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The Regulation of Growth and Protein Expression by Estrogen *In Vitro*: A Study of 8 Human Ovarian Carcinoma Cell Lines

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The effects of 17 β-estradiol (E_2) on the growth and the levels of estrogen receptor (ER), progesterone receptor (PR) and pS2 protein were examined in a range of 8 ovarian carcinoma cell lines. E_2 stimulated growth of the 3 cell lines with an ER content of 80–220 fmol/mg protein but not the 5 cell lines with ER concentrations less than 20 fmol/mg protein. After exposure to E_2, ER concentration in 2 of the 3 responsive cell lines was decreased relative to untreated cells and in 2 lines, progesterone receptors were increased. No change in steroid receptor levels was observed in cell lines with low or negligible levels of receptors. The pS2 protein was not induced by E_2 in the 5 ovarian carcinoma cell lines examined. These results indicate that E_2 can stimulate the growth of some ER-positive ovarian carcinoma cells and that these effects may be associated with changes in the cellular levels of steroid hormone receptors.

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INTRODUCTION

The majority of human ovarian adenocarcinomas are estrogen-receptor (ER)- positive (reviewed in Slotman and Rao, 1988 [1]) and experimental evidence is accumulating to indicate that, at least a proportion of, these tumours are estrogen-responsive [2–5]. This is consistent with the clinical observation that a subset of ovarian cancer patients are responsive to the anti-estrogen tamoxifen [6–8]. To define those characteristics that determine sensitivity to estrogen (and therefore anti-estrogens) in this disease, we have developed a series of ovarian cancer cell line models [2, 9]. Using these models, we have previously demonstrated that growth of the ER-positive ovarian carcinoma line PE04 line is modulated by 17 β-estradiol (E_2) *in vitro* and *in vivo*, while growth of the ER-negative PE014 line is not [2, 3]. In addition to growth responses, we are also attempting to define proteins whose expression is under estrogen control. The identification of estrogen-regulated proteins should lead to an improved understanding of the mechanisms of estrogen (and anti-estrogens) in this disease and provide possible indicators of hormonal sensitivity in clinical specimens. In breast cancer, estrogen-regulated markers, which have been shown to have utility, include the progesterone receptor (PR) [10, 11], the pS2 (or pNR-2) protein [12, 13] and procathepsin D [14, 15]. This last protein has already been demonstrated as being increased in ER-positive ovarian cancer cells after exposure to E_2 [5, 16].

In the present study, we have investigated the ability of E_2 to influence the growth of a series of ovarian carcinoma cell lines with moderate–high levels of ER (PE04, PE01, PE06) and with low or negligible levels of ER (PEA1, PEA2, PE014, PE023, PE016) [2]. The levels of ER, PR and pS2 have been measured after culture in the absence and presence of E_2 treatment in these lines to determine whether these proteins are modulated and might function as markers of estrogen sensitivity. Comparisons are made with the ER-positive ZR-75-1 and ER-negative MDA-MB-231 breast carcinoma cell lines.

EXPERIMENTAL

Cell lines

The human ovarian carcinoma cell lines (PE01, PE04, PE06, PEA1, PEA2, PE014, PE023 and PE016) were established and characterized as described

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previously [9]. Cells were routinely maintained at 37°C in a humidified atmosphere of 5% CO_2 in air in RPMI 1640 (Gibco) containing 10% heat-inactivated foetal calf serum (FCS) and supplemented with streptomycin (100 μg/ml), penicillin (100 IU/ml) and glutamine (2 mM). The breast carcinoma cell lines (ZR-75-1 and MDA-MB-231) were maintained in DMEM (Gibco) containing FCS and the same additives as for the ovarian carcinoma lines.

Growth studies

Exponentially growing cells were harvested by trypsinisation and plated in 24-well plates (Falcon) at densities of 2.5–5 × 10^4 cells/well (4 wells/experimental condition) in RPMI 1640 containing phenol red and 10% FCS. After 24 h, to allow for attachment, the medium was removed and phenol red-free RPMI 1640 containing 5% charcoal-stripped FCS [17] was added. The cells were incubated for a further 24 h and the media were removed. RPMI 1640 containing 5% charcoal-stripped FCS, with or without E_2, at concentrations ranging from 10^{-12} to 10^{-5} M was added. Medium, with or without E_2, was replenished 3 days later. Cells were trypsinized from wells after a total of 6 days exposure and counted using a Coulter Counter.

Determination of ER and PR by enzyme-immunoassay

Mid-log phase cells growing in 175 cm^2 flasks were treated with or without 10^{-10} M E_2 for 6 days as described in the growth experiments. After 6 days treatment, medium was removed and cells were harvested by scraping. The contents from 4 identical flasks were pooled to give each pellet and these were stored at −80°C until use. Cell pellets (50–200 mg) were weighed and homogenized in buffer (10 mM Tris, 0.25 M sucrose, 1 mM ethylene diammine tetraacetate, pH 8.0, plus 1% v/v monothioglycerol and 10% v/v glycerol) as previously described [18, 19]. After centrifugation at 105,000 *g*, the supernatant cytosol was assayed using the ER-EIA or PR-EIA kits provided by Abbott Laboratories (Maidenhead, Berkshire, U.K.), according to the manufacturer's instructions [20]. The protein content of the cytosol was determined by the method of Bradford [21] and receptor concentrations were expressed as fmol/mg protein. We arbitrarily consider tissue/cells containing less than 5 fmol/mg protein as "receptor-negative".

Determination of pS2 by flow cytometry

Cells were plated at a density of 5–10 × 10^4/ml (2 ml/well) into 6-well plates (Falcon) and subjected to 3 or 6 days exposure to medium with or without 10^{-10} M E_2. pS2 was then detected by flow cytometry as follows. Cells were trypsinized, fixed in 70% ethanol at 4°C for 30 min and washed twice in PBS containing 5% FCS and 0.5% Tween 20 (wash buffer). Anti-pS2 antibody (100 μl; Histo-CIS, Gif-sur-Yvette Cedex, France) was diluted 1:2 in the above buffer on ice and added to the cells for 30 min. Cells were then washed in buffer. Sheep anti-mouse FITC conjugate (Sigma, U.K.) diluted 1:20 in buffer was added for 30 min to cells on ice. Cells were then washed once in buffer, then twice in PBS, resuspended in PBS and analysed on a FACScan flow cytometer (Becton Dickinson).

RESULTS

Effect of E_2 on growth

The 8 ovarian carcinoma cell lines were treated with E_2 at concentrations ranging from 10^{-12} to 10^{-5} M for 6 days. The 3 lines with moderate–high levels of ER (PE04, PE01 and PE06) were all growth stimulated by E_2 at concentrations between 10^{-12} and 10^{-7} M (Fig. 1). Growth stimulation was maximal at concentrations of 10^{-10} and 10^{-9} M E_2 and the former concentration was selected for the protein modulation experiments below. The growth of the other lines (PEA1, PEA2, PE014, PE023 and PE016) with low to zero levels of ER were unaffected by E_2 over this range of concentrations (data

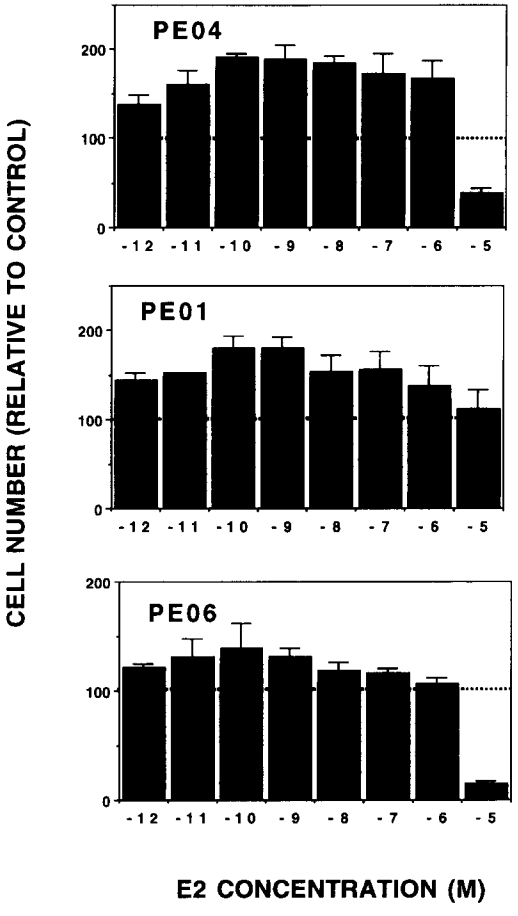

Fig. 1. Effect of 17 β-estradiol (E_2) on the growth of ER-positive ovarian carcinoma cell lines. Cells were treated for 6 days with E_2 (as described in Experimental) at the concentrations shown. Each value is the mean value of quadruplicate observations. Error bars represent standard error. The experiment shown is representative of at least 3 identical experiments.

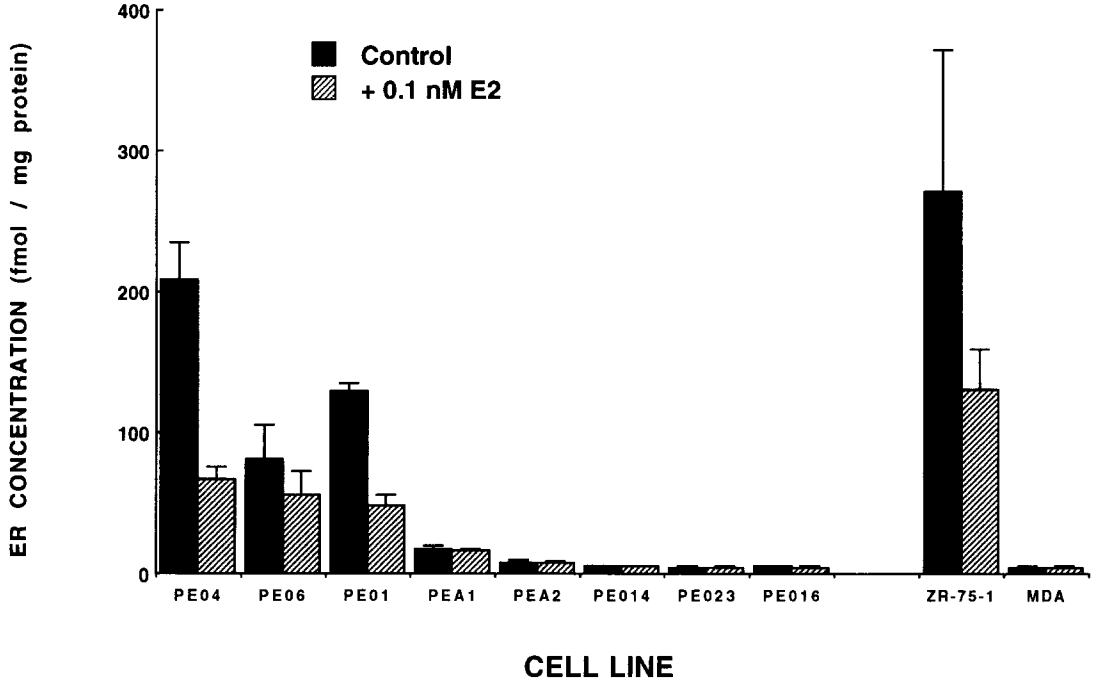

Fig. 2. Estrogen receptor content of ovarian and breast cancer cell lines in the absence and presence of 17 β-estradiol (E_2). Each point is the mean value of 3 independent measurements and error bars represent standard error. Solid bars represent values obtained on cells grown in the absence of E_2 and hatched bars are for cells grown in the presence of 10^{-10} M E_2 for 6 days (as described in Experimental).

not shown). Consistent with previous reports [22], growth of the ER-positive breast cancer ZR-75-1 cell line was stimulated by 10^{-10} M E_2 (120% increase in cell number relative to control) while growth of the ER-negative MDA-MB-231 line was unaffected (data not shown).

Effect of E_2 on ER concentrations

Two of the 3 ovarian carcinoma cell lines (PE04 and PE01) with high concentrations of ER demonstrated a reduction (approx. 50%) in the level of ER after exposure to 10^{-10} M E_2 compared to untreated cells (Fig. 2). This response was similar to that seen in the ZR-75-1 cell line (Fig. 2). The PE06 (81 fmol ER/mg protein) and the PEA1 (18 fmol ER/mg protein) lines did not show a significant reduction in ER content after exposure to E_2 compared to untreated cells. The other ovarian carcinoma cell lines examined possessed levels around the limit of detection (< 10 fmol/mg protein) in the absence or presence of 10^{-10} M E_2.

Effect of E_2 on PR concentrations

Two of the 3 ovarian carcinoma cell lines with high levels of ER (PE04 and PE06) showed an increase in the level of PR after exposure to 10^{-10} M E_2 compared to untreated cells (Fig. 3). The other ovarian lines demonstrated levels at the limit of detection of the assay. The ZR-75-1 line contained a much higher concentration of PR in the absence of E_2 (212 fmol/mg protein), than the ovarian carcinoma cell lines; PR was increased 5-fold after exposure to 10^{-10} M E_2.

Effect of E_2 on pS2 concentrations

The expression of pS2 in five of the ovarian carcinoma cell lines (PE04, PE01, PE06, PEA1 and PE014) was investigated in the presence or absence of 10^{-10} M E_2. No expression was found in these ovarian lines after either 3 or 6 days exposure to E_2. In contrast, the protein could be detected in the ZR-75-1 line and was increased by 260% in the presence of E_2 (data not shown). The ER-negative MDA-MB-231 breast cancer line did not express this protein.

DISCUSSION

This study indicates that E_2 can stimulate the growth of certain human ovarian carcinoma cell lines and this stimulation is associated with the presence of moderate–high levels of ER. These findings are similar to those found for breast cancer cell lines [23] and, as for breast cancer, suggest that the minimum requirement for ovarian tumour cells to be sensitive to estrogen is the presence of moderate numbers of ER. The ER value widely used to indicate likely sensitivity of breast cancer cells to anti-estrogen therapy (thus implying estrogen sensitivity) is 20–30 fmol/mg protein. To the best of our knowledge, no other ovarian cancer cell lines than those described in this report have been reported to have moderate–high levels of ER; therefore

these are the first systems which could act as a model for primary ovarian tumours with significant ER levels. Previous studies investigating the hormonal sensitivity of ovarian cancer have demonstrated that the BG-1 line ovarian carcinoma line which possesses an ER value of 23 fmol/mg protein is growth stimulated by E_2 [5] while the NIH:OVCAR-3 ovarian carcinoma line with an ER content of 28 fmol/mg protein is not [4] indicating that variable effects on growth are obtained around this cut-off point. A report describing clonogenic assays of human ovarian carcinoma cells obtained directly from clinical specimens indicated that 2 of 4 samples with an ER content greater than 30 fmol/mg protein responded to the anti-estrogen tamoxifen, while 0 of 14 samples with an ER value less than 30 fmol/mg were unresponsive, suggesting again that this value is a reasonable crude index of estrogen sensitivity [24]. Further experiments with clinical specimens of ovarian carcinoma are required to define a more accurate cut-off value.

Consistent with the view that E_2 is operating via the ER, levels of ER decreased in those ovarian carcinoma lines with the highest levels of ER after exposure to E_2 in a manner similar to that found for ER-positive breast cancer lines [10, 11].

For breast cancer, additional markers of estrogen response have been sought to help delineate more precisely that group of ER-positive tumors that are most likely to respond to anti-estrogen therapy. Among these are the PR and pS2 proteins [10–13]. In two of the three ER-positive ovarian carcinoma lines which were stimulated by E_2, PR was induced to a concentration of 20–30 fmol/mg protein. In the ZR-75-1 breast carcinoma line, PR levels in the absence of E_2 were 212 fmol/mg protein and this was increased to 920 fmol/mg protein after exposure to 10^{-10} M E_2. While the magnitude of the PR contents are markedly different between the ER-positive breast and ovarian carcinoma lines, the factor by which PR increases in the presence of E_2 is comparable in breast and ovarian cells. We have previously investigated the effects of exogenous E_2 on PE04 xenografts growing in nude mice and, in that system, the PR content increases from approx. 100 fmol to 800 fmol/mg protein after exposure to E_2 [3]. This increase, however, is associated with growth inhibition. The NIH:OVCAR-3 line also demonstrates an induction of PR after exposure to E_2; however, in this line there is no effect on growth [4]. Induction, therefore, of the PR in ER-positive ovarian carcinoma cell lines may indicate that protein expression is being modulated by estrogen, but does not imply that growth stimulation is also occurring. High levels of PR, therefore, in primary ER-positive ovarian tumours, may be indicative of a tumor being exposed to estrogen rather than a marker of estrogen sensitivity.

The expression of pS2 was investigated in several ovarian cancer cell lines. While expression of pS2 was detected in the ZR-75-1 breast line as previously reported, and was increased after exposure to E_2, no expression of this protein was found in the ovarian lines. Previous studies of primary ovarian tumours have identified pS2 in a subset of tumours [25–27]. Wysocki *et al.*[25] detected pS2 mRNA in 6 of 29 cases, 4 of

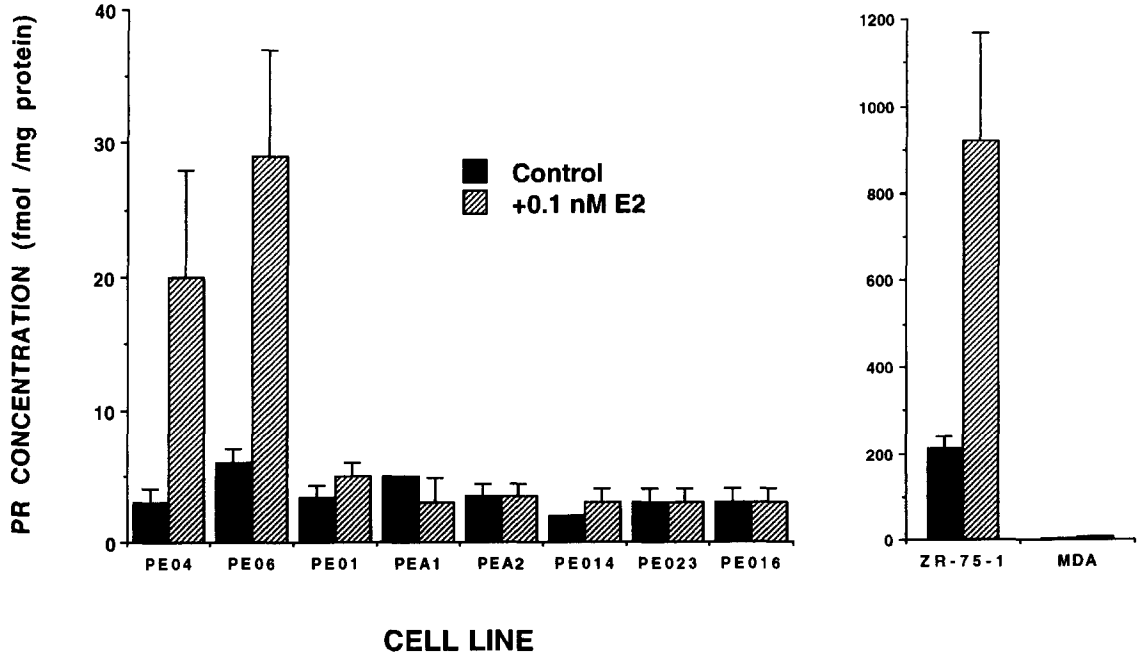

Fig. 3. Progesterone receptor content of ovarian and breast cancer cell lines in the absence and presence of 17 β-estradiol (E_2). Each point is the mean value of 3 independent measurements and error bars represent standard error. Solid bars represent values obtained on cells grown in the absence of E_2 and hatched bars are for cells grown in the presence of 10^{-10} M E_2 for 6 days (as described in Experimental).

which were of the mucinous subtype while Henry *et al.*[26] detected the protein (by immunohistochemistry) in 9 of 25 cases, of which 6 were also of the mucinous subtype, suggesting a possible association with this subtype. In contrast, Foekens *et al.*[27] using a radiometric immunoassay, were unable to detect levels above 11 ng/mg protein (the level they defined as clinically relevant) in 26 ovarian cancers. None of our cell lines was derived from a mucinous ovarian carcinoma but are derived from the serous subtype and thus our findings may be more representative of the "normal spectrum" of tumours. However, it is of interest, that in the study of Wysocki *et al.*[25], although 59% of the 29 tumours were ER-positive, 5 of the 6 demonstrating expression of pS2 had an ER concentration less than 20 fmol/mg protein suggesting that, in contrast to data for breast cancer, these two parameters are not linked. Indeed, the mucinous subtype, in contrast to the serous and endometriod forms of ovarian cancer, is more frequently ER-negative [1], again suggesting a probable lack of association of ER with pS2 in this disease.

In conclusion, we have demonstrated that the growth of a proportion of ovarian carcinoma cell lines can be stimulated by estrogen in culture. This stimulation appears to be associated with the presence of moderate–high levels of ER and may also be associated with the induction of PR. We are contributing to use these models to study other molecular pathways under control by estrogen to help identify indicators of hormonal sensitivity in clinical tumors.

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