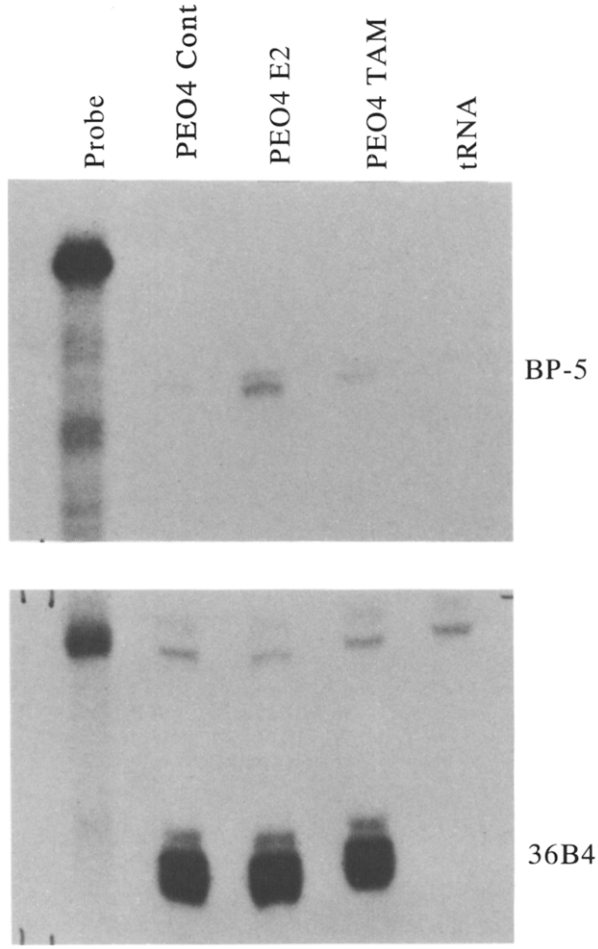

Fig. 5. IGFBP-5 ribonuclease protection assay of PE04 under three conditions: control (PE04 cont), 1×10^{-9} mol/l oestradiol (PE04 E_2) and 1×10^{-6} mol/l tamoxifen (PE04 TAM). The samples were simultaneously probed for 36B4. A 96-h exposure is shown.

TAM. PE04 cells did not express IGFBP-1 mRNA under any conditions, although IGFBP-2 to -6 could be detected (Figs 3–5 and data not shown). IGFBP-2 and -4 levels were not significantly altered from control levels by exposure to E_2 or TAM (Fig. 3). 36B4 is a cDNA that is not regulated by E_2 in hormone-responsive breast cancer cells [14] and was used as control throughout these experiments.

In contrast, BP-3 mRNA was significantly decreased by E_2. This decrease was evident after 24 and 48 h of exposure to E_2 (Fig. 4). Radioanalytic scanning of the gel demonstrated a 70% decrease in mRNA levels. ER-negative ovarian cancer cells

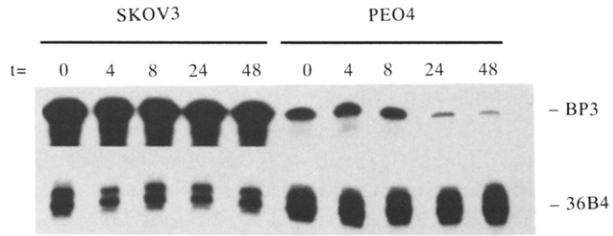

Fig. 4. IGFBP-3 mRNA ribonuclease protection assay. SKOV3 (ER-negative) and PE04 cells were treated with 1×10^{-9} mol/l E_2 over 48 h. Cells were harvested for RNA at the indicated time points. Oestrogen did not regulate SKOV3 IGFBP-3 levels, while PE04 levels were decreased. A 24-h exposure is shown.

(SKOV3) were not affected by oestrogen. In PE04, TAM had little effect on IGFBP-3 expression (data not shown). Although IGFBP-6 mRNA was easily detectable in PE04, neither E_2 nor TAM significantly regulated its expression (data not shown). In contrast, low levels of BP-5 were expressed under control conditions and E_2 exposure increased the level of RNA more than 2-fold (Fig. 5).

Detection of IGFBP expression in PE04 conditioned media

To determine if secreted IGFBPs were under similar hormonal controls, PE04 cells were grown in serum-free medium under control, E_2 and TAM conditions and the media were collected after 48 h. Western ligand blot performed demonstrated five distinct bands (Fig. 6). The bands were consistent with the reported values for an IGFBP-3 doublet (38.5 and 41.5 kD), IGFBP-2 (34–36 kD), and IGFBP-4 (~ 25 kD) [16]. We have confirmed that the species marked as IGFBP-3 and IGFBP-2 react with antibodies specific to these binding protein species in immunoblot studies [17]. The identity of the 29 kDa band is uncertain. It migrates at the reported position for IGFBP-5 [18], however, it may represent a deglycosylated IGFBP-4 species. It is possible that 29 kD band represents a proteolytic fragment of IGFBP-3, however, Ocrant *et al.* have suggested that these fragments are not detected by ligand blotting [19]. IGFBP-6 was not identified unambiguously. This binding protein is reported to have a molecular weight of 33 kD and has a higher affinity for

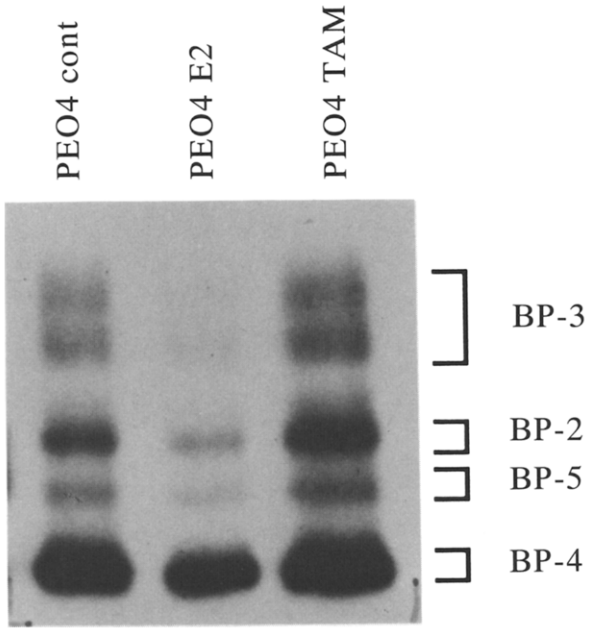

Fig. 6. IGFBP western ligand blot. PE04 conditioned media was examined after 48 h of treatment. Three conditions were examined: control (PE04 cont), 1×10^{-9} mol/l oestradiol (PE04 E_2) and 1×10^{-6} mol/l tamoxifen (PE04 TAM). The IGFBP migration position according to the literature is marked. Exposure is for 72 h.

IGF-II [20]. Thus, it may comigrate with IGFBP-2 and be less detectable with IGF-I.

The amount of conditioned media examined was adjusted for the number of cells after each treatment. E_2 treatment reduced the levels of IGFBP-2 to -5 detected in the conditioned media. TAM treatment resulted in a slight increase in the level of detectable IGFBP (Fig. 6). Radioanalytic scanning of the ligand blot demonstrated that E_2 treatment reduced IGFBP levels to between 39 and 61% of control while TAM increased these levels (Fig. 7).

Detection of IGFBP RNA in ovarian cancer tissues

We studied several ovarian cancer tumour (OCT) specimens for IGFBP expression. In these tumours, IGFBP-2 to -5 mRNA were expressed (Figs 8, 9). IGFBP-1 and -6 mRNA were not detected in any of the metastatic tissues obtained (data not shown). Thus, RNA expression of IGFBP is found not only in cell lines, but these RNA were also detected in material taken directly from patients. These differences in levels of expression may reflect different biological growth properties of the tumour.

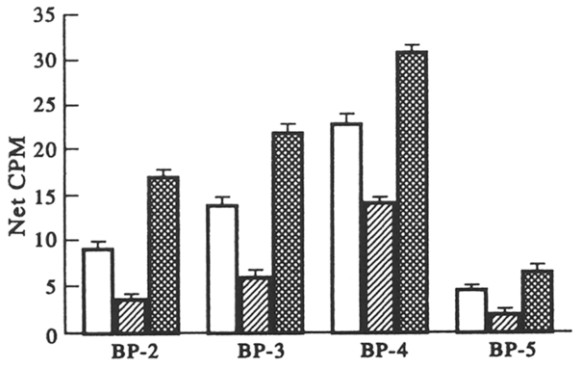

Fig. 7. IGFBP western ligand blot Ambis quantitative analysis of PE04 cells. Control treatment is represented by open boxes, oestradiol hatched, and TAM by cross-hatched boxes. Results are expressed as net counts per min.

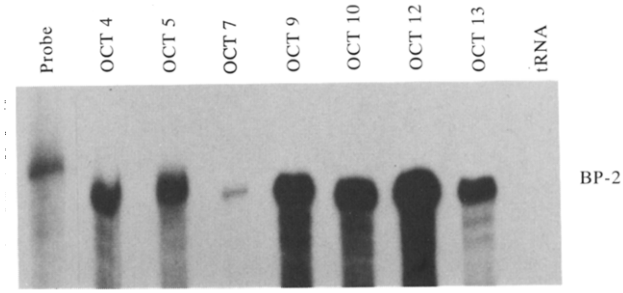

Fig. 8. IGFBP-2 ribonuclease protection assay of metastatic ovarian cancer tumours (OCT).

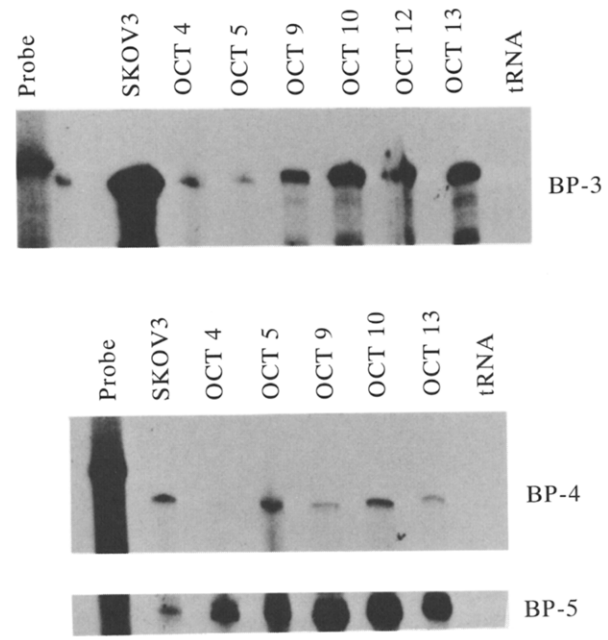

Fig. 9. IGFBP-3, -4 and -5 ribonuclease protection assay of metastatic ovarian cancer tumours (OCT). A 24-h exposure is shown.

DISCUSSION

Ovarian cancer is the most common cause of death among females with a gynaecological malignancy [21]. Epidemiological studies have implicated steroid hormones (E_2, progesterone) in the pathogenesis and growth regulation of ovarian epithelial cancers [22]. Experimental ovarian neoplasms have been induced by E_2 and a suggested association between exogenous E_2 and ovarian carcinoma has been made [23]. Since regulation of peptide growth factors by E_2 has been documented in breast cancer cells [24], we performed this study to see if similar regulation of IGF-I in ovarian cancer cells existed.

We found that IGF-I mRNA levels were not regulated by E_2, however, IGFBP mRNA and protein levels were generally decreased. Although E_2 significantly decreased levels of IGFBP in the conditioned media, the effect on RNA levels was more modest. Decreased transcription of IGFBP induced by E_2 may, in part, be responsible for the decreased IGFBP levels in the conditioned media. This seems most evident in the case of IGFBP-3 where both mRNA and protein levels were decreased by E_2 to a similar degree. Since we used a functional assay, ligand blotting, to measure IGFBP protein levels it is possible

that E_2-induced post-translational modifications could account for the decreased levels of IGFBP in the conditioned media. For example, in breast cancer cells, E_2 induces the induction of the protease cathepsin D [14] as well as protease activators such as plasminogen activator [25]. Proteases, such as plasmin, may inactivate IGFBP [26], and induction of proteases or protease activators may play an important additional role in the decreasing functional IGFBP levels.

We found increased expression of IGFBP-5 RNA after E_2 exposure, but did not document increased secretion of this protein. Levels of IGFBP-5 were low in PE04 cells and the increase in mRNA levels may not have been great enough to detect increased protein levels secreted in the conditioned media. For example, we have shown previously that TAM increased IGFBP-3 mRNA levels in breast cancer cells, yet no increased protein was detected by ligand blotting of conditioned media [9].

The precise function of each IGFBP is not known; they may serve to provide a storage pool and prolong the half-lives of the circulating IGF, inhibit the actions of the IGF by complexing with IGFBP, or to potentiate the action of the IGF [16]. Since IGFBP have been demonstrated in normal ovarian tissues [27] they are likely to play important roles in regulating, and perhaps determining, IGF action. Additionally, IGFBP from various tissues have been shown to be regulated by a variety of substances such as growth hormone, IGF-I, insulin, vasopressin, platelet-derived growth factor, epidermal growth factor, transforming growth factor-β, bombesin and steroids [28–30]. Taken together, these facts suggest that regulation of IGF BP expression by other peptide and steroid hormones may play an important role in regulating the response to IGF-I. For example, the decreased levels of IGFBP detected in the conditioned media after E_2 exposure could make the cells more responsive to IGF-I. Moreover, IGFBP mRNA were also detected in human metastatic ovarian cancer tissues. Thus, IGFBP may also play an important role in regulating ovarian cancer cell growth *in vivo*.

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