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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.

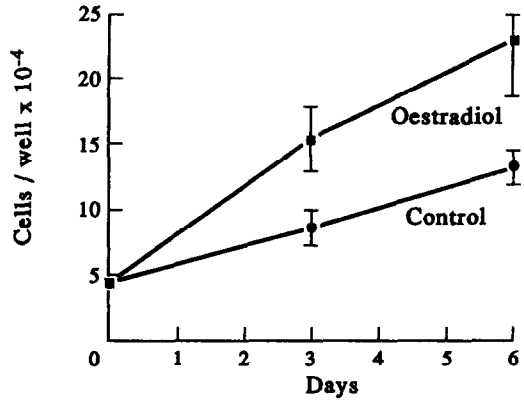

Fig. 1. Estradiol (E_2) effects on monolayer cell growth of ovarian cancer cell line PE04. 50 000 cells/well were plated in 24-well dishes and grown in oestrogen-free conditions for 48 h prior to exposure to 3×10^{-9} mol/l E_2. Points represent the mean total cell count from triplicate wells. A representative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

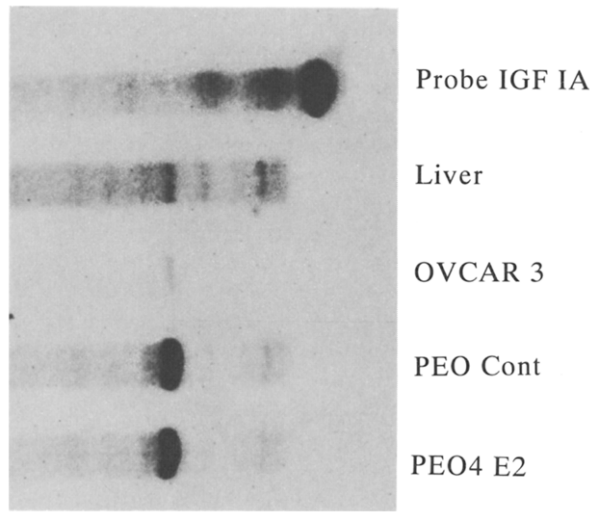

Fig. 2. IGF-IA ribonuclease protection assay of liver, ovarian cancer cell line OVCAR-3, and ovarian cancer cell line PE04 in the presence or absence of 1×10^{-9} mol/l E_2. Liver and PE04 cells express a full-length IGF-IA cDNA transcript seen as the largest band. The ovarian cancer cell lines also 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.