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SKOV3 Ovarian Carcinoma Cells Have Functional Estrogen Receptor but are Growth-resistant to Estrogen and Antiestrogens

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Estrogen receptor positive ovarian cancer is often refractile to antiestrogen therapy. Here we describe the SKOV3 human ovarian carcinoma cell line as an *in vitro* model for estrogen and antiestrogen resistant ovarian cancer. While SKOV3 cells expressed estrogen receptor (ER) mRNA and protein at a similar level as the estrogen responsive T47D breast carcinoma cell line, their growth was not responsive to estradiol (E_2) and was not inhibited by the antiestrogens OH-tamoxifen and ICI 164,384. The ER in SKOV3 cells was normal with respect to apparent K_d for binding with E_2, E_2 regulation of a transiently transfected ERE driven reporter gene, and E_2 stimulation of expression of the early growth response genes *c-myc* and *c-fos*. However, the SKOV3 cells exhibited no expression of the progesterone receptor gene (PR) even after addition of E_2, and the protein products of the estrogen responsive genes HER-2/*neu* and cathepsin D were expressed at constitutive levels that were not regulated by E_2. Therefore, estrogen resistance in these cells may be a result of constitutive expression and loss of E_2 regulation of selected growth regulatory gene products rather than a defect in estrogen activation of ER as a transcriptional regulator.

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INTRODUCTION

Ovarian carcinomas are the most lethal tumors of the female genital tract [see 1, 2]. Typically, the disease remains clinically silent until it is advanced. Improved treatment strategies and diagnosis depend on a better understanding of the molecular defects involved in this disease.

Female sex steroid hormones are believed to play a role in the growth and malignant progression of ovarian cancer. Estrogen receptor (ER) is expressed in a subset of ovarian cancers estimated to be more than 60% using radioligand binding assays [2] and about 30% by immunohistochemical analysis of epithelial cancer cells [3, 4]. In addition, estrogen stimulates the growth of several ER^+ ovarian carcinoma cell lines *in vitro* [5–7].

Estrogen regulated growth is thought to occur by altered expression of the products of differentiation and growth regulatory genes that are under direct or indirect control of the transcriptional activation function of ER [8]. for example, estrogen regulates expression of the progesterone receptor (PR), of the early growth response protooncogene c-*myc*, and the level of cathepsin D in breast cancer cells [9–11] and in ovarian cancer cells [6, 7, 12].

While many ER^+ breast cancers respond to the antiestrogen, tamoxifen [13], the response of ER^+ ovarian carcinomas to tamoxifen treatment is poor [2, 14]. The high antiestrogen resistance observed in ovarian cancer may be due, in part, to advanced disease at detection. However, antiestrogen resistance is an important clinical problem for both breast and ovarian cancer. As many as 50% of ER^+ breast cancers are initially resistant to hormonal therapy, while

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responsive cancers eventually develop resistance [13]. Cell lines that model antiestrogen resistant breast cancer have been of value for defining ER functions that are lost or altered and for screening ER targeted growth inhibitors for possible clinical use [15–17]. For example, many tamoxifen resistant breast cancer cell lines are sensitive to the pure antiestrogen, ICI 164,384 suggesting its therapeutic value [see 18]. Antiestrogen resistance in ovarian cancer is of greatest clinical significance in patients that have not received tamoxifen therapy. To date stable cell lines that mimic antiestrogen resistant ovarian cancer have not been described.

Here we report ER expression, growth response to estrogen and antiestrogens and patterns of expression of estrogen regulated genes in the SKOV3 human ovarian carcinoma cell line. Evidence is presented that SKOV3 cells are a model for stable estrogen and antiestrogen resistant ovarian cancer with functional ER.

MATERIALS AND METHODS

Chemicals and reagents were obtained from Sigma (St Louis, MO) unless specified. Reagents for sodium dodecyl sufate (SDS)–polyacrylamide gel electrophoresis (PAGE) were obtained from Bio-Rad (Richmond, CA).

Cell lines

The T47D breast carcinoma cell line and the three ovarian carcinoma cell lines: CAOV3, NIH.OVCAR-3, and SKOV3 were all obtained from American Type Culture Collection (A.T.C.C., Rockville, MA). The NIH.OVCAR-3 [19] and SKOV3 cells were adenocarcinomas isolated by cultivation of ascitic fluid from patients with ovarian cancer. PEO4 ovarian carcinoma cells, a gift of Dr Thomas C. Hamilton, were derived from the malignant ascites of an ovarian cancer patient with a recurrent mucinous ovarian adenocarcinoma [5]. The normal ovarian epithelial cell line of limited passage, IOSE.VAN [20], was obtained from Dr Nelly Auersperg. T47D and PEO4 cells were routinely maintained in RPMI 1640 (JRH Biosciences, Lenexa, KS) supplemented with 10 μg/ml of insulin. SKOV3, CAOV3 and NIH.OVCAR-3 cells were maintained in DMEM (JRH Biosciences). IOSE.VAN cells were cultured in 50% Medium 199 and 50% MCDB 105. All cell lines were cultured in medium supplemented with 10% fetal bovine serum (FBS) (Hyclone Lab. Inc., Logan, UT) and 0.5% gentamicin (GIBCO-BRL, Gaithersburg, MD) at 37°C in a humidified incubator with 5% CO_2.

cDNA probes

The 1.96 kb human estrogen receptor (hER) full length cDNA was generated from pER7 plasmid. The 2.8 kb full length human progesterone receptor cDNA was provided by Dr David Brandon and was generated by EcoRI digestion of hPR-pGEM-4 plasmid [21]. The 1 kb AvaI fragment of rat c-*fos* cDNA was recovered from pSP65-c-*fos*-Rat plasmid [22]. The 1.5 kb sstI fragment of exon 2 from the human c-*myc* gene [23] was generated from pCmycsst1.5uc plasmid, a gift from Dr Grover Bagby. The 800 bp HindIII-PstI fragment of human c-*jun* was recovered from the RSV-cJ plasmid [24].

Estrogen effects

Cells were first depleted of steroids by cultivation in phenol-red free DMEM supplemented with 5% dextran charcoal treated FBS (DCFBS) (stripped medium) with media changes every 2 days for 5 days. DCFBS was prepared as described [6]. After steroid depletion, 10 nM of 17β-estradiol (E_2) or the ethanol vehicle was added with media changes every 2 days. To test E_2 effects on expression of early growth response genes, cells grown in stripped medium for 5 days were washed with PBS, and incubated for 48 h in 1% dialyzed DCFBS to achieve quiescence prior to administration of E_2.

Western blot analysis

SDS–PAGE and Western blot analysis was conducted as previously described [25]. Protein, resolved by SDS–PAGE in a 6% polyacrylamide gel, was transferred onto nitrocellulose membranes (Costar, Cambridge, MA), which were then blocked with 5% nonfat dried milk in water. Membranes incubated with anti-ER or anti-PR monoclonal antibodies were then incubated with horseradish peroxidase conjugated to goat anti-rat IgG. Blots incubated with the previously characterized anti-HER-2/*neu* polyclonal antibodies [25] were then incubated with horseradish peroxidase conjugated goat anti-rabbit IgG (GIBCOBRL, Gaithersburg, MD). Blots were developed by enhanced chemiluminescence reagent (Amersham Life Science, Arlington Heights, IL) and exposed to X-ray film (Eastman Kodak Co., Rochester, NY).

Immunoprecipitation of cathepsin D

E_2 treated or control cells in 6 well plates were labelled with 200 μCi/ml of [^{35}S]methionine/cysteine (Amersham France) in methionine/cysteine free DMEM for 6 h. Equal amounts of trichloroacetic acid precipitable cpm from cell extracts or from media were immunoprecipitated with 5 μg/ml purified D8F5 monoclonal antibody to cathepsin D as described [6]. The washed immune complex was resolved in a 12% acrylamide gel by SDS–PAGE.

K_d determination of E_2 for ER

Hormone binding was conducted as described [26]. Aliquots of cytosol from cells cultivated in stripped medium for 7 days were incubated with [^{3}H]E_2 (New

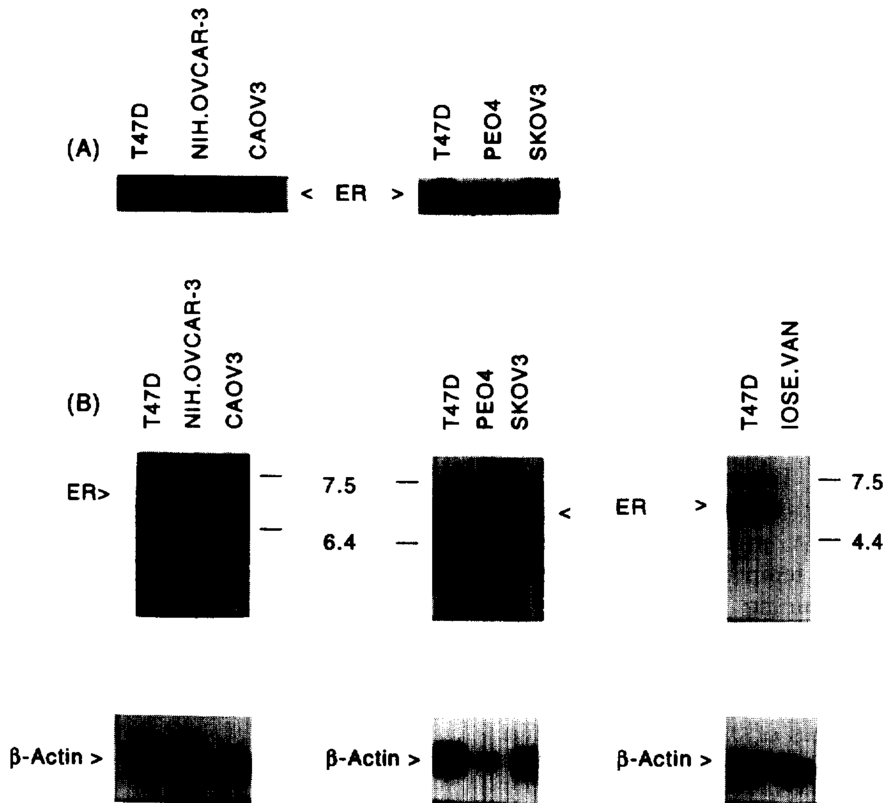

Fig. 1. Expression of ER mRNA and protein. (A) 400 μg of protein from each cell line was analyzed by Western blotting using anti-ER monoclonal antibody D75 at 1 μg/ml [44]. (B) Poly(A) RNA from each cell line was extracted and about 3 μg were analyzed by Northern blotting as described in Materials and Methods. The Northern blot was hybridized with [α-^{32}P]dCTP labeled random primed human ER cDNA probe. The blots were stripped and hybridized with ^{32}P-labeled β-actin (bottom panel) to standardize for amount of mRNA.

England Nuclear, Boston, MA) at concentrations between 0.2–4 nM, or with 200-fold excess of unlabeled diethylstilbestrol (DES). The amount of Bound E_2 was plotted as X-axis and Bound/Free as Y-axis, and the dissociation constant (K_d) was calculated as the slope of the line.

Isolation of Poly(A) RNA and Northern blot analysis

Isolation of mRNA was performed according to a procedure described by Schwab *et al.* [27]. Cells were lysed in buffer with 1% SDS and the DNA was sheared with a 21 gauge needle. The lysate was digested with

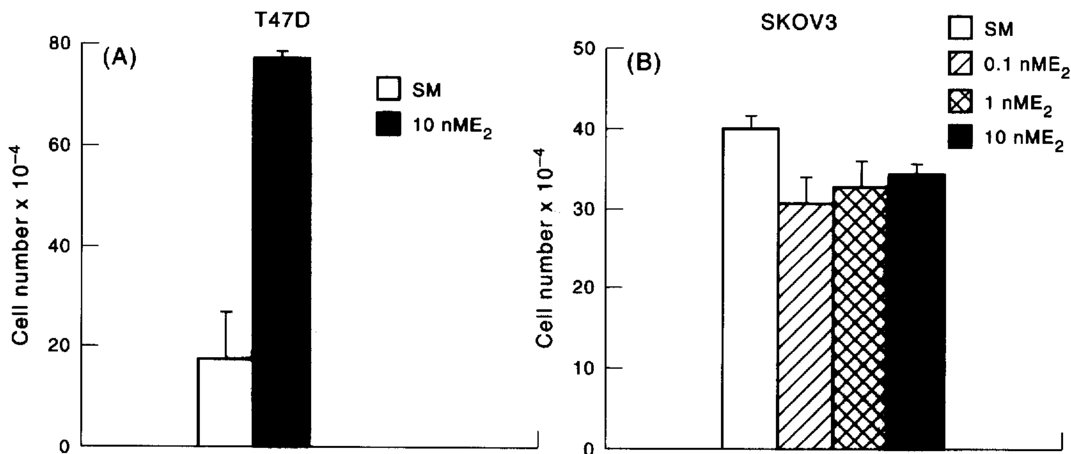

Fig. 2. Effects of E_2 on growth of SKOV3 and T47D cells. 5000 cells/cm^2 were cultured in 6-well plates in phenol red free DMEM supplemented with 5% DCFBS (stripped medium). On day 5, E_2 or the ethanol vehicle were added every 2 days in fresh medium for 7 days. Cells in triplicate wells were trypsinized and counted using a cytometer. (A) T47D cell, with stripped medium containing the ethanol vehicle (SM) or with 10 nM E_2. (B) SKOV3 cells in stripped medium with ethanol vehicle (SM), or with 0.1 nM, 1 nM, and 10 nM of E_2. Each bar represents the mean and standard deviation of the experiments conducted in triplicate.

of proteinase K, adjusted to 0.4 M NaCl, and adsorbed to Oligo-dT cellulose (Collaborative Research, Bedford, MA). The cellulose was washed in 0.4 M NaCl buffer with 0.2% SDS, with 0.1 M NaCl buffer, and the mRNA wa eluted from the column with "no salt" buffer. The eluate was adjusted to 0.2 M sodium acetate and 67% ethanol and the mRNA was precipitated. 5 μg of poly(A) RNA was spearated on a formaldehyde-denaturing gel containing 1% agarose and was then transferred onto a nytran membrane (Schleicher & Schuell, Keene, NH) by capillary action and subjected to hybridization as described [28]. cDNA probes were labeled with [α-^{32}P]dCTP (New England Nuclear, Boston, MA), using random primer DNA labelling kit (Boehringer Mannheim, Indianapolis, IN), and hybridized overnight with 0.5–1 × 10^6 cpm/ml of [α-^{32}P]dCTP-labeled cDNA probe. Membranes were washed 20 min at room temperature and at 65°C for 5 min in 2 × SSC containing 1% SDS followed by 60 min in 1 × SSC containing 1% SDS. Autoradiographs were analyzed by a laser densitometer (Bio-Rad, Hercules, CA). The membranes were stripped of probe by boiling in two changes of 0.1 × SSC for 5 min.

Transient transfection and CAT assay

Steroid depleted cells were plated overnight at about 40% confluency in 6 cm plates and then were transfected using the calcium phosphate DNA coprecipitation method [29]. The plasmid ERE-TK-CAT, obtained by subcloning the vitellogenin estrogen responsive element sequence into the Xbal and Bam HI sites of the pBL CAT 8PON vector, or the control plasmid, TK-CAT, were introduced at 1.5 μg per 6 cm plate. RSV-β-galactosidase expression plasmid at 1.5 μg per plate was used as an internal control of transfection efficiency and pSG1 carrier DNA was added to achieve 5 μg total DNA per plate. 16 h after introduction of the DNA, cells were shocked with 20% glycerol, washed, and treated with E_2 at 10 nM or control ethanol vehicle for 24 h in stripped media. CAT enzyme assays were performed in cell extracts after normalization to β-galactosidase activity. The acetylated and nonacetylated forms of ^{14}C chloramphenicol were separated by thin layer chromatography and detected by autoradiography.

RESULTS

Expression of ER mRNA and protein in ovarian cells

ER expression was examined in four ovarian cancer cell lines and in ovarian epithelial cells of limited life span, IOSE.VAN. The well characterized estrogen responsive breast carcinoma cell line, T47D [30] was used for comparison. Northern blot analysis revealed that the T47D cells and the PEO4 and SKOV3 ovarian carcinoma cells contained mRNA that hybridized with the ER cDNA probe [Fig. 1(B)]. No ER mRNA was detected in the NIH.OVCAR-3, CAOV3, or in the IOSE.VAN cells [Fig. 1(B)]. SKOV3 and T47D cells expressed similar levels of ER mRNA while the PEO4 cells contained about 5-fold higher levels when standardized to the amount of β-actin mRNA [Fig. 1(B)]. The ER protein analyzed by Western blotting was at similar levels in T47D and SKOV3 cells whereas PEO4 cells had about 3-fold higher levels standardized to total cell protein [Fig. 1(A)].

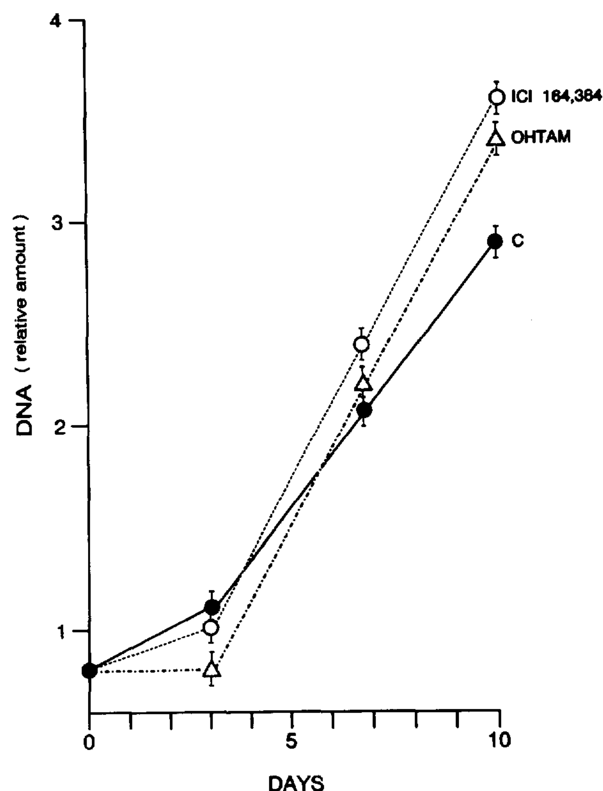

Fig. 3. Effect of antiestrogens on growth of DKOV3 cells. SKOV3 cells cultivated in stripped medium for 5 days were plated in 24-well plates and treated with control ethanol vehicle (●) with 100 nM 4OH-tamoxifen (△) or with 100 nM ICI 164,384 (○) with media changes every 2 days. At the indicated times, triplicate wells were fixed with methanol and the DNA content was assayed by the diaminobenzoic-acid fluorescence assay [45]. The DNA content is expressed in relative optical density units as mean values with standard deviations.

ER in SKOV3 cells had not been previously characterized, so we determined the affinity of E_2 binding to ER. The dissociation constant, K_d, calculated from the Scatchard plot for ER in SKOV3 cells was 2.0×10^{-10} M with a -0.84 correlation coefficient. In a duplicate experiment the K_d was 1.04×10^{-10} M for ER in SKOV3 cells. The affinity for E_2 binding to ER from T47D cells was not significantly different with a K_d calculated to be 1.8×10^{-10} M with a -0.96 correlation coefficient.

Effects of E_2 and antiestrogens on growth of SKOV3 cells

To further characterize ER function in SKOV3 cells, the effect of E_2 was tested on growth compared to T47D cells which have a similar level of ER. E_2

increased the number of T47D cells about 4-fold relative to the control [Fig. 2(A)] while three different concentrations of E_2 had no significant mitogenic effect on SKOV3 cells [Fig. 2(B)]. When the growth rate of SKOV3 cells was retarded by cultivation in serum free medium supplemented with insulin, transferrin and selenium (ITS), we were still unable to detect a proliferative effect of E_2. Antiestrogens inhibit the growth of ER^+ breast carcinoma cells even in the absence of estrogen by inhibition of growth factor induced proliferation [31]. The effects of the partial antagonist, 4-OH tamoxifen (OH-Tam), were therefore tested on the growth rate of steroid deprived SKOV cells. There was no inhibition of proliferation estimated by DNA content of cells at 3, 7, and 10 days of treatment with 100 nM OH-Tam (Fig. 3). Many tamoxifen resistant breast carcinoma cells exhibit sensitivity to the pure ICI antiestrogens [18]. However, SKOV3 cells were entirely resistant to growth inhibitory effects of ICI 164,384 (Fig. 3). The slight growth stimulation of cells treated for 10 days with antiestrogens (Fig. 3) was not observed at 10 days of treatment with higher concentrations (1 μM) of OH-Tam or ICI 164,384 (data not shown). Thus, SKOV3 cells were resistant to growth inhibition by two classes of antiestrogens.

Transcriptional activation function of ER in SKOV3 cells

To determine whether estrogen resistance may be related to a defect in the transactivation function of ER, we tested effects of E_2 on expression of an estrogen response element (ERE) driven reporter construct (ERE-TK-CAT) in transiently transfected cells (Fig. 4). E_2 treatment of transfected SKOV3 cells caused about 5-fold enhanced CAT activity compared to ethanol vehicle treated cells or compared to the E_2 treated cells transfected with the control plasmid without the ERE (TK-CAT). A similar extent of E_2-dependent stimulation of CAT activity was observed in transiently transfected T47D cells (Fig. 4). These results showed that ER had transactivation function in SKOV3 cells.

E_2 regulation of expression of gene products

The growth resistance to estrogen and antiestrogens may be from altered expression of estrogen responsive genes. For example, progesterone receptor (PR) expression is under control of estrogen in steroid target tissue [9]. E_2 induced both A and B forms of PR by about 10-fold in T47D cells [Fig. 5(A)] and there was 4–5-fold induction of 11.4 and 6.1 kb species of PR mRNA [32] by 1 h of hormone treatment [Fig. 5(B)]. However, neither PR protein or mRNA

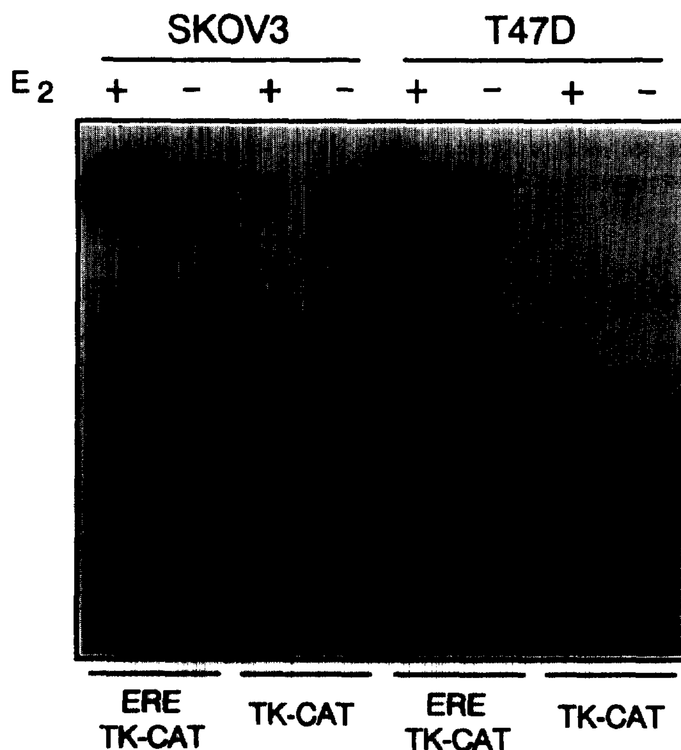

Fig. 4. Effect of E_2 on ERE-TK-CAT activity in transiently transfected SKOV3 and T47D cells. Transient transfections and reporter gene product enzyme assays were conducted as described in Methods. Steroid depleted SKOV3 cells and T47D cells in 6 cm plates were transiently transfected with 1.5 μg of the following plasmids: RSV-β-GAL, ERE-TK-CAT or the control TK-CAT plasmid. Transfected cells were treated with the ethanol vehicle or 10 nM E_2.

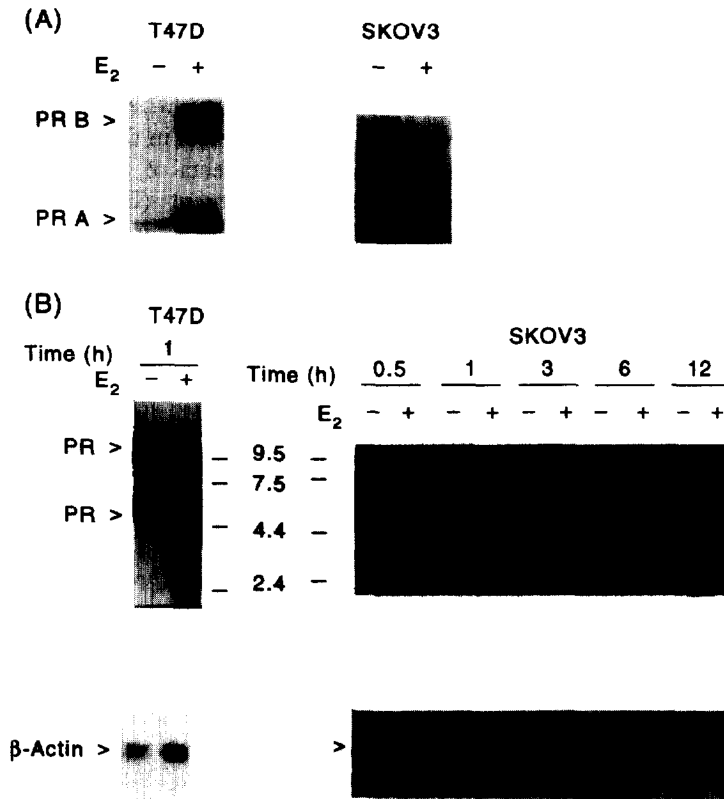

Fig. 5. E_2 effects on PR expression. (A) Steroid deprived T47D and SKOV3 cells were treated with ethanol vehicle (lane 1), or 10 nM E_2 (lane 2) for 5 days. 400 μg protein from each sample was resolved by SDS–PAGE and immunoblotted with 0.01 μg/ml monoclonal anti-PR antibody JZB39 [46]. (B) Poly(A)-selected mRNA from T47D and SKOV3 cells treated as above was analyzed by Northern blotting and probed with ^{32}P-labeled human PR cDNA probe. The blots were stripped and rehybridized with ^{32}P-labeled β-actin sto standardize for amount of mRNA.

was detected in SKOV3 cells treated up to 12 h with E_2 [Fig. 5(A, B)].

Estrogen stimulation of the secreted protease, cathepsin D, has been studied extensively in breast carcinoma cells [11]. E_2 had no effect on the level of extracellular 52 kDa procathepsin D [Fig. 6(A)] or the three intracellular forms of cathepsin D (52,48 and 34 kDa) which were constitutively produced in SKOV3

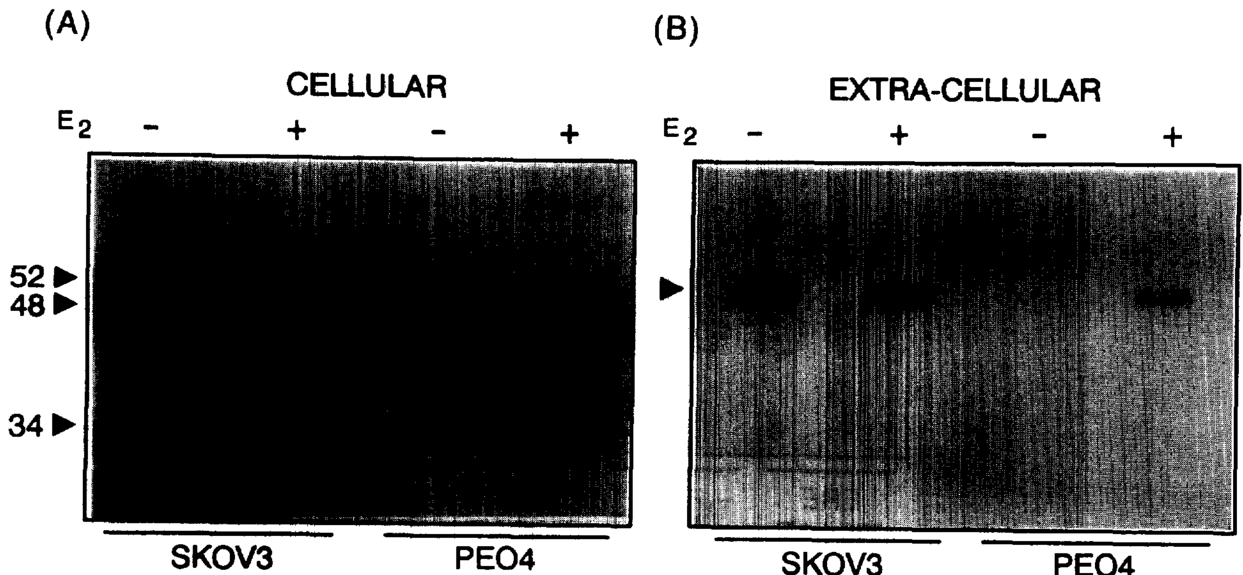

Fig. 6. E_2 regulation of intracellular and secreted cathepsin D levels. Steroid depleted SKOV3 cells and PEO4 cells treated for 2 days with 10 nM E_2 or with control ethanol vehicle were labeled with [^{35}S]methionine for 6 h. Media and cell extracts were immunoprecipitated with anti-cathepsin D antibodies and immune complexes were resolved by SDS–PAGE.

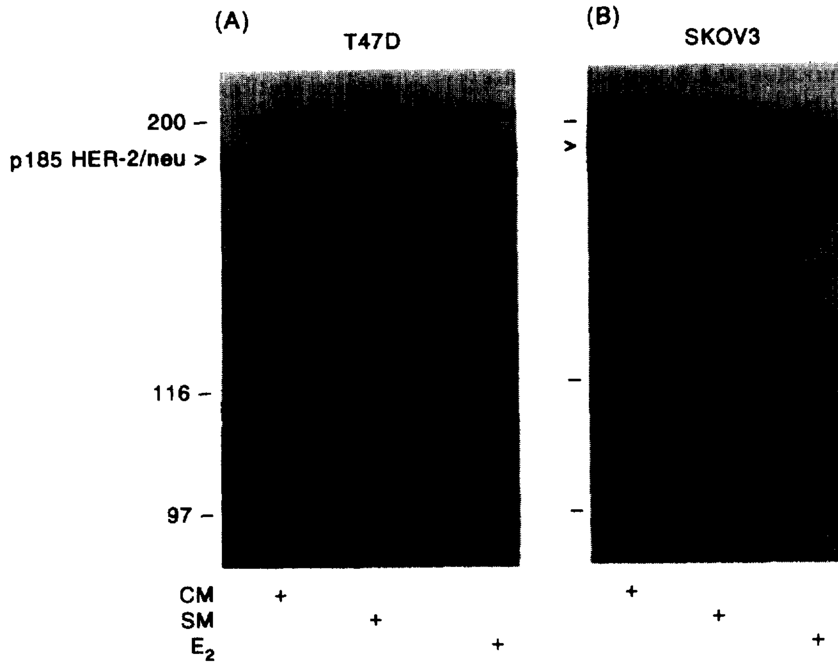

Fig. 7. E_2 regulation of p185 HER-2/neu levels. T47D (A) or SKOV3 (B) cells were maintained in Complete Media containing phenol red and 5% FCS for 9 days (lane 1) or Stripped Media for 5 days and control ethanol vehicle for 4 days (lane 2) or stripped media for 5 days and 10 nM E_2 for 4 days (lane 3). 400 μg of T47D cell extract protein or 40 μg of SKOV3 cell extract protein was subjected to Western blot analysis using anti-HER-2 antibody.

cells [Fig. 6(B)]. In comparison, PEO4 ovarian carcinoma cells had much lower levels of secreted and intracellular cathepsin D that were strongly induced by E_2 treatment [Fig. 6(A, B)]. These results showed that cathepsin D was expressed at high levels that were not regulated by E_2 in SKOV3 cells.

The product of the HER-2/*neu* protooncogene is down regulated by estrogen in responsive breast carcinoma cells [33–35]. To determine whether the levels of the HER-2/*neu* product were estrogen regulated, SKOV3 cells and T47D cells were cultivated in complete media, in stripped media, and in stripped media with E_2 and then analyzed by Western blotting with HER-2/*neu* specific antibodies. 10-fold less protein from the SKOV3 cells was used for comparison with the T47D cells since HER-2/*neu* expression is greatly amplified in SKOV3 cells [these results and 36]. There was no apparent decrease in p185/HER-2/*neu* levels following E_2 treatment of SKOV3 cells while there was a striking 20-fold decrease in the level of this protein in E_2 treated T47D cells (Fig. 6).

The early growth response protooncogenes are regulated by estrogen and other mitogenic agents [10]. Expression of c-*myc*, c-*fos* and c-*jun* was therefore investigated in the ER^+ SKOV3 cells that were cultivated in low serum (1% dialyzed, DCFBS) for 48 h to achieve quiescence. E_2 was added for the indicated times and the RNA was extracted and subjected to Northern blot analysis. A maximal 4-fold enhanced level of c-*myc* mRNA was detected at 1 h and up to 12 h of E_2 treatment compared to the control [Fig. 7(A)]. c-*fos* mRNA was strongly induced by serum and the levels rapidly declined by 1 h [Fig. 7(B)]. In addition, an approx. 2-fold increase in c-*fos* mRNA was observed at 30 min of E_2 treatment which returned to unstimulated levels by 3 h [Fig. 7(B)]. c-*jun* mRNA levels were mildly stimulated by serum, but did not appear to respond to E_2 treatment [Fig. 7(C)].

It was surprising that E_2 treatment increased c-*myc* and c-*fos* mRNA levels in the absence of a proliferative response. Therefore, SKOV3 cells were compared with T47D cells and PEO4 cells that are mitogenically responsive to estrogen. Quiescent cells were treated with E_2 for 60 min in the presence of 5 or 10% DCFBS to indicate whether serum factors may potentiate E_2 effects and then the RNA was extracted and subjected to Norther blot analysis. T47D cells had about 4.5-fold elevated c-*myc* mRNA levels relative to β-actin mRNA with no effect of different serum concentrations [Fig. 8(A)]. Although the basal level of c-*myc* in quiescent cells (1% serum) was much higher in PEO4 cells, the extent of E_2 induction of c-*myc* mRNA was similar (about 4-fold) in the PEO4 and SKOV3 cells. The SKOV3 cells were also indistinguishable from the estrogen responsive PEO4 cells by the extent of E_2 mediated increase in c-*fos* mRNA [about 3-fold, Fig. 8(B)]. There was little or no effect of E_2 on c-*jun* mRNA levels [Fig. 8(C)]. In summary, E_2 dependent increases in c-*myc* and c-*fos* mRNA levels did not correlate with growth response or with levels of ER.

DISCUSSION

To understand the function of ER in ovarian cancer, we analyzed the molecular expression and E_2-mediated functions of ER in ovarian carcinoma cell lines. ER mRNA and protein were expressed in SKOV3 and PEO4 ovarian carcinoma cell lines with no obvious expression of variant forms. No ER expression was detected in NIH.OVCAR-3, CAOV3, or in the IOSE.VAN cell line, nor did any of these cells exhibit E_2 induction of PR (Hua and Clinton, unpublished observations). However, previous studies of NIH.OVCAR-3 cells found ER expression, estrogen induction of PR, of c-*myc*, and growth in cells pretreated with tamoxifen [12]. Differences in cells or culture conditions may explain why we did not detect ER or PR expression in NIH.OVCAR-3 cells.

SKOV3 cells did not exhibit a proliferative response to E_2 nor was proliferation inhibited by the antiestrogen OH-tamoxifen. Importantly, there was complete cross resistance to the pure antiestrogen, ICI 164,384. The antiestrogen resistance of SKOV3 cells is stable since this phenotype has been maintained for 2 years of cultivation in our laboratory. In contrast many breast carcinoma cell lines selected *in vitro* for tamoxifen resistance are unstable and are not cross resistant to pure antiestrogens [17, 18]. Additional examples of hormone resistant ovarian carcinoma cells need to be studied, however, to establish whether there are some classes of tamoxifen resistant ER^+ tumors that may respond to pure antiestrogens.

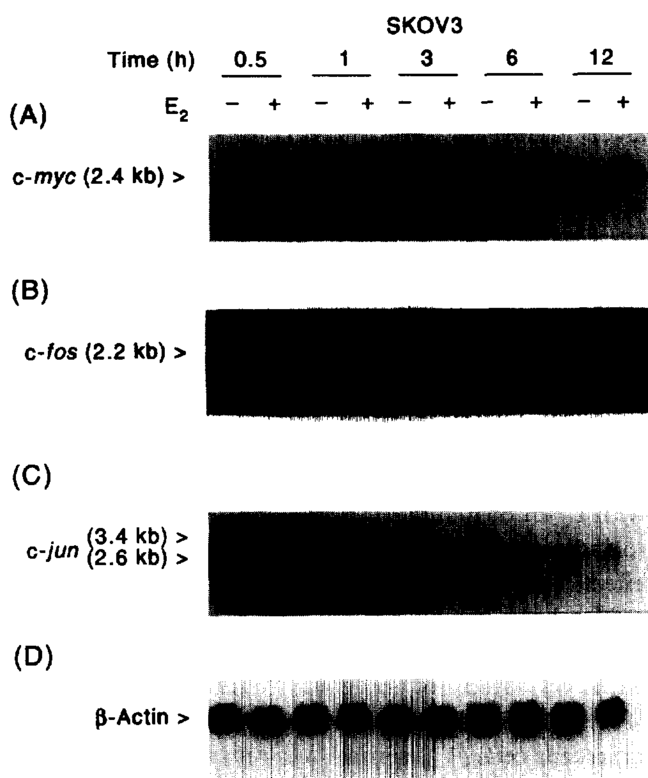

Fig. 8. E_2 regulation of c-*myc*, c-*fos* and c-*jun* expression in SKOV3 cells with time. SKOV3 cells were cultured in stripped media with 5% DCFBS for 5 days and then in 1% dialyzed DCFBS for 48 h to achieve quiescence and synchronization. Cells were treated with ethanol vehicle or 10 nM E_2 in stripped medium with 5% DCFBS for 0.5, 1, 3, 6, and 12 h. Poly(A) RNA was extracted and 5 µg of RNA was analyzed by Northern blotting. The Northern blot was hybridized, stripped and rehybridized with different [α-^{32}P]dCTP labeled random primed cDNA probes. (A) c-*myc*, (B) c-*fos*, (C) c-*jun* and (D) β-actin.

Expression of the early growth response protooncogene c-*myc* has been associated with estrogen induced growth of breast [37] and ovarian carcinoma cells [12]. Although there was no mitogenic response to E_2, SKOV3 cells had a similar extent of E_2-dependent increase (about 4-fold) in the levels of c-*myc* mRNA as the estrogen responsive T47D and PEO4 ovarian carcinoma cells. From these results we conclude that E_2 stimulation of c-*myc* expression was not sufficient to cause growth stimulation of SKOV3 ovarian carcinoma cells. Another early growth response protooncogene, c-*fos*, is strongly induced by estrogen in human endometrial carcinoma cells [38] but not in breast carcinoma cells [39]. Here we observed a small but reproducible increase in c-*fos* expression following E_2 treatment of the breast and ovarian carcinoma cells. The biological significance of small increases in c-*fos* mRNA levels is unclear.

Hormone resistance is a problem of clinical significance that has been studied extensively in breast cancer cells but has not been described in ovarian cancer cell lines. Several mechanisms could explain resistance including defective ER function. However, ER in SKOV3 cells was functional because E_2-dependent expression of the transfected ERE driven reporter gene and of c-*myc* and c-*fos* was normal compared to T47D and PEO4 cells. Detection of normal E_2 driven transactivation function also rules out the explanation that SKOV3 cells were resistant to added E_2 because they produced high levels of endogenous estrogens.

Generation of variant or mutant ER has been proposed to explain antiestrogen resistance in breast carcinoma cells [see 40]. Mutations or variant ER forms in SKOV3 cells not detected in Northern or Western blots, may affect protein–protein interactions and cross talk between ER and other signal transduction systems.

Altered expression of estrogen responsive genes in breast cancer cells has also been implicated in the development of estrogen-independent disease and antiestrogen resistance [17]. In SKOV3 cells there was no E_2 induction of the endogenous PR gene. Loss of PR induction has been observed in several antiestrogen resistant breast carcinoma cell lines [17]. Whether loss of PR induction is due to a defect in ER or in other cellular factors required for expression of PR is an interesting question that requires further investigation.

Constitutive expression of estrogen responsive gene products involved in growth and metastasis was also observed in SKOV3 cells and may be a mechanism underlying estrogen resistance. Cathepsin D, a secreted

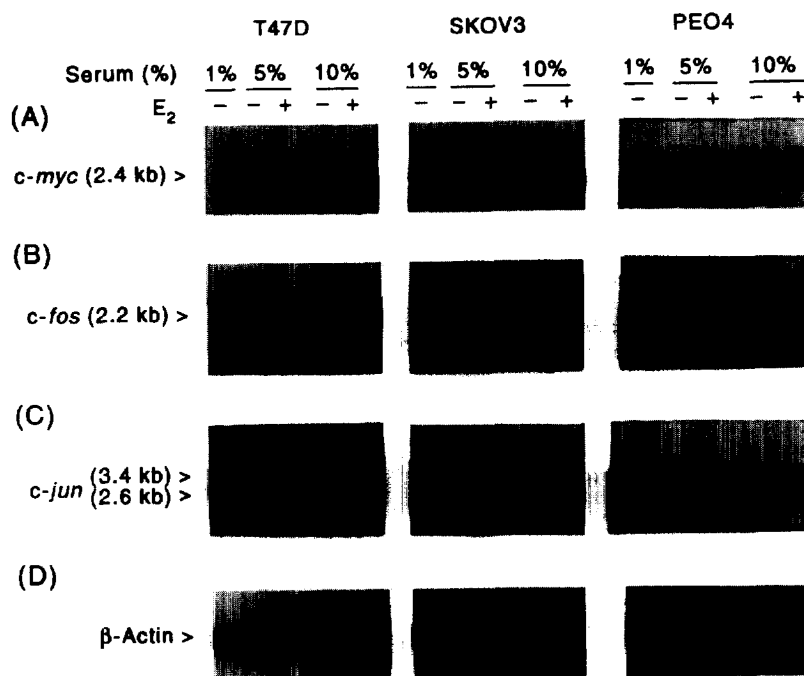

Fig. 9. E_2 regulation of *c-myc*, *c-fos* and *c-jun* expression in breast and ovarian carcinoma cells. T47D, SKOV3 and PEO4 cells were cultured as described in Fig. 8 to achieve quiescence, and then treated with ethanol vehicle or 10 nM E_2 in media supplemented with 1, 5 or 10% DCFBS for 1 h. Poly(A) RNA was analyzed by Northern blotting as in Fig. 8.

protease thought to be significant in cell growth and tumor metastasis, is E_2 regulated in responsive ovarian carcinoma cells including BG-1 cells [6] and in PEO4 cells as shown here. However, both the intracellular and secreted forms of cathepsin D were resistant to E_2 regulation and were overproduced in SKOV3 cells compared to PEO4 (Fig. 6) and BG-1 cells (data not shown). Cathepsin D is controlled by multiple effectors including growth factors [41] and is overproduced in ER negative breast cancer cells such as MDA231 cells [11]. It is possible that constant stimulation of cathepsin D expression by growth factors has bypassed estrogen regulation in SKOV3 cells.

HER-2/*neu* protein product was also overexpressed and escaped estrogen regulation in SKOV3 cells. HER-2/*neu* encodes a receptor tyrosine kinase whose overexpression in breast and ovarian cancer is associated with cancer progression [42]. ER binding to regulatory sequences in the HER-2/*neu* promoter is thought to be the mechanism for down regulation [35]. Gene amplification associated with overexpression of HER-2/*neu* in SKOV3 cells [36] may have surpassed the supply of available ER, or resulted in loss of regulatory elements that are critical for down-regulation by ER. Interestingly, ectopic expression of HER-2/*neu* in MCF-7 breast cancer cells has been found to result in antiestrogen resistance [43].

In conclusion, we favor the hypothesis that uncontrolled expression of growth regulatory genes such as HER-2/*neu* and cathepsin D may overwhelm mitogen pathways resulting in nonresponsiveness to growth regulation by estrogen and antiestrogens in SKOV3 cells. And, we propose that the SKOV3 cell line provides a model for stable antiestrogen resistance commonly observed in ER^+ ovarian cancer.

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