Cell cycle control by steroid hormones in breast cancer: implications for endocrine resistance

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INTRODUCTION

The sex steroid hormones oestrogen and progesterone, in association with peptide hormones and growth factors, play a central role in normal mammary gland development including the control of differentiated function. There is also compelling evidence that oestrogen is intimately involved in the pathogenesis of breast cancer (Henderson et al. 1988). The majority of breast carcinomas retain some degree of steroid responsiveness and this has been exploited therapeutically by the use of agents which interfere with the production or action of oestrogenic steroids e.g. aromatase inhibitors, LH-RH agonists, and antioestrogens (Santen et al. 1990). Tamoxifen, a synthetic nonsteroidal antioestrogen, is currently the preferred treatment for hormone-responsive breast cancer. Endocrine therapy, including tamoxifen treatment, induces objective responses in about one third of unselected patients and in almost 50% of oestrogen-receptor-positive (ER+) patients with advanced disease (Santen et al. 1990). More importantly a recent overview demonstrates significant reductions in risk of recurrence, increased overall survival, and reduced incidence of contralateral breast cancer after adjuvant tamoxifen treatment in early breast cancer (Early Breast Cancer Trialists' Collaborative Group 1992). The more widespread and effective use of tamoxifen, however, is limited by inherent and acquired resistance to the drug such that about 50% of ER+ tumours fail to respond on first exposure to tamoxifen and most tumours that initially respond later relapse in the presence of the drug. A number of potential mechanisms which might explain these phenomena have been proposed and these are discussed in detail in these proceedings.

Research in this laboratory in recent years has centred on the control of cell cycle progression by steroids and growth factors in normal breast epithelial cells and breast cancer cell lines, with the aim of identifying cell cycle phase-specific effects and ultimately the genes which might mediate these effects. It is hypothesised that identifying growth-regulatory molecules, their receptors and signal transduction pathways will facilitate identification of genes pivotal to the control of cell proliferation, differentiation, and cell death. Such an approach is expected to provide a deeper understanding of normal growth control in the mammary gland and to identify molecular lesions involved in the development and progression of breast cancer, including the loss of endocrine responsiveness.

This chapter summarises some recent studies on breast cancer cell cycle control by growth factors, steroids, and steroid antagonists which identify the proto-oncogenes c-myc and cyclin D1 as key genes involved in these effects. The potential implications of amplification and overexpression of these genes, a relatively common event in clinical breast cancer, on tumour phenotype and response to endocrine therapies are discussed.

HORMONAL REGULATION OF BREAST CANCER CELL CYCLE PROGRESSION

In order to study the role of individual hormones and growth factors on breast cancer cell proliferation we employed a chemically defined, serum-free medium to maintain T-47D breast cancer cell lines in a quiescent, nonproliferating state (Musgrove & Sutherland 1993a). T-47D cells were induced to reinitiate cell
cycle progression by addition of insulin (Fig. 1), IGF-I, EGF, TGFα, or basic FGF and entered S phase in a semisynchronous manner ~10h after mitogenic stimulation. Thus, as has been described for other cell systems, these growth factors can stimulate arrested cells to reenter the cell cycle and progress through G₁ phase. This model has been useful in identifying genes involved in the mitogenic response of breast cancer cells to growth factor stimulation, as described below.

Oestrogens are mitogenic for several cell types including epithelial cells of the mammary gland, uterus, and vagina. Early studies in which these models were employed revealed that oestrogens increased the rate of cell proliferation both by recruiting noncycling cells into the cell cycle and by shortening the overall cell cycle time, due predominantly to a reduction in the length of G₁ phase (Sutherland et al. 1983c). Later experiments in which synchronised breast cancer cells were used in vitro confirmed that oestrogen acts in the early part of G₁ phase (Leung & Potter 1987).

Studies with antioestrogens support this conclusion. Antioestrogen treatment of breast cancer cells in vitro leads to growth arrest, with accumulation of cells in G₁ phase (Sutherland et al. 1983a, 1983b). The use of mitotically selected cells confirmed that sensitivity to growth inhibition by antioestrogens is limited to a portion of G₁ phase, extending from soon after mitosis until mid G₁ phase. Cells elsewhere in the cell cycle are essentially insensitive, and proceed through S phase and mitosis at the same rate as untreated cells (Taylor et al. 1983, Musgrove et al. 1989). Thus oestrogens and antioestrogens regulate cell proliferation by their actions on a cell cycle control point in early to mid G₁ phase (Fig. 2).

The effects of progestins on cell-proliferation kinetics have not been as readily defined. This is due, in part, to the greater cell-type specificity of the actions of progestins (reviewed by Clarke & Sutherland 1990). For example, in uterine epithelium progestins act in a manner analogous to that of antioestrogens by inhibiting oestrogen-induced cell cycle progression early in G₁ phase. In contrast, in other tissues, particularly the mammary gland, progestins both stimulate and inhibit cell proliferation. Our analysis of the effects of progestins on breast cancer cells in vitro demonstrated both a stimulatory and an inhibitory effect on cell cycle progression (Musgrove et al. 1991). The points of action of these effects are temporally displaced within G₁ phase: the inhibitory effect occurs soon after mitosis whereas the stimulatory effect is later, although still before mid G₁ phase (Fig. 2). As a result, the inhibitory
effect is predominant in this model system (Sutherland et al. 1988, Musgrove et al. 1991).

Like antioestrogen or progesterin treatment, anti-progestin treatment of breast and endometrial cancer cells in vitro leads to growth arrest accompanied by an increase in the proportion of cells in G1 phase (Horwitz 1992, Musgrove & Sutherland 1993b). Since progestins and antiprogestins both inhibit proliferation, there has been some debate as to whether the antiprogestin effects resulted from progestin-agonist or progestin-antagonist activity. However, when the changes in cell cycle phase distribution were compared, temporal differences between growth inhibition by progestins and by antiprogestins were observed (Musgrove & Sutherland 1993b). Such data are supportive of the view that antiprogestin inhibition occurs by a process which opposes the stimulatory effect of progestin. Interestingly, antiprogestins and antioestrogens led to temporally similar changes in cell cycle phase distribution, suggesting that their actions occurred at similar points within G1 phase (Fig. 2) (Musgrove & Sutherland 1993b).

MECHANISMS OF CELL CYCLE CONTROL

The demonstration of growth-factor mediated and steroidal control of cell cycle progression at defined points within G1 phase suggests that these agents, acting via their respective receptors, are controlling the expression of key cell cycle regulatory genes, the products of which determine rates of G1 progression. Such an interpretation is compatible with current concepts of mammalian cell cycle control where environmental signals act within G1 phase to regulate rates of cell proliferation (Baserga 1990). Significant advances in our understanding of the molecular basis of cell cycle control in mammalian cells have emerged in recent years due to the discovery and functional analysis of the cell cycle regulatory cyclins, cyclin-dependent kinases (CDKs), and inhibitors of these kinases (Motokura & Arnold 1993, Pines 1994). These genes, in addition to known proto-oncogenes (e.g. c-myc) and tumour-suppressor genes (e.g. RB), provide potential targets for steroid and growth-factor action in breast epithelial cells.

Cyclins and CDKs are the regulatory and catalytic subunits, respectively, of cell cycle-regulated kinases. Mammalian cells contain multiple cyclins and CDKs (Motokura & Arnold 1993). The members of each family share sequence homology within specific motifs which are thought to have functional significance. Some cyclins are particularly closely related, e.g. cyclins D1, D2, and D3, and thus form subgroups within the cyclin family. The existence of three closely related D-type cyclins raises questions of the functional significance of apparently similar molecules. However, there is now clear evidence for tissue-specific expression and distinct roles in the control of differentiation as well as proliferation, indicating that these genes are not redundant but may have complementary functions (Inaba et al. 1992, Kato & Sherr 1993, Sherr 1993). Some cyclins are capable of binding to multiple CDKs e.g. cyclin D1 can activate CDK4 and CDK6, presumably to mediate multiple functions (Pines 1993); differential expression of the CDKs allows further scope for cell- and tissue-specific roles for particular cyclin/CDK complexes. In T-47D human breast cancer cells, the model used in the majority of our studies, CDK4 is the predominant partner for cyclin D1.

The sequential transcriptional activation of cyclin genes and consequent transient accumulation and activation of different cyclin/CDK complexes is

![Figure 2 Cell cycle phase-specific actions of steroids and steroid antagonists. Boxes represent times of action: for example, oestrogen acts during early G1 phase, whereas progestins stimulate cells in mid G1 but inhibit progress of cells upon reentry into G1 from mitosis (M).](image-url)
thought to be the central mechanism for a series of control points within the mammalian cell cycle (Fig. 3). Cyclins D1, D2, D3, and E are rate limiting for progress through G1 phase (Ohtsubo & Roberts 1993, Quelle et al. 1993, Musgrove et al. 1994). These cyclins are induced after mitogen stimulation in a sequence which is largely independent of the mitogen used or the cell type examined. Thus, cyclin D1 abundance typically increases in early G1 phase whereas cyclin E abundance increases as cells approach the G1-S phase boundary (Matsushime et al. 1991, Koff et al. 1992, Musgrove et al. 1993). Induction of cyclin D1 upon mitogen stimulation of rodent macrophages or fibroblasts is followed by activation of CDK4 in mid G1 phase (Matsushime et al. 1994). Similarly, induction of cyclin E is accompanied by activation of CDK2 late in G1 phase (Dulic et al. 1992, Koff et al. 1992, Rosenblatt et al. 1992).

Figure 3 Sequence of cyclin-CDK complex formation throughout the cell cycle. Times of maximum activity of cyclin-CDK complexes are shown schematically, based on current published data.

Microinjection studies with anticyclin D1 antibodies or antisense oligonucleotides have shown cyclin D1 to be necessary for entry into S phase (Baldin et al. 1993, Quelle et al. 1993, Lukas et al. 1994), whereas alterations in cyclin D1 expression determine rates of G1 progression (Quelle et al. 1993, Musgrove et al. 1994, Resnitzky et al. 1994). In cycling T-47D cells expressing human cyclin D1 under the control of a metal-inducible metallothionein promoter, induction of cyclin D1 resulted in an increase in the number of cells progressing through G1 and in the rate of transition from G1 to S phase, indicating that cyclin D1 is rate limiting for progress through G1 phase (Musgrove et al. 1994). Similar data obtained with the use of rodent fibroblasts (Quelle et al. 1993, Resnitzky et al. 1994) indicate that this function is likely to be universal in cells which express cyclin D1. In addition, in T-47D breast cancer cells arrested in early G1 phase after growth factor deprivation, induction of cyclin D1 was sufficient for completion of the cell cycle - a process requiring growth factor stimulation in control cells (Musgrove et al. 1994).

Together these observations provide evidence for a central role for cyclin D1 in breast cancer cell proliferation i.e. cyclin D1 is both necessary and sufficient for progression through G1 phase. This suggests that molecules which regulate cyclin D1 expression or the function of associated kinases, particularly CDK4, might also play a critical role in the control of cell cycle progression in these cells. Recently a number of endogenous inhibitors of CDK activity have been identified. These include: p16INK4, which specifically inhibits the catalytic activity of cyclin D/CDK4 complexes (Serrano et al. 1993); p27Kip1, which inhibits both cyclin D/CDK4 and cyclin E/CDK2 complexes and appears to link the functions of these kinases (Polyak et al. 1994); and p21WAF1/CIP1, a general inhibitor of cyclin/CDK complexes (Hunter 1993). The potential downstream targets for mediating environmental effects on rates of cell proliferation, including the effects of sex steroids and their antagonists, thus include cyclins, CDKs, and CDK inhibitors, as well as the immediate-early genes activated in response to mitogenic peptide growth factors.

HORMONAL REGULATION OF c-myc AND CYCLIN GENE EXPRESSION

To test the hypothesis that these genes are targets for growth-factor action and steroid hormone action in breast cancer cells, the relationship between cell cycle position and cyclin gene expression was examined after stimulation of growth-arrested T-47D cells
with insulin (Fig. 4). Cyclin D1 mRNA levels were increased within 2h of insulin addition and increased cyclin D3 mRNA levels were observed as cells progressed through G1. Increased expression of cyclin E and cyclin A coincided with entry into S phase. Sequential induction of cyclins D1, D3 and E during G1 progression in T-47D cells was also observed after treatment with other potent breast cancer mitogens, e.g. IGF-I, fetal calf serum (Musgrove et al. 1993), and bFGF (unpublished data). Furthermore, the proportion of cells which later entered S phase was correlated with the degree of induction of cyclins D1, D3, and E (Musgrove et al. 1993).

In serum-free medium oestrogens alone are not mitogenic and fail to significantly increase either cyclin D1 gene expression or the rate of cell cycle progression, although some induction of c-myc is apparent. As has previously been noted by a number of groups (for example, van der Burg et al. 1988, Stewart et al. 1990) oestrogen is mitogenic in the presence of insulin or IGF-I. In the presence of insulin, oestradiol further increased rates of cell cycle progression and this was preceded by marked increases in c-myc and cyclin D1 expression (Musgrove et al., unpublished).

Progestins induce a transient increase in the rate of cell cycle progression (Musgrove et al. 1991) which is accompanied by a transient induction of cyclin D1 mRNA. The time course of cyclin D1 induction was delayed compared with that of the immediate-early proto-oncogenes c-fos and c-myc (Musgrove et al. 1991) and maximum induction was reached at 3-6h. Progestin-stimulated cells began to enter S phase after approximately 8h treatment (Fig. 5) (Musgrove et al. 1991). Since simultaneous treatment with steroid and steroid antagonist prevents the effects of the steroid on cell cycle progression, it would also be expected to prevent the modulation of genes involved in the regulation of cell cycle progression. In experiments designed to test this proposition, the effects of antiprogestin treatment on progestin induction of c-fos were equivocal but simultaneous antiprogestin treatment prevented progestin induction of c-myc (Musgrove et al. 1991). In addition, progestin induction of cyclin D1 and stimulation of cell cycle progression were both prevented by antiprogestin, added either simultaneously or after a 3h delay (Musgrove et al. 1993). Induction of c-myc expression by progestins is transient, and by 3h c-myc mRNA levels are beginning to decline. It might be expected, therefore, that addition of antiprogestin 3h after progestin treatment would be too late to prevent the consequences of c-myc induction. Since antiprogestin addition 3h after progestin treat-

![Figure 4 Cell cycle phase-specific gene expression after growth factor stimulation. T-47D cells were growth arrested in chemically defined serum-free medium then stimulated to reenter the cell cycle by addition of 10μg/ml insulin at time 0, as in Fig. 1. Northern blot analysis was used to measure relative expression of cyclin mRNAs during cell cycle progression. Redrawn from Musgrove et al. (1993).](image-url)
ment prevents stimulation of cell cycle progression, regulation of cyclin D1 appears to be more closely connected to progestin regulation of proliferation than does regulation of c-myc.

The induction of cyclin D1 gene expression within 2h of mitogenic stimulation with steroids or growth factors is compatible with a role for this gene in early G1 phase, a time when breast cancer cells are sensitive to the growth-inhibitory effects of antioestrogens and antiprogestins (Taylor et al. 1983, Musgrove et al. 1989, Musgrove & Sutherland 1993b). Examination of cyclin expression after treatment with the antiestrogen ICI 164384, a potent inhibitor of breast cancer cell cycle progression (Wakeling & Bowler 1987, Musgrove et al. 1989, Musgrove & Sutherland 1993b) showed time-dependent decreases in the level of cyclin D1 mRNA but not cyclin D3 mRNA (Fig. 6). The decrease was similar in magnitude (50-60%) to the decrease in DNA synthesis as assessed by histone H4 expression and S phase (Musgrove et al. 1993, Watts et al. 1994). Cyclin D1 expression began to decrease within 4h of antiestrogen treatment, substantially preceding any decline in DNA synthesis (Fig. 6). Thus, the regulation of cyclin D1 expression by ICI 164384 is not merely a consequence of growth arrest.

Changes in cell cycle phase distribution occur over a similar time frame after antioestrogen or anti-

**Figure 5** Effect of progestin stimulation on c-myc and cyclin D1 gene expression. T-47D cells proliferating in insulin-supplemented serum-free medium were treated with the synthetic progestin ORG 2058 (10nM) and harvested for Northern blot analysis at intervals thereafter. Parallel flasks were treated with RU 486 (100nM) which was added 3h after progestin. The consequent effects on cyclin D1 and S phase are shown in dashed lines. Redrawn from Musgrove et al. (1993).
role for cyclin D1 in determining the rate and magnitude of cell cycle progression in these cells (Musgrove et al. 1994).

**IMPLICATIONS FOR ENDOCRINE RESISTANCE**

Resistance to endocrine therapies, particularly tamoxifen therapy, is an important clinical problem: by defining the molecular basis for the lack (or loss) of response to endocrine agents it may be possible to reverse resistance or to design treatment strategies to prevent its occurrence. It may also be possible to identify markers of phenotype that distinguish potential responders from nonresponders prior to therapy.

Many mechanisms have been proposed that could potentially account for loss of steroid sensitivity and the development of resistance to antioestrogens e.g. ER mutations and post-receptor events including changes in the production and/or sensitivity to endocrine, autocrine, or paracrine growth factors. Of particular interest in the light of our recent studies is the possible involvement of alterations in cell cycle control. The actions of steroids and their antagonists in determining the rate of breast cancer cell proliferation by direct effects on genes controlling the cell cycle implies that if the normal function of these genes was disrupted, for example by their underexpression or overexpression, or by mutation, this could contribute to autonomous growth and loss of hormone sensitivity. Since there is now strong evidence that several oncogenes, e.g. c-myc, cyclin D1, and tumour suppressor genes, e.g. RB, are downstream targets for steroid effects on cell proliferation and because aberrant expression of these genes is relatively common in breast cancer, such genes are potential contributors to the acquisition of steroid insensitivity and resistance to endocrine agents.

For example, our results demonstrating a central role for cyclin D1 in the control of breast cancer cell cycle progression (Musgrove et al. 1994) and a rapid decline in cyclin D1 mRNA levels after antioestrogen treatment of breast cancer cells (Musgrove et al. 1993, Watts et al. 1994) suggest that this cyclin may be involved in mediating antioestrogen inhibition of growth and may therefore be potentially involved in the development of antioestrogen resistance. Failure of antioestrogens to sufficiently inhibit cyclin D1 gene expression because of its constitutive overexpression or upregulation by amplification of other signalling pathways, e.g. those activated by autocrine/paracrine growth factors, or amplification and overexpression of cell surface receptors and their signalling molecules (Daly et al. 1994, Janes et al. 1994) would then result in continued cyclin D1/CDK activation and continued cell cycle progression in the presence of the growth inhibitor.
Although such mechanisms are purely speculative at this time, several studies have described the common overexpression of cyclin D1 in breast cancer and breast cancer cell lines. The frequency of amplification at 11q13, the chromosomal locus of the cyclin D1 gene, in breast cancers is about 13% (Fanti et al. 1993), but it is now clear that overexpression also occurs in the absence of gene amplification. In our study of cyclin D1 expression at the mRNA level we demonstrated that 45% of breast carcinomas overexpressed cyclin D1 when compared with histologically normal breast tissue (Buckley et al. 1993). Similarly, in two recently published studies in which immunohistochemical detection of cyclin D1 protein was employed (Bartkova et al. 1994, Gillett et al. 1994) 30-43% overexpression in breast carcinomas was demonstrated.

The relationship between cyclin D1 amplification and selected clinical and pathological parameters has been reported but to date there are limited data on the relationship of cyclin D1 overexpression with such parameters. The results of several studies have suggested a weak association between 11q13 amplification and tumour size and lymph node status, a strong association with oestrogen-receptor status, and claims that amplification is correlated with poor survival (Fanti et al. 1993). Hence cyclin D1 overexpression may identify a subset of poor-prognosis patients within the ER+ good-prognosis group. Whether cyclin D1 overexpression does predict poor prognosis and, if so, whether this might be due to resistance to hormonal therapies is currently under investigation.

Similar arguments can be raised for the involvement in endocrine resistance of other cell cycle regulatory genes including not only cyclins, CDKs, and their inhibitors but also c-myc and RB. However, these hypotheses have arisen predominantly from studies with cell lines in vitro and urgently need to be tested in well-characterised patient material where gene expression can be correlated with known responsiveness to endocrine therapy.

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