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TRANSITION OF HUMAN BREAST CANCER CELLS FROM AN OESTROGEN RESPONSIVE TO UNRESPONSIVE STATE

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Summary—An *in vitro* model system is described for studying the problem of loss of steroid sensitivity in breast cancer cells. Growth of cloned oestrogen-sensitive human breast cancer cells in the long-term absence of steroid gives rise to a population of oestrogen-insensitive cells. In ZR-75-1 cells, the effect is clonal but occurs at high frequency suggesting a mechanism affecting a wide proportion of the cell population synchronously. This does not involve any reduction in oestrogen receptor number. Furthermore, there is no coordinated loss of oestrogen-sensitive molecular markers, showing that oestrogen receptors remain not only present but functional. These growth changes are not accompanied by any loss of growth inhibition by antioestrogen. Although steroid deprivation does not result in loss of oestrogen-sensitive markers, this does not hold true for other steroids. There was a reduction in progestin, androgen and glucocorticoid regulation on transfected LTRs. Loss of steroid-sensitive growth was accompanied by changes in response to exogenous growth factors and altered endogenous growth factor mRNA production. Steroid-deprived T-47-D cells acquire sensitivity to stimulation by TGFβ and have raised TGFβ_1 and TGFβ_2 mRNA levels. ZR-75-1 cells are growth inhibited by TGFβ and have reduced TGFβ_1 mRNA levels. In MCF-7 cells, increased IGFII mRNA, following transfection, can result in an increased basal cell growth rate in the absence of steroid. These findings are discussed in relation to possible autocrine mechanisms in the loss of steroid sensitivity of breast cancer cells.

INTRODUCTION

Alteration of the steroid environment, particularly by reduction in oestrogen levels and antagonism of oestrogen action, can reduce breast tumour growth in many species [1, 2] and this forms the basis for endocrine therapy of breast cancer. However, only 30% of human breast cancers respond to such therapy and even of those, regression is invariably temporary and is followed by growth of steroid unresponsive tumours and metastatic disease. This is a major clinical problem.

Whilst the growth of both normal and tumour mammary cells can be regulated by steroid hormones, little is known about the mechanism by which the tumour cells lose this control on proliferation. The origin of mammary tumours is now accepted to be monoclonal [3] and the loss of steroid sensitivity occurs during tumour progression, at a time when the tumour cells undergo many diverse changes [reviewed 4]. It has been implied in the past that this progression to insensitivity arises as a result of a mutation event followed by cell selection [5–8] but evidence is now emerging for a phenotypic or epigenetic mechanism involving stable alterations in the programme of gene expression across a large proportion of the cell population [4, 8–12] which may not simply represent selection of the most aggressive phenotype [13].

One long-held view assumes that progression of breast cancers from steroid sensitive to insensitive state is caused by a loss of steroid receptor. Some tumours do lose receptors for oestrogen and progesterone but there remain many tumours which retain these receptors and yet do not respond to endocrine therapy [14, 15]. Indeed, it remains to be proved whether receptor loss found in some tumours is actually a cause or merely a consequence of loss of response [16]. Cells in culture can evidently lose response to steroid without any loss of receptor [17].

Loss of response could equally result from perturbations in other cell proliferation mech-

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anisms within the cell, independent of steroid receptor-related pathways. In recent years, there has been increasing evidence to indicate growth factor involvement in steroid regulation of breast cancer cell growth by both autocrine and paracrine mechanisms [18]. Steroid independence could result from uncoupled growth factor regulation by either constitutive secretion of a stimulatory growth factor or decreased secretion of an inhibitory growth factor in an autocrine or paracrine mode.

Our approach to unravel mechanisms involved in this loss of steroid sensitivity has been to study the divergence of cloned steroid-responsive breast cancer cells in tissue culture. Growth of such cells in the long-term absence of steroid results in a loss of steroid sensitivity. Originally based on the androgen-sensitive S115 mouse mammary tumour cell line [4, 9], the studies have been successfully extended to oestrogen-sensitive human breast cancer cell lines [12, 19–21] provided phenol red is removed from the culture medium [22]. This development of steroid insensitive cells from cloned steroid sensitive cells allows direct comparisons to be made within the same cell line, rather than using off-the-shelf responsive and unresponsive cell lines where the relationship between the lines is unknown. Thus, we have exploited this model system to investigate mechanisms involved in loss of steroid response, particularly in relation to the role of steroid receptors and involvement of growth factors.

LOSS OF GROWTH RESPONSE TO OESTROGEN

Growth of the human breast cancer cell lines ZR-75-1 [23] and T-47-D [24] is regulated *in vitro* by oestrogen. Removal of the weakly oestrogenic phenol red from the culture medium [22] allows further characterization, showing that ZR-75-1 cells are dependent on oestrogen for growth and are unable to proliferate in its absence, while T-47-D cells are responsive to oestrogen always growing to a limited extent in its absence [25]. Long-term growth of these cells in the absence of steroid results in loss of oestrogen-stimulated growth by a mechanism involving upregulation of the basal growth rate in the absence of steroid (Fig. 1). There is no effect on the oestrogen-stimulated growth rate. Similar patterns for this increase in basal cell growth rate were found with both the parent line and cells recloned at the start of the experiment, indicating that it does not result simply from outgrowth of unresponsive cells already present in the parent line. Furthermore, the similarity of these time courses both within and between cell lines is analogous to the ordered, reproducible series of events observed in loss of androgen/glucocorticoid sensitivity in S115 cells [9] showing that similar results are obtainable in a variety of steroid-responsive cell systems.

The role of cell selection in the loss of steroid sensitivity has long been a central question. In this respect, the ZR-75-1 cell model has been particularly useful. These cells, being dependent on oestrogen for growth, do not grow at all in the absence of steroid for 15 weeks before escaping from growth control (Fig. 1). Because of this 15-week period, it is possible to leave ZR-75-1 cells on the same culture dish without subculturing to monitor clonal growth as the cells adapt to growing autonomously. Hundreds of clones appeared simultaneously (Fig. 2). Adaptation to steroid insensitivity in these ZR-75-1 cells thus appears to be clonal but occurs at a frequency of at least one clone per thousand cells plated [12]. In addition, individual clones of cells were isolated as they appeared and oestradiol sensitivity monitored. The clones were not immediately unresponsive but turned so with time both synchronously and at the same rate as the whole parent line [12]. Since published figures indicate a mutation rate for cancer cells in culture of much lower rates than the 10^{-3} found here [26, 27], the high frequency and synchrony of appearance of loss of response in ZR-75-1 cells indicates a mechanism involving phenotypic changes across a wide proportion of the cell population.

Recent reports of genetic instability leading to gross changes in DNA content in the T-47-D cell line has been suggested to contribute to progression of these cells [28]. However, it has not been proved that this has any linkage to loss of steroid sensitivity. Our parent T-47-D cell line was a mixture of hyperdiploid and hypertetraploid cells, and loss of response was associated with development of a hyperdiploid population and loss of the hypertetraploid population (Fig. 3). However, our recloned T-47-D cell line which was hyperdiploid, did not undergo any change in DNA ploidy upon loss of steroid sensitivity (Fig. 3). This indicates that gross changes in DNA ploidy are not a prerequisite for loss of oestrogen growth response.

Removal of phenol red from the culture medium reveals that monolayer growth of hu-

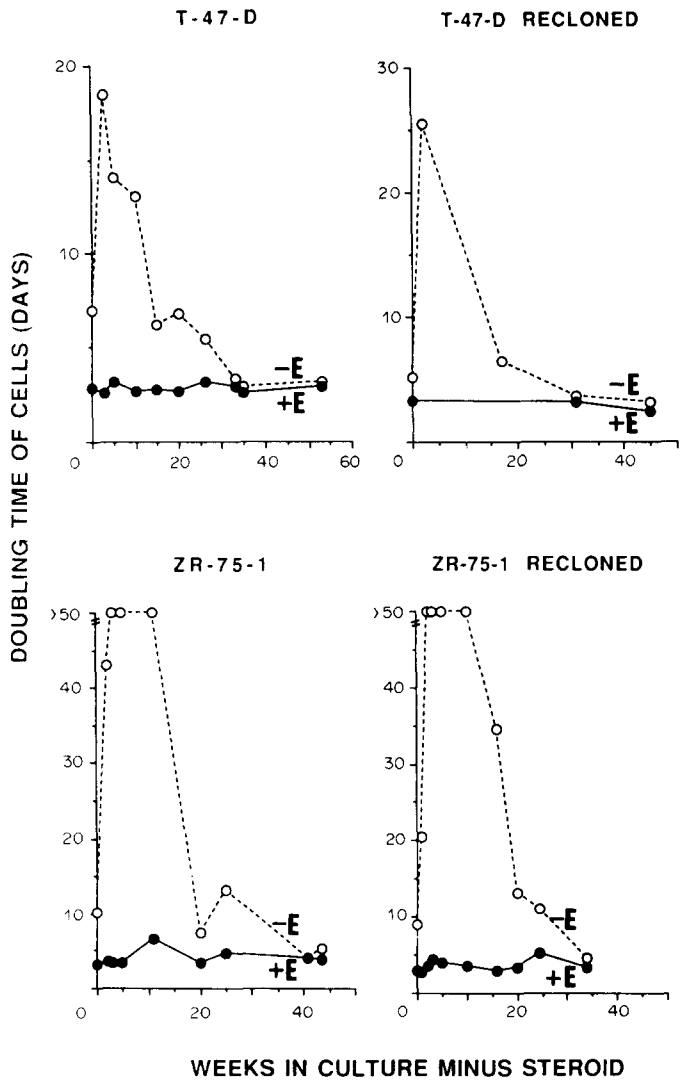


Fig. 1. Changes in the rate of proliferation of T-47-D and ZR-75-1 human breast cancer cells in monolayer culture in the short-term presence (●—● +E) or absence (○---○ −E) of 10^{-8} M oestradiol following increasing periods of long-term steroid withdrawal from stock cultures. Cells were grown long-term in phenol red-free RPMI 1640 medium with 5% DC-FCS only and aliquots of cells were assayed periodically for short-term growth rate in the same medium with or without oestradiol. Results are shown for stock parental lines and for cells recloned at the start of the experiment. Use of recloned cells ensured that effects observed were not simply the result of selecting a population of cells already present in the parental line.

man breast cancer cells is stimulated not only by oestrogen but also by glucocorticoid, progestin and androgen [21]. Loss of growth response to oestrogen is paralleled by a loss of response to these other steroids also [21]. This is in accordance with data for S115 cells, where removal of steroid resulted in loss of response not only to androgen but also to glucocorticoid [29]. In this latter model, there were cross-protective effects between the two steroids in that androgen protected not only against loss of androgen response but also glucocorticoid response and vice versa [29]. It remains to be established whether any other steroid can protect against loss of oestrogen response in the human breast cancer cells, but this will be interesting in view of the fact that androgen, glucocorticoid and progestin act at similar hormone response elements in the DNA [30, 31] but oestrogen response elements appear to be distinct [32].

MOLECULAR MARKERS

The loss of growth response to oestrogen was not associated with loss of oestrogen receptor numbers, as measured by enzyme immunoassay [12]. In fact, oestrogen receptor levels were raised after steroid deprivation, a phenomenon documented by other workers to be due to downregulation by oestrogen of oestrogen

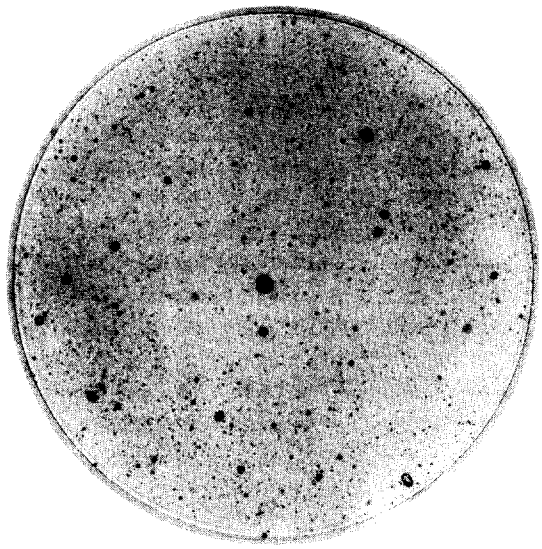


Fig. 2. Photograph of a 9 cm dia dish of recloned ZR-75-1 human breast cancer cells in monolayer culture following 13 weeks of steroid withdrawal. Cells were seeded at 20×10^5 cells per dish and grown in phenol red-free RPMI 1640 medium with 5% DC-FCS only. From this and serial dilutions, it was estimated that the rate of appearance of clones was 1 clone per 1000 cells plated.

receptor mRNA in a post-transcriptional mechanism [33].

Nor was there any coordinated loss of oestrogen receptor function as assayed by three molecular markers of oestrogen action (Fig. 4) [12]. Following steroid deprivation of ZR-75-1 cells, pS2 mRNA remained reinducible by oestradiol and a transiently transfected ERE-tk-CAT gene was oestrogen stimulated in the clones examined. Progesterone receptor remained oestrogen regulated also in ZR-75-1 clone 4 cells. Our T-47-D cells did not possess any detectable levels of pS2 mRNA and had constitutive progesterone receptor levels in excess of 1200 fmol/mg protein irrespective of steroid treatment [12]. However, use of the transient transfection assay of ERE-tk-CAT did show that this molecular marker remained oestrogen-sensitive even after long-term steroid deprivation in T-47-D cells (Fig. 4) [34]. This suggests, then, that changes in cell growth occur without any loss of receptor function, as reported also for MCF-7 [19] and S115 [17] cells.

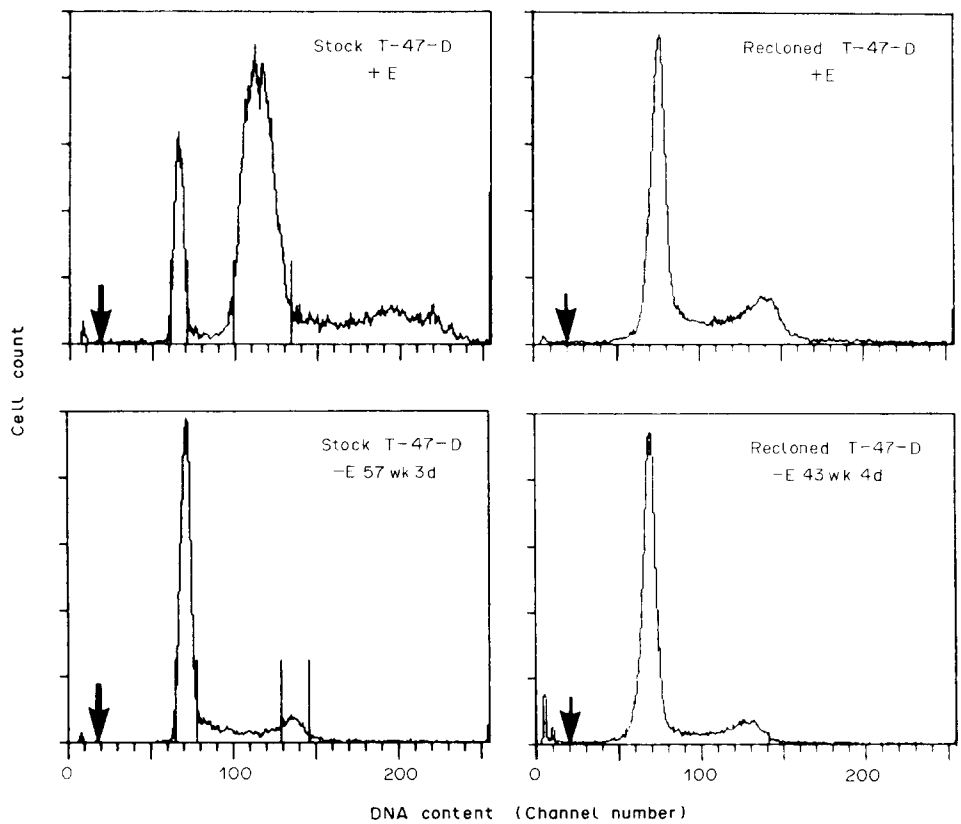
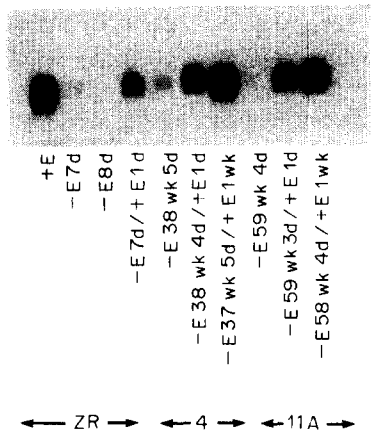


Fig. 3. DNA histograms of T-47-D human breast cancer cells which had been either oestrogen-maintained (+E) or oestrogen-deprived (−E). Oestrogen withdrawal was for the stated number of weeks (wk) and days (d). Data is presented for the stock parental line and for cells recloned at the start of the experiment. The arrows indicate the position of chicken erythrocyte DNA.

A. pS2 mRNA



B. Progesterone receptor

Cells	Steroid treatment	PR (fmol/mg protein)
ZR-75-1	+E	670
ZR-75-1	-E 1wk	50
ZR Clone 4	-E 52wk5d	56
ZR Clone 4	-E 51wk5d/+E 1wk	738
ZR Clone 11A	-E 72wk 4d	1010
ZR Clone 11A	-E 70wk 2d/+E 1wk	1359

C. Transient transfection of ERE-tk-CAT

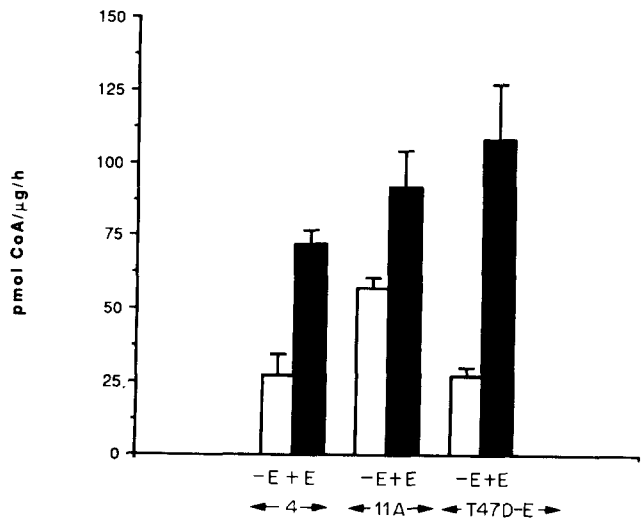


Fig. 4. Oestrogen regulation of molecular markers in ZR-75-1 and T-47-D human breast cancer cells following varying periods of steroid deprivation. (A) Northern blots of pS2 mRNA in ZR-75-1 cells grown either continuously with 10^{-8} M oestradiol (+E) or after varying periods of oestrogen deprivation/readdition as indicated (wk = weeks; d = days). Long-term steroid deprived cells were clones 4 and 11A [12]. (B) Levels of progesterone receptor (PR) in ZR-75-1 cells following varying periods of steroid withdrawal/readdition as indicated (wk = weeks; d = days). PR was measured by enzyme immunoassay (kit from Abbott Laboratories). (C) Assay of oestrogen receptor function by transient transfection with ERE-tk-CAT DNA in long-term steroid-deprived ZR-75-1 and T-47-D cells. Cells were grown for 57 weeks (ZR-75-1 clone 4), 77 weeks (ZR-75-1 clone 11A) or 76 weeks (T-47-D) without steroid, transfected for 6 h with ERE-tk-CAT and then grown for 48 h with (+E) or without (−E) 10^{-8} M oestradiol [34]. CAT activity is expressed as pmols of [^{14}C]acetyl group transferred from [^{14}C]acetyl CoA to chloramphenicol per hour and normalized per μg protein.

Although there was no general loss of oestrogen sensitivity of molecular markers, the increased levels of progesterone receptor in one clone of ZR-75-1 cells (clone 11A) does suggest that alterations in expression of individual oestrogen-regulated genes can occur. Whatever the relationship of progesterone receptor to cell growth, it would seem that loss of oestrogen-sensitive growth can be associated with either low levels of fully oestrogen inducible progesterone receptor (ZR-75-1 clone 4) or high levels of minimally inducible progesterone receptor (ZR-75-1 clone 11A). This has inevitable consequences for the use of progesterone receptor levels as a marker of oestrogen responsiveness in oestrogen-receptor-positive tumours [35].

By contrast to the retention of oestrogen-inducible genes in human breast cancer cells, in S115 cells loss of growth response is paralleled by loss of molecular markers. There is a loss of androgen and glucocorticoid induction of both endogenous [9] and transfected [17] MMTV-LTR sequences following steroid deprivation, and the irreversible loss of RNA transcribed is accompanied by increased methylation of LTR sequences in the DNA [9]. More recent studies have shown that MMTV-LTR sequences also undergo inactivation in human breast cancer cells [36]. In these experiments, T-47-D cells were stably transfected with a chimaeric LTR-C3 gene [37] which is regulated by progestin, androgen and glucocorticoid but not by oestrogen [38]. In brief, this gene is composed of the hormone regulatory sequences of the MMTV-LTR linked to the coding sequence of a marker gene not normally expressed in T-47-D cells [the rat C3(1) gene for prostatic steroid binding protein]. C3 mRNA is induced to equal extents in stock T-47-D cells by 10^{-8} M dexamethasone, 10^{-8} M testosterone and 10^{-9} M progestin R5020. However, after 22 weeks of steroid deprivation there is a selective loss of induction by the 10^{-9} M R5020 (Fig. 5). Longer periods of steroid withdrawal result in reduced induction by dexamethasone and testosterone also (Fig. 5). Since oestrogen is known to induce progesterone receptor [39], one could suppose that steroid withdrawal simply results in loss of progesterone receptor to explain the loss of progestin action, but in these cells this was not the case since progesterone receptor remained high throughout [36]. Thus, while no loss of gene regulation by oestrogen has been found to accompany the changes in growth control, this may not hold true for genes regulated by other steroids.

ANTIOESTROGEN ACTION

Loss of oestrogen-sensitive growth was not accompanied by any loss of inhibitory effects of

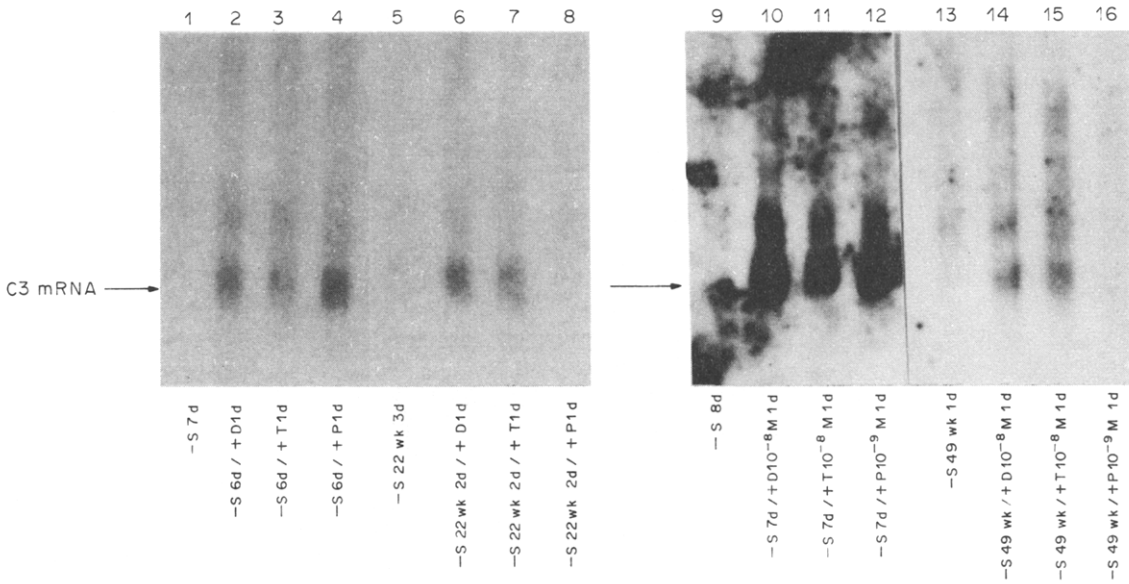


Fig. 5. Steroid hormone regulation of expression of the stably transfected LTR-C3 gene in T-47-D human breast cancer cells following various periods of steroid deprivation. Northern blot analysis of C3 RNA from cells grown without steroid (−S) followed by readdition of 10^{-8} M dexamethasone (D), 10^{-8} M testosterone (T) or 10^{-9} M progestin R5020 (P) for the number of weeks (wk) or days (d) indicated.

antioestrogens, tamoxifen and hydroxytamoxifen, in our T-47-D/ZR-75-1 cells [12, 21] or in MCF-7 cells [19]. This would suggest that the development of antioestrogen resistance is an event separable from loss of oestrogen sensitivity in progression of breast cancer cells. Since the T-47-D/ZR-75-1 cells used had been deprived of steroid for a year, this shows that antioestrogens can act in the complete absence of any oestrogenic stimulus. The mechanism for this action remains unknown except that since the antioestrogenic action was reversible with oestradiol [12], such effects are presumably still mediated via the oestrogen receptor. Vignon *et al.* [40] have shown that antioestrogens can inhibit EGF or insulin stimulated MCF-7 cell proliferation in the absence of oestrogen, although whether antagonism of such pathways occurs by antioestrogen in the oestrogen-insensitive cells remains to be investigated.

INVOLVEMENT OF GROWTH FACTORS

Thus, the question remains as to what causes the upregulation of the basal cell growth if it is not due to oestrogen receptor defects. In view of current evidence for autocrine growth regulation of breast cancer cells (see Introduction), we have studied growth factor gene expression and sensitivity in the cells during steroid deprivation to investigate whether the altered growth could result from increased endogenous growth factor production or an altered sensitivity of the cells to exogenous growth factors. Loss of oestrogen sensitivity was indeed paralleled by a change in sensitivity to serum growth factors [12, 21, 34] which in T-47-D cells resulted in acquired ability to grow faster at low serum levels [12, 34]. It remains in question as to 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 grow more rapidly in serum-free culture than their steroid-maintained counterparts [34].

Comparison of expression of genes for transforming growth factor alpha (TGFα), the insulin-like growth factors (IGFs) and the transforming growth factors beta (TGFβs) between steroid-maintained and steroid-deprived cells revealed a small upregulation of TGFα in steroid-deprived T-47-D cells [12] but the most dramatic alterations were in the TGFβ mRNAs [12, 34]. Progression to steroid autonomy was accompanied in T-47-D cells by upregulation of TGFβ_1 and TGFβ_2 mRNA, and in ZR-75-1 cells by downregulation of TGFβ_1 (Fig. 6) [12, 34]. TGFβ has been presented as a potential autocrine growth inhibitor of breast cancer cells [41] but in general it is a multifunctional growth factor where 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 [42]. Accordingly, these changes which we have observed must be considered in the light of the cell responses to TGFβ. Steroid-maintained T-47-D cells do not respond to TGFβ but steroid deprivation results in an acquired sensitivity to stimulation by exogenous TGFβ and inhibition by exogenous TGFβ antibodies [34]. Whilst, on the other hand, both steroid-maintained and steroid-deprived ZR-75-1 cells remain inhibited by TGFβ [12]. In some cells, such as NRK-49F fibroblasts, it has been reported that addition of exogenous TGFβ can autoregulate endogenous levels of TGFβ expression [43]. However, such a mechanism does not appear to contribute to the cellular responses in our steroid-deprived cells (Fig. 7). Changes in TGFβ expression after steroid deprivation could contribute, therefore, to loss of steroid sensitivity in both T-47-D and ZR-75-1 cells by upregulation of a growth stimulator in the former system and downregulation of a growth inhibitor in the latter.

The IGFs have also been implicated in growth regulation of breast cancer cells [18, 44–46] and upregulation of IGFII mRNA has been reported in some breast cancer cell lines [47]. Within our laboratory, we have found a marked difference in IGFII mRNA in two sub-lines of MCF-7 cells. MCF-7-McGrath cells [48] are dependent on oestrogen for growth, unable to grow in its absence [21], and produce undetectable levels of IGFII mRNA (Fig. 8). However, MCF-7-KO cells [48], which are responsive to oestrogen for growth but grow quite well in its absence [21], produce much higher levels of IGFII mRNA (Fig. 8). The possibility exists, therefore, that increased expression of IGFII within MCF-7 cells could result in an increased basal growth rate in the absence of steroid. To test this hypothesis, IGFII cDNA was transfected stably into the MCF-7-McGrath cells to investigate whether

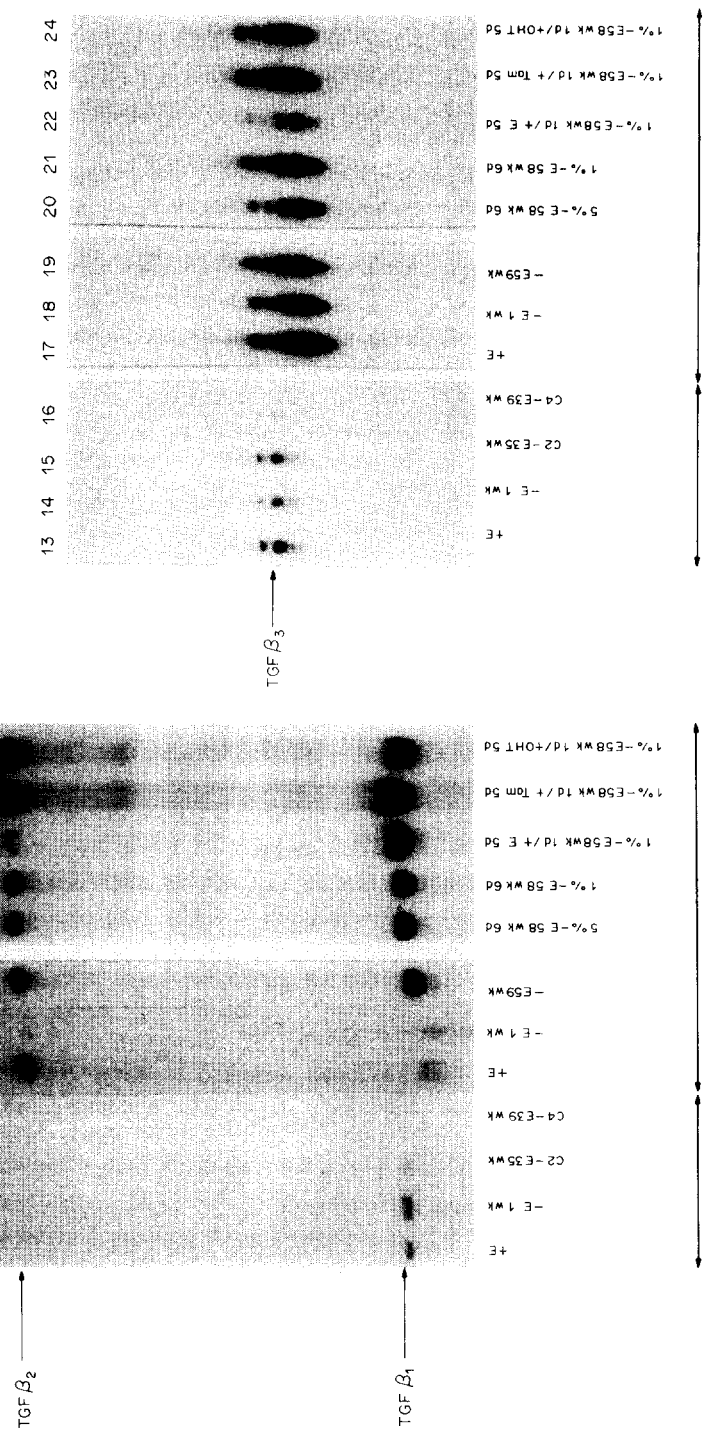


Fig. 6. Levels of mRNA for TGFβ_1, TGFβ_2 and TGFβ_3 assayed by ribonuclease protection in ZR-75-1 and T-47-D human breast cancer cells before and after short-term and long-term steroid deprivation. Cells were grown in RPMI 1640 medium lacking phenol red either continuously with 10^{-8} M oestradiol (+E) or after varying periods of oestrogen deprivation (−E)/readdition of 10^{-8} M oestradiol (+E), 10^{-6} M tamoxifen (Tam) or 10^{-8} M hydroxytamoxifen (+OHT) as indicated (d = days; wk = weeks). Long-term steroid deprived ZR-75-1 cells were either clone 2 (C2) or clone 4 (C4) [12]. All cells in tracks 1–8, 13–20 were grown in 5% DCFCS. Cells in tracks 9–12, 21–24 were grown in 5% DCFCS for 58 weeks 1 day but changed to 1% DCFCS for the 5 day test period with or without steroid.

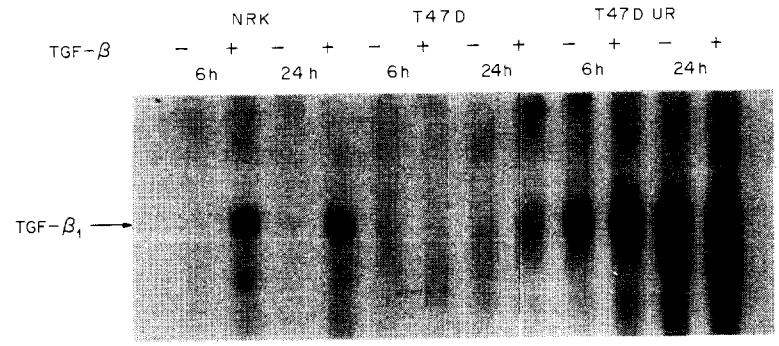


Fig. 7. Regulation of TGFβ_1 mRNA by TGFβ in NRK-49F cells, steroid-maintained T-47-D cells (T47D) and T-47-D cells deprived of steroid for 75 weeks (T47D UR). Northern blot of TGFβ_1 mRNA in cells grown with (+) or without (−) 10^{-10} M TGFβ for 6 h or 24 h. Inductions were performed in serum-free medium: phenol red-free RPMI 1640 medium with 15 mM HEPES pH 7.2, 2 μg/ml transferrin, 0.75 μg/ml fibronectin and 0.1% bovine serum albumin.

upregulation of IGFII in these cells could increase the basal cell growth rate (manuscript in preparation). The method we chose was to link the coding sequence of human IGFII [49] to the metallothionein IIA (Met IIA) promoter from which the glucocorticoid response elements had been removed, leaving only the metal response elements [50]. This gave an inducible system whereby IGFII mRNA could be upregulated within the transfected cells by metal ions. This DNA construction was co-transfected with the pSV2 neo vector [51] into MCF-7-McGrath cells and transfected cells were isolated by virtue of their resistance to G418 sulphate. Zinc treatment of the transfected cells did indeed induce IGFII mRNA (Fig. 9A) and also caused a marked increase in basal cell growth (Fig. 9B). This zinc induction of basal cell growth in the IGFII transfected cells can be inhibited by an antibody against the IGFI receptor which blocks ligand binding (αIR3 from Oncogene Science) (manuscript in preparation). It would thus appear that, in principle, upregulation of IGFII can result in an increase in basal growth rate of MCF-7 cells, although it remains to be proved whether this is a marker or a mechanism in MCF-7-KO cells.

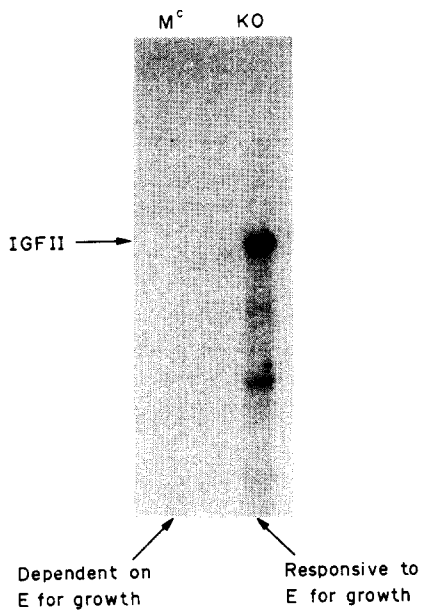


Fig. 8. Levels of IGFII mRNA assayed by ribonuclease protection in oestrogen-maintained MCF7-McGrath (M^c) and MCF-7-KO (KO) human breast cancer cells.

Consequently, alterations in growth factor production and sensitivity evidently do accompany loss of steroid growth response although in most cases, a causal relationship has yet to be demonstrated. The differences between cell lines caution against adopting too simple a model of growth factor involvement in breast tumour progression. Such differences may exist because changes observed are simply markers of loss of response and the general overriding mechanism has yet to be identified. On the other hand, if a causal role does exist, it may be that there are multiple pathways for loss of steroid sensitivity in breast cancer. Furthermore, growth factor pathways involved in the progression to steroid independence may be separate from those regulated by oestrogen in the steroid responsive cells.

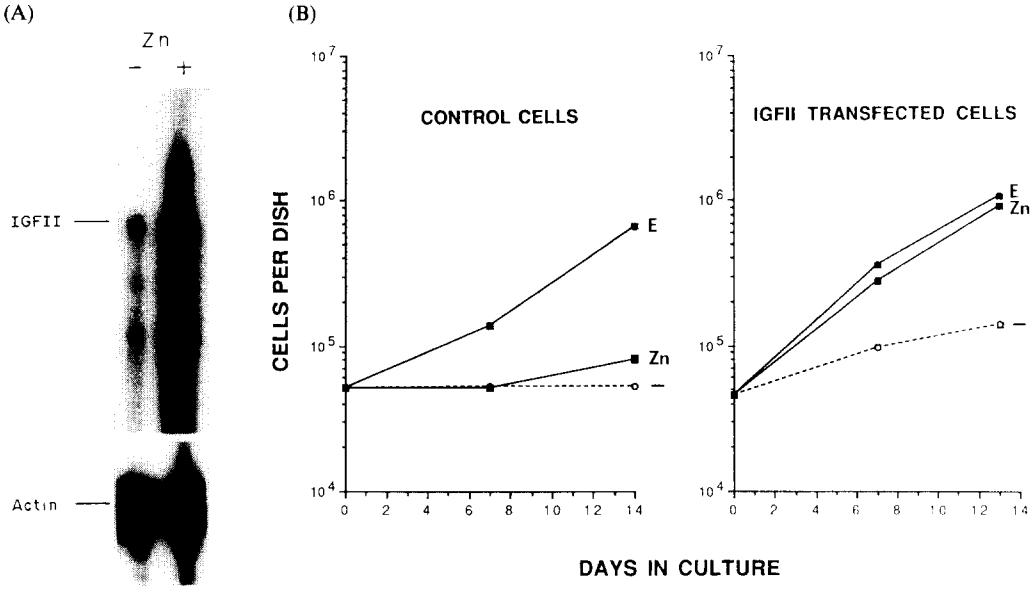


Fig. 9. Zinc induction of IGFII mRNA (A) and effects on cell growth (B) in transfected MCF-7-McGrath human breast cancer cells. A. Ribonuclease protection assay for induction of IGFII mRNA by 5×10^{-5} M zinc chloride (Zn) in cells stably transfected with MetIIA-IGFII construct. B. Growth in monolayer culture of cells stably transfected with either pSV2 neo DNA alone (control cells) or the MetIIA-IGFII construct plus pSV2 neo DNA (IGFII transfected cells). Growth was assayed in phenol red-free RPMI 1640 medium with 5% DCFCS with no addition (−), 5×10^{-5} M zinc chloride (Zn) or 10^{-8} M oestradiol (E). Bars represent SE of triplicate dishes. Where no bars are shown, error was too low for visual display.

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