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Involvement of steroid hormone and growth factor cross-talk in endocrine response in breast cancer

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Abstract

Multiple lines of evidence implicate steroid hormone and growth factor cross-talk as a modulator of endocrine response in breast cancer and that aberrations in growth factor signaling pathways are a common element in the endocrine resistant phenotype. Delineation of these relationships is thus an important diagnostic goal in cancer research, while the targeting of aberrant growth factor signaling holds the promise of improving therapeutic response rates.

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Introduction

Research examining those factors affecting the development of breast cancer has identified that steroid hormones are of pivotal importance in directing the growth of these tumours. This knowledge has been exploited clinically, with endocrine treatments which seek to perturb the steroid hormone environment of the tumour cells often promoting extensive remissions in established tumours and, furthermore, significant survival benefits for patients. Unfortunately, the beneficial actions of existing endocrine measures are, in part, counteracted by the capacity of the tumour cells to eventually circumvent the need for steroid hormones, allowing them to continue to grow and progress despite such therapy. Thus, at presentation of breast cancer, current endocrine therapies are not effective in all patients (*de novo* endocrine resistance) and initially responsive tumours will sooner or later progress despite such treatments (acquired resistance), inevitably resulting in patient relapse and ultimately death. The identification of the factors and pathways responsible for the development of these resistant conditions is therefore an important diagnostic and therapeutic goal in cancer research. It is self-evident that any accurate identification of primary endocrine resistance in advance of therapy would not only prevent unnecessary treatment morbidity in unresponsive patients, but would also significantly limit the time lost during which the disease may progress unchecked by inappropriate therapy, and the waste of financial resources associated with the ever escalating cost of drugs. Equally, a knowledge of the causes of primary and acquired endocrine resistance might allow the development of new therapeutic regimes to either prevent the evolution of these conditions, or at least delay their appearance, hence severely compromising the disease process.

A proposed model for such loss of steroid hormone sensitivity in breast cancer in both the *de novo* and acquired setting suggests that aberrations advantageous to tumour cell growth occur specifically within key growth factor signalling pathways, allowing mitogenesis to proceed highly efficiently despite the challenge of endocrine therapy. These events may totally circumvent the constraints of classical steroid hormone dependency. However, since growth factor and steroid hormone mitogenic pathways are intimately entwined, any associated phenotypic changes may also serve to exaggerate the actions of steroid hormone signalling pathways and hence enhance the agonistic qualities known to be a feature of many current antihormonal therapies. A new paradigm is thus emerging where knowledge of the expression of growth factor signalling elements may be prognostically relevant in identifying endocrine responsiveness and where appropriate anti-growth factor signalling therapeutic regimes, in combination with antihormonal measures, are thus expected to be beneficial to breast cancer patients.

In light of the above model, the present article briefly outlines the elaborate molecular biology of steroid hormone and growth factor signalling pathway interactions believed to play a central role in normal and

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neoplastic breast mitogenesis. It subsequently examines how changes often occurrent in the breast cancer phenotype could severely perturb the balance of such signalling, and thus providing a possible explanatory hypothesis for the tumour growth associated with the phenomena of *de novo* and acquired endocrine resistance. A limited discussion of how such data might be thera-peutically exploitable in breast cancer is also included.

Oestrogen receptor (ER) signalling

Multiple lines of evidence have been established that the steroid hormone oestrogen is an essential factor not only for the normal breast (Clarke *et al.* 1997), but also for the growth and development of breast cancer (Howell *et al.* 1997). The effects of oestrogens are mediated primarily through interaction with the ER, which in the absence of steroid ligand resides in a large molecular complex with multiple heat-shock proteins (Segnitz & Gehring 1995). Importantly, the receptor becomes hyperphosphorylated at several serine and tyrosine residues upon oestrogen binding (Kuiper & Brinkmann 1994), and specific conformational changes are subsequently induced in the receptor which result in dissociation of heat-shock proteins, receptor dimerisation and nuclear localisation. This favours receptor association with (i) simple oestrogen response elements (EREs) within promoters of target genes (reviewed in White & Parker 1998) and (ii) composite response elements which bind receptors in addition to other transcription factors (Diamond *et al.* 1990). Functional analysis of ER has shown it to be a modular protein (Kumar *et al.* 1987), with two transcriptional activator functions (AF), AF-1 and AF-2 (Kraus *et al.* 1995). One of the effects of ligand binding is to juxtapose the AF-1 and AF-2 domains through receptor conformational change, thereby generating a productive

Table 1 Growth factor signalling elements which influence ER signalling

1. Peptide growth factors and receptor tyrosine kinases
Peptide growth factors bind to plasma membrane-located receptors which possess intracellular tyrosine kinase domains. On ligand binding, receptor dimerisation ensues, allowing each member of the receptor pair to trans-phosphorylate its partner specifically on tyrosine residues.
2. SH2 domain-containing proteins
Many substrates for growth factor receptors contain a structural motif, Src homology 2 domain (SH2). These domains are present in dozens of intracellular proteins which either possess enzymatic activity, such as phospholipase Cα or phosphinositide-3-kinase, or function as bridging proteins (Grb2 and Shc). Bridging proteins recruit other signalling proteins, including son of sevenless (SOS) which in turn brings Ras into its active guanosine triphosphate binding form.
3. Src family of non-receptor protein tyrosine kinases
Several growth factors activate the Src family of kinases which again recruit and/or activate SH2-containing proteins, as well as phosphorylate many intracellular proteins.
4. Ras/ MAP kinase
Activated Ras translocates Raf-1 kinase to the plasma membrane, ultimately leading to a sequential activation of MAP kinase kinase signalling elements including MAP kinase (ERK1/2) and pp90rsk1.
5. PKC
Growth factor activation of phospholipase Cα increases inositol lipid turnover resulting in the generation of the second messenger, diacylglycerol. This in turn activates PKC, which phosphorylates and hence activates Raf-1 kinase.
6. Protein phosphatases
Signal transduction regulation is largely provided by the complex balance of protein kinases and their cognate phosphatases.
7. Cyclins, cyclin-dependent kinases and casein kinase II
End-point targets of growth factor signalling and key components of cell cycle regulation and survival.
8. The AP-1 complex
The activation of nuclear transcription factors by the various MAP kinase/PKC signalling pathways (ERK1/2, JNK and p38) regulate (Elk-1 and serum response factor), or indeed comprose (Fos and Jun) components of AP-1.
9. Myc
MAP kinase signalling activation of the nuclear transcription factor Myc, a key intermediate early response gene involved in growth response.

association (Kraus *et al.* 1995, Pratt & Toft 1997). Interestingly, while the activity of AF-2 appears to be largely ligand-enabled, showing relatively strict specificity for oestrogens, there is increasing evidence that AF-1 activity is constitutive, a feature likely to derive from considerable ligand-independent (notably growth factor pathway-mediated) influences. Contributory growth signalling through other members of the steroid hormone nuclear transcription family utilise similar intracellular mechanisms to those described for the ER.

Growth factor signalling

Growth factor-induced signal transduction proceeds via a cascade of protein phosphorylation steps which serve to relay stimuli into cellular responses. Those events ultimately result in the induction/activation of multiple nuclear transcription factors, with an equally diverse range of target response elements, notably those in the promoters of genes orchestrating cell-cycle regulation and survival. Important elements in growth factor signalling also believed to influence, either directly or indirectly, ER signalling include those listed in Table 1.

Cross-talk between ER and growth factor signalling pathways (Fig. 1)

Oestrogens stimulate positive elements of growth factor signalling pathways (Fig. 1-1)

Oestrogen sensitivity and endocrine response have been extensively investigated in experimental models of human breast cancer both *in vitro* and *in vivo*. Based on these studies (reviewed in Gee *et al.* 1996, Nicholson & Gee 1996), it is becoming increasingly evident that oestrogens can promote the autocrine expression of growth factor signalling pathway components, notably transforming growth factor (TGF)-α (Bates *et al.* 1988), insulin-like growth facor-II (IGF-II) (Brunner *et al.* 1993) and growth factor receptors (e.g. epidermal growth factor (EGF) receptor (EGFR) (Bertois *et al.* 1989, Chrysogelos *et al.* 1994) and IGF-I receptor (Freiss *et al.* 1990)), in oestrogen-responsive (MCF-7 and T47-D) and oestrogen-dependent (ZR-75-1) human breast cancer cell lines. Such actions, which are often antagonised by antioestrogens (reviewed in Gee *et al.* 1996, Nicholson & Gee 1996), could significantly supplement the cellular growth responses directly primed by oestrogens (Cho & Katzenellenbogen 1993, Smith *et al.* 1993).

In addition, it appears that oestrogens directly stimulate (while antioestrogens inhibit) the tyrosine kinase activities both of the EGFR-related protein c-erbB-2 (Matsuda *et al.* 1993) and of c-src (Migliaccio *et al.* 1993), the activation of which can provide important mitogenic signals to epithelial cells through the recruitment of the p21ras/mitogen-activated protein (MAP) kinase pathway.

Oestrogens inhibit negative elements of growth factor signalling pathways (Fig. 1-2)

As well as the positive influences exerted by oestrogens on growth factor signalling pathways detailed above, it is notable that in parallel they diminish (while antioestrogens induce) the expression of the growth inhibitory factor TGFβ (Knabbe *et al.* 1987) in several oestrogen-responsive human breast cancer cell lines.

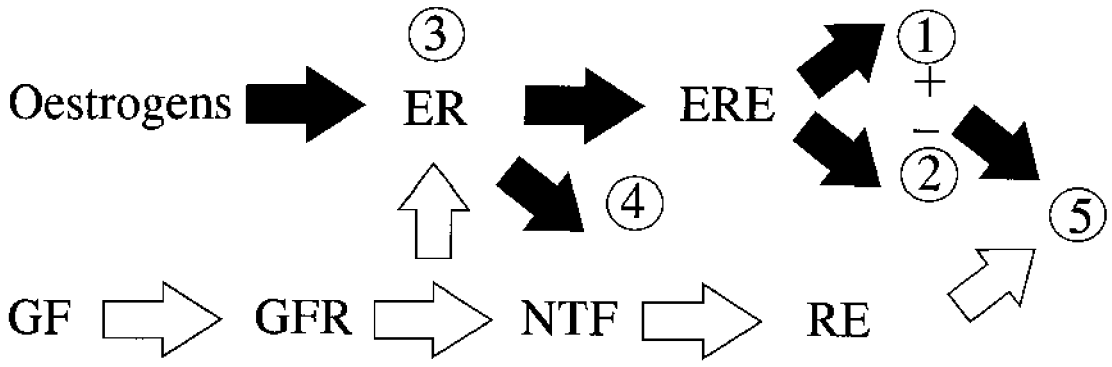

Figure 1 Cross-talk between ER and growth factor signalling pathways.
GF, growth factor; GFR, growth factor receptor; NTF; growth factor-induced nuclear transcription factor; RE; response element regulated by growth factor signalling pathways.

Additionally, however, it is of particular significance that oestrogens can inhibit expression of tyrosine phosphatases in ER-positive breast cancer cells to increase growth factor mitogenic activity, while both steroidal and non-steroidal antioestrogens increase such enzyme activity. Tamoxifen, for example, inhibits the mitogenic activity of EGF by promoting significant dephosphory-lation of EGFR, an effect believed to be ER mediated (Freiss *et al.* 1990, Freiss & Vignon 1994). It appears that such EGFR dephosphorylation is accomplished via an increase in tyrosine phosphatase activity, as evidenced not only by an effective inhibition by sodium orthovanadate, but furthermore by a time- and dose-dependent increase in membrane phosphatase activity with the antioestrogen (Freiss & Vignon 1994). In this light, two tyrosine phosphatases have been identified which appear to be regulated by antioestrogens, LAR and FAP-1 respectively (Freiss *et al.* 1998). Significantly, antisense inhibition of FAP-1 expression abolishes the antioestrogen-mediated inhibition of growth factor mitogenic activity, although the 'pure' antioestrogen ICI 182,780 appears to retain inhibitory activity under these conditions, suggesting that the effects of this compound are FAP-1 independent (Freiss *et al.* 1998).

ER is a target for growth factor-induced kinase activity (Fig. 1-3)

Numerous studies have now shown that the ER is subject to phosphorylation and activation by several peptide growth factors (e.g. IGF-I (Aronica & Katzenellenbogen 1993), EGF, TGFα (Bunone *et al.* 1996) and heregulin (Pietras *et al.* 1995)), events which can subsequently initiate ERE-mediated gene expression (Lee *et al.* 1997). Significantly, these elements appear to differentially activate AF-1 and AF-2, with the former being more responsive to EGF and TGFα signalling (Bunone *et al.* 1996), and IGF-I preferentially targeting the latter (Gangolli *et al.* 1997). While activation by these factors occurs most efficiently in the presence of oestrogens, their promotion of AF-1 and AF-2 responses is certainly adequate for investigating transcription in the absence of the hormone. An emerging concept for steroid hormone receptors is therefore that they function not only as direct transducers of hormonal effects but, as members of the cellular nuclear transcription factor pool, also serve as key points of convergence for multiple signal trans-duction pathways (McDonnell *et al.* 1995).

Three serine residues (amino acids 104, 106 and 118) located within the AF-1 domain of ER, plus Ser-294, match the consensus sequence specific amino acid recognition motif targeted by a family of serine/threonine/proline-directed kinases which include cyclin-dependent kinases and MAP kinases. Ser-104, -106 and -118 are phosphorylated upon oestrogen treatment (Ali *et al.* 1993, Le Goff *et al.* 1994), and mutation of these residues to alanine markedly decreases ligand-dependent transactivational activity. More detailed studies have recently confirmed that phosphorylation of Ser-118 is required for full activity of AF-1, and that MAP kinase is capable of this (Kato *et al.* 1995). Research shows the major oestradiol-induced phosphorylation site on the ER is Ser-167, and that this is phosphorylated by casein kinase II (Arnold *et al.* 1994, Tzeng & Klinge 1996, Castano *et al.* 1997) and pp90rsk1 in a hormone-dependent manner (Joel *et al.* 1998). Furthermore, a direct role for the cyclin A/cdk2 complex in phosphorylating the ER at a residue somewhere between amino acids 82 and 121 has also been suggested (Trowbridge *et al.* 1997). Similarly, protein kinase C (PKC)-δ targets the receptor at Ser-122 (Lahooti *et al.* 1998). Finally, the ER is also phosphorylated by p60c-src on tyrosine residues, in particular at Tyr-537 in the human protein (Arnold *et al.* 1997). This residue is located within the hormone-binding domain of the receptor and may serve to recruit coactivators (White & Parker 1998), a class of molecules which in turn serve as substrates for cellular kinases and hence growth factor signalling. The bulk of evidence from other signal regulatory systems suggests the likely existence of phosphastases regulate ER-phosphorylating reactions; however the precise identities of these enzymes as yet await discovery.

Like oestrogens, antioestrogens can induce ER phosphorylation, albeit inefficiently (Ali *et al.* 1993, Aronica & Katzenellenbogen 1993), and it is likely that this property contributes to the partial oestrogenicity exerted by some antioestrogens. It is also of particular significance that while antiestrogens appear to block AF-2 functions efficiently, they do not always prevent AF-1 activity (Berry *et al.* 1990). Since AF-1 functions are subject to ligand-independent regulation by growth factor signalling pathways, it has been suggested that inter-actions between elements of these pathways and antioestrogen-bound ER are necessary for the partial agonism of antioestrogens, and may represent a means whereby the oestrogenicity of these compounds may be exaggerated under conditions of excess growth factor stimulation (Kato *et al.* 1995, Bunone *et al.* 1996).

ER interacts positively with growth factor-induced nuclear transcription factors (Fig. 1-4)

As stated previously, an important feature of growth factor signalling is its potential to activate several profiles of nuclear transcription factors which subsequently serve to promote the expression of genes participating in a diversity of end-points, including cell cycle progression. Thus, the MAP kinase (ERK1/2) pathway leads not only to the phosphorylation of ER, but directly activates Elk-1

(Gille *et al.* 1995). This latter transcription factor subsequently forms a ternary complex with serum response factor which primes Fos expression via the c-fos serum response element (Gille *et al.* 1995). Similarly, jun kinase (JNK) (also a member of the MAP kinase family) phosphorylates the c-Jun protein, which subsequently heterodimerises with Fos (Minden *et al.* 1994). The resultant complex, AP-1, is of central importance since it directly targets the 12-O tetradecanoyl-phorbol-13 acetate-responsive element, a sequence found in the promoters of many genes involved in a plethora of cellular end-points (Pfahl 1993).

In this light, it has been reported that oestrogens can significantly enhance growth factor-induced AP-1 activity (Philips *et al.* 1993), a feature believed to be a consequence of productive protein/protein interactions between the ER and the AP-1 complex (Rochefort 1995). Indeed, αER appears able to activate genes containing AP-1 sites in their promoters (Webb *et al.* 1995), providing a mechanism whereby ER signalling may be markedly diversified. Initial studies suggested that antiestrogens antagonised growth factor-induced AP-1 activity, with maximal inhibition by pure antiestrogens (Philips *et al.* 1993). However, subsequent investigations (albeit performed in uterine cells) have suggested that the tamoxifen/ER complex may also act agonistically on promoters regulated by the AP-1 site (Webb *et al.* 1995).

Steroid hormone and growth factor signalling pathways influence common growth regulatory genes (Fig. 1-5)

In order for cells to proliferate, they initially need to be recruited into the cell cycle and then be induced to progress through it. These outcomes are orchestrated by at least two series of events which can be jointly influenced by steroid hormone and growth factor signalling pathways (Musgrove *et al.* 1993): firstly, the induction of intermediate early response genes, such as c-fos (Morishita *et al.* 1995, Duan *et al.* 1998), c-jun (Morishita *et al.* 1995, Mohamood *et al.* 1997) and c-myc (Dubik & Shiu 1992, Musgrove *et al.* 1993), and secondly, the regulation of G1 cyclins (e.g. cyclin D1) and their partner kinases and inhibitors which are involved in restriction point control (Musgrove *et al.* 1993, Lukas *et al.* 1996). Joint activation of these pathways by oestrogens and growth factors would at a minimum reinforce mitogenic signals to responsive cells, and might even result in syner-gistic interactions between these overlapping pathways.

Breast cancer phenotype and its potential influence on endocrine response

The above data generated largely from model systems convincingly show that many points of convergence exist for oestrogen- and growth factor-mediated signalling pathways and hence that mitogenic responses potentially proceed more efficiently in a mixed oestrogen and growth factor milieu. However, the questions arise as to whether growth factors and their downstream signalling pathway components are detectable in clinical breast cancer and whether there is any evidence that aberrations in their signalling pathways may modify *in vivo* endocrine response. An increasing body of evidence is certainly supportive of an important role for many components of the erbB signalling pathway.

TGFα

Enhanced production of TGFα has been observed in transformed rodent and human fibroblast and epithelial cells, where it may function as a downstream intermediary in the transformation pathway elicited by oncogenes (Salomon *et al.* 1990). It has been suggested that TGFα may act to induce hyperplastic responses in transformed breast cells, and thereby act as a promotional agent in combination with a normal background of mutational events (Matsui *et al.* 1990, Sandgren *et al.* 1990). Certainly, TGFα has been demonstrated to be present in readily detectable amounts in clinical breast cancer specimens (Ciardiello *et al.* 1991, Lundy *et al.* 1991, Umekita *et al.* 1992), where its increased expression has been related to primary endocrine insensitivity in ER-positive disease (Nicholson *et al.* 1994*a*). Furthermore, our recent examination of sequential clinical breast cancer biopsy specimens obtained during tamoxifen treatment is also supportive of elevated TGFα protein expression being involved in acquired endocrine resistance in ER-positive disease, while diminished expression appears to be a therapeutic feature of those patients exhibiting a good quality and longer duration of initial response (JMW Gee, JFR Robertson & RI Nicholson, unpublished observations).

The EGFR and c-erbB2 receptor tyrosine kinases

Clinical data emerging in the late 1980s and early 1990s have convincingly shown a significant inverse relationship between the expression of the EGFR (reviewed in Klijn *et al.* 1992), and to a lesser extent c-erbB-2 protein (Nicholson *et al.* 1993, 1997), with the degree of endocrine sensitivity in breast cancer. Thus, while patients whose tumours expressed low levels of EGFR frequently benefited from antihormonal drugs such as tamoxifen, women whose tumours contained high numbers of binding sites for EGF/TGFα (Nicholson *et al.* 1989) or which demonstrated high levels of cell membrane-assoc-iated EGFR immunostaining (Nicholson *et al.* 1994*b*) rarely responded. Although to some degree these associations may be explained by the inverse relationship known to

exist between the oestrogen and epidermal growth factor receptors, nevertheless some direct involve-ment of the EGFR in growth responses has been suggested, with increased EGFR level directly correlating both with elevated tumour proliferation and poor prognosis (Nicholson *et al.* 1997).

Intracellular components of the MAP kinase pathway

In clinical specimens, Sivaraman *et al.* (1997) demonstrated that hyperexpression of MAP kinase is a feature of clinical breast cancer. In this light, our own recent studies using specific antibodies which detect fully activated (i.e. dually phosphorylated) ERK1/2 have shown a highly significant relationship between their increased activation and a poorer quality and shorter duration of response to the antioestrogen tamoxifen, as well as with a reduced survival time in ER-positive patients (JMW Gee, JFR Robertson & RI Nicholson, unpublished observations). Similarly, elevated levels and/or activity of additional intracellular molecules comprising or regulating the MAP kinase signalling pathway, including pp60c-src (Lehrer *et al.* 1989, Lawrence & Niu 1998), Grb2 (Daly *et al.* 1994), RHAMM (Wang *et al.* 1998), Ras (Dati *et al.* 1991, Archer *et al.* 1995, Bland *et al.* 1995), Raf (Callans *et al.* 1995), PKC (Gordge *et al.* 1996) and the transcription factors c-myc (Borg *et al.* 1992, Kreipe *et al.* 1993, Shiu *et al.* 1993), PEA3 (Benz *et al.* 1997) and ESX (Chang *et al.* 1997) have also been noted in malignant breast.

AP-1 signalling

As previously stated, an important element in growth factor-induced cell proliferation is the induction and activation of the AP-1 complex (Davis 1995) and elevated expression of AP-1 activity has been observed in some human breast tumours, as compared with normal adjacent tissue (Linardopoulos *et al.* 1990). The Jun component of AP-1 is thus reported to be elevated in breast cancer (Tiniakos *et al.* 1994), and importantly there is an increasing body of *in vitro* and *in vivo* evidence to implicate the nuclear transcription factor Fos in the control of many processes associated with the neoplastic breast cell, most notably in its acquisition of endocrine independency and invasive capabilities. Thus, we have demonstrated significant associations between elevated Fos protein expression and increased proliferation, *de novo* endocrine insensitivity (Gee *et al.* 1995) and furthermore a worsened patient outlook in clinical breast cancer (Gee *et al.* 1995), also noted by Bland *et al.* (1995). Furthermore, our recent examination of sequential clinical breast cancer biopsy specimens obtained during tamoxifen treatment is also supportive of elevated Fos protein

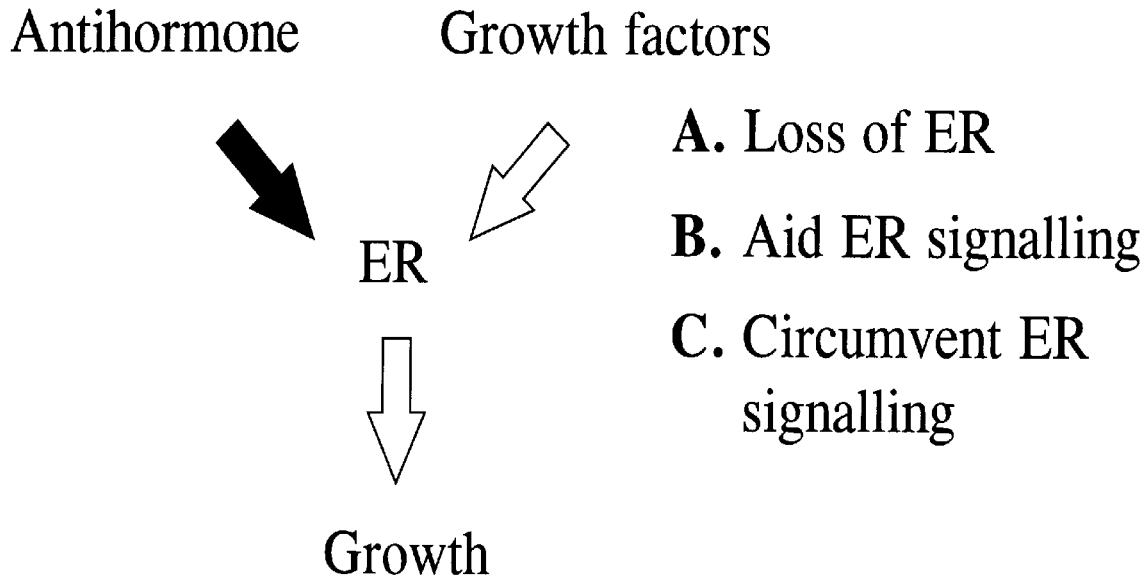

Figure 2 Endocrine resistant disease.

expression being involved in both primary and acquired endocrine resistance (Gee *et al.* 1999), while diminished Fos expression appears to be a therapeutic feature of patients with a good quality and longer duration of response. Our clinical findings demonstrating therapeutic increases in the Fos component of the AP-1 complex associated with endocrine resistance are mirrored by limited *in vitro* studies. As such, increased AP-1 DNA binding activity has been observed to be a feature of tamoxifen-resistant ER-positive breast cancer cells *in vitro* (Dumont *et al.* 1996), whilst prolonged tamoxifen exposure appears to render this antioestrogen agonistic in such cells via its augmentation of the phorbol ester-inducible expression of a chimeric AP-1 response (Astruc *et al.* 1995, Badia *et al.* 1995).

Derived model of endocrine response in breast cancer

As detailed above, there is now substantial evidence that alterations in expression and/or activity of many growth factor signalling elements, notably those comprising the erbB pathway, are not uncommon in clinical breast cancer. Additionally, many of these aberrations appear to accurately mark (and hence may potentially be causative of) *de novo* or acquired endocrine resistance. In this light, a simple working model for the transition of endocrine responsive cancers such as those of the breast to endocrine insensitivity/resistance is suggested as follows.

Endocrine responsive disease

Input signals generated by steroid hormones and stimulatory growth factors are processed by endocrine-responsive, ER-positive cells to ultimately induce/activate several classes of nuclear transcription factors (e.g. steroid hormone receptor, AP-1, Myc etc.). Such inductive events are not autonomous: they are markedly strengthened by the close interplay of the relevant signalling pathways, a feature also noted in normal breast. The sum effect of this activation is to influence patterns of gene expression, leading to the efficient gain of positive influences on cell-cycle progression (e.g. via cyclin D1), with a parallel suppression of negative influences (e.g. TGFβ). In the presence of adequate steroid hormone and growth factor input signals, cells are subsequently recruited into the cell cycle and successfully proceed through it, resulting in growth. Such pathways are also central to mitogenic events in the normal breast.

Thus, although it is likely that cross-talk between both steroid and growth factor pathways increases mitogenic efficiency, substantial reduction in the input signals and hence in resultant cell proliferation can be elicited either by treatment with antihormones or via depletion of the oestrogenic environment alone, thereby potentially leading to excellent initial tumour remissions. These data certainly support the clinical existence of a population of strictly steroid hormone-dependent tumours, deriving their mitogenic stimuli primarily from the influences of the oestrogen-activated ER.

Endocrine resistant disease

Three mechanisms are postulated for the development of endocrine resistance (Fig. 2).

Loss of ER

ER negativity is predictably associated with *de novo* endocrine resistance, comprising some 30% of tumours at presentation. It is as yet unknown if such a phenotype arises from aberrant loss of the steroid hormone receptor, or if such tumours merely arise from the outgrowth of the ER-negative/EGFR-positive cells known to exist in the normal mammary epithelium (Walker *et al.* 1991, 1992). Whatever the mechanism, it is believed that regulation of such tumours is severed from the oestrogenic environment and that they are initially wholly dependent on growth factor signalling for their mitogenic responses (Nicholson *et al.* 1997).

Retention of ER but aberrant growth factor signalling alters steroid hormone sensitivity

In such tumours it is postulated that changed growth factor signalling elements within the cancer phenotype significantly impinge on ER-directed events such that cells are able to survive and prosper under conditions where only limited amounts of oestrogens are available to the receptor or where it is occupied by antihormonal drugs. Such a mechanism may contribute to both *de novo* and acquired endocrine resistance and is consistent with the (re)-expression of ER-regulated genes in these conditions and the favourable clinical responses noted in many patients challenged with a second-line endocrine therapy.

Retention of ER but aberrant growth factor signalling circumvents the need for steroid hormone-driven growth responses

Here aberrations in growth factor signalling elements are envisaged as occurring at points which are downstream of those directly affecting ERs, but which are part of the cellular response pathways used by both growth factors and steroid hormones for promoting tumour growth. Such cancers would not normally be expected to respond to second-line endocrine measures.

New therapeutic targets

Based on the above model for loss of endocrine response in breast cancer, several therapeutic approaches can be envisaged which may delay the appearance of, or even

treat, endocrine insensitivity/resistance, hence severely compromising the disease process. These include the targeting of the following.

ER: pure antioestrogens and anti-steroid hormone receptor regimes

The most efficient theoretical means of eliminating the influences of pathway 'cross-talk' occurrent via ER would be to reduce cellular levels of this receptor. In this light, we (Nicholson *et al.* 1995*a*), and others (Gibson *et al.* 1991, Dauvois *et al.* 1992), have recognised ER down-regulation as a property of pure antioestrogens which is not shared by other antihormonal drugs. Indeed, these agents are certainly more potent than tamoxifen at promoting tumour remissions in several models of human breast cancer, additionally inhibiting growth factor-induced cell proliferation in both oestrogen-sensitive and -resistant ER-positive breast cancer cells (Nicholson *et al.* 1995*a,b*). In parallel, pure antioestrogens appear highly effective in diminishing the cellular ER level and associated expression of oestrogen-regulated mRNAs and proteins in clinical breast cancer specimens (DeFriend *et al.* 1994, McClelland *et al.* 1996), and can furthermore promote long-lasting tumour remissions in patients who have developed tamoxifen resistance (Howell *et al.* 1995, 1996).

Importantly, however, the ER protein is eventually re-expressed at significant levels within pure antioestrogen-treated cells, an event which coincides with an up-regulation of oestrogen-regulated gene expression and the development of a pure antioestrogen-resistant state (Larsen *et al.* 1997). Although the mechanisms associated with the regain of these cellular functions are as yet unknown, ER re-expression coincident with the development of resistance may again imply that additional therapeutic benefit may accrue from re-instigating receptor loss. In this light, we are currently exploring the feasibility of using gene inhibition strategies to further reduce the intracellular activity of the ER. These strategies employ anti-sense technologies (to inhibit ER expression) and ER-dominant negative mutants (to interfere with ER protein function). Certainly, the transient expression of a truncated version of the ER protein (DNER-1), which notably lacks the C-terminal hormone binding and AF-2 domains of the receptor, reduces the ability of the wild-type ER protein to transactivate ERE reporter gene constructs in co-transfected ER-positive breast cancer cell lines (TA Madden & RI Nicholson, unpublished observations).

Growth factor receptors: anti-ligand and receptor regimes

Given the role established for erbB tyrosine kinase receptors in the regulation of breast cell proliferation, a number of approaches have been used to reduce the signalling primed by EGFR and c-erbB-2 within cancer cells.

Several groups have successfully employed immunotherapy with monoclonal antibodies specifically targeting the EGFR or c-erbB-2 proteins to disrupt their subsequent signalling and prevent autocrine loops (Ennis *et al.* 1991, Baselga *et al.* 1996, Kolibaba & Druker 1997). Growth inhibition of EGFR and c-erbB2 overexpressing tumours can thereby be enabled both *in vitro* and *in vivo* (Dean *et al.* 1994, Eccles *et al.* 1994). Indeed, phase II clinical trials with a humanised anti-erbB-2 antibody performed on node-positive breast cancer patients showed an overall response rate of 12% (Baselga *et al.* 1996). Additionally, such antibodies (or antibody fragments) have also been used to deliver drugs, radiation or prodrug-activating enzymes (Harris 1997), in each instance with some evidence of therapeutic benefit. Finally, toxin conjugates of relevant ligands that damage erbB-expressing cells have also been described (Ennis *et al.* 1991, Tang *et al.* 1994, Fiddes *et al.* 1995, Jeschke *et al.* 1995, Siegall *et al.* 1995, Osborne & Coronado-Heinsohn 1996), as have appropriate antisense mRNA strategies (Casalini *et al.* 1997).

Other ways to exploit tumour dependency on growth factor signalling has been to (i) block ligand binding to receptors, using agents such as the trypanocidal drug suramin (Eisenberger *et al.* 1995) or its derivatives (Gagliardi *et al.* 1998) and (ii) use cell-permeable low molecular weight inhibitors specific to a particular tyrosine protein kinase (Kelloff *et al.* 1996). Potential inhibitors of protein kinases include ATP analogues and peptide-based inhibitors, including those competing for the SH2 domain (reviewed in Lawrence & Niu 1998). Of particular promise are the tyrosine-specific protein kinase inhibitors, notably tyrophostins (Lawrence & Niu 1998) and more recently quinazoline derivatives (Ward *et al.* 1994, Wakeling *et al.* 1997), while several naturally occurring compounds may also be relevant (e.g. erbstatin (Toi *et al.* 1990, Umezawa 1995, Wiechen & Dietel 1995), lavendustin A (Onoda *et al.* 1989) and genistein (Clark *et al.* 1996)). Encouragingly, quinazolines not only specifically block the growth-promoting effects of EGFR ligands applied exogenously in culture (Fry *et al.* 1994, Wakeling *et al.* 1996, Jones *et al.* 1997), but have also been shown in a number of cases to reduce basal growth under serum-free conditions (Jones *et al.* 1997). Therefore, while it appears that many such cells are certainly capable of synthesising and secreting ligands which can activate the EGFR in an autocrine manner, it is nevertheless likely that such pathways may be equally susceptible to the inhibitory properties of these new compounds, where they may also fortuitously instigate programmed cell death. Additionally, EGFR-selective tyrosine kinase inhibitors

when used at high concentrations *in vitro* can (i) block the cellular actions of oestrogens on breast cancer cells (Wakeling *et al.* 1996), (ii) reduce the growth of ER-positive oestrogen-growth independent (i.e. resistant) cells (Nicholson *et al.* 1995*b*) and (iii) show additive inhibitory properties when combined with antioestrogens (RI Nicholson, unpublished observations).

Signal transduction pathways

An extensive biological evaluation of the cellular effects of various inhibitors of individual downstream elements in growth factor signalling pathways is being undertaken by numerous groups (see Heimbrook & Oliff 1998). However, of particular interest are Ras protein inhibitors, which either inactivate the enzymes that catalyse its post-translational modification (e.g. farnesyl protein transferase (Kohl *et al.* 1995, Reuveni *et al.* 1997)) or which act to lower Ras mRNA levels through the use of anti-sense oligonucleotides and ribozymes (Monia *et al.* 1992, Kawada *et al.* 1997, Scherr *et al.* 1997). Many naturally occurring compounds also inhibit Ras function, often by preventing association with the membrane, including a Vinca alkaloid (Umezawa *et al.* 1994), squalene (found in olive oil (Newmark 1997)), diallyl disulfide (found in garlic (Singh *et al.* 1996)) and damnacanthal (Hiramatsu *et al.* 1993).

Ras-inhibitory compounds, like EGFR-selective tyrosine kinase inhibitors, might be expected to influence both steroid hormone and growth factor signalling, as would pharmacological and anti-sense inhibitors of PKC (Philip & Harris 1995, Melner 1996, Kobayashi *et al.* 1997, McGraw *et al.* 1997, Geiger *et al.* 1998), Raf (Monia 1997), MAP kinase (Alessi *et al.* 1995, Amundadottir & Leder 1998) and c-src (Hori *et al.* 1992, Levitzki 1996). In our own hands, inhibition of MAP kinase activity by the compound PD098059 is effective not only as a means of reducing growth factor-driven proliferative responses within ER-positive breast cancer cells, but also to inhibit ER activation of ERE reporter gene constructs following transient transfection. Equally, PD098059 blocks (i) MAP kinase-induced expression of the early intermediate response gene Fos, (ii) the subsequent activation of AP-1-mediated signalling (see below), (iii) productive associations between steroid hormone and growth factor signalling pathways in driving gene responses and (iv) oestrogen and growth factor-promoted proliferation of the cells.

Nuclear transcription factors

Potentially useful anti-tumour effects can be generated in breast cancer cells *in vitro* through the inhibition of AP-1 signalling. Relevant inhibitory agents include glucocorticoids (Jonat *et al.* 1990, Kerppola *et al.* 1993, Huang *et al.* 1997) and retinoids (Nicholson *et al.* 1990), as well as AP-1 dominant negative (Olive *et al.* 1997) and anti-sense strategies (Holt *et al.* 1996). In a number of instances, it has been shown that compounds such as all-trans retinoic acid not only have anti-proliferative activity, as mediated by inhibition of AP-1 activity, but importantly that they may also be potent inducers of apoptosis (Mangiarotti *et al.* 1998). We have observed that all-trans retinoic acid efficiently blocks growth factor-mediated expression of Fos protein and AP-1 activity in breast cancer cells *in vitro*. Such inhibition appears sufficient to prevent the growth-promoting effects of oestrogens and furthermore aids the inhibitory effects of antioestrogens (RA McClelland & RI Nicholson, unpublished observations). Such data certainly imply significant and therapeutically exploitable cross-talk between these pathways, and that combination therapy of antihormones and retinoids may be appropriate. It is noteworthy that several laboratories have developed synthetic retinoids that can selectively target AP-1 signalling without activating transcription of retinoid-regulated genes (Chen *et al.* 1995, Nagpal *et al.* 1995, Fanjul *et al.* 1996, Agadir *et al.* 1997, Huang *et al.* 1997). Indeed, since such compounds can synergise with glucocorticoids to efficiently repress phorbol ester-induced AP-1 activity (Chen *et al.* 1995), they may find an expanding role in the therapy of those endocrine responsive and unresponsive cancers which show increased reliance on AP-1 signalling. Finally, many naturally occurring microbial and plant extracts and their derivatives may be of future use. Of particular note are the momordins (Lee *et al.* 1998) and curcumin (diferuloylmethane). These agents inhibit AP-1 activity (Bierhaus *et al.* 1997, Pendurthi *et al.* 1997, Xu *et al.* 1997), the latter compound inducing an unstable, hyperphosphorylated Fos protein (Huang *et al.* 1995) to inhibit proliferation and elicit programmed cell death (Chen & Huang 1998).

Summary and conclusions

Signalling of steroid hormone and growth factor pathways and their key components is far from simplistic, with an elaborate molecular and protein biology and a diverse regulation encompassing a network of phosphorylation cascades. It is becoming increasingly apparent that there are additional layers of complexity to such signalling, with the pathways being intimately linked rather than autonomous. Indeed, several points of productive cross-talk between steroid hormone- and growth factor-directed pathways have now been identified in oestrogen-responsive cells which are believed to markedly reinforce their individual cellular effects on growth and gene responses. It is thus postulated that aberrations occurrent in growth factor signalling pathways could dramatically influence/circumvent steroid hormone action. Certainly, altered elements of growth factor signalling pathways are a relatively common phenotypic characteristic of clinical and experimental breast cancer, a feature which correlates

with the development of endocrine insensitivity in both the *de novo* and acquired setting. A projected paradigm, therefore, is that inhibitory agents (either synthetically or naturally derived) directed towards reducing the influence of growth factors, or of their intracellular signalling pathway components, may prove of clinical benefit in the therapy of breast tumours exhibiting resistance to anti-hormonal measures or may delay the appearance of these deleterious conditions. With the recent and contin-ued expansion of available technologies and an increasing battery of available pharmacological and molecular therapeutic agents, such targeting of aberrant growth factor signalling is now becoming a genuine possibility, and may eventually be applicable to many tumour types.

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