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Interaction of Growth Factors During Progression Towards Steroid Independence in T-47-D Human Breast Cancer Cells

Roger J. Daly, Roger J.B. King, and Philippa D. Darbre

Cellular Endocrinology (R.J.D., P.D.D.) and Hormone Biochemistry Laboratories (R.J.B.K.), Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, England

When deprived of steroid in the long term, T-47-D human breast cancer cells lose estrogen sensitivity of cell growth. This loss of response results from an increased basal growth rate in the absence of steroid, not from a loss of estrogen-stimulated growth, and it occurs without any loss of estrogen receptor number or function. Growth factor gene expression and sensitivity have been investigated in this model system in an attempt to unravel the molecular mechanisms underlying the progression to steroid autonomy. The transition was accompanied by a decreased dependence on added serum and by a loss of the stimulatory effects of insulin and basic fibroblast growth factor, but also by an acquired sensitivity to stimulation by transforming growth factor-β (TGF-β). An increase in TGF-β_1 mRNA was detected following loss of steroid sensitivity. There was no increase in epidermal growth factor (EGF) receptor number. These findings are discussed in relation to current knowledge concerning the mechanisms by which estrogens stimulate breast cancer cell proliferation.

Key words: estrogen, EGFR, bFGF, insulin, TGF-β

A major problem in the endocrine therapy of breast cancer concerns the inevitable progression of the tumor cells from a state of steroid sensitivity to insensitivity. Only one-third of clinical cases of metastatic breast cancer respond to endocrine therapy [1,2], and, even in these cases, regression is often temporary and followed by the development of hormone-independent tumors refractory to such treatment. Our approach to unravel mechanisms involved in this loss of steroid sensitivity has been to study the divergence of cloned breast cancer cells in vitro. Studies with S115 mouse mammary tumor cells, which are responsive to androgen and glucocorticoid, have shown that long-term steroid deprivation in tissue culture results in progression from steroid sensitivity to insensitivity by an ordered series of reproducible phenotypic events [3]. This steroid deprivation model system has now been successfully applied also to loss of estrogen sensitivity in

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human breast cancer cells [4–7] provided that phenol red is removed from the culture medium [8]. However, it has recently been shown that MCF-7 sublines unresponsive to growth stimulation by estradiol in vitro may still exhibit an estrogen-responsive phenotype in vivo [9].

Current theories of estrogen action in breast cancer propose that, in estrogen-responsive breast cancer cells, estrogen stimulation of cell growth is mediated by the production of autocrine/paracrine growth factors [10]. Estrogen treatment of MCF-7 human breast cancer cells results in increased transforming growth factor-α (TGF-α)- [11] and an IGF-I-related activity [12,13], whereas administration of antiestrogens increases production of growth inhibitory TGF-β [14]. Conflicting results have been obtained concerning the role of epidermal growth factor (EGF) in estrogen stimulation of growth of breast cancer cells [11,15,16]. Recently it has been shown that IGF-II mRNA is estrogen regulated in T-47-D cells [17]. Progression to steroid independence is suggested to result from constitutive secretion of autocrine growth factors [18]. Transfection of MCF-7 cells with the v-Ha-ras oncogene resulted in estrogen independence [19] and increased secretion of TGF-α, TGF-β, and IGF-I activity [20], although the phenomena may not be linked [21,22], and overproduction of TGF-α alone does not lead to steroid autonomy [23]. Furthermore, in two rodent models, loss of hormone sensitivity is accompanied by decreased TGF-α levels [24]. Synergistic actions of growth factors may prove of great importance [25]. In an attempt to understand the changes in growth control that accompany the transition to steroid independence, we have studied growth factor gene expression and sensitivity as T-47-D human breast cancer cells lose estrogen response following steroid deprivation. Our studies performed within the same cell line will complement other reports comparing different cell lines.

MATERIALS AND METHODS

Culture of Stock Cells

The T-47-D human breast cancer cells were kindly provided by the originators of the cell line to our institute [26] and were of about the same passage generation as in previous experiments [27]. Stock T-47-D cells were grown routinely as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) (containing phenol red) supplemented with 5% fetal calf serum (FCS) (Flow Laboratories, Irvine, Scotland) and 10^{-8} M estradiol in a humidified atmosphere of 10% carbon dioxide in air at 37°C. Cells were subcultured at weekly intervals by suspension with trypsin (see below).

The MCF-7 McGrath cells were kindly provided by Dr. Kent Osborne [28]. The cell lines BT-20, HBL-100, and MDA-MB-231 were obtained from the American Type Culture Collection. Growth was as for the T-47-D cells except for the addition of insulin (10 μg/ml) to MCF-7 McGrath stock cultures and the omission of estradiol for the latter three cell lines.

Stock Cultures for Loss and Recovery of Response Experiments

At the start of each experiment, a new vial of cells was thawed from liquid nitrogen, which ensured that control cells of the starting passage number were available for comparison at any time. Freshly thawed cells were grown for 2 weeks as stock cultures with estrogen (see earlier) and then switched to phenol red-free RPMI 1640 medium (Gibco Bio-Cult, Glasgow, Scotland) containing 5% dextran-charcoal (DC)-

FCS. DC treatment of FCS was as described previously [29] except that the procedure was done twice rather than just once. Cells were subcultured using phenol red-free 0.06% trypsin/0.02% EDTA (pH 7.3) as necessary—after 1 week of steroid deprivation, thereafter every 2–3 weeks during the period of low growth, then increasing until eventually once per week.

For recovery of response, estrogen was added back to the steroid-depleted medium so that culture conditions were kept the same throughout except for the presence or absence of estrogen. Cells were assayed from these cultures for estrogen-responsive growth and molecular parameters.

Cell Growth Experiments

Cells were suspended from stock plates by treatment with phenol red-free 0.06% trypsin/0.02% EDTA (pH 7.3), added to an equal volume of RPMI 1640 lacking phenol red with 5% DC-FCS, and counted on a hemocytometer. Cells were added to the overall required volume of medium RPMI 1640 lacking phenol red/5% DC-FCS at a concentration of 0.2×10^5 cells/ml and plated in monolayer in either 5 ml aliquots into 5 cm plastic tissue culture dishes or 2.45 ml aliquots into 3.5 cm plastic tissue culture dishes. The medium was changed after 24 h so that dishes contained the appropriate serum concentration, steroid, growth factor, and/or growth factor antibody. For serum-free culture, cells were still plated in 5% DC-FCS but after 24 hr were washed several times with RPMI 1640 medium alone and then grown in RPMI 1640 medium containing 15 mM Hepes (BDH Chemicals Ltd.), 2 μg/ml transferrin (Sigma), 0.75 μg/ml fibronectin (Sigma), and 0.1% bovine serum albumin (Sigma fraction V). Steroids were dissolved in ethanol and added to give an ethanol concentration of 0.01% in culture. Insulin (Sigma) was dissolved in 6 mM HCl, basic fibroblast growth factor (bovine pituitary, Collaborative Research Inc.) in water, and TGF-β_1 (porcine platelet, R. & D. Systems), in 4 mM HCl/1 mg/ml BSA. TGF-β blocking antibody (R. & D. Systems) was reconstituted according to manufacturers instructions. Growth factor solutions were diluted 1 in 1,000 or 10,000 in culture, and control dishes contained vehicle alone. Culture medium was changed routinely every 3–4 days.

Cell Counting

All cell counts were done in triplicate on triplicate or quadruplicate dishes, and results were calculated as the mean ± SE. Doubling time of the cells was calculated as described previously [3]. Cells in monolayer were washed with saline in situ and lysed in 2 ml 0.01 M HEPES buffer/1.5 mM $MgCl_2$ plus four drops of Zaponin (Coulter Electronics Ltd., Harpenden, England) for 5 min. The nuclei released were counted in Isoton (Coulter Electronics Ltd.) in a model ZB1 Coulter counter.

Preparation and Analysis of RNA by Northern Blotting

A minimum of 3 ×14 cm dishes of cells were used for each RNA preparation. Cells were washed in situ with phosphate-buffered saline (PBS) and harvested with a rubber policeman into ice-cold PBS. The cells were pelleted and whole-cell RNA was made by the guanidinium-cesium chloride method [30].

Total cellular RNA was subjected to electrophoresis in 1.5% agarose-formaldehyde gels [30] and was transferred onto Hybond-N membranes (Amersham International, Amersham, England). Northern blots were hybridized to a 1.05 kb Eco RI

fragment corresponding to λβ CI [31] or a 960 bp skeletal muscle actin cDNA ^{32}P-labeled by primer extension (kit from Amersham International) using 10^6 cpm/ml hybridization buffer. Hybridization was at 42°C for 18 hr in 5×SSPE/5×Denhardt's solution/50% formamide/0.5% sodium dodecyl sulfate (SDS)/20 μg/ml salmon sperm DNA. Blots were washed at a stringency of 0.1×SSPE/0.1% SDS at 65°C for 30 min and autoradiographed on Kodak XAR film using intensifying screens at −70°C.

Immunoassay of Estrogen and Progesterone Receptors

Cells from two 14 cm dishes were washed in situ with PBS and harvested with a rubber policeman into ice-cold PBS. Cells were pelleted and stored frozen in liquid nitrogen. For estrogen receptor assays, freeze-fractured cell pellets were homogenized in the ratio 1:8 parts buffer (10 mM Tris HCl, 1 mM EDTA, 2 mM dithiotheitol (DTT), 10% glycerol, 0.5 M NaCl, 4 mM leupeptin, pH 7.4) at 4°C for ten passes through a Teflon-glass homogeniser. Homogenates were centrifuged at 40,000 rpm for 1 h at 4°C in a Beckman 50Ti rotor, and cytosols were stored at −70°C.

For progesterone receptor assays, cytosols were prepared in the same way but using a different buffer: 10 mM Tris HCl, 1.5 mM EDTA, 5 mM sodium molybdate, 1 mM monothioglycerol. The high levels of NaCl and DTT used in the estrogen receptor cytosol interfered with the progesterone receptor antibody assay. Estrogen receptor and progesterone receptor assays were performed using commercially available enzyme immunoassay kits following the manufacturer's instructions (Abbott Laboratories).

Transient Transfection Assays Using Estrogen Response Element (ERE) Thymidine Kinase (tk)-CAT

Function of the estrogen receptor in long-term steroid-deprived cells was assayed by estrogen regulation of expression of an estrogen-sensitive gene transfected transiently into the cells. The construct used consisted of the ERE of the vitellogenin A2 gene from −331 to −295 bp cloned into the pBLCAT2 vector [32] upstream of the tk promoter.

Efficiency of transfection and normalization of results was by β-galactosidase assays [33]. The construct used (termed pJ3-β gal) consisted of the coding region of the β-galactosidase gene linked at the 5′ end to SV40 promoter/enhancer sequences and at the 3′ end to Simian virus 40 (SV40) splice/poly-A signals.

Cells were grown in monolayer culture in 5 cm tissue culture dishes in DMEM lacking phenol red with 5% DC-FCS from a density of 5×10^5 cells/dish for 3 days. Cells were then transfected for 6 h with 10 μg of ERE-tk-CAT DNA and 0.5 μg of pJ3-β gal DNA per dish using the calcium phosphate coprecipitation method [34]. Cells were washed in phenol red-free DMEM, shocked with 25% glycerol in DMEM for 1 min, and incubated overnight in phenol red-free RPMI 1640 medium with 5% DC-FCS. The following day, the medium was changed to 5% DC-FCS in RPMI 1640 containing either 10^{-8} M estradiol or ethanol vehicle alone. Cells were harvested 48 h later, and assays for CAT activity [35] and β-galactosidase activity [33] were performed as described previously.

EGF Receptor Assays

EGF receptor assays were performed on intact cells by the method of Murphy et al. [36] using ^{125}I-EGF and multipoint Scatchard analysis.

RESULTS

Steroid Deprivation Model

Under conditions of long-term steroid deprivation, T-47-D human breast cancer cells lose their growth response to estradiol (Fig. 1). Loss of response resulted not from a reduction in the estrogen-stimulated growth rate but rather from an up-regulation of growth in the absence of steroid, such that after 32 weeks of steroid deprivation the doubling time of the cells was the same in the presence and in the absence of steroid. The initial sharp decline in growth without steroid is due to loss of estrogen memory effects from previous growth in estrogen-containing medium [27]. After an initial increase in estrogen receptor (ER) levels during the first week of withdrawal, the cells retained the same amount of salt-extractable ER between 1 week and 58 weeks of steroid deprivation (Fig. 1).

To assess whether the estrogen receptor remained functional, molecular markers of estrogen sensitivity were followed during steroid deprivation. Transient transfection of an exogenous estrogen-sensitive gene (ERE-tk-CAT) was used as a molecular marker of estrogen receptor function. Even after long-term steroid deprivation, a fourfold estrogen induction of CAT activity was observed (Fig. 2). Control experiments with estrogen maintained cells showed a two- to sixfold induction of CAT activity (data not shown). Progesterone receptor [37] and pS2 mRNA [38,39] were not useful as markers of

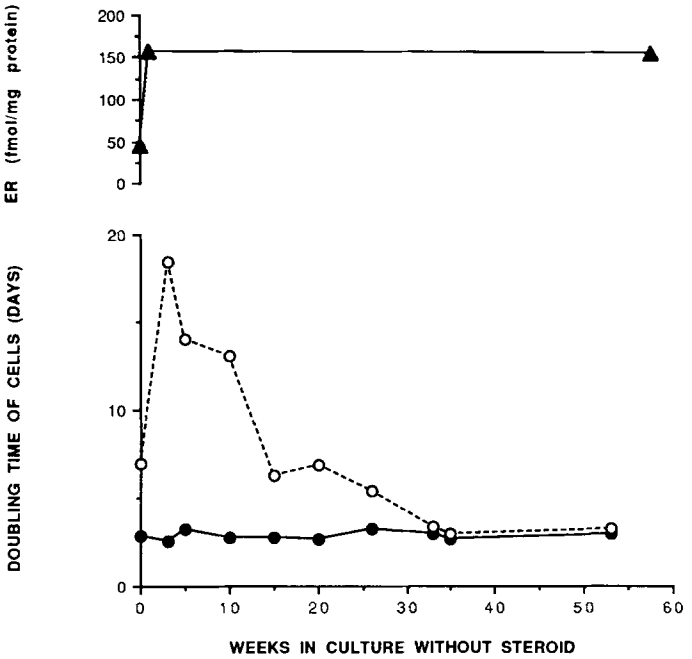

Fig. 1. Changes in the rate of proliferation and estrogen receptor (ER) content of T-47-D human breast cancer cells in monolayer culture following increasing periods of steroid withdrawal from stock cultures. Proliferation rate is expressed as the doubling time of the cells, estrogen receptor in fmol/mg protein. Cells were grown for increasing lengths of time in 5% DC-FCS without added steroid or phenol red, and at intervals cell proliferation rates were assessed in the short-term presence (●) or absence (○) of 10^{-8} M estradiol and estrogen receptor levels measured in the absence of steroid (▲).

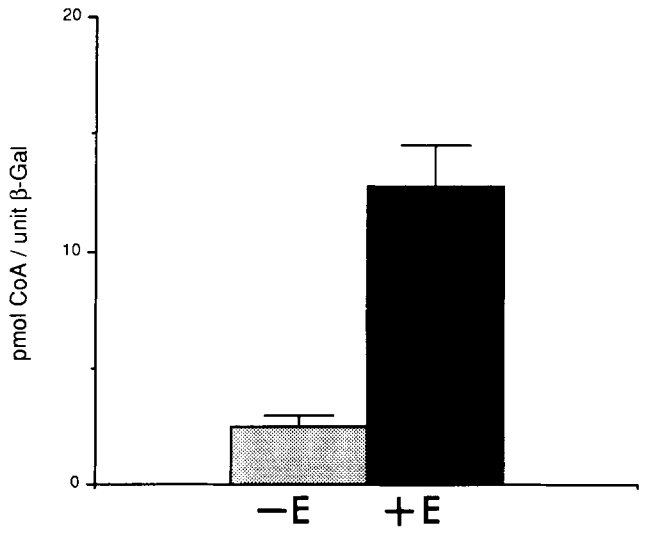

Fig. 2. Assay of estrogen receptor function by transient transfection with ERE-tk-CAT DNA in long-term steroid-deprived T-47-D human breast cancer cells in monolayer culture. Cells were grown for 76 weeks in 5% DC-FCS without added steroid or phenol red, transfected for 6 h with ERE-tk-CAT and pJ3-β gal DNA, and then grown for 48 h with (+E) or without (−E) 10^{-8} M estradiol. CAT activity is expressed as pmols of ^{14}C-acetyl group transferred from ^{14}C-acetyl CoA to chloramphenicol per hour and normalized per unit of β-galactosidase activity.

estrogen response in these cells. Progesterone receptor levels were high (1,400–1,450 fmol/mg protein) in stock cells and were not affected to any great extent by estrogen deprivation or readdition [7]. pS2 mRNA was not detectable in our T-47-D cells [6,7].

Parallel Changes in Sensitivity to Exogenous Growth Factors

Long-term steroid deprivation resulted in an alteration in sensitivity of the cells to serum concentration (Fig. 3). Although the steroid-deprived cells respond to increased serum by a small stimulation of growth, they grow faster in low serum than their estrogen-maintained counterpart. This was most marked in the absence of estradiol.

Proliferation of stock estrogen-maintained T-47-D cells is stimulated in a dose-dependent manner by both bFGF (Fig. 4) and insulin (Fig. 5). Interestingly, stimulation by these growth factors is seen in both the presence and the absence of estradiol. However, at optimal levels of bFGF, estradiol was still able to stimulate proliferation. In responsive cells, the insulin dose-response curve was changed in the presence of estradiol such that maximal effects were seen with a tenfold lower concentration of insulin. Long-term steroid deprivation resulted in loss of these responses to bFGF and insulin (Figs. 4, 5). Effects of bFGF were tested in both 5% DC-FCS (Fig. 4) and 1% DC-FCS (data not shown). Stimulatory action of bFGF in the absence of estradiol was greater at 1% than at 5% serum, such that 100 ng/ml bFGF increased the proliferation rate above that with estradiol, but the effect of bFGF on estrogen-stimulated growth in 1% serum was not tested. However, all response to bFGF was lost in the steroid-deprived cells at 1% as well as at 5% DC-FCS. Response to insulin was lost in the steroid-deprived cells not only in 5% DC-FCS but also in serum-free conditions (data not shown).

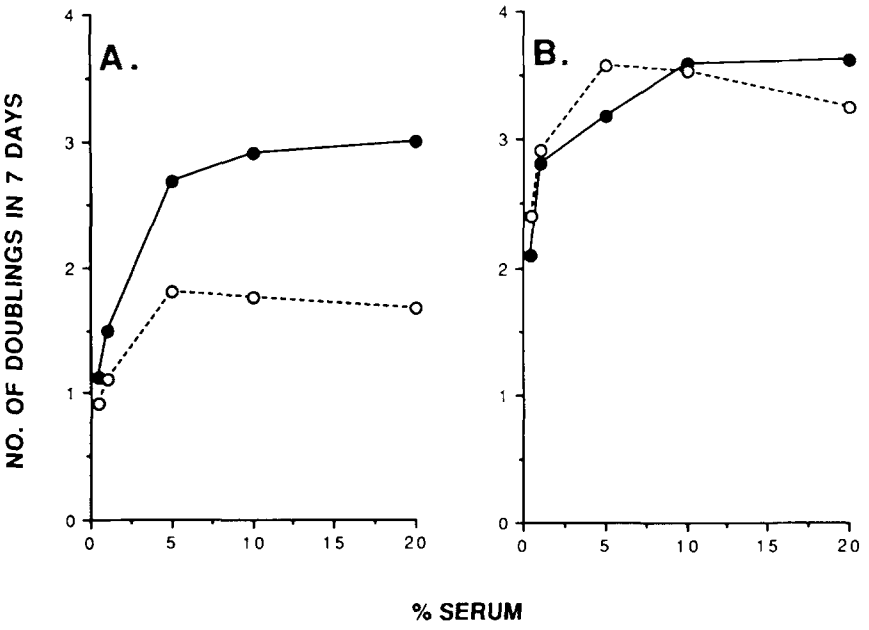

Fig. 3. Serum sensitivity of T-47-D human breast cancer cells in monolayer culture following 0 weeks (A) or 66 weeks (B) of steroid deprivation. Cells grown long-term without steroid were cultured in 5% DC-FCS without added steroid or phenol red. Growth rate was assessed over 7 days at varying concentrations of DCFCS either with (●) or without (○) 10^{-8} M estradiol.

Our stock T-47-D cells showed no response to TGF-β in either 5% DC-FCS (data not shown) or 1% DC-FCS (Fig. 6, columns 1–4). Long-term steroid deprivation of these cells resulted in an acquired sensitivity to TGF-β such that it gave a small stimulation of growth both in the absence ($P < 0.1$) and presence ($P < 0.05$) of estradiol. The more significant stimulation in the presence of estradiol may be due to inhibition of TGF-β production by this steroid, as reported by Knabbe et al. [14]. This stimulation was seen in 1% DC-FCS (Fig. 6, columns 5–8) but not in 5% DC-FCS (data not shown). This stimulation was reproduced but not enhanced when serum-free conditions were employed (data not shown). For comparison, administration of TGF-β (20 pM) to stock ZR-75-1 human breast cancer cells in 5% DC-FCS resulted in a 30% inhibition of growth in both the presence and the absence of estradiol (data not shown). By contrast, addition of a neutralizing TGF-β antibody to long-term steroid-deprived cells resulted in a small inhibition of growth in the absence ($P < 0.05$) but not in the presence ($P > 0.1$) of estradiol.

Growth Factor Gene Expression

T-47-D cells maintained in the long-term absence of steroid exhibited elevated TGF-β_1 mRNA levels compared with stock cells (Fig. 7). Although large amounts of TGF-β_1 mRNA were also detected in the steroid-independent, ER-negative breast cancer cell lines BT-20, HBL-100, and MDA-MB-231, high levels were also observed in MCF-7 McGrath cells, which are steroid dependent for growth [6].

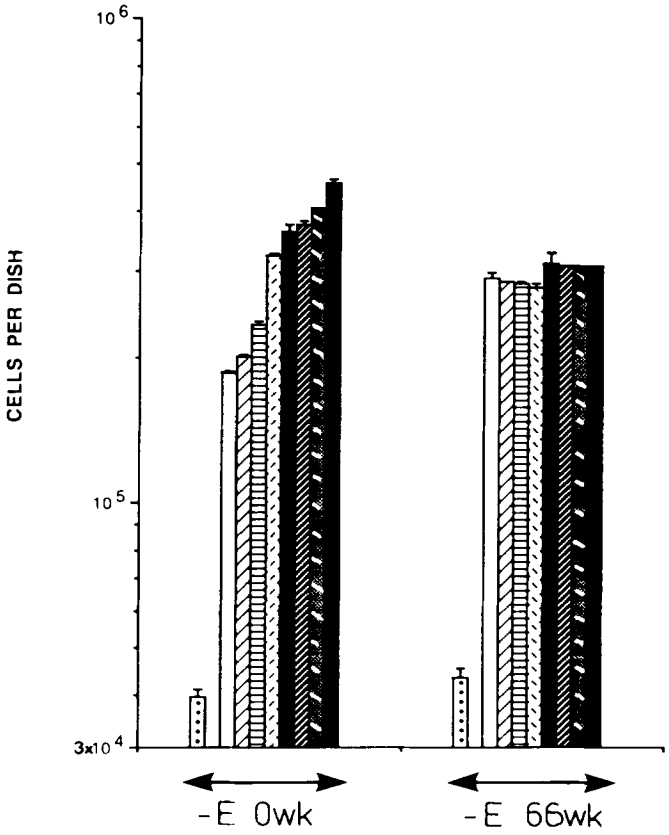

Fig. 4. Sensitivity to basic fibroblast growth factor (bFGF) of T-47-D human breast cancer cells in monolayer culture following 0 weeks or 66 weeks of steroid deprivation. Cells grown long-term without steroid were cultured in 5% DC-FCS without added steroid or phenol red. Cells per 3.5 cm culture dish were counted on day 0 (from left to right, first bar) and then after 12 days (stock cells) or 7 days (steroid-deprived cells). Cells were grown in 5% DC-FCS without any addition (second bar), 1 ng/ml bFGF (third bar), 10 ng/ml bFGF (fourth bar), 100 ng/ml bFGF (fifth bar), 10^{-8} M estradiol (sixth bar), 10^{-8} M estradiol + 1 ng/ml bFGF (seventh bar), 10^{-8} M estradiol + 10 ng/ml bFGF (eighth bar), 10^{-8} M estradiol + 100 ng/ml bFGF (ninth bar). Marks show SE of triplicate dishes. Where no error marks are shown, error was too low for visual display.

EGF Receptors

Radioreceptor assays indicated about 2,000 sites/cell for EGF receptors in stock estrogen-maintained T-47-D cells. Even after 70 weeks of steroid deprivation, this value did not change.

DISCUSSION

After long-term steroid deprivation, T-47-D breast cancer cells were found to exhibit the same growth rate in the presence and in the absence of added estradiol. This loss of response resulted from an up-regulation of the basal growth rate in the absence of steroid. Continued presence of estrogen receptors measurable by enzyme immunoassay

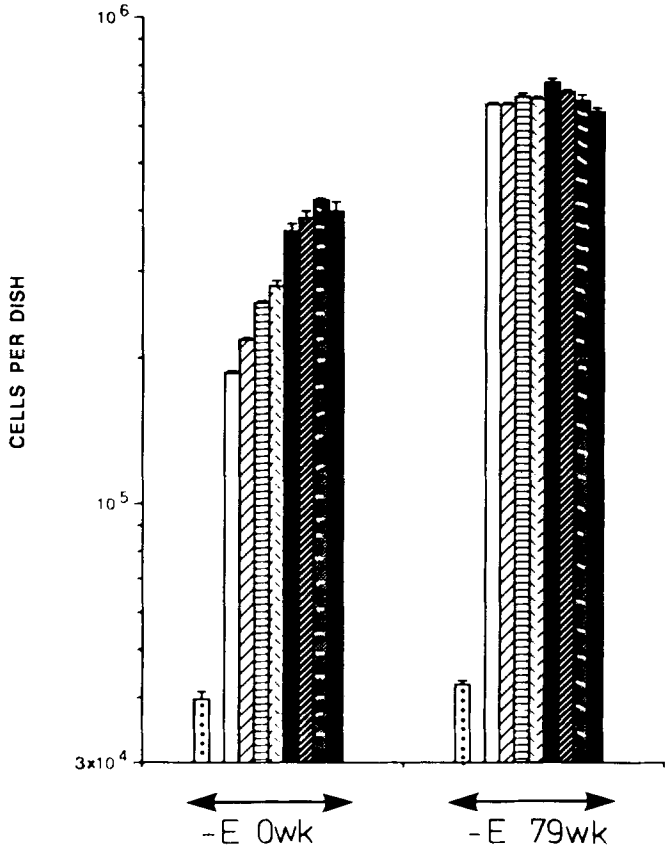

Fig. 5. Sensitivity to insulin of T-47-D human breast cancer cells in monolayer culture following 0 weeks or 79 weeks of steroid deprivation. Cells grown long-term without steroid were cultured in 5% DC-FCS without added steroid or phenol red. Cells per 3.5 cm culture dish were counted on day 0 (from left to right, first bar) and then after 12 days (stock cells) or 7 days (steroid-deprived cells). Cells were grown in 5% DC-FCS without any addition (second bar), 100 ng/ml insulin (third bar), 1 μg/ml insulin (fourth bar), 10 μg/ml insulin (fifth bar), 10^{-8} M estradiol (sixth bar), 10^{-8} M estradiol + 100 ng/ml insulin (seventh bar), 10^{-8} M estradiol + 1 μg/ml insulin (eighth bar), 10^{-8} M estradiol + 10 μg/ml insulin (ninth bar). Marks show SE of triplicate dishes. Where no error marks are shown, error was too low for visual display.

and continued induction of an estrogen-sensitive molecular marker (CAT activity) shows that these changes in cell growth occurred without any loss of estrogen receptor number or function. However, not all estrogen-regulated growth properties were lost; growth of the cells could still be inhibited by tamoxifen, and this inhibition could be reversed with estradiol [7].

The involvement of genotypic/phenotypic mechanisms and the role of cell selection in tumor progression has long been a central question. In this respect, we have argued previously for a phenotypic/epigenetic mechanism for loss of androgen response in S115 mouse mammary tumor cells [3,40]. Similarly, loss of estrogen response following steroid deprivation of recloned steroid dependent ZR-75-1 human breast cancer cells occurs at a frequency of approximately 1 in 10^3 [6,7], indicating changes across a wide proportion of the cell population. However, the mechanism for the observed

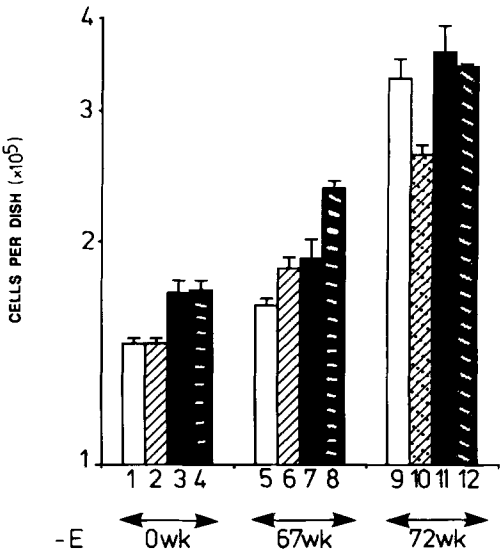

Fig. 6. Effects of TGF-β (columns 1–8) and TGF-β neutralizing antibody (columns 9–12) on growth of T-47-D human breast cancer cells in monolayer culture following 0 weeks or long-term steroid deprivation. Cells grown long-term without steroid were cultured in 5% DCFCS without added steroid or phenol red. Cells per 3.5 cm culture dish were counted on day 0; $9.6 \pm 0.3 \times 10^4$ (−E 0 wk), $4.7 \pm 0.2 \times 10^4$ (−E 67 wk), $5.4 \pm 0.1 \times 10^4$ (−E 72 wk) and then after 7 days (columns 1–8) or 8 days (columns 9–12). Cells were grown in 1% DC-FCS (columns 1–8) or under serum-free conditions (columns 9–12) without any addition (columns 1,5,9), 10^{-10} M TGF-β (columns 2,6) 10^{-8} M estradiol (columns 3,7,11), 10^{-8} M estradiol + 10^{-10} M TGF-β (columns 4,8), 25 μg/ml TGF-β neutralizing antibody (column 10), 10^{-8} M estradiol + 25 μg/ml TGF-β neutralizing antibody (column 12). Marks show SE of triplicate dishes.

loss of estrogen sensitivity in T-47-D cells remains open, especially in view of the recently reported genetic instability in this cell line [41,42]. This instability may be reflected in the fact that the stock, estrogen-maintained T-47-D cells used in this study exhibit constitutive progesterone receptor expression and an absence of pS2 mRNA and consequently already differ in the expression of two estrogen-regulated genes compared with some T-47-D lines [6,41,42] and other estrogen-responsive breast cancer cell lines [10]. Whatever the mechanism involved in loss of steroid sensitivity, steroid deprivation of T-47-D cells provides a model system for comparing responsive and unresponsive cells derived from the same cell line.

The loss of estrogen sensitivity was not associated with the development of a classical estrogen-independent cell line, since the adapted cells retained estrogen receptor. In addition, there was no increase in EGF receptor levels, another parameter associated with ER-negative cell lines [43] and tumors [44]. Elevated levels of mRNA for the nuclear protooncogene c-*myc* have also been detected in ER-negative cell lines [45] and tumors [10], but we found no increase in c-*myc* mRNA in steroid-deprived T-47-D cells (data not shown). This would suggest that loss of estrogen receptor and up-regulation of EGF receptor and c-*myc* mRNA are not prerequisites for development of steroid insensitivity but instead may occur as later events in tumor progression.

In view of current evidence for the involvement of growth factors in autocrine/paracrine regulation of mammary epithelial cell proliferation (see Introduction), growth factor gene expression and sensitivity were studied in these cells during steroid depriva-

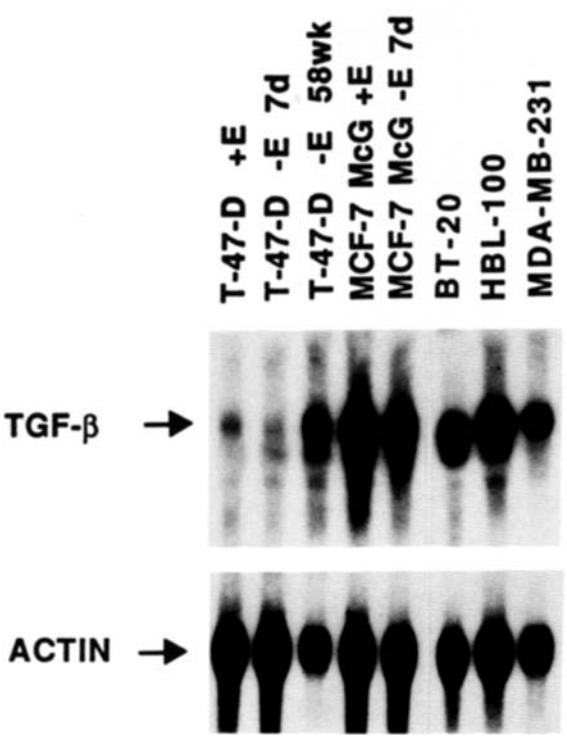

Fig. 7. Expression of TGF-β_1 mRNA in human breast cancer cell lines. Northern blots of whole cell RNA (10 μg) from T-47-D and MCF-7 McGrath cells grown either continuously with 10^{-8} M estradiol (+E) or after varying periods of estrogen deprivation (−E) (d, days; wk, weeks). Estrogen receptor-negative cell lines, BT-20, HBL-100, MDA-MB-231, were grown without added steroid. Blots were hybridized to an actin probe as a control.

tion in an attempt to unravel the molecular mechanisms underlying these changes in growth control. Loss of sensitivity to estrogen in T-47-D cells appeared to be paralleled by a change in sensitivity to serum growth factors and an acquired ability to grow faster at low serum levels. It remains in question whether this reflects an increased sensitivity to lower concentrations of serum growth factors or development of an independence from them. However, the latter possibility is supported by the fact that steroid-deprived T-47-D cells proliferate more rapidly in serum-free culture than their steroid-maintained counterparts.

Stock T-47-D cells were unaffected by TGF-β in accordance with other reports on this cell line [46,47]; by contrast, bFGF and high concentrations of insulin had stimulatory effects, also in line with results from other laboratories [47,48]. Loss of sensitivity to estrogen was paralleled by a loss of response to both insulin (although IGF-I receptors could still be detected on steroid-deprived T-47-D cells; data not shown) and bFGF. However, acquisition of steroid autonomy was paralleled by an acquired sensitivity to stimulation by TGF-β, inhibition by anti-TGF-β neutralizing antibody, and increased TGF-β_1 mRNA. TGF-β has been presented as a potential autocrine growth inhibitor of breast cancer cells [14], but TGF-β is a multifunctional growth factor and the nature of its action on any target cell depends not only on the cell type but also on its state of

differentiation and on other growth factors present [49–52]. This change in sensitivity to TGF-β may be due to an increase in TGF-β receptor number, and we are currently investigating this possibility. In this context, Arteaga et al. [46] could not detect TGF-β receptors on stock T-47-D cells grown in their laboratory. The level of active TGF-β protein secreted by the cells is also under investigation as TGF-β production by breast cancer cell lines has been shown to be posttranscriptionally regulated [14]. Although Arteaga et al. [46] reported increased secretion of TGF-β by ER-negative breast cancer cell lines relative to ER-positive lines, it is clear from our data that expression of TGF-β_1 mRNA does not correlate simply with loss of steroid sensitivity; large amounts of this mRNA are produced both by the ER-negative cell lines investigated and steroid-dependent MCF-7 McGrath cells.

Since T-47-D cells also produce mRNAs for two other autocrine growth factors, TGF-α [53] and IGF-II [17], we have also investigated the levels of these two mRNAs in stock and steroid-deprived T-47-D cells. Interestingly, no up-regulation of these mRNAs (relative to actin mRNA) was observed in the steroid-deprived cells [7]. Therefore, the only change in growth factor gene expression we have observed during loss of steroid sensitivity is a large increase in TGF-β_1 mRNA. We believe that this, coupled with an acquired sensitivity to stimulation by TGF-β, probably represents a component of a more complex, interacting system.

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