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Estrogen Regulation of Transforming Growth Factor-α in Ovarian Cancer

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Transforming growth factor alpha (TGFα) may be induced by estrogen in estrogen responsive systems and can contribute to the growth-modulatory effects of this hormone. To test whether TGFα contributes to estrogen-regulated growth in ovarian cancers, we have compared the effects of 17β-estradiol (E_2) and TGFα in a range of ovarian carcinoma cell lines. Addition of E_2 to the estrogen receptor (ER)-positive cell lines (PE01, PE04 and $PE01^{CDDP}$) produced a 2–4 fold increase in TGFα protein concentrations in media conditioned by the cells. Both E_2 and TGFα stimulated the growth of the PE01 and PE04 lines and inhibited the growth of the $PE01^{CDDP}$ line. Furthermore, the E_2-mediated growth effects could be reversed by an epidermal growth factor (EGF) receptor-targeted antibody. E_2 also down-regulated EGF receptor expression in ER-positive cell lines. In a series of primary ovarian tumors, higher concentrations of ER were associated with an increased percentage of tumors expressing TGFα mRNA and a decreased percentage expressing EGF receptor protein. All these data are consistent with E_2 increasing production of TGFα in ER-positive ovarian cancer and this in turn acting through the EGF receptor to modulate growth in an autocrine manner. © **1998 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

There is an accumulating body of evidence to indicate that the growth of a percentage of ovarian adenocarcinomas may be regulated by estrogen. Approximately 50% of primary ovarian carcinomas contain estrogen receptors (ERs) and recent clinical studies indicate that 10–20% of all ovarian cancer patients respond to the anti-estrogen tamoxifen [1–3]. Experimental studies using cell line models show that the growth of ovarian cancer cells with moderate-high concentrations of ERs is stimulated by 17β-estradiol (E_2) and that the cells increase their expression of progesterone receptor (PR) and procathepsin D in response to this hormone [4–7]. These estrogen-inducible responses are analogous to those found in estrogen-sensitive breast cancer and have been proposed as indicators of functional regulation. One of the suggested mechanisms by which estrogen regulates growth of breast cancer cells is via increased expression of transforming growth factor α (TGFα) which acts through the epidermal growth factor (EGF) receptor. A number of studies have demonstrated that the expression of TGFα is regulated by estrogen in breast cancer cell lines [8] and furthermore that strategies targeting either TGFα (antisense neutralisation [9, 10]) or the EGF receptor (blocking antibodies [8, 11], blocking peptide [12] or tyrosine kinase inhibitor [13]) not only block the growth-stimulatory effect of TGFα but also that of E_2. This is consistent with the hypothesis that a TGFα/EGF-R autocrine pathway is involved in the mediation of estrogen-induced growth.

A recent analysis of hormone receptor status and TGFα concentrations in primary ovarian carcinomas indicated that in serous ovarian tumors the median concentration of TGFα in ER+/PR+ tumors was higher than that observed in other subgroups [14]. This is consistent with the concept that steroid hormones may influence TGFα in ovarian tumors. We have therefore investigated the hypothesis that estro-

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gen regulates production of TGFα in human ovarian cancer cell lines and that this mediates, at least in part, the mitogenic action of estrogen.

MATERIALS AND METHODS

Ovarian cancer cell lines

The human ovarian carcinoma cell lines PEO1, PE04 and PE014 were established and characterised as described previously [15, 16]. Briefly, both PEO1 and PEO4 lines were derived from ascitic fluid samples, PEO1 before and PEO4 after the onset of clinical resistance to *cis*-platinum combination chemotherapy in a single patient. The $PEO1^{CDDP}$ variant was established by *in vitro* exposure of PEO1 cells to *cis*-platinum [17]. PEO14 cells were derived from the ascitic fluid collected from a patient prior to treatment [16]. The ER content of the cell lines as measured by enzyme-immunoassay (Abbott ER-EIA) were 130 fmol/mg (PE01), 209 fmol/mg (PE04), 149 fmol/mg ($PE01^{CDDP}$) and 5 fmol/mg (PE014) [7].

Growth assays

Cells growing in log-phase were harvested by trypsinisation and seeded in 24 well plates (Falcon) at densities of 2.5×10^4 (PEO1, PEO4 and $PE01^{CDDP}$) and 5×10^4 (PEO14) cells/well in quadruplicate in RPMI 1640 (Gibco BRL, Paisley, Scotland) containing 10% heat-inactivated foetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 μg/ml). The cell lines were maintained routinely at 37°C in a humidified atmosphere of 5% CO_2 in air. After 24 h the medium was removed and the cells washed twice with phosphate-buffered saline (PBS). Culture was then continued in phenol red-free RPMI 1640 containing 5% double charcoal-stripped foetal calf serum (dcs-FCS), penicillin (100 U/ml), streptomycin (100 μg/ml) and glutamine (2 mmol/l) for 24 h. The medium was removed and replenished by fresh phenol red-free RPMI plus additives. Growth factor and hormone additions were made at this time point, designated day 0, and thereafter fresh medium plus growth factor/hormone additions were made on day 2. Cells were harvested on days 0, 2 and 5 and counted using a Coulter counter (Coulter Electronics, Luton, England).

Monoclonal antibody ior egf r3

The EGF-R monoclonal antibody ior egf r3 was supplied by the Center for Molecular Immunology, Havana, Cuba and was produced as described by Fernandez and colleagues [18]. Briefly, an EGF-R fraction was purified from solubilised human placenta using EGF-affinity chromatography. A crude placental membrane fraction was solubilised in 20 mM Tris/HCl, pH 7.4, containing 10% glycerol/1% Triton X-100. After centrifugation at 100,000*g* for 1 h, the supernatant was applied to EGF-sepharose. The EGF-R fraction was eluted with 5 mM ethanolamine, pH 9.7, containing 10% glycerol/0.1% Triton X-100. This EGF-R fraction was subsequently used for immunisation. The monoclonal antibody ior egf r3 has been tested for its ability to block ^{125}I-EGF binding to its receptor in human placental membranes. Half-maximal inhibition of EGF binding was achieved at 5×10^{-8} M [18].

Measurement of TGFα production

PEO1, $PEO1^{CDDP}$, PE04 and PE014 cells were grown in 175 cm^2 flasks (Falcon) to 70% confluence. Phenol-red free RPMI 1640 (50 ml) was added to each flask for 72 h in the absence or presence of 10^{-10} M E_2. Media from two flasks were pooled, concentrated 100-fold by freeze-drying and dialysed and TGFα content was measured using a radio-immunoassay kit specific for TGF-α (Peninsula Laboratories, St. Helens, U.K.). Values obtained were expressed as pg/ml.

Measurement of EGF-R by ligand binding

Confluent cells in 175 cm^2 culture flasks were harvested by cell scraping, sonicated in Tris-buffered saline (TBS, pH 7.4), centrifuged at 105,000*g* for 30 min at 4°C and resuspended in TBS. The protein content was determined by Bradford assay [19]. Cell preparations (100 μl) were incubated with 200 μl of unlabelled EGF (final concentrations of 0–300 nmol/l, Sigma) and 100 μl of [^{125}I]-EGF (approximately 2 nmol/l, Du Pont U.K.) for 90 min at 26°C. Ice-cold IgG (0.5% w/v, Sigma) was added to terminate the reaction. Following the addition of polyethylene glycol (25% w/v), bound and free [^{125}I]-EGF were separated by centrifugation and the resulting pellet counted in a Packard Gamma Counter. The number of binding sites and dissociation constants of binding were determined by Scatchard analysis [20] and binding was expressed as fmol/mg protein.

Measurement of ER in ovarian cancers

Primary ovarian tumors, collected at initial debulking surgery for suspected ovarian cancer, were snap frozen in liquid nitrogen and stored at −180°C until use. Tumor histology was assessed on paraffin sections and classified according to WHO criteria. Tissue fragments (50–200 mg) were weighed and homogenized in buffer (10 mM Tris, 0.25 M sucrose, 1 mM EDTA, pH 8.0 at 22°C, plus 1% monothioglycerol and 10% v/v glycerol). After centrifugation at 105,000*g*, the supernatant cytosol was assayed by enzyme-immunoassay kit (Abbott, Basingstoke, U.K.) according to the manufacturer's instructions. The protein content of the cytosol was determined as described above and receptor concentrations were expressed as fmol/mg protein.

Reverse-transcription PCR

400 mg of tumor tissue was homogenised using a tissue dismembranator at −20°C. Total cellular RNA was extracted from the pulverized tissue using the lithium chloride–urea method [21]. Pelleted RNA was resuspended in diethyl pyrocarbonate-treated water and the concentration and purity assessed by measuring absorbance at 260 and 280 nm. RT-PCR for TGFα and γ-actin were carried out using a Techne PHC-3 thermocycler. For the reverse transcription assay, 20 μg aliquots of total cellular RNA were reverse-transcribed by incubation with 300 ng of a random hexamer oligonucleotide with 2 mM each of dATP, dTTP, dCTP and dGTP (Pharmacia, U.K.) and 200 units of Superscript reverse transcriptase (Life Technologies, Paisley, U.K.) for 1 h at 42°C in a total volume of 20 μl. For all PCR reactions, 0.2–1 μl of reverse-transcribed RNA was added to 100 ng of each primer in a volume of 50 μl. The reverse transcriptase was inactivated by heating to 94°C for 10 min and then cooled rapidly to 4°C. PCR reactions were performed in a final volume of 100 μl containing the following: 0.5 units of *Taq* polymerase (Promega, Southampton, U.K.), 1.25 μM dATP, dTTP, dCTP and dGTP, 100 ng of each primer, 50 mM potassium chloride, 10 mM Tris–HCl, 0.1% Triton-X and 2.5 mM magnesium chloride. The amplification reaction was carried out over 40 cycles with the following parameters: step 1, 94°C for 38 s; step 2, 50°C for 53 s; step 3, 72°C for 68 s. For the final cycle, the 72°C step was extended to 7 min to ensure that all transcripts were full length. The primers used were: TGFα sense, 5′-GTAAAATGGTCCCCTCGG-3′, TGFα antisense, 5′-GTGATGATAAGGACAGCCAGGG-3′, γ-actin sense, 5′CAAGTTCTACAATCCAGTGC-3′, γ-actin antisense, 5′-ACGAGACCACCTTCAACTCC-3′. PCR products were visualised after electrophoresis on polyacrylamide gels and staining with ethidium bromide. Tumors were scored as positive for TGFα when a PCR product of the correct molecular size was amplified and identified following electrophoresis. Molecular size was assessed in samples by using a 100 bp ladder (Gibco, U.K.). PCR of γ-actin was performed to establish the integrity of transcribed RNA. Further confirmation of the identity of the TGFα transcript sequence was obtained by Southern blot analysis using a specific [^{32}P]5′-end labelled probe (5′-TAATGACTGCCCAGATTCCCAC-ACT-3′) targeted to a unique sequence within the transcript.

RESULTS

Secretion of TGFα protein by ovarian carcinoma cell lines and its modulation by 17β-estradiol

Levels of TGFα in media conditioned by the ER-positive PEO1, PEO1^{CDDP} and PEO4 cell lines for 72 h were measured at between 1 and 2 pg/ml; these values were increased by treatment with E_2 and TGFα concentrations in surrounding media were 1.9-fold, 4.3-fold and 4.0-fold higher, respectively, than in media from untreated cells (Fig. 1). For the ER-negative PEO14 cells, basal TGFα secretion was very low (<0.5 pg/ml) and did not change significantly following the addition of E_2 (Fig. 1).

Effects of TGFα and E_2 on cell growth

The effects of TGF-α and E_2 on the growth of the cell lines were examined next in order to see if the type of growth-response was similar for these two modulators. Addition of TGFα (10^{-12}–10^{-8} M) to PEO1, PEO4 and PEO14 ovarian cancer cells grown in DCS–FCS significantly increased growth at all concentrations of added TGFα (Fig. 2). Effects were concentration-dependent. Cell growth was significantly stimulated with maximal growth-stimulation observed at 10^{-10} M in PEO1 and PEO14 cells and 10^{-8} M in PEO4 cells. In contrast, TGFα significantly inhibited the growth of PEO1^{CDDP} cells; this was also concentration-dependent with maximal inhibition observed between 10^{-10} and 10^{-8} M (Fig. 2).

The profile of growth-responses to E_2 in the 3 ER-positive cell lines was analogous to that obtained with

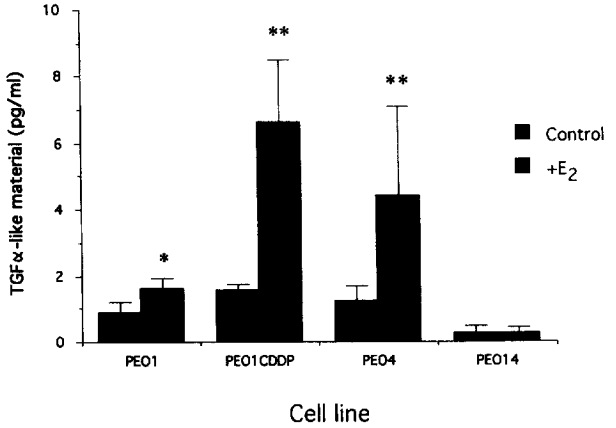


Fig. 1. Effect of E_2 on TGFα concentrations in media conditioned by human ovarian carcinoma cell lines. Cell lines were cultured in the presence or absence of 10^{-10} M E_2 for 72 h. Conditioned media were then collected, concentrated and analysed by radioimmunoassay. Values shown represent the mean of 3 separate estimations obtained from duplicate values and error bars signify standard error of the mean. Comparison between no added E_2 vs 10^{-10} M E_2; statistical significance, $^{**}p < 0.05$, $^{*}p = 0.05$ evaluated by Student's *t*-test. ER content of the cell lines: PE01, 130 fmol/mg protein; PE01^{CDDP}, 149 fmol/mg protein; PE04, 209 fmol/mg protein; PE014, 5 fmol/mg protein.

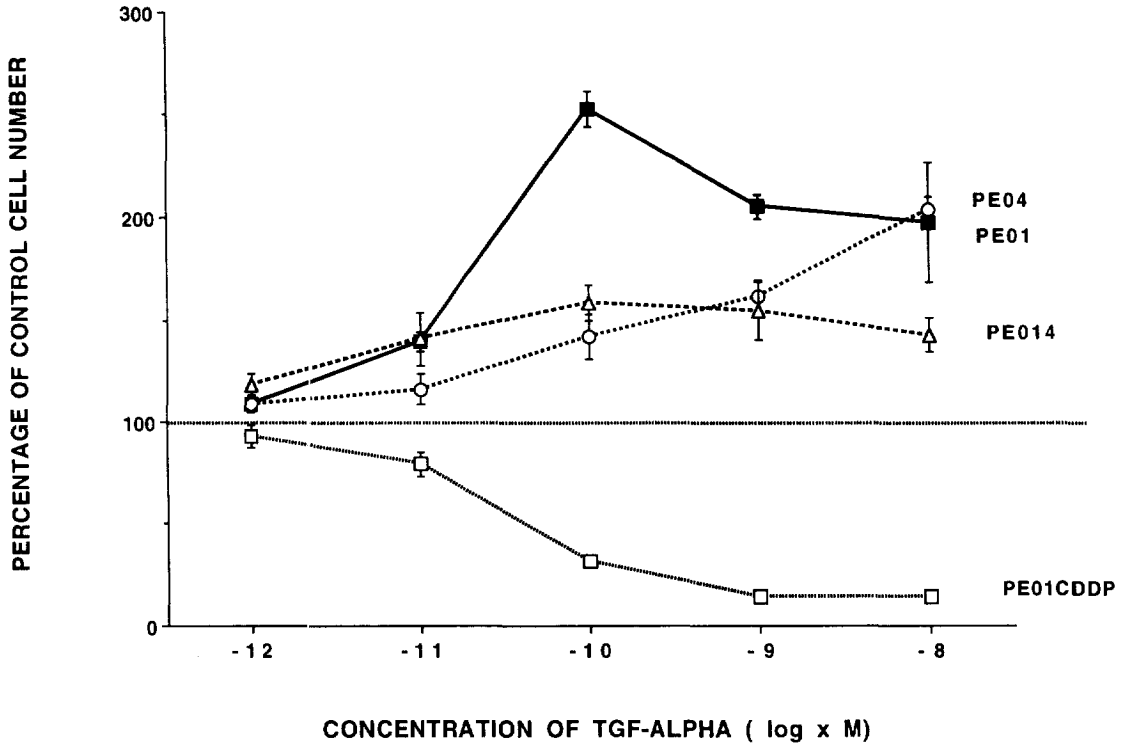


Fig. 2. Effect of TGFα on cell line growth. Cells were grown in phenol red-free medium supplemented with 5% dcs FCS and treated with the concentration of TGFα indicated over a period of 5 days. The graph shows the results from a typical experiment which is representative of at least 3 identical experiments. Each value represents the mean of quadruplicate values; error bars denote standard error of the mean.

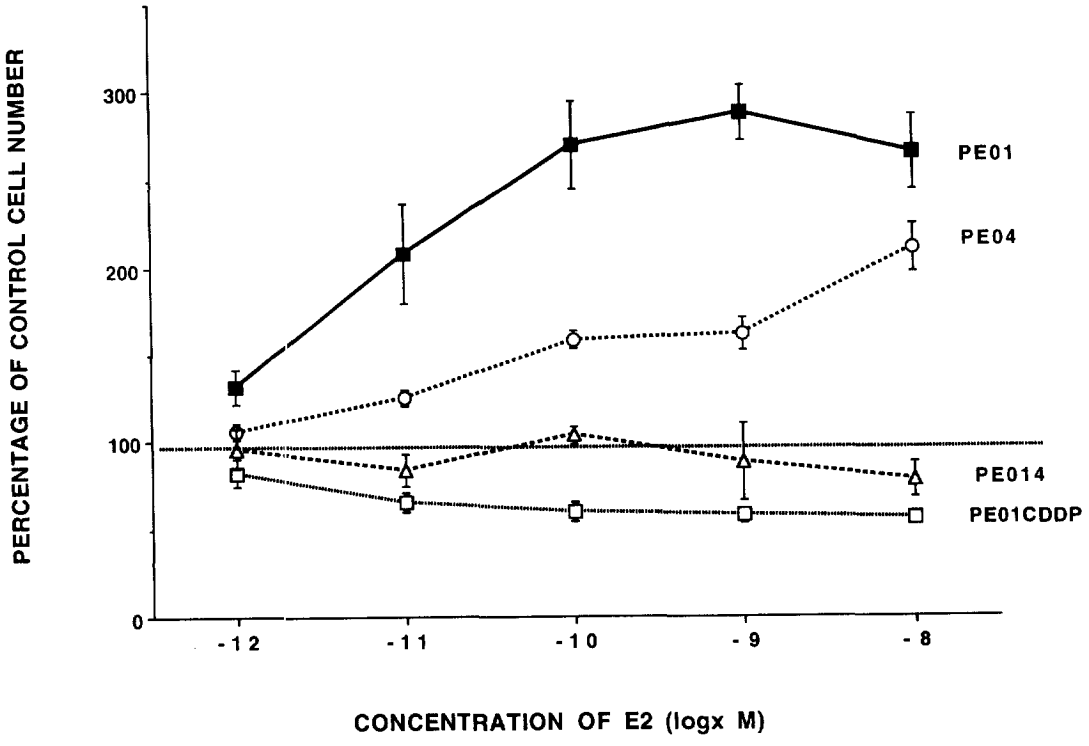


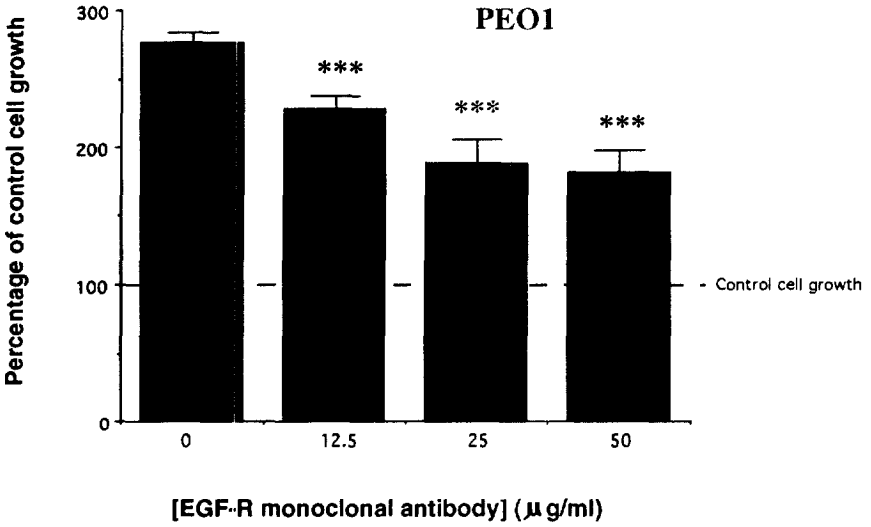
Fig. 3. Effect of E_2 on cell line growth. Cells were grown in phenol red-free medium supplemented with 5% dcs FCS and treated with the concentration of E_2 indicated over a period of 5 days. The graph shows the results from a typical experiment which is representative of at least 3 identical experiments. Each value represents the mean of quadruplicate values; error bars denote standard error of the mean.

TGFα in that E_2 stimulated cell growth in the PEO1 and PEO4 cell lines while inhibiting growth in the $PEO1^{CDDP}$ line, all effects being concentration-dependent (Fig. 3). However, E_2 (10^{-12}–10^{-8} M) had no significant effect on growth in the ER-negative PEO14 cell line (Fig. 3).

Modulation of TGFα and E_2 induced growth effects by EGF-R monoclonal antibody

To test whether the estrogen induction of TGFα might mediate estrogen-induced growth-response, the effects of an antibody (ior egf r3) on TGFα- and E_2-modulated growth were studied. This antibody targets the EGF receptor and prevents binding of TGFα to the EGF receptor.

The antibody inhibited the TGFα-stimulated (10^{-10} M) growth of PE01 cells in a concentration-related manner (Fig. 4), the reagent used at a concentration of 50 μg/ml reduced TGFα-stimulated growth by 50%. In an analogous manner, the TGFα-inhibition (10^{-10} M) of growth in $PEO1^{CDDP}$ cells, was significantly attenuated by ior egf r3 in a concen-



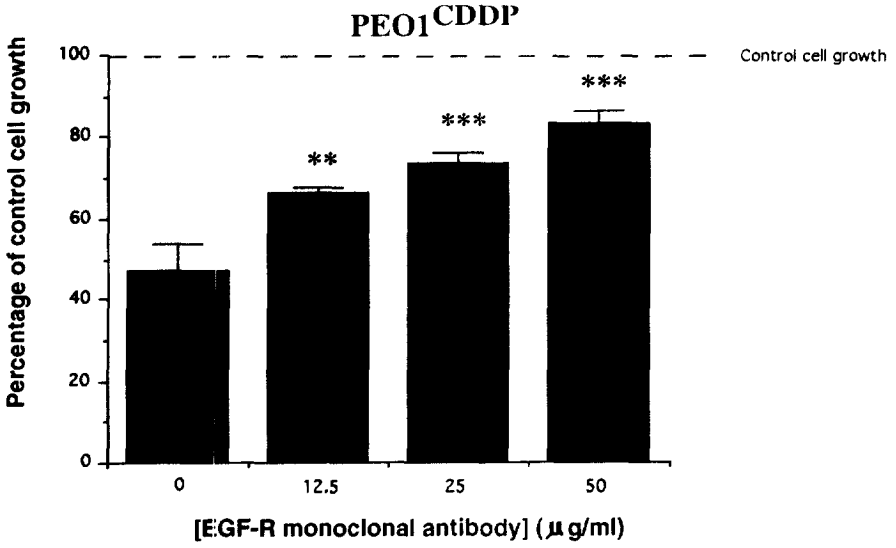


Fig. 4. Effect of an EGF receptor targeted antibody (ior egf r3) on TGFα-stimulated PE01 and TGFα-inhibited $PE01^{CDDP}$ cell growth. Cells were grown in phenol red-free medium supplemented with 5% dcs FCS and treated with the concentration of antibody indicated over a period of 5 days. The graph shows the results from a typical experiment which is representative of at least 3 identical experiments. Each value represents the mean of quadruplicate values; error bars denote standard error of the mean. Statistical comparisons were made with respect to the TGF-α stimulated group: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (Student's t-test).

tration-related manner (Fig. 4), the presence of ior egf r3 at a concentration of 50 μg/ml reduced inhibition by 70%.

The effect of ior egf r3 on estrogen modulated-growth of PE01 cells is shown in Fig. 5. The addition of ior egf r3 (100 μg/ml) reduced E_2-stimulated (10^{-10} M) growth in PEO1 ovarian cancer cells (Fig. 5) and conversely inhibited E_2-induced (10^{-10} M) growth inhibition of $PEO1^{CDDP}$ cells (Fig. 5). In the absence of E_2, ior egf r3 inhibited

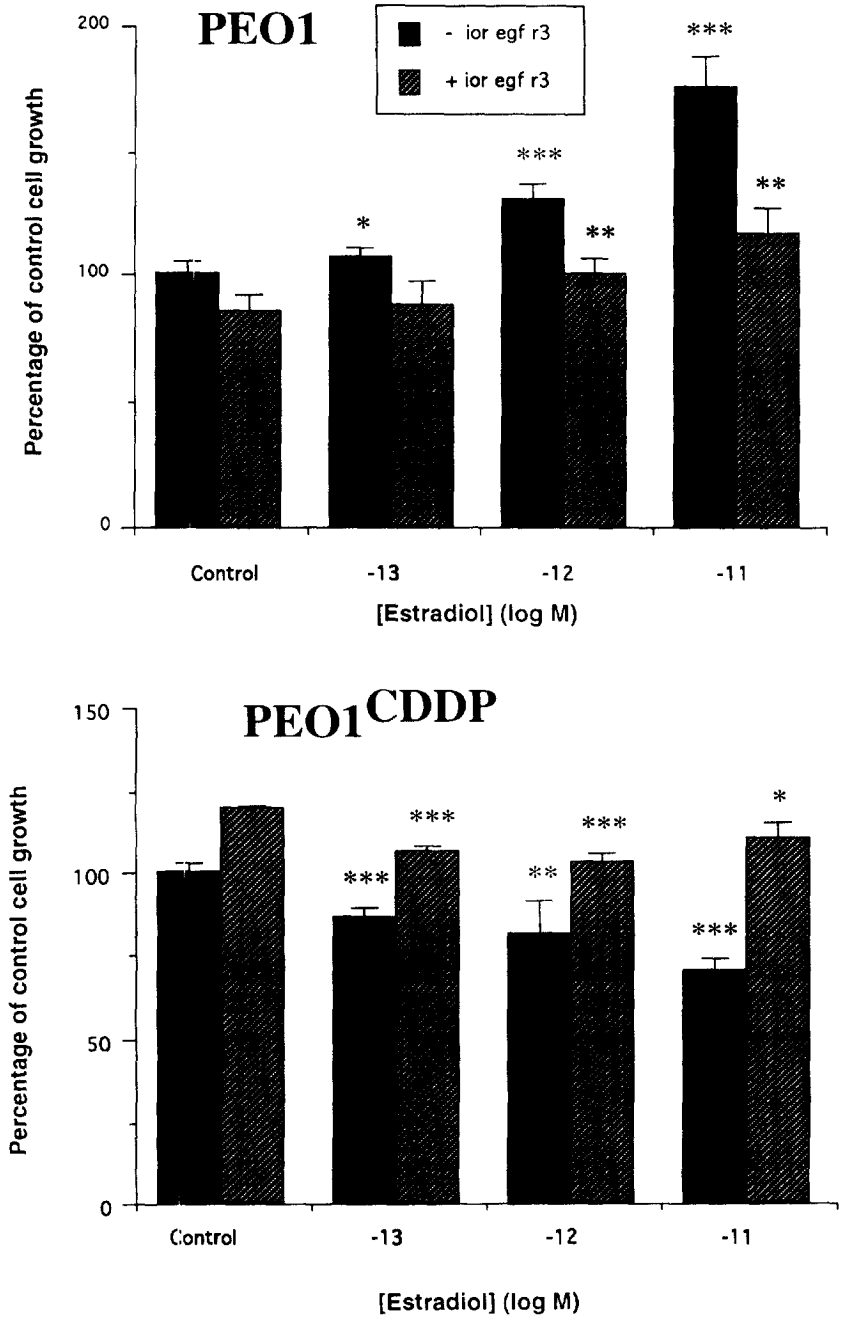


Fig. 5. Effect of an EGF receptor targeted antibody on E_2-stimulated PE01 and E_2-inhibited $PE01^{CDDP}$ cell growth. Cells were grown in phenol red free medium, supplemented with 5% dcs FCS and treated with the concentration of E_2 indicated over a period of 5 days. The graph shows the results from a typical experiment which is representative of at least 3 identical experiments. Each value represents the mean of quadruplicate values; error bars denote standard error of the mean. Statistical comparisons were made with respect to the control groups depending on whether antibody was present or not: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (Student's *t*-test). For the control groups, addition of antibody produced a significant growth inhibition in the PE01 cell line ($p = 0.0024$; Student's *t*-test) and a significant stimulation ($p < 0.0001$; Student's *t*-test) in the $PE01^{CDDP}$ line.

growth of PEO1 ($p = 0.0024$; Student's t-test) and stimulated growth of $PEO1^{CDDP}$ ovarian cancer cells ($p < 0.0001$; Student's t-test).

Downregulation of EGF receptor by E_2

Since TGFα is known to downregulate the EGF receptor, it was of interest to see whether E_2 would produce a similar effect in cell lines showing a growth response to estrogen. PE01, PE04 and PE014 cells were cultured in phenol red free RPMI 1640 containing 5% DCS-FCS in the presence or absence of E_2 (10^{-8} M) for 5 days. Results from Scatchard analysis of binding data indicated both high ($K_d \leq 1$ nM) and low affinity ($K_d > 1$ nM) EGF binding sites in all 3 lines. PE01 and PE04 cells exposed to E_2 demonstrated reduced levels of both high and low affinity receptors compared to cells not treated with E_2 (Fig. 6). For the ER-negative PE014 cell line, culture with E_2 had no effect on EGF receptor levels (Fig. 6).

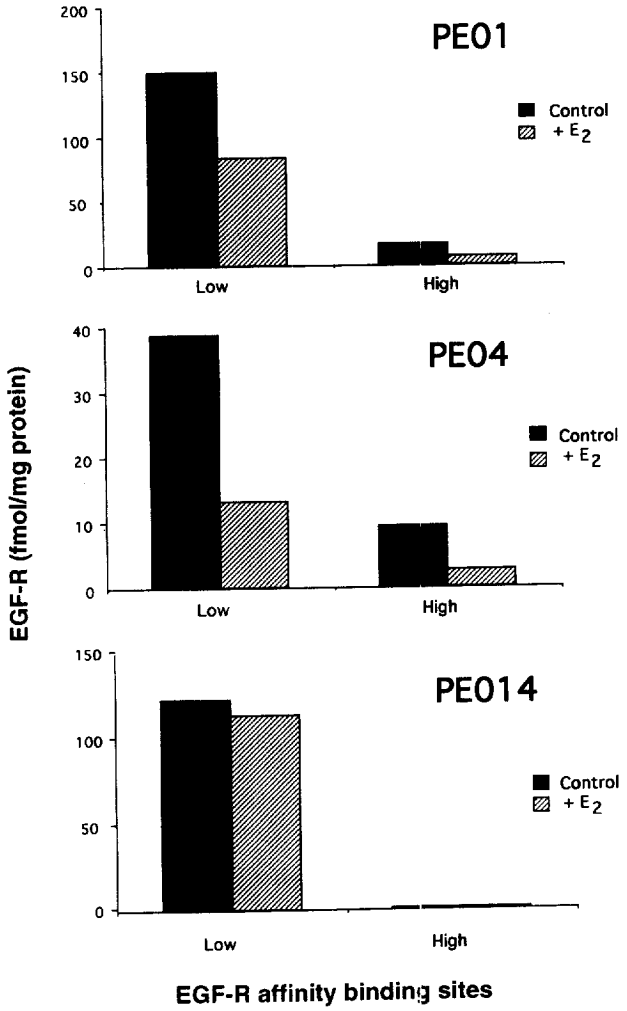


Fig. 6. Effect of E_2 on the high and low affinity EGF receptor levels in PE01, PE04 and PE014 cell lines. Cell lines were grown in phenol red-free medium supplemented with 5% dcs FCS and treated with 10^{-8} M E_2 over a period of 5 days. EGF receptors were measured by ligand-binding assay as described in Section 2 and analysed by Scatchard analysis. Biphasic curves indicated high ($K_d \leq 1$ nM) and low affinity ($K_d > 1$ nM) binding sites.

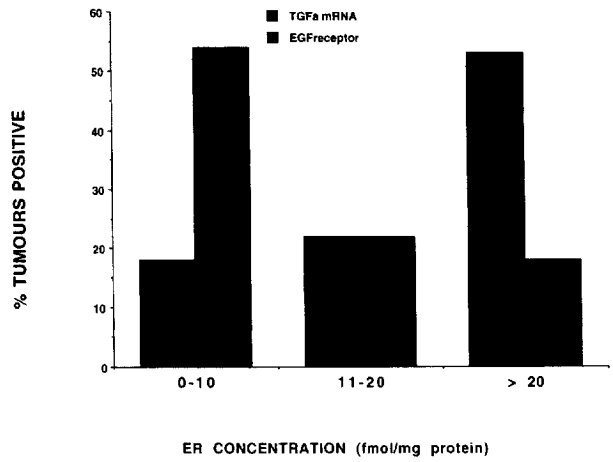


Fig. 7. Relationship between ER content and the production of TGFα mRNA and EGF receptor protein in primary ovarian tumors. With increasing ER concentration there are trends towards an increasing percentage of tumors expressing TGFα mRNA ($p = 0.014$; X^2 test for trend) and a decreasing percentage expressing EGF receptor protein (≥20 fmol/mg protein) ($p = 0.037$; X^2 test for trend).

Association of TGFα mRNA and EGF receptor protein expression and ER content in primary ovarian carcinomas

To investigate whether TGFα expression might be regulated by E_2 in primary ovarian tumors, the association between ER content and the presence of TGFα mRNA expression was investigated in a series of 56 primary ovarian carcinomas. While only 18% (3 of 17) of ovarian carcinomas with an ER content of 0–10 fmol/mg protein and 22% (2 of 9) with a content of 11–20 fmol/mg protein expressed TGFα mRNA, 53% (16 of 30) with an ER content>20 fmol/mg protein expressed this factor (X^2 for trend, $p = 0.014$) (Fig. 7). Conversely the percentage of ovarian cancers expressing a moderate level (20 fmol/mg protein) of EGF receptor decreased with increasing ER content, consistent with possible down-regulation of the EGF receptor by estrogen (X^2 for trend, $p = 0.037$) (Fig. 7).

DISCUSSION

In this study we have investigated whether the growth-modulatory effects of E_2 in ovarian cancer cells may be mediated via TGFα. This was aided by the use of model systems in which E_2 stimulated, inhibited or had no effect on the growth of these cell lines. E_2 stimulated growth in two of these lines and inhibited a third; TGFα produced the same profile of responses, consistent with it contributing to this effect. All 3 ER-positive lines examined secreted more

TGFα (2–4 fold) in the presence of E_2 than in its absence indicating that E_2 can increase expression of this factor. Blockade of the EGF receptor with an inhibitory antibody (thereby preventing interaction with TGFα) not only inhibited the effects of TGFα but also those of E_2. Finally, E_2 downregulated expression of the EGF receptor in responding cell lines (with ER ≥ 30 fmol/mg protein) but not in the non-responsive ER-negative cell line. All of these results are compatible with increased production of TGFα in response to E_2, which in turn acts through the EGF receptor to modulate growth. Extension of this study into primary ovarian cancers indicated that tumors with an increased ER content were more likely to express TGFα mRNA and less likely to express EGF receptor which is in agreement with the view that estrogen up-regulates TGFα expression but down-regulates EGF receptor expression.

These data are novel for ovarian cancer, however previous studies of estrogen-dependent breast cancer cell lines have yielded similar findings. Synthesis of TGFα mRNA and protein are induced by estrogen in ER-positive breast cancer cells 2- to 8-fold depending on cell line and culture conditions [22] and this compares well with the 2- to 4-fold induction in these ovarian cancer cell lines. A variety of strategies targeting either TGFα or the EGF receptor have been studied in breast cancer systems as approaches to determine the involvement of TGFα in the regulation of growth by estrogen. Introduction of an antisense TGFα mRNA expression vector into ER-positive T47D [9] or MCF-7 or ZR-75-1 [10] breast cancer cells reduced expression of TGFα mRNA and protein and also prevented induction of TGFα and most of the growth stimulation in response to E_2. A tyrosine kinase inhibitor, RG 13022, with a degree of specificity for the EGF receptor also has the potential to block cell proliferation induced by EGF, TGFα or E_2 in breast cancer cells as does the synthetic peptide analogue of TGFα, [(Ac-D-hArg(Et)2^{31}), $Gly^{32,33}$] HuTGF-alpha (31–43)NH_2 [13]. Antibody blockade of the EGF receptor has also been shown to inhibit estrogen-driven growth in breast cancer cell lines although this is often only a partial block [8, 11].

While these observations in breast cancer systems point to the role of TGFα contributing to estrogen-regulated growth, there are also a number of experiments which do not support such a view. For example, MCF-7 breast cancer cells transfected with the TGFα gene (thereby resulting in high constitutive expression of TGFα) still require estrogen for growth in nude mice [23]. Similarly, cell lines expressing high levels of TGFα such as the MDA-MB-231 line fail to support the growth of estrogen-dependent MCF-7 cells *in vivo* [24]. Data from cell lines such as CAMA-1 suggest that alternative pathways may also be involved. This breast cancer cell line is estrogen-responsive yet is EGF receptor-negative and insensitive to TGFα indicating that growth factors other than TGFα are perhaps involved in estrogen-regulated growth [25]. Estrogen has been shown to regulate expression of insulin-like growth factor I (IGF-I) [26], IGF-II [27] and also their binding proteins [28, 29], platelet-derived growth factor [30] and TGF-β [31] in breast cancer systems and these are all likely to be involved in estrogen-regulated growth control. Recently, another member of the EGF family, amphiregulin, has also been shown to be regulated in breast cancer systems [32]. Regulation of these factors by estrogen has not been explored in ovarian cancer but it seems probable that TGFα is only one growth factor of many that may be under endocrine control. It is also likely that the different growth factors will contribute to varying degrees in individual tumors or cell lines, depending on the levels of expression of the factor and its receptor.

Since the ovarian cancer cell lines studied here express both TGFα and the EGF receptor and also demonstrate a growth-response to exogenously added TGFα, it is feasible that TGFα may be acting as an autocrine regulator in these models. In the absence of added growth factor or hormone, the blocking of the EGF receptor by antibody inhibited PE01 and stimulated $PE01^{CDDP}$ cell growth which is consistent with the presence of a degree of TGFα autocrine-controlled growth. Evidence for an autocrine loop involving TGFα and the EGF receptor in cultured cells from primary ovarian cancers has previously been obtained with the help of blocking antibodies [33] and the cell line data obtained here support such a role.

The growth inhibitions obtained with E_2 and TGFα against the $PE01^{CDDP}$ cell line also indicate that under certain circumstances these agents may be growth inhibitory to ovarian cancer cells. Further studies are currently in progress to identify the mechanism by which this inhibition occurs.

In conclusion, these are the first data to demonstrate that not only in breast cancer but also in ovarian cancer cells, TGFα may be an important mediator of estrogen-induced growth. Such pathways may provide potential targets for new therapeutic strategies in ovarian cancer.

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