Breast Cancer Research and Treatment **31**: 237–248, 1994.

Hormonal carcinogenesis in breast cancer: cellular and molecular studies of malignant progression

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Key words: malignant progression, estrogen, carcinogenesis, hormone dependence, breast cancer

Summary

We have established and characterized a series of variant cell lines in which to identify the critical factors associated with E2-induced malignant progression, and the acquisition to tamoxifen resistance in human breast cancer. Sublines of the hormone-dependent MCF-7 cell line (MCF7/MIII and MCF7/LCC1) form stable, invasive, estrogen independent tumors in the mammary fat pads of ovariectomized athymic nude mice. These cells retain expression of both estrogen (ER) and progesterone receptors (PGR), but retain sensitivity to each of the major structural classes of antiestrogens. The tamoxifen-resistant MCF7/LCC2 cells retain sensitivity to the inhibitory effects of the steroidal antiestrogen ICI 182780. By comparing the parental hormone-dependent and variant hormone-independent cells, we have demonstrated an altered expression of some estrogen regulated genes (PGR, pS2, cathepsin D) in the hormone-independent variants. Other genes remain normally estrogen regulated (ER, laminin receptor, EGF-receptor). These data strongly implicate the altered regulation of a specific subset or network of estrogen regulated genes in the malignant progression of human breast cancer. Some of the primary response genes in this network may exhibit dose-response and induction kinetics similar to pS2, which is constitutively upregulated in the MCF7/MIII, MCF7/LCC1 and MCF7/LCC2 cells.

Introduction

Carcinogenesis involves three phases, initiation, promotion and progression. Initiation occurs with the first irreversible genetic change within a target cell. Promotion results from the reversible stimulation of the initiated/transformed target cell. Progression describes the process through which the transformed cell acquires additional genetic/epigenetic changes that confer additional growth advantages on specific tumor subpopulations. In breast cancer, the etiologic factors responsible for driving the process of carcinogenesis are largely unknown. However, the steroid hormones, primarily the estrogens and progestins, are widely implicated as critical initiating and/or promoting agents (re-

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viewed in reference [1]). It has been suggested that hormonal carcinogenesis in the human breast reflects the duration of the exposure of breast tissue to estrogens [2].

Many breast tumors appear to follow a predictable clinical pattern. Initially tumors appear to be locally confined, and responsive to endocrine therapy and/or cytotoxic chemotherapy. However, those tumors that are not cured by local therapy (e.g. surgery, radiotherapy) ultimately progress to a phenotype characterized by the presence of metastases that are resistant to both endocrine and cytotoxic modalities. The apparent progression of cells to a more malignant phenotype may reflect responses to selective pressures (e.g. immunological, nutrient deprivation, therapeutic intervention) [3]. Breast cancer is often characterized by a long latency/dormancy [4, 5], and there is a direct association between increasing breast tumor size and increasing aggressivity [6]. These observations reflect the greater number of cell divisions since transformation required to produce the larger tumors. In these tumors, there is a greater probability that some cells will have acquired the necessary phenotypes for increased malignancy [3].

In ongoing studies, we have begun to address several hypotheses related to the malignant progression of breast cancer. We initially hypothesized that an *in vivo* selection of the mammary fat pads of ovariectomized nude mice would provide a highly relevant physiological selection. Critical tumor-host interactions, which could significantly influence tumor progression, would be at least partly mimicked by selecting *in vivo* [7]. Thus, we should be able to apply selective pressures to cells exhibiting an 'early' breast cancer phenotype, and isolate variants with a more progressed phenotype.

We also hypothesized that the estrogen (E2) induced progression of human breast cancer is the result of the induction and/or repression of specific genes, and that these genes are constitutively induced/repressed in E2-independent tumors. Thus, if we could generate relevant variants by *in vivo* selection, we should be able to use these variants to study perturbations in the expression of E2-regulated genes. If we could establish that known E2-regulated genes exhibit differential regulation among the parental and variant cells, we should be able to use the variants to identify the genes that are functionally responsible for conferring hormone-independent growth.

Clonal evolution and malignant progression

There are three critical phenotypic attributes that contribute to the progressed phenotype in human breast cancer. If hormone-dependent cells did not become hormone-independent (i.e. capable of growing in the absence of E2 or in a very low E2 environment), there would be no hormone-responsive tumors in postmenopausal women. On the basis of their response to endocrine manipulation, approximately 70% of breast tumors in postmenopausal women apparently exhibit a hormone-responsive phenotype. Acquisition of an invasive and metastatic phenotype is the second critical phenotypic characteristic. If tumors did not become metastatic, they would be readily cured by local therapy (i.e. surgery and radiotherapy). Finally, if cells did not acquire a multihormonal resistant (MHR) and multidrug resistant phenotype (not necessarily MDR1-mediated multidrug resistance), metastatic disease would be cured by systemic therapy (i.e. endocrine therapy and/or cytotoxic chemotherapy). It is the acquisition of a hormone-independent, multidrug and multihormone resistant phenotype by metastatic tumors that is primarily responsible for the failure of current systemic therapies. Most hormone-independent and metastatic breast tumors become multihormone and multidrug resistant.

The factors that control the process of tumor progression in breast cancer, and the genes responsible for conferring the critical phenotypic changes associated with progression, are unknown. It has been suggested that progression reflects the systematic selection of clonal populations that progressively acquire additional, advantageous phenotypic changes [8, 9]. There are two potential predictions arising from this clonal evolutionary hypothesis. Many tumors may be monoclonal in origin, and cells representative of an 'early' phenotype should respond to appropriate selective pressures by pro-

ducing cells with a more aggressive or progressed phenotype.

The monoclonal origin of breast cancer

Early in development, each cell in a female randomly inactivates one of its X chromosomes. Studying X-linked polymorphic enzymes, for example in combination with PCR-based restriction fragment length polymorphic analyses [10–12], it is possible to determine whether a cell has inactivated its maternal or paternal X chromosome. If breast tumors are polyclonal in origin, then some cells will possess an inactivated maternal X chromosome, and others an inactivated paternal X chromosome. However, studies of breast tumors in patients where the origin of each X chromosome can be readily identified, demonstrate that each cell has inactivated the same X chromosome [11, 12]. Thus, it seems clear that most breast tumors are monoclonal in origin. Phyllodes tumors of the breast, which are considered to be of stromal origin, exhibit monoclonal transformed stromal cells with a polyclonal epithelial component [10].

Acquisition of a more aggressive phenotype: appropriate models

There have been few studies in solid tumors that address the prediction of the clonal evolution hypothesis that 'early' cell populations can acquire a more progressed phenotype. To directly address this aspect of the clonal evolution hypothesis in breast cancer, we have subjected cells representative of an early breast cancer phenotype to an appropriate selective pressure, and isolated and characterized the resultant variants. We have previously described in detail the characteristics of a hypothetical breast cancer precursor cell [13]. Such a cell may be represented by the ER positive epithelial cells in normal mature human breast [14–16], which occur as single cells and are more prevalent in the lobular structures than the interlobular ducts [15]. These potentially hormone-responsive cells may be responsible for the proliferative and secretory functions associated with pregnancy and lactation in normal glandular breast tissue [13].

MCF-7 human breast cancer cells closely represent one early phenotype in the process of breast cancer progression. The cells express high levels of ER and PGR, low levels of EGF-R, and are E2-dependent for growth both *in vitro* and *in vivo* [7]. When growing in E2-supplemented ovariectomized athymic nude mice, MCF-7 cells form poorly invasive [17] and non-metastatic tumors [18–21]. These tumors are adenocarcinomas, exhibiting some degree of apparent differentiation and glandular organization [17], and this is often indicative of an early phenotype.

MCF-7 cells are inhibited by drugs representative of each of the major structural classes of antiestrogens [7], and are sensitive to a variety of cytotoxic drugs, including adriamycin [22–24], melphalan [23, 25], methotrexate and 5-fluorouracil [23] when growing *in vitro*. The hormone-dependent, antiestrogen and multidrug sensitive, poorly invasive and non-metastatic phenotype of MCF-7 cells, clearly defines these cells as representing an appropriate early phenotype in which to study the process of malignant progression in human breast cancer [3, 13].

Appropriate selective pressures for the study of malignant progression in human breast cancer

MCF-7 cells were derived from a pleural effusion in a postmenopausal breast cancer patient [26]. Since the serum E2 levels in ovariectomized nude mice are comparable to those in postmenopausal women [18, 27], these animals provide an appropriate *in vivo* endocrine environment for the selection of E2-independent variants from the E2-dependent MCF-7 human breast cancer cells. Various studies have demonstrated the validity of using an orthotopic site of implantation for the study of tumorigenesis and metastasis [28–31]. The mouse mammary fat pad provides the appropriate orthotopic site for the implantation of human breast cells.

Results

We have performed an extensive series of analyses to isolate and characterize variant populations of the putative 'early' phenotype exhibited by MCF-7 cells. Subsequently, we have used these variants to establish a potential role for E2-regulated genes in the acquisition of a hormone-independent and more aggressive phenotype.

In vivo *selection for hormone-independence and increased metastatic potential*

We wished to obtain direct evidence that human breast cancer cells respond to appropriate selective pressures by producing cells that express a progressed phenotype. MCF-7 cells were selected for growth following orthotopic implantation into the mammary fat pads of ovariectomized, NCr *nu/nu*, athymic nude mice [7, 32]. Proliferating tumors were identified after prolonged selection (approximately 6 months), and reestablished *in vitro* [7]. Cells selected after one passage *in vivo* were designated MCF7/MIII. MCF7/MIII cells were subjected to a second equivalent *in vivo* selection. The resultant tumors were reestablished *in vitro* and designated MCF7/LCC1 [33].

Since the cells were obtained by an *in vivo* selection, MCF7/MIII and MCF7/LCC1 cells were screened for the presence of mouse cells. Each variant was examined for the presence of mouse chromosomes and for immunoreactivity with antimouse antisera. These studies clearly demonstrate the absence of mouse cells, and the presence of only human chromosomes [7, 33].

MCF-7 origin of the hormone-independent MCF7/MIII and MCF7/LCC1 variants

Polymorphic enzyme and karyotype analyses were performed to confirm the MCF-7 origin of the variants. The polymorphic enzymes were analyzed were adenylate kinase (EC 2.7.4.3), esterase D (EC 3.1.1.1), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), glyoxalase (EC 4.4.1.5), lactate dehydrogenase, mitochondrial malic enzyme (EC 1.1.1.40), phosphoglucomutase-1 (EC 2.7.5.1) and phosphoglucomutase-3 (EC 2.7.5.1). Karyotype and isozyme analyses were performed by Dr. Ward Peterson (Children's Hospital of Michigan, Detrout, MI). The patterns of polymorphic enzyme and marker chromosome expression clearly identify the MCF7/MIII and MCF7/LCC1 cells as being derived from MCF-7 cells [7, 33].

Selection or adaptation: evidence from karyotype analyses

Analyses of a sufficient number of individual karyotypes enables an estimation of chromosome number range (Table 1). These studies indicate a progressive narrowing of the chromosome number range with each *in vivo* selection. These observations are consistent with the clonal expansion hypothesis, suggesting the emergence of a subpopulation of cells with an ability to grow in the low E2-environment. However, we are unable to determine whether this reflects selection of a preexisting E2-independent subpopulation within the parental MCF-7 cells, or an adaptation of a MCF-7 subpopulation to E2-independent growth. Either condition could give rise to our ability to isolate E2-independent cells from MCF-7 populations.

It is reasonable to assume that the emergence of the E2-independent cells, whether the result of adaptation or selection, was the result of a growth advantage of the variants over E2-dependent cells. Since MCF-7 cells have been consistently maintained in the presence of estrogenic stimuli, there has been no growth advantage conferred upon the hormone-independent cells. This is evident from

Table 1. The chromosome number range of the MCF-7 variants. Data was obtained from karyotypes analyses performed by Dr. Ward Peterson (Children's Hospital of Michigan, Detroit, MI)

Cell line	Chromosome number range	Reference
MCF-7	58–98	[7]
MCF7/MIII	65–75	[7]
MCF7/LCC1	66–73	[33]

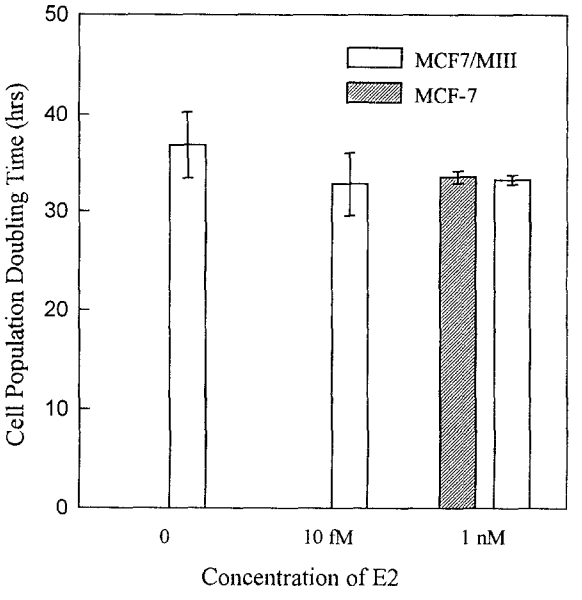

Fig. 1. Cell population doubling times of MCF-7 and MCF7/MIII cells growing in IMEM without phenol red and supplemented with 5% steroid stripped calf serum. It is reported that these culture conditions contain ≤ 10 fM of E2 [36]. Thus, the final concentration of E2 in the 10 fM-treated cells is likely to be in the range of 10–20 fM. Data adapted from Clarke *et al.* Proc Natl Acad Sci USA 86: 3649–3653, 1989 [7].

Fig. 1, which clearly demonstrates that *in vitro*, MCF-7 and MCF7/MIII cells growing in the presence of E2 have equivalent cell population doubling times. In the absence of E2, the MCF7/MIII but not MCF-7 cells proliferate.

A definitive delineation between selection and adaptation in this system would provide little useful information. At some point during the evolution of the MCF-7 tumor, adaptation to E2-independent growth must have occurred. This adaptation arose either in a subpopulation during the growth of the original tumor in the patient, or during the selection *in vivo* in the nude mouse. The original MCF-7 tumor arose in a postmenopausal women [26]. Thus, it seems likely that the primary tumor contained hormone-independent cells, and that these cells are present in the parental MCF-7 cells, perhaps in relatively small numbers.

MCF7/MIII and MCF7/LCC1: a hormone-independent, hormone-supersensitive, or antiestrogen resistant phenotype

MCF7/MIII [7, 34] and MCF7/LCC1 cells [33] are refractory to the mitogenic effects of E2 *in vitro*. This could represent either a fully hormone-independent, hormone-supersensitive, or hormone-unresponsive (antiestrogen resistant) phenotype. We wished to determine which of these phenotypes best described the MCF-7 variants.

A putative estrogen supersensitive MCF-7 variant has been previously described [35]. A hormone-supersensitive phenotype could grow in the low E2 environment of an ovariectomized nude mouse, or the very low (≤ 10 fM) E2 concentrations present in charcoal stripped serum [36]. A common mechanism for producing an increased sensitivity to a ligand requires an increased expression of hormone receptors. Thus, the degree of receptor occupancy required to induce a biological response is achieved at lower ligand concentrations. As is apparent from the ligand binding data in Table 2, the levels of ER expression in the hormone-independent MCF-7 variants are equivalent to the parental cells.

To further address the possibility of a supersensitive phenotype, we determined the ability of MCF7/LCC1 cells to respond to E2 while growing in a serum-free, chemically defined medium, devoid of serum steroids and phenol red [37]. MCF7/LCC1 cells proliferate rapidly in this medium, and were maintained successfully for several passages. In serum-free media, MCF7/LCC1 cells are unresponsive to the mitogenic effects of exogenous E2 (unpublished observations).

Table 2. Estrogen and progesterone receptor expression in parental and variant MCF-7 cells. Numbers represent hormone binding sites per cell × 10^3. Cells treated + E2 were treated with 1 nM E2 for 48 hours prior to the receptor assay. Whole cell binding assays were performed as previously described [23]

Cell line	ER	PGR-E2	PGR + E2	Reference
MCF-7	120 ± 20	5 ± 2	47 ± 12	[7]
MCF7/MIII	239 ± 95	10 ± 3	60 ± 6	[7]
MCF7/LCC1	117 ± 16	29 ± 7	75 ± 21	[33]
MCF7/LCC2	91 ± 1	6 ±1	17 ± 2	[42]

Since antiestrogens primarily function through their interactions with ER [38], a supersensitive phenotype would be expected to exhibit a concurrent sensitization to antiestrogens. We determined the dose response relationship among a series of antiestrogens representative of each of the major structural classes, i.e. triphenylethylene (TAM), benzothiophene (LY117018), steroidal (ICI 164,384) and other nonsteroidal (nafoxidine) antiestrogens. Using an anchorage independent assay, we observed equivalent dose response curves in MCF-7 and MCF7/MIII to each of these antiestrogens [34]. Similar data were obtained with MCF7/LCC1 cells [33]. MCF7/MIII cells also retain sensitivity to the inhibitory effects of LHRH analogues *in vivo* [39].

Growth of the MCF-7 variants is unlikely to reflect increased endogenous estrogen biosynthesis

The hormone-independent phenotype of MCF7/MIII and MCF7/LCC1 could reflect an ability of these cells to synthesize their own estrogens. This could be the result of P450 aromatase activity [40], or an increased release of estrogen sulfates by the sulfatase enzyme [41]. To address this possibility, we have investigated the dose response relationship among MCF-7, MCF7/MIII cells and the ER negative MDA-MB-231 cells to the aromatase inhibitors aminoglutethimide and 4- hydroxyandrostenedione, and the sulfatase inhibitor danazol. Preliminary data indicate equivalent dose response relationships among all three cell lines to each agent. Definitive proof awaits direct assessment of the activities of the P450 aromatase and sulfatase enzymes. These studies are currently in progress.

MCF7/MIII and MCF7/LCC1 cells represent a hormone-independent and responsive phenotype

The data from our various studies indicate that MCF7/MIII and MCF7/LCC1 cells are neither antiestrogen resistant, nor hormone-supersensitive. We have chosen to designate this phenotype as being a hormone-independent and hormone-responsive phenotype. This reflects their ability to grow *in vivo* and *in vitro* without E2, but their retained sensitivity to the ER-mediated antiproliferative effects of antiestrogens *in vivo* and *in vitro*. By these criteria, the parental MCF-7 cells are designated hormone-dependent, and ER-negative cells (e.g. MDA-MB-231) are designated hormone-unresponsive.

Selection for antiestrogen resistance in vitro

The development of resistance to antihormonal therapies represents another major phenotypic characteristic of malignant progression in breast cancer [3, 38]. Since MCF7/MIII and MCF7/LCC1 cells are responsive to antiestrogens, we wished to isolate a population resistant to antiestrogens. Following a stepwise selection *in vitro* against 4-hydroxytamoxifen, we established a stably resistant population designated MCF7/LCC2 [42]. These cells retain ER and PGR expression, and exhibit hormone-independent growth both *in vitro* and *in vivo*. MCF7/LCC2 cells are resistant to the inhibitory effects of 4-hydroxytamoxifen when growing both *in vitro* and *in vivo* [42]. However, MCF7/LCC2 cells are not cross resistant to the steroidal antiestrogens ICI 182,780 [42] and ICI 164,384 [43]. These data would predict that patients failing TAM would respond to a steroidal antiestrogen. Data from a phase I trial of ICI 182,780 clearly demonstrate responses in patients that have failed TAM [44]. Thus, the pattern of antiestrogen responsiveness exhibited by the MCF7/LCC2 cells accurately predicted for a pattern of clinical response.

Phenotypic changes associated with malignant progression – increased invasiveness

One of the critical characteristics of malignant progression is the acquisition of an invasive and metastatic phenotype [3, 13]. The selective pressures that confer a survival advantage upon cells with a metastatic phenotype remain unclear. Metastasis is a multistep process, requiring the tumor to provide access to the vasculature through neovascularization, and some tumor cells to be invasive (e.g. able

to degrade basement membrane), motile (e.g. able to intravasate and extravasate), and capable of proliferation at a secondary site [45, 46].

We would expect to observe increased invasiveness in the variants for several reasons. Invasiveness, as estimated by the ability to digest an artificial basement membrane *in vitro*, is E2-regulated in MCF-7 cells [34, 47]. Since MCF7/MIII and MCF7/LCC1 cells have acquired hormone-independent growth, they also may exhibit altered expression of other E2-regulated functions. The physical restriction of inoculation into the mammary fat pad might provide a selective pressure that would favor locally invasive cells.

Both MCF7/MIII and MCF7/LCC1 cells can invade locally from solid mammary fat pad tumors, and produce primary extensions on the surface of intraperitoneal structures including liver, pancreas, and diaphragm [17]. Furthermore, both variants are significantly more invasive than MCF-7 cells by two or more fold, when compared using the Boyden chamber assay of *in vitro* chemoinvasive potential [7, 33].

The artificial basement membrane used to assess chemoinvasion *in vitro* contains Matrigel, a type IV collagen matrix containing various factors including laminin and heparan sulfate proteoglycans. Laminin receptor expression is E2-regulated in MCF-7 cells [48]. However, we observe no increase in either the level of laminin attachment, or laminin receptor mRNA expression in the MCF7/MIII or MCF7/LCC1 cells [17]. We also have estimated the level of secretion of type IV collagenase and the E2-regulated cathepsin D. Secreted type IV collagenolytic activity is not altered in the variants [17]. However, cathepsin D secretion is significantly increased in the MCF7/MIII, MCF7/LCC1 and MCF7/LCC2 cells [17, 43]. The role of cathepsin D expression in conferring the increased invasiveness remains to be determined [49].

Phenotypic changes associated with malignant progression – increased metastatic potential

Access to vascular tissues is critical for metastatic dissemination. While we have not attempted to quantitate the degree of vascularization in parental and variant tumors, their ability to produce viable tumors in excess of 1 mm^3 strongly implies an induction of tumor vasculature. Individual MCF7/MIII and MCF7/LCC1 tumors exhibit clear neovascularization on gross examination, but this is not readily apparent in all tumors.

There have been no clear reports of metastatic or invasive spread from either ZR-75-1 or T47D hormone-dependent cells growing *in vivo*. Two studies report the presence of secondary lung deposits arising from MCF-7 tumors with frequencies of 15% [50] and 40%, respectively [51]. Tumor growth and metastasis are hormone-dependent [50, 51]. However, the majority of studies do not report metastases from parental MCF-7 tumors [18–21, 52].

Following MCF7/MIII or MCF7/LCC1 inoculation into mammary fat pads, both lymphatic and hematogenous dissemination are observed [17]. This can result in the establishing of pulmonary, bone, and lymph node metastases [17]. The pattern of metastasis by MCF7/MIII and MCF7/LCC1 cells closely resembles that frequently observed in breast cancer patients. However, the interexperimental incidence of metastases, and the time from cell inoculation to the appearance of metastatic disease are variable. The current incidence of macroscopic metastases ($\leq 10\%$) is too low and unpredictable for these variants to provide a reproducible model for metastasis in human breast cancer [17]. Nevertheless, the data clearly support the hypothesis that appropriate *in vivo* selection can produce variants with a more aggressive/progressed phenotype.

Perturbations in the expression of E2-regulated genes – phosphoinositide turnover

Many growth factors and oncogenes, including TGF-α and the *ras* oncogene, can utilize phosphatidyl inositol as a substrate for transduction of hormone and growth factor signals initiated by receptor-ligand interactions [53]. A MCF-7 variant selected in the presence of increasing concentrations of the antineoplastic agent adriamycin ($MCF\text{-}7^{ADR}$) produces E2-independent and E2-unresponsive tumors *in vivo* [22]. These cells exhibit a 60-fold in-

crease in the hydrolysis of phosphoinositides when compared with the parental cells [54]. We have directly determined steady state [^{3}H] myo-inositol turnover in the MCF-7 and MCF7/LCC1 cells. While MCF7/LCC1 cells have acquired E2-independent growth, this is not accompanied by elevated steady state levels of inositol phosphate turnover. Furthermore, while E2 increases inositol phosphate turnover in MCF-7 cells, MCF7/LCC1 cells are refractory to induction by E2 [33].

Altered gene expression associated with progression to E2-independence: role of E2-regulated genes

By studying the levels of expression and E2-regulation of several genes and proteins in the E2-independent variants, we have clearly demonstrated that E2-induced progression, and progression to E2-independence are associated with the altered regulation of specific genes. The data in Table 2 clearly demonstrate that expression of ER, which is E2-regulated in MCF-7 cells, remains largely unaltered as cells acquire an E2-independent phenotype. In marked contrast, expression of baseline levels of PGR protein in the absence of E2 appear to increase as cells become stably E2-independent. However, PGR mRNA levels are not upregulated [33]. The increased PGR protein expression in MCF7/LCC1 cells probably reflect posttranslational effects.

Induction of pS2 mRNA expression by E2 is considered a primary estrogenic response in MCF-7 cells [55]. The baseline level of pS2 mRNA expression in MCF7/LCC1 [33] and MCF7/LCC2 cells [43], growing both *in vitro* and *in vivo* in the absence of E2, is significantly elevated above that observed in MCF-7 cells. Cells growing *in vitro* appear to be effectively E2-unresponsive for induction of pS2, suggesting a constitutive expression. In contrast, expression of pS2 mRNA *in vivo* retains some E2-inducibility. This reflects the apparent ability of E2 to increase cell proliferation *in vivo* but not *in vitro* [33]. Expression of TGF-α mRNA in the absence of E2 remain unaltered in the E2-independent MCF-7 variants. These cells also retain the ability of E2 to increase expression of these mRNAs [21]. The maximum levels of expression of TGF-α and EGF-receptor in the E2-independent variants never exceeds that observed in their respective hormone-dependent parental cells (manuscript in preparation).

Discussion

A comparison of the phenotypic perturbations acquired by the MCF-7 variants has raised a series of novel issues in breast cancer biology [3, 13, 34, 56]. For example, it appears that hormone-independence and antiestrogen resistance are independent phenotypic characteristics [3, 34]. This is clearly apparent from the antiestrogen sensitivity of the MCF7/MIII and MCF7/LCC1 cells [33, 34]. The independence of these phenotypes is further substantiated by the analysis of the phenotypes of breast tumors. The antiestrogen responsive tumors in postmenopausal women also are hormone-independent and hormone-responsive by our criteria.

Despite the hormonal regulation of various aspects of the invasive/metastatic cascade, MCF-7 cells selected for hormone-independent growth *in vitro* do not appear to exhibit the increased metastatic potential of the MCF7/MIII and MCF7/LCC1 cells [7, 17]. Thus, hormone-independence and increased metastatic potential are independently acquired processes [3]. The validity of this assumption gains some support from the observation that a significant proportion of breast tumors in postmenopausal women do not, at least initially, exhibit detectable metastatic spread. Some of these breast cancer patients can survive 30 years or more, being apparently cured by surgery [57].

Acquired resistance to cytotoxic drugs also appears to occur independently of increased metastatic potential and hormone independence [13]. For example, the dose response relationships for methotrexate (non-gp170 substrate) and colchicine (gp170 substrate), are comparable in MCF-7 and MCF7/LCC1 cells [13]. Breast cancer is generally considered, at least initially, to be one of the more drug responsive solid tumors. Many metastatic tumors respond to a variety of cytotoxic regimens, including those incorporating cyclophosphamide,

methotrexate, 5-fluorouracil, adriamycin and vincristine [58]. While many tumors ultimately become resistant to these agents, their initial responsiveness is consistent with drug resistance being acquired independently of either hormone-independence or metastatic potential.

Biological relevance of the MCF7/MIII, MCF7/LCC1 and MCF7/LCC2 phenotypes

Since the plasma E2 levels in castrated female nude mice are equivalent to that found in postmenopausal women [18, 27], selection in the mammary fat pads of these mice should increase the potential to isolate variants with appropriate phenotypes. The variants we have isolated are ER and PGR positive, express low EGF-R levels [7], are antiestrogen sensitive [34], and invasive [17]. This phenotype closely reflects the phenotype expressed by many ER/PGR positive human breast tumors in postmenopausal women. Thus, when compared with their parental cells, these variants provide a novel model for investigating the critical aspects of E2-induced progression in ER positive, E2-responsive, antiestrogen resistant tumors.

E2-independent growth, resistance to triphenylethylenes, and a high metastatic potential, are three of the most critical attributes that breast tumor cells must acquire in order to survive and colonize sites distant to the primary tumor [3]. For example, the acquisition of an E2-independent phenotype is essential for the progression of human breast tumors. Fully E2-dependent tumors would proliferate only slowly, or not at all, in postmenopausal women. Antiestrogen responsive tumors, whether or not they were metastatic, would have a high potential for being cured by treatment with antiestrogens if they did not acquire antiestrogen resistance or a multihormone resistant (MHR) phenotype. ER positive tumors that did not invade and metastasize in a very low estrogen environment could be readily cured by local therapy. MCF7/MIII, MCF7/LCC1 and MCF7/LCC2 cells exhibit various aspects of these critical phenotypic attributes in a manner that closely reflects the human disease.

The role of E2-regulated genes in acquired hormone-independence – gene networks

We have demonstrated that the expression of many E2-regulated genes are unaltered by acquisition of hormone-independent growth (ER, TGF-α, EGF-receptor, laminin receptor, PGR mRNA). However, other genes clearly exhibit increased expression in the absence of E2 (PGR protein, cathepsin D secretion, pS2 mRNA). These data provide clear support for the hypothesis that hormone-independent growth is the result of the altered expression of a specific subset or network of E2-regulated genes. Preliminary data from 2-dimensional gel electrophoretic analyses of global patterns of protein expression provide further support for this hypothesis. We have observed two distinct patterns of expression, a progression-like pattern (E2-induced in MCF-7, constitutive in MCF7/LCC1) and a suppressor-like pattern (E2-repressed in MCF-7, constitutively repressed in MCF7/LCC1) [59]. We are currently identifying a series of hormonally-regulated and differentially expressed proteins, which appear to be associated with hormone-independent and antiestrogen resistant growth.

We have observed that only a relatively small subset of hormone-regulated genes/proteins are associated with acquired hormone-independence [33, 59]. Steroids frequently induce gene expression in apparent 'networks' [60], each network exhibiting different characteristics often in terms of dose response relationship, time course of induction, level of regulation (transcriptional e.g. pS2 [33] or post-translational e.g. PGR [33]), and biological function. Thus, we hypothesize that the acquisition of hormone-independence is associated with a small network of genes that are regulated by E2 and responsible for E2-induced mitogenesis. Some of the primary response genes in this network may exhibit dose-response and induction kinetics similar to pS2 [55], since pS2 is constitutively upregulated in the MCF7/MIII, MCF7/LCC1 and MCF7/LCC2 cells [33, 43]. The secondary genes in this network may exhibit regulation characteristics comparable to cathepsin D [17, 43].

The altered regulation of pS2 mRNA [33, 43] and PGR protein but not mRNA [33] illustrate some of

the problems associated with identifying the functionally relevant genes. pS2 is E2-inducible in MCF-7 cells [55] and overexpressed in the variants [33, 43], but is probably not functionally responsible for any of the phenotypic changes induced in the variants. There are almost certainly additional genes that are differentially regulated in this manner, but functionally irrelevant. The altered expression of PGR protein but not mRNA indicate that some critical events may occur at the level of altered protein expression/function, and will not be amenable to the now standard molecular biologic analyses. The challenge is to identify only those genes/proteins that are involved in conferring the phenotypic characteristic of interest.

We may be able to predict some of the probable functions of the critical genes. Since hormone-independence is functionally defined as an ability to proliferate in the absence of hormone, some genes/proteins will be involved in regulating (directly or indirectly) cell cycle progression. Many of the critical control points in cell cycle regulation are thought to be the result of altered protein function, perhaps as the substrates of cell cycle dependent kinases [61]. The expression of some of the critical genes, particularly those downstream of the primary/master regulatory genes, may be the consequence of altered cell cycle regulation e.g. genes that are expressed only during specific phases of the cell cycle. Since TAM treatment and hormone-withdrawal are associated with the induction of apoptosis [62], some genes may be closely associated with the regulation of programmed cell death.

Conclusions

We have found the phenotypic changes acquired by the MCF7/MIII, MCF7/LCC1 and MCF7/LCC2 cells to be stable over prolonged periods. For example, the MCF7/MIII cells have retained their phenotype for over 5 years. Even when routinely maintained in the absence of selective pressures (i.e. in the presence of E2), MCF7/MIII remain hormone-independent and hormone-responsive. This observation strongly suggests that the events that conferred the phenotype are inheritable, and may reflect increased genetic instability and/or 'epigenetic instability' [56]. Should the altered gene network have become activated through epigenetic instability, it may be a potentially reversible process. We are currently investigating the ability of various agents to restore the hormone-dependence and antiestrogen responsivity to these MCF-7 variants.

When generating animal models of human disease, there is often a tendency to attempt to fit the disease to the model, rather than the model to the disease [63]. Extensive characterization of the phenotypes of the MCF-7 variants have identified only changes that are reflected in the human disease. We have yet to observe characteristics in the variants that are not observed in many human breast tumors. Ultimately, the validity of this approach and the utility of the resultant variants will depend upon the ability to use these variants to predict accurately responses not previously reported in the human disease, and to identify the genes/mechanisms responsible for conferring critical phenotypic characteristics. The antiestrogen resistant phenotype of the MCF7/LCC2 cells has already successfully predicted a clinical phenotype (non-crossresistance between triphenylethylene and steroidal antiestrogens) that was not previously reported. We have begun to isolate and identify differentially regulated proteins apparently associated with critical phenotypic perturbations [59]. However, the relevance of these proteins to the human disease remains to be determined.

Acknowledgements

This work was supported in part by Public Health Service grants: NIH R01-CA58022 (R. Clarke), NIH R55-CA51782 (R. Clarke), U01-CA51908 (R. Clarke), the Lombardi Cancer Research Center Animal Core Facility funded through NIH 1P30-CA51008 (R. Clarke), and a research grant (A.I.C.R. # 90BW65) from the American Institute for Cancer Research (R. Clarke).

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