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14. ABSTRACT

The factors driving resistance to antiestrogens are unknown. Comparing the transcriptomes of antiestrogen responsive and resistant MCF-7 variants by serial analysis of gene expression, we have implicated several genes, including the human X-box binding protein-1 (XBP-1). XBP-1 is a cAMP response element (CRE) binding protein associated with estrogen receptor (ER) expression in gene expression profiles of human breast cancers. **We hypothesize** that overexpression of XBP-1 and/or activation of CRE contribute functionally to the ability of responsive cells to survive the metabolic stresses induced by exposure to antiestrogens. **We also hypothesize** that measuring expression of the XBP-1 protein will assist in better identifying antiestrogen resistant and/or responsive tumors. **Aim 1:** We will further study the likely functional role of XBP-1/CRE by overexpression through transfection into responsive cells, and inhibiting expression in resistant cells using novel CRE oligonucleotide decoys, antisense and/or ribozymes. Effects of these molecular manipulations on responsiveness to antiestrogens will be studied *in vitro* and *in vivo*. **Aim 2:** We will explore the prognostic and predictive significance of XBP-1 expression in a unique series of human breast cancer biopsies. Thus, we will assess the extent to which XBP-1 is an independent prognostic factor, and whether it is associated with response to antiestrogens. Where possible, we will explore whether XBP-1 expression data allow us to build better predictive/prognostic models.

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Nov 29, 2005

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INTRODUCTION

Antiestrogens are effective in premenopausal and postmenopausal patients, and in the chemopreventive, adjuvant and metastatic settings (1), probably through the induction of growth arrest/apoptosis (1). The triphenylethylene TAM, a partial agonist, is the most widely used antiestrogen. Long term TAM use reduces the incidence of contralateral breast cancer (antagonist) and primary breast cancer in high risk women (antagonist), maintains bone density (agonist) and increases the risk of endometrial carcinomas (agonist) (2). Newer antiestrogens include the "pure antagonist" ICI 182,780 (Faslodex; no agonist activity). In patients that had previously shown a response to TAM but recurred, Faslodex produces a response rate significantly higher than the response rate for crossover to another triphenylethylene (Toremifene) following TAM failure (3).

Antiestrogen Resistance. Most breast tumors that initially respond to TAM recur and require other endocrine or cytotoxic therapies (4). Despite over 10 million patient years of experience with TAM, the precise mechanisms that confer acquired resistance are unknown (1). Absence of ER expression is clearly important for *de novo* resistance (1). ER expression is *not* lost in most breast tumors that acquire antiestrogen resistance (5). Currently, there is little compelling evidence that expression of ER splice variants and mutant ER contribute significantly to antiestrogen resistance in patients (1,6). While the importance of wild type ERα is established as a mediator/predictor of antiestrogen responsiveness, that of ERβ remains unclear. ERα may be the predominant species in most ER+ breast tumors (7,8), and is associated with a better prognosis (9). ERβ is associated with a poorer prognosis, absence of PgR, and lymph node involvement (8,10). One small study reported higher ERβ mRNA levels in resistant tumors (11). However, this association could not be separated from that between ERβ and a more aggressive phenotype (8,10). Some studies report activities independent of ER function, which may initiate events that are necessary but not sufficient for antiestrogen-induced effects (1). Our research team has recently reviewed in detail the potential mechanisms of antiestrogen resistance in ER+ tumors (12).

Implicating XBP-1 in Antiestrogen Resistance. Initially, we explored differences in the transcriptomes of the MCF7/LCC1 (antiestrogen sensitive) and MCF7/LCC9 cells (antiestrogen resistant – resistant to both TAM and Faslodex) by serial analysis of gene expression (SAGE) as previously described (13), using the "SAGE" software (Dr. Kinzler, Johns Hopkins University). Most genes identified are not differentially expressed between MCF7/LCC1 and MCF7/LCC9 cells. Differentially expressed genes were selected by (a) the Tags compared represent $\leq$2 genes, (b) a Tag found in either the MCF7/LCC1 or MCF7/LCC9 SAGE library must represent $\tilde{}$0.10% of the database, and (c) fold difference ~2-fold. Evidence that a gene is expressed in breast cancers also was considered. No single criterion was considered an absolute requirement for selection. Among the genes we identified were cathepsin D, nucleophosmin (NPM), tumor necrosis factor (TNF) and XBP-1 (14).

To confirm the altered expression of XBP-1, we first performed Western analysis on proteins from MCF7/LCC1 and MCF7/LCC9 cells. We initially detected a ~5-fold induction of XBP-1 protein in MCF7/LCC9 cells, comparable with the 4-fold induction in mRNA levels (14). Measuring protein levels and/or protein bound to responsive elements can be poor indicators of the functional activation of transcription factors. Since XBP-1 activates CREs, we measured directly CRE transcriptional activation using a CRE promoter-firefly luciferase reporter assay

(PathDetect *in vivo* signal transduction pathway *cis*-reporting system; Stratagene). Cells were transiently transfected with the appropriate plasmids using Qiagen's Superfect reagent. Normalization of transfection efficiency was made to a *Renilla* luciferase reporter driven by the constitutive cytomegalovirus promoter (Promega's Dual-luciferase reporter assay). The basal CRE activity is significantly increased in MCF7/LCC9 cells compared with MCF7/LCC1 cells (14).

Upregulation of CRE activation would be of limited use to cells if it could be inhibited by Faslodex-occupied ERs. Thus, we assessed the ability of Faslodex to affect CRE activation using the promoter-reporter assay. Faslodex treatments (10 nM) were administered for 48 hrs post-transfection. Faslodex treatment does not alter the transcriptional regulatory activities of the CRE promoter in either responsive MCF7/LCC1 or resistant MCF7/LCC9 cells (14). These data further imply a functional role for XBP-1 in acquired resistance to Faslodex. In responsive cells, the inability to induce CRE in the presence of Faslodex allows for the dominance of growth inhibitory signals leading to growth arrest/apoptosis. Resistant cells may survive growth inhibition/apoptosis by upregulating signaling through CREs. Since CRE-activation is required for MCF-7 cell proliferation (15), some breast cancer cells may survive antiestrogen treatment by upregulating factors that are not affected by ER-mediated signaling, e.g., XBP-1/CRE.

BODY

This is a final report and a request for a no-cost extension is pending. Thus, an "amended final" report will be submitted at the end of the no-cost extension period. This "amended final" report will provide all the data generated to that point. In this report we present only the work completed in the previous 12-months.

KEY RESEARCH ACCOMPLISHMENTS

We have continued to make good progress and have nearly completed most of the Tasks as proposed. We have made changes to Task 3 to change the direction in a more productive manner and obtained significant new data from gene expression microarray studies. These new studies provide powerful mechanistic insights not initially envisioned in the application. We are on target to complete most of the unfinished work and have requested a no-cost extension to help us do so.

Bulleted List of Research Accomplishments

- Performed small *in vivo* pilot study
- Published the first study on XBP-1 expression in breast tumors
- Performed gene expression microarray studies on MCF7/XBP-1 cells
- Completed "low-end" analyses of microarray data and identified genes implicated in driving XBP-1's effects on cell cycle and apoptosis
- Selected first round of candidate genes for validation

- Validated differential expression of three genes (ERα, Bcl2, CYP19/aromatase) by Western or real-time PCR

- Designed and built XBP-1 siRNA constructs

- Selected the siRNA construct that is most effective in reducing XBP-1 activity in CRE promoter-reporter assays

Nota Bene: Please note that we propose a change of emphasis for Task 3 in the SOW. This is discussed under Task 3 and the new data we have generated to be consistent with the modification also are presented under Task 3 (below).

RESEARCH ACCOMPLISHMENTS

TASK 1: Overexpress XBP-1 in antiestrogen sensitive cells

This aim has been completed except for the *in vivo* study. A small preliminary experiment was inconclusive with respect to XBP-1 conferring full estrogen-independence *in vivo* in MCF7/XBP-1 cells. Thus, we are in the process of repeating this study and including the T47D clones (these were not adequately characterized when we did the preliminary study in the MCF-7 model). If we do not get full estrogen-independence, we may not pursue these studies because the incidence of estrogen-independent tumors may require larger numbers of animals to obtain statistical power. If we need to give estrogen supplementation to increase tumor incidence, the study design also becomes more complex. We would prefer to use any remaining resources to explore the exciting cellular signaling data we have generated in the past year (see Task 3; below).

TASK 2: Inhibit XBP-1 expression in antiestrogen resistant cells

We had some delays in getting these experiments initiated and some technical problems with personnel changes and medical leave; these delays were reported in last year's report. To try and recover time on this Task, we decided to take an siRNA approach. We thought that this approach also would be more specific and provide more readily interpretable data than the primary approach initially proposed (CRE oligos). However, it took more time than anticipated to design, obtain, screen, and finally identify the most effective siRNA construct. We have now identified

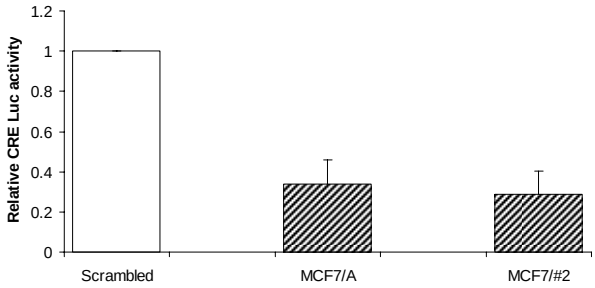

Fig 1: Activity of XBP-1 siRNA in a CRE promoter-reporter assay. Data represent mean ± SE for three experiments in two different MCF-7/XBP-1 clones.

the most active siRNA, which reduces XBP-1 activity by approximately 70% (Fig 1). In these experiments we used 5 nM hXBP-1 siRNA or scrambled sequence control. Cells were cotransfected with 0.4 µg of CRE- luciferase plasmid and 0.1 µg of a plasmid containing the *Renilla* luciferase gene (control). We observed significant suppression of CRE activity in the XBP-1 transfectants (MCF/A, p=0.032; MCF/#2, p=0.025). We hope to be able to use the siRNA to assess directly the effects of inhibiting XBP-1 in the MCF7/XBP-1 and T47D/XBP-1 cells and in the LCC1 and LCC9 cells that provided the initial observations in support of this award (14).

TASK 3: Explore the molecular events that confer XBP-1's ability to affect endocrine responsiveness (modified Task for Statement of Work)

TASK 3: Timing of acquired increase in XBP-1 expression and CRE activation (original task)

While we were progressing with these proposed studies, we had the opportunity to perform gene expression microarray experiments on the MCF7/XBP-1 cells, which is the best characterized of our transfected models. After discussion by the research team and an evaluation of our progress and data on this task, we felt that this would likely be a much more productive and informative research direction and better use of our remaining resources. Thus, we chose to perform these studies as a higher priority; this should be seen as a modification of the SOW and associated Task 3, since these were not proposed as a primary goal in the initial application.

Experimental Design: We collected high quality total RNA from 6 independent cultures (cell populations gown on different days from different stocks); three from MCF7/XBP-1 cultures and three from the vector control cultures. MIAME 1.1 compliant data were collected as proposed by the Microarray Gene Expression Data (MGED) Society (http://mged.org). RNA quality was assessed by several measures, including RIN number (16), OD readings, and visual inspection of the electropherograms from Agilent Bioanalyzer analyses. RNA was prepared, labeled and hybridized to U133A Affymetrix GeneChips using standard and/or manufacturer recommended procedures.

Data Preprocessing and Analysis: We have not yet completed the detailed analysis of this data set. We take an in-depth approach that includes the use of commercial, published freeware, and in-house methods, many of which are computationally intensive and require time on our institutional computational grid to complete. We here present the results of our initial "low-end" analyses, which already have identified exciting and potentially critical new insights into the role of XBP-1 in driving the phenotypes we have described above and in our previous reports.

The raw gene expression data were preprocessed using the RMA software available through the Bioconductor project. For further data analysis, we used either existing or in-house routines programmed in the R (GNU S) statistical software development environment, and in-house algorithms developed and implemented in MatLab (MathWorks). Some of our in-house methods are already published (17-22). Quality control (QC) measures include the "spiked-in" controls recommended by Affymetrix. Data quality was assessed using various tools including those recommended by Affymetrix and by a series of additional QC measures developed in our laboratory. These measures test the quality of each major step in the extraction, preparation and labeling of RNA, and the quality of the hybridization and overall digitized data as obtained post-hybridization.

The basic approach for data analysis is to first apply a series of predesigned filters to generate a reduced dimensional data set enriched in the most informative signals (by excluding those genes least likely to be differentially expressed between the two experimental groups). For studies into gene network signaling, these filters generally comprise a series of univariate measures. These measures are applied without correction for multiple comparisons to minimize the proportion of false negative data. For the "low-end" analysis, the filters applied resulted in a reduced data set containing genes that exhibit $\geq$1.5 fold change, $p<0.05$ (pairwise univariate comparisons) and genes with intensity $\geq$log2(10) in both control and experimental groups.

Data visualization before and after dimensionality reduction is facilitated by multidimensional scaling as estimated using Principal Component Analysis (PCA) and our Discriminant Component Analysis (DCA) method (18). This ensures that the global structure of the data has not been compromised during the reduction procedures. In this experiment, the control and XBP-1 transfected data appeared largely linearly separable in 3-dimensional principal component or discriminant component space before and after dimensionality reduction (not shown). Gene ontology analysis (several tools) was then used to annotate the genes that remained after filtering and to assist in a simple and quick intuitive analysis to identify those broad cellular/molecular functions most likely affected by XBP-1 expression. The more detailed and more time-consuming analysis of the reduced dimensional data space is in progress.

Results: The reduced dimensional data set comprised 401 genes. The primary gene functions that are represented among these genes are shown in Table 1. The categories are those defined in the Gene Ontology (GO) database (http://www.geneontology.org). Since some genes have multiple functions, and some categories are subsets of others, the sum of the percentages is greater than 100%.

CATEGORY	n	%
physiological process	208	51.87
binding	153	38.15
cellular process	140	34.91
catalytic activity	98	24.44
signal transducer activity	51	12.72
transcription regulator activity	42	10.47
development	34	8.48
transporter activity	34	8.48
molecular_function unknown	22	5.49
biological_process unknown	21	5.24
cellular_component unknown	17	4.24
extracellular	13	3.24
regulation of biological process	11	2.74
enzyme regulator activity	10	2.49
structural molecule activity	7	1.75
chaperone activity	6	1.50
motor activity	5	1.25
unclassified	115	28.68

Table 1: Gene functions of the 401 genes. N=number of genes associated with a specific function; %=proportion of genes associated with a specific function.

Since we have previously shown that XBP-1 affects both apoptosis and cell cycle distribution, we looked specifically at those genes associated with these two functions. The data for these genes are provided in Tables 2 (apoptosis) and 3 (cell cycle); genes (gene symbols are those approved by the human gene ontology nomenclature committee; HUGO) or signaling of particular interest are bolded in these two tables. Within these groups we found several genes already implicated in our earlier work. For example, in the apoptosis functional category we found evidence of altered tumor necrosis factor (TNF) signaling (Table 2). This is consistent with the implied role for TNF in our initial study that first implicated XBP-1 (14). We have recently implicated Bcl2 and Bcl2-family members in endocrine resistance (manuscript in

SYMBOL	GENE NAME
DDX41	DEAD (Asp-Glu-Ala-Asp) box polypeptide 41
TNFRSF11B	**tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)**
TNFRSF21	**tumor necrosis factor receptor superfamily, member 21**
IL24	interleukin 24
NOTCH2	Notch homolog 2 (Drosophila)
PDCD4	programmed cell death 4 (neoplastic transformation inhibitor)
BCL2	**B-cell CLL/lymphoma 2**
PHLDA2	pleckstrin homology-like domain, family A, member 2
WWOX	WW domain containing oxidoreductase
VDAC1	voltage-dependent anion channel 1
BECN1	beclin 1 (coiled-coil, myosin-like BCL2 interacting protein)
GULP1	GULP, engulfment adaptor PTB domain containing 1
PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1
TNFAIP8	**tumor necrosis factor, alpha-induced protein 8**

Table 2: Differentially expressed genes associated with apoptosis as annotated in the GO database.

preparation), and Bcl2 is upregulated in the XBP-1 overexpressing cells. Since Bcl2 also is included among the cell cycle genes (Table 3), we selected Bcl2 as a high priority gene for independent validation. Of further relevance is the overexpression of ERα (ESR1; Table 3). In a prior annual report, we described the ability of XBP-1 to form heterodimers with ERα. Thus, confirming the upregulation of ERα also was identified as a high priority gene for independent validation. Other genes also are consistent with their role in breast cancer, *e.g.,* investigators at our institution have shown the importance of altered c-myc expression (23,24), which is implicated here in Table 3.

SYMBOL	GENE NAME
PAFAH1B1	platelet-activating factor acetylhydrolase, isoform Ib, alpha subunit 45kDa
CSPG6	chondroitin sulfate proteoglycan 6 (bamacan)
TFDP1	transcription factor Dp-1
G22P1	thyroid autoantigen 70kDa (Ku antigen)
ESR1	**estrogen receptor 1 (ERα)**
MACF1	microtubule-actin crosslinking factor 1
APBB2	amyloid beta (A4) precursor protein-binding, family B, member 2 (Fe65-like)
RAB8A	RAB8A, member RAS oncogene family
S100A6	S100 calcium binding protein A6 (calcyclin)
NFYC	nuclear transcription factor Y, gamma
TOP1	topoisomerase (DNA) I
CRK	v-crk sarcoma virus CT10 oncogene homolog (avian)
NOTCH2	Notch homolog 2 (Drosophila)
BCL2	**B-cell CLL/lymphoma 2**
NFIB	nuclear factor I/B
POLE3	polymerase (DNA directed), epsilon 3 (p17 subunit)
MYC	**v-myc myelocytomatosis viral oncogene homolog (avian)**
REPRIMO	candidate mediator of the p53-dependent G2 arrest

Table 3: Differentially expressed genes associated with the cell cycle as annotated in the GO database.

In addition to the genes listed here, we found several others of more immediate interest and relevance. Of these, the array data suggested a significant induction of CYP19 (aromatase). Since aromatase is the enzyme responsible for the final step in estrogen biosynthesis, and a major target for endocrine therapy in breast cancer (aromatase inhibitors), we also prioritized independent validation of this observation.

Validation of prioritized genes from the initial analyses: We generally prefer evidence that the protein levels of candidate genes are differentially expressed, since this can be a more robust measure for validation than simply measuring mRNA. However, there are relatively few good antibodies for CYP19. Thus, we perform Western hybridization analyses to validate differential expression of the Bcl2, and ERα, and real-time PCR (RT-PCR) to validate the CYP19 (aromatase) data. For Bcl2 and ERα, protein levels also are often useful indicators of activity. The most compelling evidence for altered aromatase activity would be obtained from a functional assay for enzyme activity. We have recently established a new collaboration with Dr. Angela Brodie at the University of Maryland; Dr. Brodie is a world expert in the study of aromatase. We hope to obtain definitive evidence for the upregulation of aromatase activity within the next few weeks.

As can be seen in the data presented below, we have successfully confirmed the increased expression of Bcl2 protein, ERα protein, and aromatase mRNA in the XBP-1 transfected cells. There is some evidence of regulation of Bcl2 by Faslodex in the MCF7/XBP-1 cells (Fig 2). The low levels in the controls are not always visible but are detected by the software used to analyze

the digitized images. These observations may reflect some actions of Faslodex through its effects on ERα and endogenous Bcl2, since this also occurs in the controls. A similar trend is evident for Tamoxifen. Since in neither case does Bcl2 expression fall below that in controls, the upregulation of Bcl2 by XBP-1 may contribute to the endocrine resistance phenotype seen in the transfectants.

The data in Fig 3 show that the XBP-1 transfectants express a significantly higher level of ERα. This is potentially important, since XBP-1 can also bind to ERα (reported last year).

The data with aromatase are particularly striking. Expression of endogenous aromatase is relatively low in wild-type MCF-7 cells, so we included aromatase transfected cells kindly provided by Dr. Shiuan Chen (City of Hope, Duarte, CA) (25). Over expression of XBP-1, which results in about a 3-fold induction in activity in the CRE reporter-promoter assay (prior annual report), produces an 8-fold induction in endogenous aromatase mRNA expression, a level of expression equivalent to that seen in the aromatase transduced cells (MCF7/AR; Fig 4.).

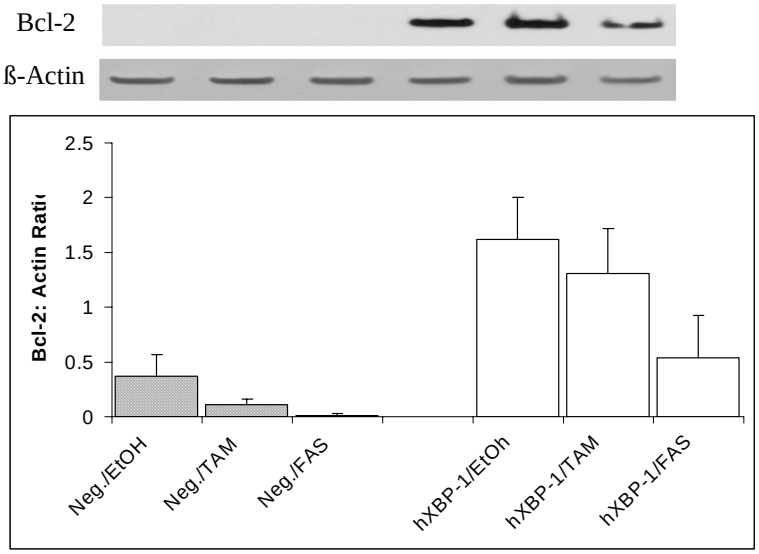

Fig 2: Bcl2 is upregulated in MCF7/XBP-1 cells. Tamoxifen (TAM) has no effect on Bcl2 expression. While Bcl2 levels are lower in cells treated with Faslodex (FAS), the levels are still comparable to those in untreated control cells. The top panel shows a typical blot.

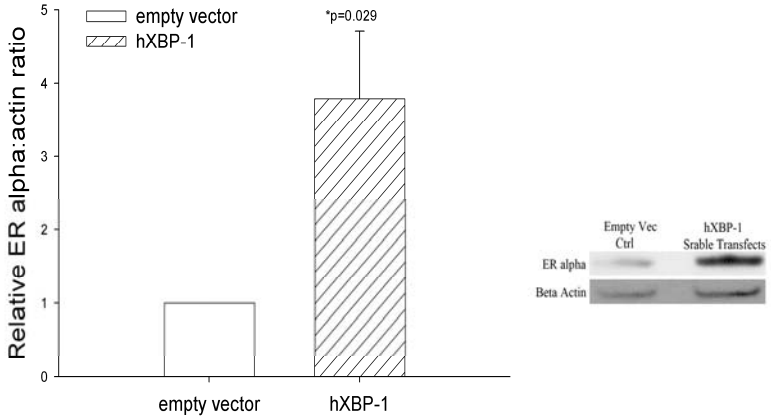

Fig 3: ERα expression is significantly increased in T47D/XBP-1 cells.

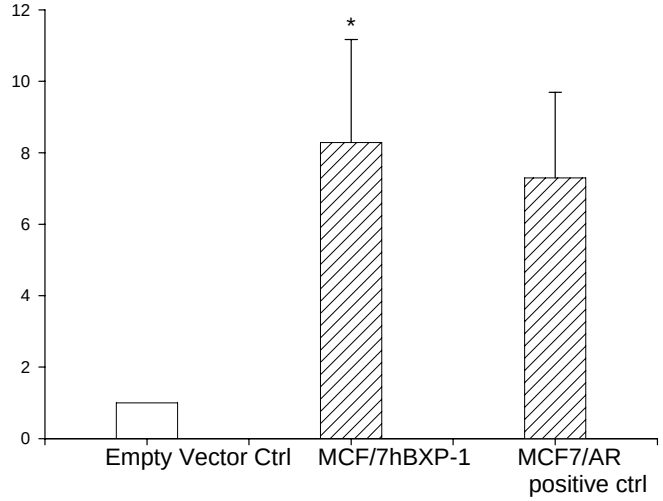

Fig 4: RT-PCR analysis of CYP19/aromatase expression in MCF-7/XBP-1 and empty vector control cells. The positive control is MCF-7 cells transfected with the CYP19/aromatase cDNA. *$p<0.05$.

TASK 4: Explore XBP-1 expression in clinical samples

We have completed and published the first study on the clinical samples and the preprint is included with this report. We received frozen material for study but the accompanying clinical information was incomplete. To perform additional studies, we have identified two other sources of material from collaborators at Northwestern University and at the University of Lund (Sweden). Both collaborators have different breast cancer studies but the cases will allow us to complete any remaining experiments. These cases are prearrayed on tissue microarrays, which should make the experiments relatively straightforward. However, we have not yet received these and are still in the process of exchanging the necessary paperwork. It is not clear if these will be in hand in time to be formally completed by the end of the pending request for a no-cost extension. Nonetheless, we are fully committed to completing these studies as proposed in the application.

REPORTABLE OUTCOMES

We have now published some of our data and presented other data at meetings and in abstracts. Another paper is in preparation and will likely be submitted by February. Publications in the past 12 months are listed below.

1. Zhu, Y., Singh, B., Hewitt, S., Liu, A., Gomez, B., Wang, A. & Clarke, R. "Expression patterns among proteins associated with endocrine responsiveness in breast cancer: interferon regulatory factor-1, human X-box binding protein-1, nuclear factor kappa B, nucleophosmin, estrogen receptor-alpha, and progesterone receptor." *Int J Oncol*, in press.

2. Riggins, R.B., Bouton, A.H., Liu, M.C. & Clarke, R. "Antiestrogens, aromatase inhibitors, and apoptosis in breast cancer." *Vit Horm*, 71: 202-237, 2005.

3. Bouker, K., Zhu, Y., Riggins, R., Zwart, A., Nehra, R., Gomez, B. & Clarke, R. "Resistance to endocrine therapy in breast cancer." *Department of Defense Breast Cancer Research Program Meeting,* 4th Era of Hope, Philadelphia, Pennsylvania, 2005 (abstract).

4. Gomez, B.P., Riggins, R., Zhu, Y., Zwart A. & Clarke, R. "Human X Box Binding Protein in Antiestrogen Resistance". *Department of Defense Breast Cancer Research Program Meeting*, 4th Era of Hope, Philadelphia, Pennsylvania, 2005 (abstract).

5. Gomez, B.P., Riggins, R.B., Zhu, Y., Zwart, A., Wang, A & Clarke, R. "Human X box binding protein-1 (XBP-1) induces antiestrogen resistance via deregulation of cell cycle progression and the intrinsic apoptotic pathway." AACR Annual General Meeting, 2006 (abstract - submitted).

CONCLUSIONS

To date, our data are consistent with a potentially important role for XBP-1 in breast cancer. We have successfully overexpressed XBP-1 in MCF-7 cells, shown that XBP-1 binds to ERα, and induces estrogen-independence and antiestrogen resistance. We have optimized the use of tissue microarrays and demonstrated the detectable presence of XBP-1 protein in breast tumors. We have performed gene expression microarrays on hXBP-1 transfected and control cells and identified several key leads to explain how hXBP-1 may function. Of particular interest and relevance is the notable induction of CYP19 (aromatase) mRNA, which directly implicates hXBP-1 as a potential player in affecting responsiveness to aromatase inhibitors.

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APPENDICES

1. Zhu, Y., Singh, B., Hewitt, S., Liu, A., Gomez, B., Wang, A. & Clarke, R. "Expression patterns among proteins associated with endocrine responsiveness in breast cancer: interferon regulatory factor-1, human X-box binding protein-1, nuclear factor kappa B, nucleophosmin, estrogen receptor-alpha, and progesterone receptor." *Int J Oncol*, in press.

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Expression patterns among interferon regulatory factor-1, human X-box binding protein-1, nuclear factor kappa B, nucleophosmin, estrogen receptor-alpha and progesterone receptor proteins in breast cancer tissue microarrays

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Abstract. Interferon regulatory factor-1 (IRF-1), human X-box binding protein-1 (hXBP-1), nuclear factor kappa B p65 (NFκB p65) and nucleophosmin (NPM) have been implicated in a signaling network of endocrine responsiveness. Expression of these proteins was measured by immunohistochemistry in tissue microarrays of 54 breast tumors. Correlations between each protein and established prognostic markers were assessed by Spearman's rank order correlation coefficient and partial correlation coefficient analyses. Moderate/strong staining is seen for hXBP-1 (79% of tumors) and NFκB p65 (57%). NPM exhibits nuclear staining (95%); IRF-1 exhibits both cytosolic (IRF-1c; 90%) and nuclear staining (IRF-1n; 51%). IRF-1c is associated with age (p=0.034); IRF-1n and PgR expression are correlated (p=0.014). NFκB p65 shows a borderline association with S phase (p=0.062). Coexpression of IRF-1c and hXBP1 (p=0.001), IRF-1c and NFκB (p=0.002), and hXBP-1 and NFκB (p=0.018) is observed. An inverse correlation exists between IRF-1n and NFκB (p=0.034). All four proteins are detected in breast tumors and their expression patterns support their role(s) in a key signaling network.

Introduction

Endocrine therapy, usually either the antiestrogen, Tamoxifen (TAM), ovariectomy or more recently an aromatase inhibitor or one of the newer selective estrogen receptor modulators (SERM) or 'pure' antiestrogens, is an effective means to manage hormone-dependent breast cancer (1-3). An understanding of the mechanisms of resistance to endocrine therapies could identify better ways to predict responsiveness. We have previously hypothesized that endocrine responsiveness is affected by a complex gene network, rather than the activity of only one or two genes or signaling pathways (4-6). To identify the key components of such a network, we first derived variants of the MCF-7 human breast cancer cell line with different estrogen (7,8) and antiestrogen response profiles (9,10). Initial transcriptome and proteome analyses of these variants implicate several genes in endocrine resistance, including interferon regulatory factor-1 (IRF-1) (11,12), nuclear factor kappa B p65 (NFκB) (11,13), human X-Box binding protein-1 (hXBP-1) (11) and nucleophosmin (NPM) (14,15), which appear to function as part of a broader gene expression network (Fig. 1).

In the proposed network, NPM is predicted to inhibit IRF-1 activity, which reduces the ability of IRF-1 to activate apoptosis, most likely through inducing a caspase cascade. Inhibition of IRF-1 activity also may eventually contribute to increased activity of the survival factor NFκB (Fig 1). Increased NFκB may, in turn, induce a second survival factor, hXBP-1 (16). Evidence from experimental models has begun to show the likely functional relevance of the altered IRF-1 (12), NFκB (11,13,17), and hXBP-1 activities (Gomez BP, *et al*, Proc Am Assoc Cancer Res, abs. 3498, 2004) in affecting endocrine responsiveness. Studies to identify other members of this network and their interrelationships are currently in progress. The known functions of the key components of the network are described below.

IRF-1 is a transcription factor that exhibits tumor suppressor activities in several cancers (18,19). In breast cancer cells, IRF-1 signaling can reduce both the rate of cell proliferation and the incidence of human breast cancer xenografts in athymic nude mice (manuscript submitted). We have shown that a

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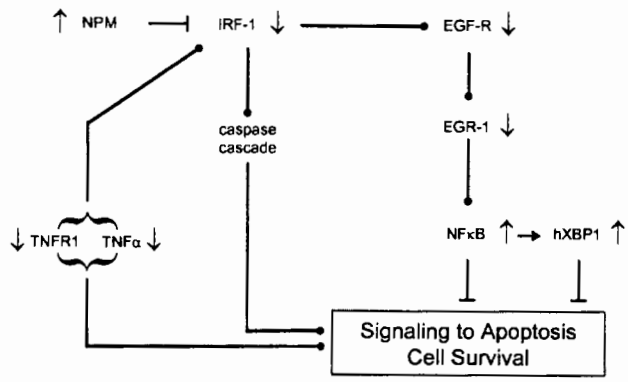

Figure 1. Components of a putative signaling network associated with endocrine responsiveness in breast cancer cells (adapted from ref. 6). ↑, increased; ↓, decreased; ⊥, blocks; —•, reduced ability to affect target. The network component incorporates the known protein/protein interactions between NPM and IRF-1 (NPM binds to IRF-1 and inhibits IRF-1 activity) and the predicted regulation of hXBP-1 by NFκB. Down-regulation of IRF-1 activity would reduce the activity of IRF-1/NFκB heterodimers that are known to regulate the transcription of several genes implicated in breast cancer, such as RANTES, VCAM-1, and IL-6. Down-regulation of TNFR1 (tumor necrosis factor receptor 1) and its ligand, TNFα (tumor necrosis factor alpha), were previously described (11), signaling from this complex is a major inducer of IRF-1 transcription. A full description of this network component and its anticipated function can be found in ref. 6.

dominant negative IRF-1 blocks antiestrogen-induced apoptosis in sensitive breast cancer cells and reduces their antiestrogen sensitivity (12); a similar role for IRF-1 has been recently reported in normal mammary cells (20). These activities of IRF-1 are probably mediated through its proapoptotic effects, which can occur in a p53-dependent or -independent manner (21,22) and involve its ability to induce a caspase cascade that includes caspase-1 (20,22), caspases-3/7 (20,23), caspase-8 (24) and/or Fas ligand (25). Caspases are known to affect antiestrogen responsiveness (26). Lower levels of IRF-1 protein have been reported in high-grade ductal carcinoma *in situ* or invasive ductal carcinoma of the breast when compared with adjacent normal breast epithelium (27).

The NFκB p50/p65 heterodimer complex comprises two homologous proteins encoded by different genes; the p105 precursor of p50 (NFκB1) is on chromosome 4, while the p65 (RelA) gene is on chromosome 11. The predominant form in human breast cancer cell lines is NFκB (p50/p65); another member of the family (p52) is also expressed in some breast cancers (28). NFκB (p50/p65) is implicated in several critical cellular functions including cell survival (29); these functions are often cell context specific (30). We have shown differential expression and activation of NFκB expression with both acquired antiestrogen resistance (11) and estrogen independence in breast cancer cells (13). Other studies also show increased expression of NFκB in endocrine-resistant breast cancer cell lines (31,32). We have begun to establish the functional relevance of these observations. For example, estrogen-independent cells significantly up-regulate NFκB; when its inhibitor IκBα is overexpressed in these cells their xenografts regress upon estrogen withdrawal (13). Anti-estrogen-resistant cells are more sensitive to growth inhibition by parthenolide, a small molecule inhibitor of NFκB, than their antiestrogen sensitive parental cells (11). Furthermore, parthenolide can reverse the antiestrogen resistance phenotype and synergistically interact with antiestrogens *in vitro* (17).

As a member of the ATF/CREB transcription factor family that activate promoters containing specific cyclic AMP responsive elements (CRE) (33), hXBP-1 regulates the expression of several tissue-specific genes, including tissue inhibitor of metalloproteinases, osteopontin and osteocalcin (34). Potentially downstream of NFκB activation in some cells (35), hXBP-1 is associated with increased proliferation and reduced apoptosis (16), which implies a survival function. Changes in cAMP concentrations and CRE activation have been widely implicated in carcinogenesis and endocrine signaling, including affecting signaling from ERα and PgR (36). While the role of hXBP-1 in the normal/neoplastic breast has not been studied in detail, hXBP-1 is part of a cluster of genes associated with some ERα-positive breast tumors (37,38) and a recent study suggests it may be expressed in breast cancer cells (39). We have previously implicated increased expression of hXBP-1 in acquired antiestrogen resistance (11). More recently, we have shown the ability of hXBP-1 to induce an estrogen-independent phenotype and to confer antiestrogen resistance (unpublished data).

The oncogenic nucleolar phosphoprotein, NPM, is a DNA-binding protein (40) that inhibits the ability of the YY1 (41) and IRF-1 transcription factors to regulate gene expression (42). NPM also serves as a substrate for several important serine-threonine kinases, including protein kinase C (43), $p34^{cdc2}$ kinase (44,45) and casein kinase II (46). Insulin, which is a major mitogen for breast cancer cells, also increases NPM phosphorylation (45). Overexpression of NPM is sufficient to transform NIH/3T3 fibroblasts (47), and chromosomal translocations fusing NPM to either an anaplastic lymphoma kinase (48) or the retinoic acid receptor-α (49) have been reported in some cancers. We have shown that NPM is induced by estradiol (14) and expressed at higher levels in estrogen-independent breast cancer cells (11); a putative estrogen responsive element in the NPM promoter has now been recently described (50). In breast cancer patients, auto-antibodies to NPM are lower in patients treated with the anti-estrogen Tamoxifen and increase six months prior to recurrence (15). Of particular relevance is the reduced expression of IRF-1 and concurrent increased expression of NPM, an endogenous IRF-1 inhibitor, in antiestrogen-resistant breast cancer cells (11).

We have now measured the expression of IRF-1, NFκB (p65), hXBP-1 and NPM in breast cancer specimens from women diagnosed at our institution. Using tissue microarrays and immunohistochemistry, we asked if these proteins could be detected in breast cancer, whether their expression might be correlated with other known prognostic markers, and whether the four proteins are expressed in patterns consistent with their known functions and/or our putative gene expression network. We find several proteins to be either coexpressed or inversely expressed in patterns consistent with our network hypothesis.

Materials and methods

Tissue specimens. Tissue microarrays were constructed using fifty-four, untreated, primary breast cancer cases diagnosed

between 1998 and 1999 from the breast cancer tumor bank at the Lombardi Comprehensive Cancer Center Histopathology Shared Resource at Georgetown University Medical Center. The cases were initially selected to determine the number of cores from a tumor needed to give the same estimation of ERα and PgR positivity as the entire section (51). Hence, the proportion of steroid hormone receptor-positive specimens (81% ERα; 44% PgR) are higher than might be expected from a random sampling of breast cancer cases. While we cannot exclude the possibility of some selection bias in these cases, this selection should have identified cases most relevant to our initial hypothesis, implicating the proteins of interest in endocrine responsiveness (52). Differentiation/nuclear grade (53), DNA index (54) and S Phase (55) were determined as previously described. The categories for each end-point in Table I were selected prior to data analysis and are consistent with other studies (56,57). All material and information was collected and used in accordance with approved Institutional Review Board protocols. Clinical-outcome data and additional prognostic marker data are not available for these cases; available data are shown in Table I.

Table I. Patient/tumor characteristics.

	n	Range
Age	54	36-85 (56)[a]
<50 years	25	
≥50 years	29	
Tumor grade	49	0-2
Grade 1	13	
Grade 2	26	
Grade 3	10	
Tumor size	54	0.2-6.8 (1.35)
<2 cm	39	
≥2 cm	15	
Lymph nodes	40	0-4
Negative (0)	28	
Positive (≥1)	12	
DNA index	47	1-2.89
<1.5%	27	
≥1.5%	20	
S-phase	28	1.64-27.00 (5.09)
<5% (low)	14	
≥5% (high)	14	

[a]Values in parentheses are median values.

Tissue microarrays. Tissue microarrays were constructed with a Beecher Instruments manual tissue arrayer (Beecher Instruments, Inc., Sun Prairie, WI) as previously described (58,59). The instrument punches holes in the recipient paraffin block and acquires tissue cores from the donor block. Briefly, a thin-walled needle with an inner diameter of 0.6 mm was held in an X-Y precision guide. The cylindrical sample was retrieved from the selected region in the donor block and extruded directly into the recipient block with defined array coordinates. A solid steel wire, which closely fits the tube, was then used to transfer the tissue cores into the recipient block. The transfer was made under direct visual control with a stereotactic microscope using an additional bright light source. This cycle was repeated to obtain the appropriate number of cores. An adhesive-coated tape system (Instrumedics, Inc., Hackensack, NJ) was then used to cut 5 μm sections of the tissue microarray block. The microtome knife cut underneath tape placed over the block surface. Thin tissue sections adhered to the tape, which was then rolled on an adhesive-coated microscope slide to transfer the section onto the slide. For this study, tissue microarrays were built with 480 cores from fifty-four breast carcinomas. Regions of invasive carcinoma were marked on each hematoxylin-and-eosin-stained slide. Ten cores were made from these areas of the paraffin block for 42 cases; for 12 additional cases, five cores were made. Thus, either 10 or 5 cores represented each tumor.

Antibodies and immunohistochemistry. The following commercial antibodies were used: ERα (ER1D5, Immunotech) (60), erbB2 (CB11; Zymed, San Francisco, CA) (61), hXBP-1 (sc-7160; Santa Cruz); IRF-1 (sc-497; Santa Cruz) (27), and NFαB p65 (sc-109; Santa Cruz) (28). The NPM monoclonal antibody was kindly donated by Dr P-K Chan (62). Tissue microarray sections were deparaffinized in two 5-min changes of xylene and rehydrated through graded alcohol to distilled water. Immunohistochemistry was performed by a standard biotin-streptavidin-horseradish peroxidase method (63,64). Briefly, microarrays were treated with 1% H_2O_2 in methanol for 30 min to block endogenous peroxidase activity. Before applying the primary antibody, microarrays were boiled for antigen retrieval in 10 mM citrate buffer (pH 6.0) for a total 10 min. Microarrays were washed in phosphate-buffered saline containing 3% biotinylated goat antiserum to the appropriate IgG and 0.3% Triton X-100 (pH 7.4) for 30 min. Subsequently, tissue microarrays were incubated with the primary antibody at a 1:500 dilution (or as appropriate for the antibody) in PBS for 48 h at 4°C. After several washes, microarrays were treated with the appropriate secondary antibody (1:800; Vector Laboratories, Burlingame, CA) for 2 h, followed by a 1 h incubation with streptavidin-peroxidase conjugate (Vector Laboratories). Antigen-antibody complex was visualized by incubation with the VIP Kit (DAB Kit; Vector Laboratories). Finally, microarrays were counter-stained with either methyl green or hematoxylin, mounted and examined. All immunostaining was first optimized in single tissue slides. Negative controls were obtained using a standard method where microarrays are processed as described above but without incubation with the appropriate primary antibody.

Data analysis. The level of specific immunostaining, as determined relative to negative controls, was measured as an ordinal variable according to the nominal scale 0, 1+, 2+, 3+; where 0 is undetectable, 1+ refers to weak (barely perceptible) staining, 2+ to moderate staining, and 3+ to strong staining.

For nuclear staining, the scale applied was 1 = 0-25% of nuclei with detectable staining, 2 = 26-50%, 3 = 51-75%, 4 = ≥76%. The average score for all cores representing a tumor was used for data analysis. The relationships among staining values for each protein were compared using Spearman's rank order correlation coefficient analysis. All statistical tests are two-sided. We considered comparisons where $p<0.05$ to be statistically significant; estimates of $p\geq0.05$ and $p\leq0.10$ were considered to indicate borderline statistical significance and potential biological relevance; comparisons where $p>0.10$ were considered to be insignificant.

Pairwise correlation analyses could not account for the possibility that the associations of IRF-1n or IRF-1c may confound each other, since the expression of these two IRF measures may be correlated. To address this issue, we applied a novel use of partial correlation coefficient analysis, the partial correlations being calculated as shown in Eq 1:

$$r_{xy.z} = \frac{r_{xy} - (r_{xz})(r_{yz})}{\sqrt{(1-r_{xz}^2)(1-r_{yz}^2)}} \qquad \textbf{Eq 1}$$

Where $r_{xy.z}$ = the correlation coefficient between x and y while controlling for the correlations between x and z and between y and z.

Partial correlations are most widely applied in the analysis of small signaling networks of 3-5 variables, and allow an estimate of the correlation between two variables while controlling for a third, fourth and/or fifth. Since we make functional assessments based upon cellular location, the use of partial correlations appears reasonable in the context of IRF-1n and IRF-1c. For the correlations between IRF-1c or IRF-1n and age, ER, PgR, NFκB and hXBP-1 the partial correlations were calculated with either IRF-1c or IRF-1n as the controlling variable.

Results

ERα and PgR expression. Measurements of ERα and PgR expression are the most widely used predictive factors in directing breast cancer therapy. The specimens in this study were originally selected to study ERα and PgR expression (51) and these two proteins are coexpressed in a substantial proportion of breast cancers. The expression of ERα (81%) and PgR (44%) using our criteria (Table II), and their significant coexpression in the tumors used in this study (Table V; $p<0.001$) implies that the samples are likely to be broadly representative of ERα-positive breast cancers and appropriate for exploring protein expression patterns in cases likely to be selected for endocrine therapy.

IRF-1 expression. As a putative tumor suppressor, we might expect activated IRF-1 protein to be in the nucleus (IRF-1n) and inactive protein to be in the cytosol (IRF-1c). Whether these relationships are true for the IRF-1 signals we have measured is not known but we might expect the inactive form to predominate. In this context, and consistent with its putative tumor suppressor activities, the primary form of IRF-1 in breast tumors in this study appears to be IRF-1c (Fig. 2A). Of the tumors, 90% express detectable (2+ or 3+) IRF-1c in their neoplastic cells, almost half of which have 3+ IRF-1 staining in the cytosol. In contrast, only 51% of the tumors in our study express detectable IRF-1n in >50% of the tumor cells and no tumors express IRF-1n in >75% of cells (Tables II and III). While 98% of the specimens express both detectable IRF-1c and IRF-1n, only 2% express IRF-1c alone and none express only IRF-1n. The inverse relationship between IRF-1n and IRF-1c ($p=0.088$), while of borderline statistical significance, suggests that some breast tumors may differentially regulate the activation state of IRF-1 (Table V). This potential correlation raises the possibility that some associations implicating IRF-1c or IRF-1n may be confounded by the effect of the other. Our observations also are broadly consistent with a study reporting higher levels of IRF-1 protein in adjacent normal breast epithelium when compared with high-grade ductal carcinoma *in situ* or lymph node-positive invasive ductal carcinoma of the breast (27).

Table II. Immunohistochemical staining scores of five proteins detected in the cytosol.

Score	ERα	PgR	NFκB	[b]IRF-1c	hXBP-1
0	[a]9	22	2	0	1
1+	1	7	18	4	9
2+	5	3	14	21	27
3+	37	20	13	17	10
Total	52	52	47	42	47
[c]Detected	81% (42/52)	44% (23/52)	57% (27/47)	90% (38/42)	79% (37/47)

[a]Values represent the number of cases in each category; scoring categories are described in Materials and methods. [b]IRF-1c, IRF-1 cytoplasmic staining. [c]Detected, proportion of cases with weak or stronger cytosolic staining.

NFκB expression. We measured NFκB p65 expression, which is the predominant form of NFκB in human breast cancer cells (28) and the form associated with both estrogen independence (13) and acquired antiestrogen resistance (11). While active in breast cancer cell lines, NFκB p65 has been reported as being cytosolic (potentially inactive) whereas NFκB p50 has been reported to be primarily nuclear (active) in a prior study of n=17 breast tumors (28). While the pattern of NFκB p65 staining is broadly consistent with this observation in many of our breast tumors (Fig. 2C), we found 57% of the tumors to express detectable (2+ or stronger) NFκB in their neoplastic cells (Table II).

hXBP-1 expression. Increased expression of hXBP-1, a nuclear transcription factor that activates cyclic AMP responsive elements (33), is associated with some forms of acquired anti-estrogen resistance (11). hXBP-1 expression is detected in 79% of the breast tumors in this study (Table II), with the strongest staining seen in the cytosol (Fig. 2E). This observation

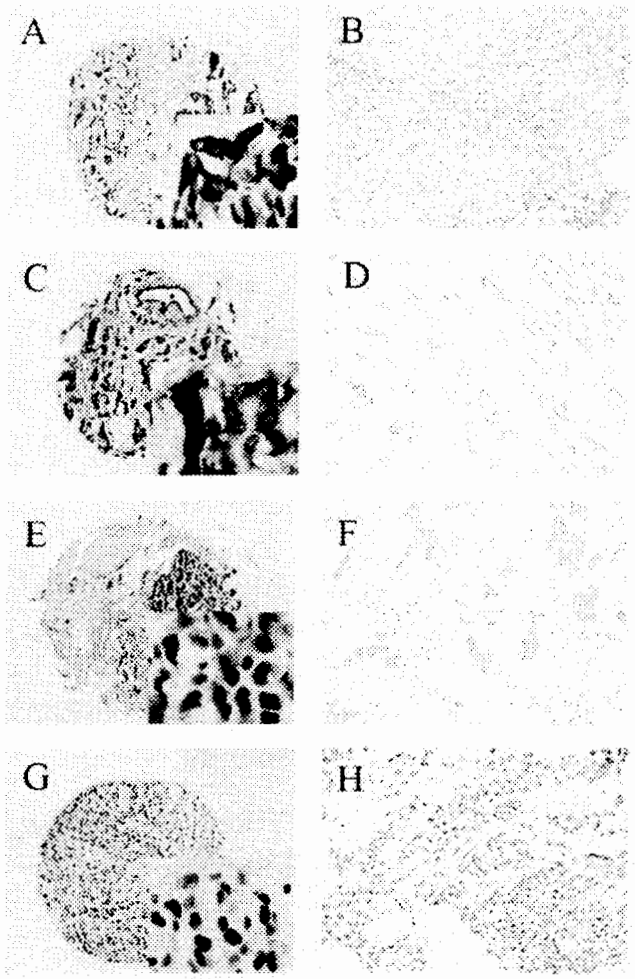

Figure 2. Representative immunostaining of IRF-1, NFκB p65, hXBP-1 and NPM in breast cancer. (A) IRF-1 staining where the inset shows typical patterns of cytosolic and nuclear staining; (B) Control for IRF-1 staining; (C) NFκB p65 staining where inset shows the primarily cytosolic staining pattern; (D) Control for NFκB p65 staining; (E) hXBP-1 staining where inset shows the primarily cytosolic staining pattern; (F) Control for hXBP-1 staining; (G) NPM staining where the inset shows the nuclear staining pattern for NPM; (H) Control for NPM staining. Figures of tissue microarray cores are at x10 magnification; inset at x100 magnification; control at x40 magnification.

is consistent with a small study of hXBP-1 expression in primary breast cancers (n=11) and breast cancer cell lines (n=5). In this recent study, expression was detected in all tumors and cell lines studied but hXBP-1 was almost undetectable in non-cancerous breast tissue (33).

NPM expression. NPM is a nucleolar phosphoprotein that is induced by estradiol (14) and expressed at higher levels in breast cancer cells with acquired antiestrogen resistance (11). In breast cancer patients, autoantibodies to NPM increase six months prior to recurrence and are lower in patients treated with TAM (15). Consistent with its nucleolar localization in cell culture, NPM staining is strongly nuclear in breast tumors (Fig. 2G). Of the breast cancers in this study, 95% express NPM in >50% of their neoplastic cell nuclei, the majority expressing NPM in >75% of their cells (Table II).

Table III. Immunohistochemical nuclear staining scores of IRF-1 and NPM.

Score	IRF-1n	NPM
1	2	1
2	22	1
3	25	3
4	0	39
Total	49	44
[c]>50%	51% (25/49)	95% (42/44)

[a]Values represent the number of cases in each category; scoring categories are described in Materials and methods. [b]IRF-1n, IRF-1 nuclear staining. [c]>50%, proportion of cases where data are available that exhibit >50% of cell nuclei staining positive relative to the negative controls (NPM and IRF-1n are data for nuclear staining).

Correlation among proteins and patient/tumor characteristics. Several correlations among existing prognostic markers are known and are apparent in our data set (Table IV). Both PgR-positive (p=0.03) and ERα-positive tumors (borderline) are associated with a greater degree of differentiation and better prognosis (65). Borderline relationships between DNA index and both PgR-positivity and ERα-positivity (inverse correlation), and between NFκB and S phase (direct correlation) are also evident. A higher incidence of ERα-positive tumors is seen in older women (66) but our study was probably underpowered to detect this relationship. Nonetheless, the significant association between IRF-1c and age (Table 1V; p=0.034) and the potential association between IRF-1c and ERα (p=0.079), may reflect the underlying relationship between ERα and age. We found no other associations among IRF-1, NFκB, hXBP-1 and NPM with either tumor grade, tumor size, DNA index, lymph node status, or S-phase fraction.

Correlation among protein expression patterns. Expression of several of the four proteins is correlated in breast tumors. Since our study is limited in size and power, we present those associations that reach conventional statistical significance and those where the association is of borderline statistical significance but of potential biological relevance. The data in Table V show coexpression of ER and IRF-1c (borderline), PgR and IRF-1n (p=0.014), IRF-1c and hXBP1 (p=0.001), IRF-1c and NFκB (p=0.002), and hXBP-1 and NFκB (0.018). Inverse correlations were seen between NPM and erbB2 (not shown; p=0.016), IRF-1n and NFκB (p=0.034), IRF-1n and IRF-1c (borderline), and IRF-1n and hXBP-1 (borderline). We estimated the partial correlations for each IRF-1n and IRF-1c correlation of interest; no effect is present when the sign and magnitude of the partial correlation coefficient is comparable to the original correlation coefficient. In each case, the partial coefficients were very similar to the original coefficients and shared the same sign. Hence, IRF-1c and IRF-1n are not antecedent, intervening, or suppressing variables

Table IV. Correlation among proteins and patient/tumor characteristics.

	ERα	PgR	IRF-1c	IRF-1n	NFκB	hXBP-1	NPM
Age	-	-	P=0.034 (r=0.28)	-	-	-	-
Tumor grade	P=0.067 (r=0.22)[a]	P=0.028 (r=0.28)	-	-	-	-	-
Tumor size	-	-	-	-	-		
Lymph nodes	-	-	-	-	-	-	-
DNA index	P=0.077 (r=0.22)	P=0.086 (r=-0.21)	-	-	-	-	-
S phase	-	P=0.016 (r=0.41)	-	-	P=0.062 (r=0.33)	-	-

[a]Values in parentheses are Spearman rank correlation coefficients. Comparisons where $p<0.05$ are statistically significant; estimates of $p\geq0.05$ and $p\leq0.10$ are considered of borderline statistical significance and of potential biological relevance; comparisons where $p>0.10$ were considered to be insignificant.

Table V. Correlation among protein expression patterns.

	ERα	PgR	[b]IRF-1c	IRF-1n	NFκB	hXBP-1
PgR	P<0.001 (r=0.46)[a]	1				
IRF-1c	P=0.079 (r=0.23)	-	1			
IRF-1n	-	P=0.014 (r=0.32)	P=0.088 (r=-0.21)	1		
NFκB	-	-	P=0.002 (r=0.44) (r=0.42)	P=0.034 (r=-0.27) (r=0.22)	1	
hXBP-1	-	-	P=0.001 (r=0.49) (r=0.40)	P=0.082 (r=-0.21) (r=-0.23)	P=0.018 (r=0.31)	1
NPM	-	-	-	-		-

[a]Values in parentheses are Spearman rank correlation coefficients; comparisons where $p<0.05$ are statistically significant; estimates of $p\geq0.05$ and $p\leq0.10$ are considered of borderline statistical significance and of potential biological relevance; comparisons where $p>0.10$ were considered to be insignificant. Values in parentheses are the estimated partial correlation coefficients. Use of partial correlation coefficients in networks can be found in De la Fuenta, *et al*: Bioinformatics 20: 3565-3575, 2004. [b]IRF-1c, cytoplasmic staining; IRF-1n, nuclear staining.

for the correlations indicated, they exhibit respectively with hXBP-1 or NFκB (Table V).

Discussion

One approach to exploring the potential relevance of observations from experimental models is to determine whether similar relationships may also arise in tumors from patients. While not directly informative in a mechanistic sense, identification of expression patterns in tumors that reflect patterns seen in xenografts and cell cultures can support mechanistic observations in these models. Furthermore, such studies may identify candidate biomarkers for further investigation. We have explored the expression levels and patterns of coexpression of a subset of four genes (IRF-1, NFκB p65, hXBP-1, NPM) implicated in endocrine resistance

from our prior studies in experimental models (11-14).

Of the four genes we have previously implicated, IRF-1, NFκB and hXBP-1 are transcription factors and NPM is a DNA-binding nucleolar phosphoprotein. Knowledge of a signal's cellular localization can provide mechanistic insight and all four proteins would be expected to exhibit some degree of nuclear staining that could reflect active protein. For example, NFκB is maintained in the cytosol in an inactive state, complexed with members of the IκB family (67). However, correctly identifying subcellular localization by immunohistochemistry can be confounded by fixation artifacts, leading to nuclear antigen redistribution during tissue processing. A fixation artifact is responsible for the apparent cytosolic localization of the NPM-anaplastic lymphoma kinase fusion antigen (68) but NPM staining is robust and primarily nuclear in our breast cancer specimens. In contrast, the activation state of hXBP-1 and NFκB (p65) is difficult to determine because the staining is primarily cytosolic and any weak nuclear staining was not sufficient for further analysis. In this study, we chose to focus on the localization of IRF-1, which exhibits readily detectable nuclear and cytosolic staining patterns that appear inversely correlated (borderline; p=0.088). Furthermore, these patterns of staining for IRF-1 also make biological sense when considered in the context of putative active (nuclear) and inactive (cytosolic) states (see below).

Since there are only very limited published data on the expression of IRF-1, NFκB, hXBP-1 and NPM in breast cancer, we first determined whether we could detect these proteins and estimate the extent to which they are expressed in this series of predominately ER+ breast tumors. All four proteins are detectable in the cases used in this study; NPM expression is detected in >25% of the neoplastic cells in almost all the breast cancers (95%). Consistent with recent reports, IRF-1 also is detected in breast tumors (90%) (27,69), as is hXBP-1 (79%) (39). NFκB (p65) expression is the least frequently detected among the four proteins yet is detectable in 57% of the tumors. Thus, these four proteins are present in high proportions of breast cancer and are candidate biomarkers that merit further evaluation as both independent biomarkers and as a possible panel to be concurrently measured.

We have previously hypothesized that the proteins of interest are associated with affecting endocrine responsiveness (6,11,12). Acquired antiestrogen resistance primarily occurs in tumors that continue to express sufficient levels of ERα to be considered ERα-positive. While the primary form of *de novo* endocrine resistance is the absence of both ERα and PgR, a significant proportion of *de novo*-resistant tumors also are ERα-positive (52). The functional importance of continued receptor expression in either acquired or *de novo* endocrine resistance is unclear, but we might expect to find some of our network members to be coexpressed in the breast tumors used in this study (6).

A significant positive association between IRF-1n and PgR (p=0.014) and a borderline positive association between ERα and IRF-1c (p=0.079) are evident. Those PgR-positive tumors that coexpress IRF-1n may have a better prognosis and/or a better response to antiestrogens. For example, we have recently shown that the ability of the steroidal antiestrogen ICI 182,780 (Faslodex; Fulvestrant) to signal apoptosis is mechanistically related to its ability to regulate IRF-1 expression and function in breast cancer cells (12).

We could not confirm coexpression of ERα and hXBP-1 (p=0.244), an association predicted from hierarchical cluster analysis of cDNA expression microarray data from human breast tumors (37,38). Several explanations for this outcome are possible. The nature of the signals from gene expression microarrays that measure mRNA and tissue microarrays that measure protein are very different. It also is not clear how closely the levels of mRNA and protein are related for hXBP-1. Furthermore, some of the associations/relationships identified in gene expression microarray studies were found by simple hierarchical clustering and these may not be correct or complete. The use of these clustering methods to identify gene expression patterns from within the very high dimensional data spaces generated by gene expression microarrays has been seriously questioned (70,71).

hXBP-1 expression is positively correlated with IRF-1c expression (p=0.001) but inversely associated with IRF-1n (borderline; p=0.082). These observations suggest a balance between IRF-1's inhibitory activity and hXBP-1's mitogenic activity. For example, tumors where hXBP-1 activity predominates may have a poor prognosis and/or poor response to antiestrogens. Some antiestrogen-resistant cells exhibit down-regulated IRF-1 activation and up-regulated hXBP-1 activity (western; promoter-reporter data) (11,12).

Expression of hXBP-1 and NFκB (p65) are positively correlated (p=0.018). If we assume that NFκB is inactive because of its cytosolic location, the coexpression of hXBP-1 might compensate for any lack of NFκB in affecting cell survival since both are antiapoptotic. However, hXBP-1 expression appears to be downstream of NFκB, at least in plasma cell differentiation (35), implying a potential induction of hXBP-1 by NFκB. If this occurs in breast cancer cells and NFκB is active, as suggested by the potential correlation between NFκB and S-phase (p=0.062), it may explain the coexpression of hXBP-1 and NFκB in Table V. We also cannot exclude the possibility that NFκB p50 and/or NFκB p52 expression are activated and may compensate for any loss of NFκB p65 activity (28).

IRF-1 and NFκB proteins form heterodimers that can regulate gene expression and we might expect to find these coexpressed in the same tumors. We found a significant coexpression of NFκB and IRF-1c (p=0.002) and an inverse association between IRF-1n and NFκB (p=0.034). Where both proteins are primarily sequestered in the cytosol, the ability of IRF-1:NFκB heterodimers to regulate gene transcription could be inhibited. Several genes regulated by these heterodimers are implicated in breast cancer, including RANTES (regulated upon activation, normally T-Expressed and presumably secreted) (72), VCAM-1 (vascular cell adhesion molecule-1) (73) and IL-6 (interleukin-6) (74). RANTES expression correlates with a poor prognosis in breast cancer (75). VCAM-1 is involved in angiogenesis and metastasis in breast tumors (76), and an autocrine production of IL-6 is associated with drug resistance in breast cancer cells (77). The inverse relationship between IRF-1n and NFκB suggests that some tumors may have activated IRF-1 in the absence of active NFκB; such tumors may have a good prognosis and/or be sensitive to antiestrogens.

We obtained limited expression data for erbB2 (not shown). We detected a significant inverse association between erbB2 and NPM (p=0.016), suggesting that the oncogenic properties of NPM may be important in erbB2 non-overexpressing breast tumors, which represent the majority of breast cancer. No association was seen between erbB2 and either IRF-1, hXBP-1 or NFκB.

The present study represents the first analysis of the coexpression patterns of a subset of genes associated with acquired endocrine resistance in breast cancer cells. We could not adequately assess the activation state of each of the proteins and clinical-outcome data are not available in this data set. Despite these limitations, the data clearly show that all four proteins are detectable in a high proportion of the breast tumors used in this study. The data are consistent with a role for IRF-1, NFκB, hXBP-1 and NPM and their interactions in breast cancer, and are broadly supportive of the proposed component of a larger signaling network as outlined in Fig. 1. Further analysis of the expression patterns of IRF-1, NFκB, hXBP-1 and NPM as potential biomarkers for further defining endocrine response profiles in some breast cancer patients is warranted.

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7

Antiestrogens, Aromatase Inhibitors, and Apoptosis in Breast Cancer

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Antiestrogens have been the therapeutic agents of choice for breast cancer patients whose tumors express estrogen receptors, regardless of menopausal status. Unfortunately, many patients will eventually develop resistance to these drugs. Antiestrogens primarily act by preventing endogenous estrogen from activating estrogen receptors and promoting cell growth, which can ultimately lead to tumor cell death. Understanding the mechanisms by which antiestrogens cause cell death or apoptosis is critical to our efforts to develop ways to circumvent resistance. This article focuses on antiestrogen-induced apoptosis both *in vitro* and *in vivo*. We review the clinical utility of both antiestrogens and aromatase inhibitors and their apoptogenic mechanisms in cell culture models. Among the key signaling components discussed are the roles of Bcl-2 family members, several cytokines, and their receptors, p53, nuclear factor kappa B (NFκB), IRF-1, phosphatidylinositol 3-kinase (PI3K)/Akt, and specific caspases. Finally, we discuss the evidence supporting a role for apoptotic defects in acquired and *de novo* antiestrogen resistance.

I. INTRODUCTION

Breast cancer will affect one in eight women in the United States this year, making it the second-most common cause of cancer-related death in women (Jemal *et al.*, 2004). Significant progress has been made in our ability to treat and manage this disease, with both local and systemic therapies associated with an overall survival benefit in some women (Early Breast Cancer Trialists Collaborative Group [EBCTCG], 1992, 1998a). One of the most notable advances has been the development of targeted therapies that inhibit estrogen action, a major proliferative stimulus in the breast (Hilakivi-Clarke *et al.*, 2002). Indeed, estrogen present within breast tumors is the most biologically active (17β-estradiol), and the average concentration (approximately 1.2 nM) should be sufficient to occupy all ERs in a breast tumor if biologically available for receptor binding (Clarke *et al.*, 2001).

Although ovariectomy has been used to treat premenopausal women for over 100 years (Beatson, 1896), for the last several decades, antiestrogens have been the drug of choice for all patients—irrespective of menopausal status—whose tumors express estrogen receptors (ERs). Antiestrogens primarily act by preventing endogenous estrogen from activating ER and

promoting cell growth. At present, the most widely used antiestrogen is Tamoxifen (TAM), which is generally well tolerated and effective in approximately 50% of all ER-positive breast cancers (EBCTCG, 1992, 1998b). However, the remaining 50% of ER-positive breast cancer patients do not respond to TAM, and many that show an initial response will later develop resistance (Clarke *et al*., 2001).

More recently, evidence has emerged to indicate that third-generation aromatase inhibitors, which block estrogen biosynthesis, may be as effective as TAM in treating some postmenopausal women (Dixon *et al*., 2003; Miller, 2004). Whether aromatase inhibitors or the newest generation of antiestrogens such as Faslodex (ICI 182,780, Fulvestrant) will replace TAM as the first-line endocrine therapy of choice remains to be seen. Nonetheless, it is already clear that various patterns of both cross-resistance and cross-sensitivity among specific antiestrogens and aromatase inhibitors exist in breast tumors. Even in cases in which there is initial cross-sensitivity, for example, where response and then failure to an antiestrogen is followed by response to a second-line aromatase inhibitor, the overall response rates and duration of responses is frequently lower when the aromatase inhibitor is given in the second line than when administered as a first-line agent. Thus, there are very likely to be mechanisms of action and resistance that are common to both antiestrogens and aromatase inhibitors. This review examines the molecular mechanisms of endocrine therapy, focusing primarily on antiestrogens and on the changes that occur in programmed cell death (apoptosis).

II. ESTROGEN AND ERS

ERs belong to a large nuclear receptor superfamily that exerts its effects by regulating the transcription of target genes (Mangelsdorf *et al*., 1995). Two mammalian ERs are known—ERα and the more recently identified ERβ—which share a similar domain structure with a central DNA-binding region flanked by activation function 1 and 2 (AF-1 and AF-2) domains. The carboxy-terminal AF-2 is dependent on ligand stimulation; binding of estrogen induces distinct conformational changes that allow ER to bind DNA at consensus estrogen response elements (EREs), subsequently turning on estrogen-dependent gene transcription and cell proliferation. Estrogen binding also allows the recruitment of ER coactivators that subsequently attract histone acetyltransferases—a class of chromatin remodeling enzymes that allows transcriptional activation to proceed (Hall *et al*., 2001). Ligand-occupied ER can function as a coregulator protein by interacting with the transcriptional machinery at binding sites for other transcription factors such as SP-1, AP-1, or NFκB (Kushner *et al*., 2000; McDonnell *et al*., 2002). Ligands other than estrogen (including antiestrogens; see following)

can result in alternative receptor conformations, changes in coactivator or corepressor recruitment, and differential gene transcription at EREs and other sites.

In contrast, the amino-terminal AF-1 is ligand independent and typically regulated by growth factor signaling. Epidermal growth factor (EGF) and insulin-like growth factor 1 are two of several growth factors that have been shown to regulate ER-dependent transcription independent of estrogen stimulation, most likely through the induction of p44/42 extracellular signal–regulated kinase/mitogen-activated protein kinase (MAPK), which can phosphorylate ERα on serine residue 118 within the AF-1 domain (Bunone *et al.*, 1996; Kato *et al.*, 1995). Another serine/threonine kinase (protein kinase A) can be activated by cyclic AMP and phosphorylate ERα on Ser236 (Aronica and Katzenellenbogen, 1993; Cho *et al.*, 1994). Phosphorylation of Ser118 and Ser236 by MAPK, protein kinase A, or other kinases has been demonstrated to increase transcriptional activity of ERE-containing reporter genes. Although the precise contribution of ER phosphorylation events to breast tumorigenesis and the regulation of ER activity *in vivo* are still a subject of debate (Atanaskova *et al.*, 2002; Joel *et al.*, 1998; Lannigan, 2003; Murphy *et al.*, 2004), it is clear that growth factor signaling is a key component of breast cancer. Clarifying the cross talk that occurs between the growth factor and ERs will be an important step toward understanding breast cancer biology and improved management of this disease.

Another significant gap in our knowledge of breast cancer etiology is the true role of ERβ (Speirs, 2002; Speirs *et al.*, 2004). Although structurally similar to ERα in the DNA-binding and ligand-binding domains, ERβ appears to lack AF-1 function and exhibits activities distinct from those of ERα (Hayashi *et al.*, 2003; Kuiper *et al.*, 1996; Mosselman *et al.*, 1996). Notably, when the two receptors are expressed together in breast cancer cells, ERβ inhibits ERα transcriptional activity (Hall and McDonnell, 1999), and in human breast tumors, there appears to be an inverse correlation between ERα and ERβ expression (Bieche *et al.*, 2001). Furthermore, stable expression of ERβ in the T47D breast cancer cell line results in the suppression of estrogen-induced cell growth (Strom *et al.*, 2004). It has been suggested that differential recruitment of coregulatory molecules is one mechanism by which ERα and ERβ function differently in breast tissues (Muramatsu and Inoue, 2000). Another possibility is extranuclear distribution of ERβ, which has recently been localized to the mitochondria in cells of neuronal and cardiac origin (Yang *et al.*, 2004).

Accumulating evidence indicates that ERα can participate in novel, transcription-independent signaling pathways outside the nucleus (Falkenstein *et al.*, 2000). Some groups have observed ERα localized at the plasma membrane, specifically in association with lipid microdomains containing the scaffolding protein caveolin 1 (Chambliss *et al.*, 2000; Kim *et al.*, 1999;

Razandi *et al.*, 2002), whereas others have noted an association with G protein-coupled receptors, the nonreceptor protein tyrosine kinase c-Src, or members of the MAPK pathway such as B-Raf and Ras (Migliaccio *et al.*, 1996, 2000; Razandi *et al.*, 1999; Singh *et al.*, 1999; Wyckoff *et al.*, 2001). The potential functions of ER outside the nuclear environment are not clear. A report by Razandi et al. (2003) showed that these plasma membrane–associated receptors can interact with signaling pathways involving EGF receptor (EGFR) and MAPK. G protein-coupled receptors appear to be activated in response to estrogen, leading to the activation of Src, several matrix metalloproteases, and the release of surface-bound EGF from MCF-7 breast cancer cells. Other data support the idea that cytoplasmic ERα links to the MAPK pathway; estrogen treatment of MCF-7 cells results in phosphorylation of the adapter protein Shc, which stimulates extracellular signal–regulated kinase via the activation of Grb2, Sos, and Ras (Song *et al.*, 2002). MAPK-mediated transcriptional activation can also lead to cell growth (Roovers and Assoian, 2000). Song et al. (2004) have recently shown that insulin-like growth factor 1 receptor also plays a critical role in the rapid recruitment of ERα to the plasma membrane following E2 stimulation. Therefore, the actions of ERα through both transcriptional and nontranscriptional means appear to be critical for the control of cell proliferation, and deregulation of any one of these pathways could contribute to the aberrant cell growth seen in breast cancer.

AU1

III. ANTIESTROGENS

The primary mechanism of action of an antiestrogen is competition with estrogen for binding to the ER. Since the first report that ovariectomy led to a reduction in breast tumor mass in premenopausal women (Beatson, 1896), endocrine manipulation or antiestrogen-mediated inhibition of breast cancer cell growth have been some of the most successful targeted approaches in the treatment of estrogen-dependent breast tumors (Clarke *et al.*, 2001, 2003). The most common antiestrogen is the nonsteroidal triphenylethylene TAM, an ER partial antagonist that exhibits tissue selectivity for its antagonist and antagonist activities (Clarke *et al.*, 2001).

The most potent metabolite of TAM is 4-hydroxytamoxifen (OH-TAM), which binds to the ligand-binding region of ERα with high affinity. In bone, uterine, and cardiovascular tissues, TAM functions as a positive regulator of ER function. However, TAM induces a different conformational change than that of estrogen-bound ER—the coactivator recognition groove of ER is blocked when the receptor is bound to TAM (Shiau *et al.*, 1999), and it has been shown that the corepressor N-CoR binds to ER occupied by TAM (Jackson *et al.*, 1997). In breast cancer cell lines, this can result in the inhibition of estrogen-responsive gene expression and cell growth, as well as

the stimulation of apoptosis or programmed cell death (Jordan, 1990; Musgrove *et al*., 1993). Other nonsteroidal antiestrogens such as raloxifene behave similarly to TAM both *in vitro* and *in vivo*.

The steroidal or ''pure'' antiestrogen Faslodex (ICI 182,780; Fulvestrant) inhibits estrogen-dependent events through a different mechanism (Howell, 2001). Faslodex is a full antagonist and enhances the ubiquitin-mediated degradation of ERα (Dauvois *et al*., 1992; Nawaz *et al*., 1999). Interest in Faslodex as a first-line endocrine therapy is increasing. For example, Faslodex has shown significant activity as a second-line agent in women who developed TAM resistance following an initial response, and Faslodex is at least as active as the aromatase inhibitor anastrazole in inducing an objective response while enhancing overall patient survival (Howell and Dowsett, 1997; Howell *et al*., 1995, 2002; Osborne *et al*., 2002).

Regardless of mechanism, the ultimate goal of endocrine or antiestrogen therapy is to induce breast tumor regression. This can occur either by the inhibition of cell growth (cytostasis) or by the active induction of apoptosis (cytotoxicity), and both cytostatic and cytotoxic effects of antiestrogens are observed in human tumors. The dysregulation of several molecular mechanisms and signal transduction pathways may contribute to the antiestrogen resistance phenotype. We have begun to establish several components of a broader signaling network associated with antiestrogen action and resistance (Clarke *et al*., 2003). However, a comprehensive assessment is outside the scope of this review, and we instead focus on the regulation of apoptosis in response to antiestrogens.

IV. AROMATASE INHIBITORS, ESTROGEN INDEPENDENCE, AND ANTIESTROGENS

Aromatase inhibitors have been available for clinical use for several decades. Aminoglutethimide was the first such agent used in the management of invasive breast cancer, with overall response rates that are broadly comparable to those associated with ovariectomy, the progestins, and TAM (EBCTCG, 1998b; Smith *et al*., 1981). However, the nonselective nature of aminoglutethimide led to substantial toxicity, and thus to its positioning as a second-line treatment on metastatic disease progression on TAM. Second- and third-generation aromatase inhibitors have since been developed, which have greater specificity for the aromatase enzyme, and thus a more favorable safety and toxicity profile. Miller (1997) has separated the newer-generation aromatase inhibitors into two classes: those that are steroidal and compete for substrate binding (type 1; examples are formestane, exemestane), and those that are nonsteroidal (type II; examples are fadrozole, anastrazole, letrozole). Both type 1 and type 2 agents have comparable activity to TAM in various treatment settings, but letrozole appears to most

effectively decrease circulating estrogen levels by inhibiting activity by almost 99% *in vivo* (Miller, 2004).

In premenopausal women, the primary site of estrogen biosynthesis is the ovary; in postmenopausal women, adipose tissue is the most active source of aromatase activity, although muscle mass may be a greater overall contributor to plasma estrogen levels (Miller, 2004). Antiestrogens function at the level of the ER, and thus exhibit equivalent efficacy irrespective of menopausal status. The selective aromatase inhibitors function at the level of the aromatase enzyme, such that their efficacy depends on the cessation of ovarian function either biologically (i.e., by natural menopause) or medically (i.e., by elective ovariectomy or the use of LHRH agonists). In some women, peripheral aromatization of serum androgens can account for up to 50% of circulating estrogens (Kirschner *et al*., 1982). Most serum estrogens in postmenopausal women are present as the sulfated metabolite and as such are biologically inactive. Because estrogen sulfotransferases that sulfate estrogens also are detected in breast tumors (Adams *et al*., 1979), the presence of biologically active estrogens further requires activity of the steroid sulfatase (STS gene; chromosome Xp22.32; EC 3.1.6.2.) (Entrez Gene, 2004b). Indeed, we have shown that expression of STS is sufficient to support the growth of estrogen*dependent breast tumors (James *et al*., 2001).

The activity of aromatase inhibitors is not surprising, given the estrogen dependence of many breast tumors, the association of increased serum estrogen concentrations with breast cancer risk, and the high concentrations of estradiol present in tumors in postmenopausal women (Clarke *et al*., 2001). The target of these drugs is the aromatase enzyme (CYP19A1 gene; chromosome 15q 21.1; EC 1.14.14.1), which is part of the cytochrome P450 complex (Entrez Gene, 2004a). Molecular oxygen and NADPH are used by the enzyme to perform three hydroxylations that convert C19 steroids (androgens), usually androstenedione but also testosterone, to C18 steroids (estrogens). When testosterone is the substrate for aromatase, the product is estradiol. If the substrate is androstenedione, the product is estrone, a steroid with a relative binding affinity for ER approximately 60% that of estradiol (Kuiper *et al*., 1997). The final conversion of estrone to estradiol, the primary estrogen present in breast tumors, is catalyzed by the 17β-hydroxysteroid dehydrogenase type 1 enzyme (HSD17B1 gene; chromosome 17q11-q21; EC 1.1.1.62) (Ensemble, 2004). HSD17B1 is readily detected, and occasionally amplified, in breast tumors (Gunnarsson *et al*., 2003). Many breast tumors and adipose tissue within the breast express both aromatase (Goss *et al*., 2003; Miller, 2004) and HSD17B1 (Gunnarsson *et al*., 2003), which almost certainly contributes to the high intratumoral concentrations of estradiol in many breast tumors (Clarke *et al*., 2001). Inhibitors of the HSD17B1 or STS enzymes may have significant clinical activity either as single agents, in combination with antiestrogens, or in combination with aromatase inhibitors.

Paradoxically, the long-term use of some of the most effective aromatase inhibitors can induce aromatase such that there is sufficient estrogen biosynthesis to overcome the effects of the drug (Goss *et al*., 2003). Other potential mechanisms of resistance to the aromatase inhibitors include mutations in the aromatase gene and excessive exposure to exogenous estrogens (Miller, 2004). Estrogen hypersensitivity also may contribute to resistance to aromatase inhibitors (Santen *et al*., 2001), particularly if aromatase expression is induced. However, it seems unlikely that hypersensitivity or increased estradiol production would be adequate to overcome TAM activity because of the excess of antiestrogenic metabolites present in most tumors treated with this drug. Using intratumoral TAM and TAM metabolite levels rather than serum concentrations, which may not fully reflect tissue exposures, we have estimated that antiestrogenicity exceeds estrogenicity by at least two orders of magnitude in many breast tumors (Clarke *et al*., 2003). Other resistance mechanisms are clearly shared by antiestrogens and aromatase inhibitors, most notably the lack of ER expression.

Because both antiestrogens and aromatase inhibitors can interfere with the activation of ER by estradiol, it might be expected that similar mechanisms of sensitivity and resistance would exist. Nonetheless, many investigators use the term estrogen (or hormone) independence synonymously with TAM (antiestrogen) resistance. It is clear from studies in experimental models that these are very different phenotypes. Human breast cancer cell lines selected for their ability to grow in the absence of estrogens frequently retain expression of functional ER and sensitivity to antiestrogens (Clarke *et al*., 1989; Katzenellenbogen *et al*., 1987). These observations directly reflect the clinical situation in which patients with hormone receptor-positive tumors respond to an antiestrogen as first-line therapy and then to an aromatase inhibitor as second-line therapy (Rose, 2003). Evidence also indicates that in tumors the converse is true (Smith *et al*., 1981).

These experimental and clinical phenotypes clearly establish that estrogen/hormone independence is a phenotype that can be fully separated from antiestrogen resistance. Resistance to aromatase inhibitors and antiestrogens can also be separated; for example, erbB2 overexpression has been associated with a decreased likelihood of response to TAM, but it has no compelling effect on the prediction of responsiveness to aromatase inhibitors (Ellis *et al*., 2001). There also is clear evidence of cross resistance, as is seen with ER negativity. Although estrogen/hormone independence may be more correctly applied to tumors resistant to aromatase inhibitors, it may be more correct, and more useful, to define resistance phenotypes more specifically. We have previously identified four endocrine phenotypes that reflect the diversity of responsiveness patterns and suggest applying the term multihormone resistant to tumors that are cross resistant to both antiestrogens and aromatase inhibitors (Clarke and Brünner, 1995; Clarke *et al*., 2003).

V. ANTIESTROGENS VERSUS AROMATASE INHIBITORS AS ENDOCRINE THERAPIES

Endocrine therapy has established utility in treating hormone receptor–positive invasive breast cancer, as well as in the setting of chemoprevention. In terms of existing disease, TAM has been the gold standard of therapy for over 30 years. Nonetheless, the activity and favorable toxicity profile of the third-generation aromatase inhibitors, as used in treating metastatic disease, have led to clinical investigations into their efficacy in early-stage disease and chemoprevention. Large, randomized clinical trials have looked at anastrazole (Baum *et al*., 2002, 2003), letrozole (Goss *et al*., 2003), and exemestane (Coombes *et al*., 2004) in place of, or in sequence with, TAM as adjuvant therapy. Early results from each of these trials indicate a survival benefit from the use or addition of aromatase inhibitors, although longer follow-up is needed to demonstrate durability of benefit and to better characterize the long-term toxicities associated with these newer agents. In addition, TAM is an accepted means of prevention in women at high risk for developing breast cancer (Fisher *et al*., 1998), as well as in women with a personal history of ductal carcinoma *in situ* (Fisher *et al*., 1999). The use of other agents, such as raloxifene and various aromatase inhibitors, is recommended only in the context of clinical trials (Chlebowski *et al*., 2002; Leonard and Swain, 2004). However, the three large randomized clinical trials of adjuvant endocrine therapy have demonstrated a significant reduction in the development of contralateral breast cancer, indicating a putative and encouraging chemopreventive effect (Baum *et al*., 2002, 2003; Coombes *et al*., 2004; Goss *et al*., 2003).

Anastrozole, letrozole, and exemestane have at least comparable activity to TAM as first-line endocrine therapy in the metastatic setting (Bonneterre *et al*., 2000; Mouridsen *et al*., 2001). The preferential use of these agents is increasing because of data indicative of higher objective response rates and improved disease-free survival, as well as concerns regarding the risk of venous thromboembolic disease and other toxicities uniquely associated with TAM. In these cases, TAM is therefore administered as a second-line therapy. There is also increasing amounts of data supporting the use of aromatase inhibitors in the preoperative setting, which may lead to increased opportunities for breast-conserving local therapy, as well as suitable treatment alternatives for those who are not candidates for surgery or chemotherapy (Dixon, 2004; Dixon and Miller, 2003; Ellis, 2000).

AU4

The role of the steroidal and nonsteroidal aromatase inhibitors in relation to such new "pure" antiestrogens as Faslodex is less clear. There is limited evidence of cross-resistance with TAM, and the response rates in studies of Faslodex after progression on TAM have led to its approval by the Food and Drug Administration for use in the treatment of metastatic breast cancer after disease progression on TAM (Howell, 2001; Howell *et al*., 1995). Other investigations comparing Faslodex and anastrazole as

second-line endocrine therapy for advanced disease indicate equivalence with a slightly more favorable side-effect profile (Howell *et al*., 2002; Osborne *et al*., 2002; Robertson *et al*., 2003), and studies comparing Faslodex with exemestane are ongoing. The intramuscular route of administration for Faslodex—as opposed to the oral administration of these other agents—may be favorable in the context of ensuring patient compliance, but issues regarding injection-site discomfort and bleeding need to be addressed as well.

Standard recommendations regarding the clinical roles of steroidal and nonsteroidal aromatase inhibitors, the selective ER modulators, and the pure antiestrogens will likely change over the next several years. Improved knowledge about the underlying mechanisms of endocrine therapy responsiveness and resistance will be instrumental toward this end, particularly with the anticipated ability to predict which subset of patients will benefit most from a given therapeutic intervention. For example, an aromatase inhibitor may be the endocrine treatment of choice in the small proportion of ER+ tumors that overexpress erbB2, irrespective of disease stage at diagnosis (Ellis *et al*., 2001). A similar preference may be established for breast tumors that activate similar or interrelated signaling pathways, such as EGFR overexpression. The ability to combine erbB2 or EGFR inhibitors with antiestrogens, perhaps on failure of an aromatase inhibitor, offers further intriguing opportunities for future study. AU5

Results from combining antiestrogens and aromatase inhibitors have been largely disappointing. A major synergistic interaction may be unlikely, given the potency of each class of drug alone and their common targeting of ER mediated events. Studies combining aminoglutethimide with TAM have not shown any major advantage compared with either drug alone. Studies with the more recent aromatase inhibitors have been even less supportive of such drug combinations. Pharmacokinetic interactions between TAM and Arimidex and letrozole result in poorer responses in the combination arm compared with the aromatase inhibitor alone (Dowsett *et al*., 1999, 2001b). Nonetheless, it remains possible that the correct combination of antiestrogen and aromatase inhibitor administered in the appropriate schedule could still prove better than either drug alone. For example, although the agonist activities of TAM are often best exhibited in the absence of estrogen (Clarke *et al*., 2001), the ability of Faslodex to induce ER degradation (Dauvois *et al*., 1992) could overcome estrogen hypersensitivity or increased aromatase expression resulting from long-term exposure to an aromatase inhibitor.

VI. APOPTOSIS

Apoptosis is a complex and highly regulated cellular process driven by biochemical and morphological changes that ultimately lead to DNA fragmentation and cell death. Regulation of apoptosis is essential throughout

the life of all organisms, which are constantly striving for a balance between cell proliferation and cell death. Dysregulation of apoptosis can shift this balance in favor of aberrant cell growth, a hallmark of cancer. Two major signaling pathways lead to apoptosis: the cell surface receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway (Delhalle *et al.*, 2003; Hersey and Zhang, 2003). Although these processes are initiated by different means, they are not mutually exclusive—both require the activation of caspases, a family of cysteine proteases that cleave their target proteins at specific peptide residues.

The extrinsic pathway is initiated in response to extracellular signals. Proapoptotic ligands, such as tumor necrosis factor α (TNFα), TNF-related apoptosis-inducing ligand (TRAIL), or Fas-ligand (FasL), bind to their cognate receptors and can induce multimerization (Locksley *et al.*, 2001). Activated TNF, TRAIL, or Fas receptors subsequently recruit adapter proteins to their intracellular death domains, which in turn recruit and assist in the activation of initiator caspases 8 and 10. The intrinsic pathway is more often induced in response to intracellular stimuli, such as DNA damage. Subsequently, proapoptotic members of the Bcl-2 family of proteins are relocalized to the mitochondrial outer membrane. Mitochondrial membrane permeability is then compromised, leading to the release of cytochrome c, which binds to the apoptotic protease-activating factor and serves to activate initiator caspase 9. Both the intrinsic and extrinsic apoptotic pathways result in the activation of effector caspases 3 or 7 (Earnshaw, 1999; Strasser *et al.*, 2000).

A. EVIDENCE FOR ANTIESTROGEN-INDUCED APOPTOSIS *IN VIVO*

Antiestrogens have the ability to elicit cell cycle arrest or apoptosis. In the last several years, a great deal of progress has been made in understanding the apoptotic pathways used by breast cancer cells and how antiestrogens impinge on this process (Mandlekar and Kong, 2001). From a clinical perspective, the induction of apoptosis is an important component of breast cancer regression. If antiestrogens solely arrested tumor cells in the G_0 phase, it is likely that some cells would eventually escape this inhibition and resume proliferation. In addition, cells within the tumor mass would not be actively eliminated, beyond the turnover normally expected, and improvements in survival might not be expected. However, there is clear evidence that antiestrogen therapy reduces breast tumor size and increases overall survival (EBCTCG, 1992, 1998b), indicating that apoptosis is a key feature of these drugs' activity *in vivo*. Both TAM and Faslodex are capable of inducing apoptosis; Raloxifene may be less effective (Dowsett *et al.*, 2001a; Ellis *et al.*, 1997).

B. APOPTOSIS PATHWAYS IN BREAST CANCER MODEL SYSTEMS

Much of what we know about the mechanisms of antiestrogen-induced apoptosis comes from the study of a few breast cancer cell line model systems (Table I). Both the MCF-7 and T47D cell lines were derived from metastatic pleural effusions of invasive ductal carcinoma (Clarke *et al*., 2001). Another ER-positive and hormone-dependent cell line arising from an invasive ductal carcinoma metastasis is ZR-75-1. Because it is from these three lines that the majority of antiestrogen resistance models have been generated (Clarke *et al*., 2001), it is important to understand the major classical apoptotic pathways that are affected by estrogen, estrogen withdrawal, and antiestrogen treatment of the sensitive (parental) cells.

1. Cell Surface Receptors

The TNF, TRAIL, and FasL receptors are three key mediators of the extrinsic apoptotic pathway, and their ligand-dependent activation generally results in rapid cell death (Locksley *et al*., 2001). Expression and function of these receptors has been described in the three most widely used models of estrogen-dependent breast cancer, the human cell lines MCF-7, T47D, and ZR-75-1. In MCF-7 cells, TNF stimulation leads to apoptosis that proceeds via the cleavage of Bak, a Bcl-2 family member (Suyama *et al*., 2002); various clones of MCF-7 cells exhibit different degrees of response to TNF-mediated apoptosis (Burow *et al*., 1998). In ZR-75-1 cells, TNF inhibits growth of hormone-dependent cell lines by inducing cell death that is potentially mediated by changes in c-myc expression (Mueller *et al*., 1996). However, not all estrogen-dependent cells respond to TNF by inducing apoptosis; TNFR activation in T47D cells leads instead to cell cycle arrest at the G1/S checkpoint (Pusztai *et al*., 1993).

Breast cancer cell lines also exhibit divergent responses to FasL. T47D cells exhibit surface expression of both FasL and the Fas receptor, and stimulation with exogenous FasL leads to apoptosis (Keane *et al*., 1996; Ragnarsson *et al*., 2000). ZR-75-1 cells also undergo Fas-dependent cell killing when treated with the CH-11 activating antibody (Tong *et al*., 2001). Although MCF-7 cells also express FasL (Gutierrez *et al*., 1999), there are conflicting reports as to whether these cells are sensitive or resistant to Fas-dependent apoptosis. Mullauer et al. (2000) report that MCF-7 cells are not sensitive to Fas activation by CH-11, despite their high levels of FasL expression. In contrast, the FasL present in conditioned media from normal mammary epithelial cells is reported to induce death in MCF-7 cells (Toillon *et al*., 2002).

Apoptosis induced by TRAIL/Apo2L occurs primarily in cancer cells, whereas normal cells are relatively unaffected. Thus, the TRAIL pathway is a target of significant interest for the development of new cancer therapies

TABLE I. Expression of Apoptosis-Related Molecules in MCF-7, T47D, and ZR-75-1 Cell Lines

Apoptotic regulators	MCF-7	T47D	ZR-75-1
Cell surface receptors			
TNF, TNF-R	Expressed; stimulation results in PCD	Expressed; stimulation results in G1/S block	TNF expressed only when stimulated with exogenous TNF
TRAIL	Resistant	Resistant	Resistant
Fas, FasL	Resistant	Sensitive; FasL upregulated by Tam	Sensitive
Bcl-2 family members			
Bax	Expressed	Expressed; up-regulated by antiFas Ab	Expressed
Bak	Down-regulated by E2	Expressed	
Bik	Induced by E2 deprivation and antiE2		
Bcl-2	Up-regulated by E2, down-regulated by antiE2	Undetectable basal expression; up-regulated by E2	Expressed; up-regulated by E2
Bcl-xL	Expressed	Expressed	Expressed
Caspases			
Caspase 3	Negative	Expressed	Expressed
Caspase 7	May substitute for caspase 3	Expressed	Expressed
p53	Wild type	Nonfunctional; Phe194 mutation	Wild type
PI3K/Akt	Expressed; activation results in ER downregulation and antiE2 resistance	Expressed; Akt activation prevents Fas-mediated PCD	Expressed; activation results in ER downregulation
NFκB	Upregulated in antiE2-resistant variants	Expressed	Expressed

Abbreviations: E2, estrogen; antiE2, antiestrogen; PCD, programmed cell death. See text for all other abbreviations and citations.

(Fulda and Debatin, 2004). Although parental MCF-7, T47D, and ZR-75-1 cells are all resistant to TRAIL-mediated cell killing (Keane *et al*., 1999), sensitivity to TRAIL can be restored. For example, one consequence of TRAIL treatment is activation of the prosurvival nuclear factor kappa B (NFκB) transcription factor, and inhibition of NFκB can restore TRAIL-induced cell death (Nakshatri *et al*., 2004). Overexpression of interferon gamma (IFNγ) can also enhance sensitivity to TRAIL-dependent apoptosis in MCF-7 cells (Ruiz *et al*., 2004), probably by activating the transcription factor and tumor suppressor interferon regulatory factor 1 (IRF-1) (Clarke *et al*., 2004). Involvement of IRF-1 is of particular interest because we have recently shown that IRF-1 expression is down-regulated in antiestrogen-resistant MCF-7/LCC9 cells (Gu *et al*., 2002) and that a dominant-negative IRF-1 blocks the proapoptotic effects of the steroidal antiestrogen Faslodex in MCF-7 and T47D cells (Bouker *et al*., 2004) (discussed below).

Exposure of MCF-7, T47D, or ZR-75-1 cells to antiestrogens has the potential to affect signaling via the TNF and Fas/FasL pathways. In T47D cells, TAM up-regulates expression of surface FasL (Nagarkatti and Davis, 2003). Estradiol treatment of MCF-7 and T47D breast cancer cells also can increase FasL expression, and TAM decreases FasL expression (Mor *et al*., 2000). MCF-7 cells that have been subjected to long-term estrogen deprivation undergo apoptosis when treated with estradiol, which correlates with increased expression of FasL (Song and Santen, 2003). It is not clear what factors contribute to these conflicting results. FasL upregulation appears to play a major role in immune evasion. Activated T-cells expressing the Fas receptor can be killed by breast cancer cells that have up-regulated FasL (Gutierrez *et al*., 1999), implying that FasL upregulation is not necessarily beneficial to inducing the apoptosis of breast cancer cells *in vivo*.

Estradiol treatment of MCF-7 cells can abolish TNF-mediated apoptosis via effects on downstream apoptotic mediators such as Bcl-2, and this can be reversed by exposure to Faslodex (Burow *et al*., 2001). Faslodex treatment enhances cell death induced by TNF under these conditions, and expression of both the TNF receptor and TNF receptor–associated death domain are increased by TAM or Faslodex treatment of MCF-7 cells (Smolnikar *et al*., 2000). Cotreatment of MCF-7 cells with TNF and TAM also increases cell death (Matsuo *et al*., 1992). Together, these data imply a significant degree of cross talk between TNF- and antiestrogen-induced apoptosis.

2. Bcl-2-Related Molecules

Members of the Bcl-2 family of proteins perform either antiapoptotic or proapoptotic functions focused on the maintenance or disruption of mitochondrial membrane integrity (Gross *et al*., 1999). Family members contain one or more Bcl-2 homology (BH) domains, BH1–BH4. Although the antiapoptotic Bcl-2 and Bcl-XL contain all four BH domains, the proapoptotic family members such as Bax and Bak generally lack BH4 or,

like Bik, contain only BH3 domains. A comprehensive discussion of all 20 Bcl-2 family members is outside the focus of this review; therefore, we focus on those genes most strongly implicated in affecting antiestrogen responsiveness.

The proapoptotic Bax and Bak molecules are expressed in MCF-7, T47D, and ZR-75-1 cells (Leung *et al*., 1998; Mooney *et al*., 2002; Niu *et al*., 2001; Tong *et al*., 2001). In MCF-7 cells, Bak is cleaved and thereby activated during the process of TNF-initiated apoptosis (Suyama *et al*., 2002). Bak expression decreases on estradiol stimulation and increases in MCF-7 cells stably transfected with the aromatase gene when this activity is blocked by aromatase inhibitors (Leung *et al*., 1998; Po *et al*., 2002; Thiantanawat *et al*., 2003). However, others have reported that neither Bax nor Bak protein levels in these cell lines are affected by estrogen or antiestrogens such as TAM and Faslodex (Gompel *et al*., 2000; Kandouz *et al*., 1999; Salami and Karami-Tehrani, 2003; Zhang *et al*., 1999). Thus, although Bax and Bak are expressed in these model systems, there is no clear consensus on what role they may play in antiestrogen-induced apoptosis.

The BH3-only protein Bik has recently been suggested to play a critical role in the antiestrogen-induced apoptosis of breast cancer cells (Hur *et al*., 2004). Bik mRNA is upregulated in MCF-7, T47D, and ZR-75-1 cells following exposure to Faslodex, but detectable levels of Bik protein are only observed in MCF-7. Bik could also be induced by culturing MCF-7 cells under estrogen-deprived conditions, and small inhibitory RNA directed against Bik effectively eliminated Faslodex-mediated apoptosis (Hur *et al*., 2004). Moreover, Frasor et al. (2003) have reported that Bik is one of several genes down-regulated by estradiol and up-regulated by Faslodex, but not the nonsteroidal antiestrogens Raloxifene or OH-TAM, indicating that this molecule may be one mediator of apoptosis in response to steroidal antiestrogens.

Activities of the antiapoptotic factors Bcl-2 and Bcl-XL have been widely studied in breast cancer cell lines. The major mechanism by which these molecules inhibit apoptosis is by forming dimers with proapoptotic Bcl-2 family members and preventing cytochrome c release from the mitochondria. Although Bcl-XL appears to be expressed basally in all three cell lines (Simoes-Wust *et al*., 2000), Bcl-2 protein is undetectable in T47D cells (Butt *et al*., 2000; Elstner *et al*., 2002). However, estradiol can up-regulate Bcl-2 in T47D, ZR-75-1, and MCF-7 cells (Gompel *et al*., 2000), and others have shown that TAM and Faslodex can decrease Bcl-2 expression (Burow *et al*., 2001; Kandouz *et al*., 1999; Somai *et al*., 2003; Thiantanawat *et al*., 2003; Zhang *et al*., 1999). This correlates with the induction of apoptosis (Zhang *et al*., 1999) and occurs at the level of transcription, as luciferase expression driven by the Bcl-2 promoter and two consensus EREs within the Bcl-2 coding region is increased twofold in the presence of estradiol and inhibited on addition of Faslodex to MCF-7 cells (Somai *et al*., 2003). It is also

important to note that induction of apoptosis in the rat mammary gland by ICI 182,780 correlates with the down-regulation of Bcl-2 (Lim *et al*., 2001). Hence, it would seem that Bcl-2 is an important estrogen-regulated gene in these breast cancer cells and that attenuation of this prosurvival signal is directly involved in the apoptotic response to antiestrogens.

3. Caspases

Regardless of the means by which apoptosis is induced, caspases are responsible for initiation of the physical changes associated with cell death. Initiator and effector caspases are synthesized in an inactive form, and when cell death signals are received, these molecules are activated by proteolytic cleavage (Earnshaw, 1999; Strasser *et al*., 2000). The range of substrates is varied and includes proteins involved in DNA repair, cell cycle progression, and invasion or metastasis, as well as other caspases.

Caspases 3 and 7 are the two most commonly studied effector caspases. MCF-7 cells lack expression of caspase 3 because of a 47–base pair deletion that results in exon skipping, whereas ZR-75-1 and T47D cells are caspase 3 competent (Janicke *et al*., 1998). MCF-7 cells have clearly adapted to this deficiency by using other members of this family such as caspases 9, 7, or 6. Liang et al. (2001) have demonstrated that MCF-7 cells treated with neocarzinostatin, a mitotic inhibitor, undergo apoptosis that depends on the activation of caspase 9, followed by 7 and 6. In contrast, MCF-7 cells exhibit caspase 6 activation (but not caspase 7 activation) in response to the protein kinase inhibitor staurosporine (Mooney *et al*., 2002). Thus, specific caspase activation cascades may be dependent on the apoptotic stimulus.

TAM at a dose of 5 μM has been shown to modestly increase the activity of caspases 8 and 9 in MCF-7 cells, with no effects on caspase 3-like substrates (Mandlekar *et al*., 2000). Salami and Karami-Tehrani (2003) have also reported that 1 μM TAM, although capable of inducing cell death, is not sufficient to produce cleavage of caspase 3–like substrates. However, Fattman et al. (1998) showed that TAM treatment could induce the cleavage of poly-(ADP-ribose) polymerase, a caspase 3/7 substrate. In response to aromatase inhibitors, MCF-7 cells stably transfected with the aromatase gene exhibit strong activation of caspases 9, 6, and 7, accompanied by poly-(ADP-ribose) polymerase cleavage, whereas TAM and Faslodex treatment also activates these enzymes, but to a lesser degree (Thiantanawat *et al*., 2003). In T47D cells, TAM treatment induces caspase 3 activity (Ellis *et al*., 2003a). Because caspase 8 is frequently considered to be activated by extrinsic cell death signals (whereas caspase 9 participates in intrinsic apoptosis), current data indicate that antiestrogens may affect both apoptotic signaling pathways, and future studies should attempt to clearly define which pathway predominates.

4. p53

The tumor suppressor gene p53 and its family members p63 and p73 are global regulators of cell cycle arrest and apoptosis, and p53 is inactivated in about 30% of all breast cancers. In response to DNA damage, ionizing radiation, or cytotoxic drugs, expression of p53 is stabilized, and transcription of p53 target genes is increased. Target genes include those that promote either cell cycle arrest, such as the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$, or apoptosis, such as the proapoptotic Bcl-2 family member Bax. p53 has also been shown to inhibit the expression of Bcl-2 (Haldar *et al*., 1994) or relocalize to the mitochondria and prevent Bcl-2-or Bcl-X_L-mediated survival functions (Marchenko *et al*., 2000). Thus, in the presence of a growth-inhibitory signal, p53 may determine whether a cell initiates growth arrest or cell death.

Although MCF-7 and ZR-75-1 cells contain wild-type p53, T47D cells express a mutated form containing a phenylalanine substitution at codon 194 (Strano *et al*., 2000). This residue lies in the central core of the molecule, and mutations here affect the DNA binding capacity of p53. Strano et al. (2000) have also shown that p53 Phe194 in T47D cells can bind to p63 or p73 and inhibit transcription of their target genes. Given its central role in tumor suppression, it might be predicted that p53 is involved in breast cancer cell responses to antiestrogens, but the current data are contradictory.

Some studies have shown that estradiol treatment increases p53 expression in MCF-7 (Guillot *et al*., 1996; Lilling *et al*., 2002) and T47D cells (Dinda *et al*., 2002), the latter in spite of their mutant p53. Lilling et al. (2002) also reported that TAM and estrogen depletion reduced p53 levels, and Faslodex can reverse p53 induction by estrogen in T47D cells (Dinda *et al*., 2002). In transcriptional studies of the P1 promoter of p53, which was transiently expressed in MCF-7 cells, Hurd et al. (1999) found that estrogen and TAM but not Faslodex could induce P1 transcriptional activity. In contrast, Fattman et al. (1998) observed no increase in MCF-7 cell p53 levels in response to TAM, despite a significant induction of apoptosis and retinoblastoma protein dephosphorylation. Zhang et al. (1999) similarly reported no induction of p53 by TAM in this cell line.

In light of these conflicting results, it is also important to consider the intracellular distribution of p53, and not only its expression level. When intracellular localization was examined in MCF-7 cells, p53 was exported to the cytoplasm following estrogen exposure, and Faslodex reversed this effect (Molinari *et al*., 2000). Interestingly, Lilling et al. (2002) observed that p53 is primarily cytoplasmic in the TAM-resistant MCF-7/LCC2 cell line. In summary, any role for p53 in antiestrogen-induced apoptosis is complex, and further investigations are needed to clarify the relative importance of expression and subcellular localization.

The role of p53 in the apoptotic response to antiestrogens *in vivo* is also unclear. Some investigators have reported a significant correlation between p53 mutation and a poor response to TAM (Berns *et al*., 2000), whereas others have found no association between response and p53 expression or mutation (Askmalm *et al*., 2004; Berry *et al*., 2000).

5. PI3K/Akt and NFκB

Two other major contributors to prosurvival and antiapoptotic signaling in breast cancer cells are PI3K and NFκB. PI3K and its major downstream target Akt are activated following stimulation of a wide range of growth factor receptors and G protein-coupled receptors, and inappropriate activation or amplification of these molecules has been linked to many cancers including those of the breast (Luo *et al*., 2003). As a consequence, the effect of chemotherapeutics, including antiestrogens, on the PI3K/Akt axis is an important consideration. Stimulation of MCF-7 cells with estrogen rapidly induces PI3K/Akt activity through the ErbB2 receptor signaling pathway, and this signaling could be inhibited by both TAM and Faslodex (Stoica *et al*., 2003). Campbell et al. (2001) report that Akt is protective against TAM-induced apoptosis. PI3K is also important for estrogen-induced cell cycle progression of MCF-7 cells (Castoria *et al*., 2001), and estrogen induction of c-fos transcription is also dependent on PI3K (Duan *et al*., 2002). However, in MCF-7 and ZR-75-1 cells, recent evidence indicates that PI3K activation interferes with the ER's ability to repress the transcription of genes that induce cell invasion and motility (such as interleukin 6) by reducing ER expression (Bhat-Nakshatri *et al*., 2004). Taken together, this could constitute a feedback loop in which the initial estrogen stimulation is later controlled or down-regulated by activated PI3K. In T47D cells, PI3K activation can inhibit cell death induced by FasL (Gibson *et al*., 1999).

One of the many targets of activated PI3K and Akt is NFκB, although this is only one mechanism by which NFκB can be stimulated. The NFκB family of transcription factors contains five members that form dimers and regulate the transcription of various genes including cytokines, cell adhesion molecules, the proproliferative proteins c-myc and cyclin D1, and several inhibitors of apoptosis (Chen and Greene, 2004). Inhibitors of the NFκB pathway show promise as anticancer agents (Epinat and Gilmore, 1999), as constitutive NFκB activity is widely observed in many tumor types (Baldwin, 2001). We and others have shown that NFκB activity increases in breast cancer cells as they acquire the ability to grow in the absence of estrogen or in the presence of antiestrogen (Nakshatri *et al*., 1997; Pratt *et al*., 2003), and that expression of the p65 RelA subunit of NFκB is increased in MCF-7/LCC9 antiestrogen-resistant cells (Gu *et al*., 2002). Therefore, NFκB appears to play a critical role in the cellular response to antiestrogens.

VII. ANTIESTROGEN RESISTANCE AND DEFECTS IN APOPTOSIS

Because antiestrogens such as TAM and Faslodex can induce apoptosis *in vitro* and *in vivo* (Fig. 1), development of resistance to these agents may coincide with defects in cell death signaling or execution. There are two major classes of antiestrogen resistance: acquired resistance and intrinsic or *de novo* resistance. *De novo* resistance could be attributed to a lack of both ER and PR expression, but this does not account for the many ER+ or PR+ tumors that do not respond to antiestrogens. In those cases in which resistance is acquired, ER expression is, for the most part, retained (Clarke *et al.*, 2003). Alternative growth pathways—and coinciding defects in apoptosis—are likely to be responsible for at least some cases of acquired antiestrogen resistance.

A. APOPTOSIS IN ANTIESTROGEN-RESISTANT TUMORS

Evidence suggests a link between expression of apoptotic factors and the efficacy of endocrine therapy in patients. In some studies, it is necessary to separate those cases that recur because they have a poorer overall prognosis,

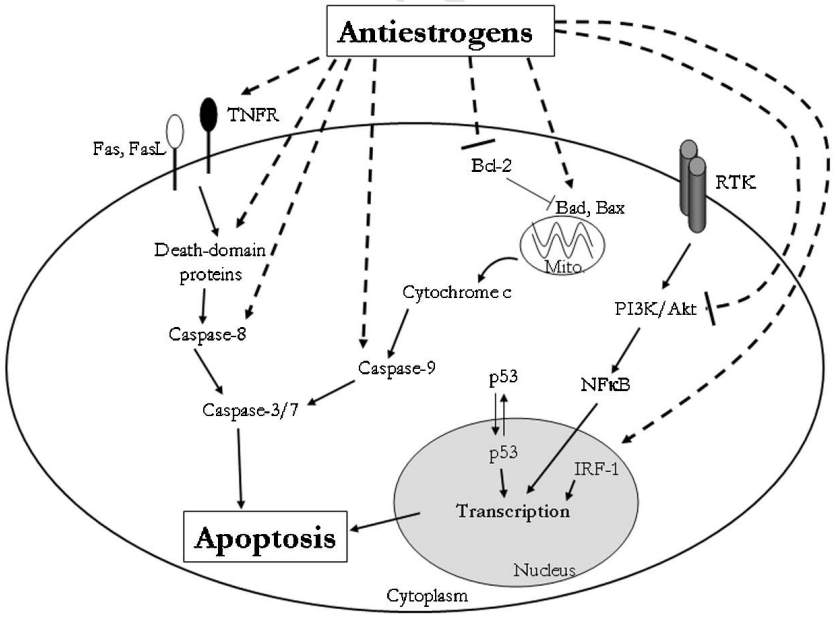

FIGURE 1. Antiestrogen effects on apoptotic signaling in breast cancer cells. Abbreviations: RTK, receptor tyrosine kinase; Mito., mitochondria. Other abbreviations have been defined in the text.

rather than simply acquired resistance to the treatment administered. Nonetheless, phosphorylated Akt, which is a key mediator of PI3K signaling, is associated with breast cancer recurrence and metastasis to distant sites (Perez-Tenorio and Stal, 2002). An association between p53 mutation and a poor response to TAM also has been reported (Berns *et al.*, 2000). However, the role of p53 in affecting antiestrogen responsiveness is unclear. Elevated expression of wild-type p53 has also been shown to be associated with a suboptimal antiestrogen response (Bottini *et al.*, 2000; Daidone *et al.*, 2000). It is suggested that this is because of the association of p53 with a dedifferentiated phenotype, but given the complexity of p53 signaling, there are likely to be additional explanations. For example, nuclear versus cytoplasmic localization of p53 may play a role in its ability to promote apoptosis in response to antiestrogens (Lilling *et al.*, 2002).

The role of Bcl-2 in affecting antiestrogen responsiveness also is not clearly defined. Some groups have reported that decreased Bcl-2 is correlated with reduced TAM effectiveness (Daidone *et al.*, 2000; Gasparini *et al.*, 1995; Silvestrini *et al.*, 1996), which is somewhat surprising considering the pro-survival functions of Bcl-2. In contrast, Cameron et al. (2000) showed that Bcl-2 levels can fall after 3 months of TAM therapy, but only in those patients who respond positively to the treatment. Although apoptosis levels within breast tumors are high after the first 24 hours of treatment, cell death is markedly decreased at the 3-month time point, and within the residual cancer cell population there are higher levels of Bcl-2 (Ellis *et al.*, 1998). Given these observations, other markers of apoptotic activity should be more closely studied to determine their connection to antiestrogen-induced apoptosis *in vivo*.

B. APOPTOSIS IN MODELS OF ACQUIRED ANTIESTROGEN RESISTANCE

We have begun to examine a network of apoptotic signaling, identified in several human breast cancer cell lines and variants. Here, we focus on two molecules with functional interactions (IRF-1 and NFκB) that are strongly implicated as key nodes in a broader gene network associated with signaling from ER activation/inactivation to cell cycle progression and apoptosis. Despite their ability to form heterodimers and selectively regulate the transcription of genes such as iNOS (Saura *et al.*, 1999), IRF-1 and NFκB clearly exhibit opposing activities; in general, IRF-1 is proapoptotic and NFκB is antiapoptotic.

1. IRF-1

The transcription factor IRF-1 is a major mediator of type I and II IFN signaling. Activation of IFN receptors results in the activation of the Janus kinase/signal transducer and activation of transcription (JAK/STAT)

pathway, which directly leads to the induction of IRF-1 expression (Kroger *et al.*, 2002). In addition to its role in the immune response, IRF-1 also exhibits tumor suppressor activities in cancer cells; expression is frequently decreased or lost in tumors and cell lines of hematopoietic origin (Sillman *et al.*, 1993). IRF-1 knock-out mice exhibit greatly increased tumor formation induced by the oncogenes myc and ras (Nozawa *et al.*, 1999)—two oncogenes implicated in breast cancer. One immunohistochemical study (Doherty *et al.*, 2001) has also shown that IRF-1 expression is decreased in neoplastic versus normal human mammary tissue, indicating that this molecule may be involved in breast tumorigenesis.

We first reported that expression of IRF-1 is down-regulated in the MCF7/LCC9 cells (Gu *et al.*, 2002). This cell line is estrogen-independent, and although it was selected for resistance to the steroidal antiestrogen Faslodex 182,780, MCF7/LCC9 cells are also cross-resistant to OH-TAM (Brünner *et al.*, 1997). We have now shown that IRF-1 plays an important functional role in the apoptotic response to the antiestrogen Faslodex (Bouker *et al.*, 2004). IRF-1 mRNA levels and transcriptional activity are significantly repressed in the MCF7/LCC9 cell line, compared to MCF-7 cells. Furthermore, although expression of IRF-1 is induced by Faslodex in antiestrogen-sensitive (MCF-7) cells, this regulation is lost in the resistant MCF7/LCC9 cells. Expression of IRF-1 in antiestrogen-resistant MCF7/LCC9 cells could be rescued by treating cells with the cytotoxic drug Adriamycin, indicating that global transcriptional regulation of IRF-1 is not defective. To address the mechanism of action of IRF-1 in antiestrogen response, we generated MCF-7 and T47D cells that stably overexpress a dominant negative IRF-1 (dnIRF-1) (Bouker *et al.*, 2004). The dnIRF-1 construct lacks the carboxyl-terminal transcriptional activation domain, successfully inhibits IFNγ-stimulated transcription, and significantly reduces antiestrogen sensitivity in both cell lines. Importantly, this occurs via a reduction in Faslodex-induced apoptosis, but not cell cycle arrest, strongly linking IRF-1 function to the apoptotic action of this antiestrogen (Bouker *et al.*, 2004).

IRF-1 is known to play a role in cell death in response to other cytotoxic agents. IFNγ can induce cell death in MCF-7 cells, and IRF-1 has been shown to contribute to apoptosis via enhanced expression and activity of caspase 8 (Ruiz-Ruiz *et al.*, 2004). IFN-mediated sensitization of MCF-7 cells to apoptosis induced by TRAIL correlates with increased activity of IRF-1 (Clarke *et al.*, 2004). IRF-1 is also known to cooperate with the tumor suppressor p53 (Tanaka *et al.*, 1996). As discussed above, MCF-7 and MCF7/LCC9 cells express normal p53, while T47D cells contain mutated p53. Because dnIRF-1 abrogates the apoptotic response to antiestrogens in both the MCF-7 and T47D cell lines, either IRF-1 is functioning independently of p53 or IRF-1-dependent apoptosis in MCF7 and T47D cells is regulated by different signaling pathways. We are currently examining in

detail these and other molecular features of antiestrogen- and dnIRF-1-regulated apoptosis in several antiestrogen-resistant model systems.

2. NFκB

NFκB is one of many targets downstream of the PI3K/Akt pathway and has been linked to the development of estrogen independence and antiestrogen resistance in breast cancer cells (Nakshatri *et al*., 1997; Pratt *et al*., 2003). In performing expression analysis of the antiestrogen-resistant MCF-7/LCC9 cells, we determined that expression of the p65 RelA subunit of NFκB is increased twofold, while NFκB-dependent transcriptional activity is increased up to 10-fold in the resistant cells (Gu *et al*., 2002). Furthermore, MCF7/LCC9 cells are selectively sensitive to growth inhibition by parthenolide, a small molecule inhibitor of NFκB. This led us to hypothesize that NFκB is a key effector of antiestrogen-induced apoptosis, and that the MCF7/LCC9 cells may be more dependent on NFκB-mediated signaling following the acquisition of resistance.

Subsequent studies have supported this hypothesis. Upstream of NFκB, the inhibitor of kappa B kinase complex phosphorylates the inhibitor IκB, allowing for the release of the NFκB dimer and its transition to the nucleus (Chen and Greene, 2004). The inhibitor of kappa B kinase complex is composed of two catalytic subunits (α and β) and a regulatory subunit known as inhibitor of kappa B kinaseγ, or NEMO. Further analysis of the NFκB pathway in the MCF7/LCC9 cells revealed that expression of NEMO is also significantly increased when compared with the antiestrogen-sensitive MCF7/LCC1 cells, which may partially explain the observed increase in NFκB transcriptional activity (Riggins *et al*., submitted). In further studies with parthenolide, we confirmed that MCF7/LCC9 cells were sensitive to this inhibitor, and although MCF7/LCC9 cells do not respond to Faslodex, the combination of parthenolide and Faslodex results in a synergistic fourfold inhibition of cell growth. Importantly, the synergistic effect of parthenolide and Faslodex is a result of a significant increase in apoptosis and has no effect on cell cycle regulation (Riggins *et al*., submitted). Given the emerging interest in NFκB inhibitors as anticancer therapies, and the fact that parthenolide has shown safety in phase I clinical trials (Curry *et al*., 2004), we propose that further preclinical studies rigorously investigate the combination of antiestrogens and parthenolide in the treatment of ER-positive breast cancer.

AU6

AU7

VIII. DE NOVO ANTIESTROGEN RESISTANCE

De novo or intrinsic resistance to antiestrogens could be the consequence of several different events, only one of which is development of an ER-negative tumor. Amplification or overexpression of genes that promote

antiestrogen-resistant growth or disrupt antiestrogen-induced apoptosis could confer resistance. Although a number of growth-regulatory molecules have been implicated in antiestrogen response *in vitro* and *in vivo*, including EGFR, erbB2, ERβ, cyclin E, and cyclin D1 (Dorssers *et al*., 2001), very few have independent predictive or prognostic power for determining a patient's chances of responding favorably to endocrine therapy. Two such candidates are the breast cancer antiestrogen resistance 1 and 3 (BCAR1 and BCAR3) genes.

BCAR1 and BCAR3 were discovered in an *in vitro* random insertion mutagenesis screen for antiestrogen resistance genes performed by Dorssers et al. (1993). Estrogen-dependent ZR-75-1 cells were infected with retroviruses, selected in media containing 1 μM TAM, and resistant clones were isolated and analyzed for common integration sites. BCAR1, the first locus, was identified as the human homologue of the rat protein p130Cas (Cas) (Brinkman *et al*., 2000). Stable transfection of ZR-75-1 cells with BCAR1 cDNA permits the growth of these cells in the presence of either TAM or Faslodex, but not conventional cytotoxic drugs such as doxorubicin, 5-fluorouracil, or methotrexate. In a study of BCAR1 in material from 937 primary human breast tumors, almost 10% exhibited strong staining (van der Flier *et al*., 2000a). These patients exhibited significantly poorer survival and increased rates of nonresponse to TAM. Moreover, high BCAR1/Cas expression was an independent predictor of reduced disease-free survival and nonresponse to TAM even when factors such as age and menopausal status were excluded.

Subsequent studies have begun to address the mechanism of BCAR1/Cas action in antiestrogen response. Van der Flier et al. (2000b) also examined tumor material from patients with acquired TAM resistance and from those who had not received endocrine therapy. They observed no significant change in BCAR1 levels between these two groups, further distinguishing BCAR1 as a predictor for *de novo* rather than acquired resistance. Immunohistochemical studies of normal and neoplastic mammary tissue revealed that BCAR1 is expressed in the luminal epithelium and vasculature, but not in the stroma or myoepithelium (van der Flier *et al*., 2001). The BCAR1-positive population was also immunoreactive for ER, indicating that the BCAR1/Cas effects on antiestrogen-resistant growth occur in the same population of cells targeted by the antiestrogens.

Although the molecular mechanisms of BCAR1/Cas-mediated antiestrogen resistance are still being uncovered, a great deal is known about the function of this protein in other contexts. Cas is an adapter molecule containing multiple protein–protein interaction domains that has been implicated in such diverse cellular processes as migration and invasion, survival, proliferation, oncogenic transformation, and bacterial engulfment (Bouton *et al*., 2001). In the case of breast cancer and antiestrogens, interaction of Cas with the nonreceptor protein tyrosine kinase c-Src may be of particular

importance. Cas, which is both a substrate and an activator of Src, is tyrosine phosphorylated on multiple residues in response to stimuli that activate Src (Casamassima and Rozengurt, 1997; Ojaniemi and Vuori, 1997), and Src appears to be the preferred kinase that phosphorylates Cas (Ruest *et al*., 2001). Moreover, the carboxyl terminus of Cas contains binding sites for both the Src-homology 2 and 3 (SH2 and SH3) domains, allowing Src binding and conformational change leading to increased kinase activity (Burnham *et al*., 1999, 2000). Between 30% and 70% of breast cancers have been reported to overexpress c-Src (Koster *et al*., 1991; Ottenhoff-Kalff *et al*., 1992), and these tumors' elevated kinase activity appears to derive from overexpression rather than activating mutations (Biscardi *et al*., 1998; Verbeek *et al*., 1996). Thus, Cas-mediated Src activation could play a major role in proliferation and antiestrogen resistance.

BCAR3 can also independently induce estrogen independence (Yu and Feig, 2002) and TAM or Faslodex resistance in MCF-7 and ZR-75-1 cells (van Agthoven *et al*., 1998). This gene is also known as NSP2 (Lu *et al*., 1999) and AND-34, which is the murine homologue (Cai *et al*., 1999). The function of BCAR3/AND-34 is less well-defined than that of BCAR1. Although it has been suggested that this molecule has guanine nucleotide exchange factor activity toward several Ras family GTPases (Gotoh *et al*., 2000), other groups have questioned these findings (Bos *et al*., 2001; Quilliam *et al*., 2002). More recently, it has been reported that AND-34 activates the Rac GTPase as well as transcription from the cyclin D1 promoter (Cai *et al*., 2003). Importantly, BCAR3/AND-34 physically associates with BCAR1/Cas (Cai *et al*., 1999), and we have recently shown that Cas and AND-34 synergistically promote Src kinase activation and cell migration (Riggins *et al*., 2003). Enhancement of cell motility is dependent on Src kinase activity and coincides with a redistribution of Cas to the plasma membrane. Hence, the interaction of Cas and AND-34 has significant functional consequences for the cell, and the collaboration of these molecules in the promotion of breast cancer and antiestrogen resistance, as well as their potential suppression of apoptosis, should be further studied.

IX. SUMMARY AND FUTURE DIRECTIONS

It is clear that many aspects of antiestrogen- and aromatase inhibitor–induced apoptosis, as well as how defects in the apoptotic pathway may be contributing to resistance, are poorly understood. Further studies in several key areas will help to increase our understanding of these problems and greatly improve the clinical management of breast cancer. One is the examination of apoptosis mechanisms in the current cell culture models of antiestrogen resistance, which should uncover important molecular targets that can be validated *in vivo*. Alongside this effort, we should also design new

clinical studies that can identify biomarkers of response versus nonresponse in breast cancer patients treated with antiestrogens and aromatase inhibitors. Ellis et al. (2003b) have begun a phase II trial of the aromatase inhibitor letrozole with the goal of analyzing changes in gene expression during the first month of neoadjuvant therapy before surgery. Preliminary data from this study indicate dramatic changes in mediators of both apoptosis and the cell cycle, and it is hoped that the complete array of data from this and other trials will form the basis for an expression profile that will be able to accurately predict which patients will respond favorably to endocrine therapy.

Our ultimate goal is to prevent or reverse resistance to antiestrogens. With a greater understanding of the mechanisms of apoptosis that are at work in breast cancer cells, we should also be able to design combination therapies that inhibit ER activities as well as the function of those molecules that contribute to the resistant phenotype. Awada et al. (2003) have recently reviewed many of the new treatments being studied in metastatic breast cancer. Several of these, such as the Bcl-2-specific antisense Genasense, PI3K pathway inhibition, the proteasome inhibitor Velcade, or adenoviral delivery of wild-type p53, may prove useful in combination with TAM, Faslodex, or aromatase inhibitors. It is hoped that these and other targeted therapies will increase the clinical efficacy of antiestrogens and greatly improve survival and quality of life for breast cancer patients.

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Abstracts

RESISTANCE TO ENDOCRINE THERAPY IN BREAST CANCER

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The precise mechanisms of resistance to endocrine therapies are unclear. Antiestrogen-stimulated growth can occur but is a minor phenotype when compared with antiestrogen unresponsiveness. Absence of estrogen receptor (ER) expression is the most common *de novo* resistance mechanism but is uncommon in acquired resistance. We have developed a series of variant cell lines that exhibit differences in their responsiveness to estrogens and antiestrogens. Using these variants and both genomic and proteomic approaches, we have begun to test our hypothesis that endocrine responsiveness is conferred by a complex signaling network that exhibits components of ER-dependent and ER-independent signaling, protein interactivity, and signaling redundancy.

Genomic (gene expression microarray and serial analysis of gene expression) studies identified several candidate genes including interferon regulatory factor-1 (IRF-1), nuclear factor kappa B (NFκB), and the human X-box binding protein-1 (hXBP-1). Proteomic (2-dimensional gel electrophoresis) analyses implicated nucleophosmin (NPM). We have observed changes in both basal expression and the ability of ER-mediated events to regulate the expression of these genes. Our recent mechanistic studies strongly implicate signaling through IRF-1, and its two protein partners NPM and NFκB, in functionally affecting the ability of breast cancer cells to induce apoptosis in response to antiestrogens. For example, the proapoptotic effects of ICI 182,780 (Faslodex; Fulvestrant) are blocked by a dominant negative IRF-1 (dnIRF-1), a function also performed by endogenous NPM expression. We show that IRF-1 acts as a tumor suppressor gene in breast cancer: overexpression reduces tumorigenicity in nude mice while dnIRF-1 increases tumorigenicity. A small molecule inhibitor of NFκB (parthenolide) restores sensitivity to ICI 182,780 in resistant cells. Overexpression of hXBP-1 confers resistance to both ICI 182,780 and Tamoxifen.

We detect each of these proteins in breast tumor tissue microarrays (all are detectable in >50% of breast cancers), often in patterns consistent with our network. For example, NFκB may regulate hXBP-1 expression and these are coexpressed ($p=0.018$); an inverse relationship is seen between the prosurvival NFκB and the tumor suppressor IRF-1 ($p=0.034$). Thus, our mechanistic and translational studies are consistent with a novel gene expression network, regulated by ER-mediated events, that affects signaling to apoptosis. Network components may be useful as biomarkers and/or molecular targets.

X-BOX BINDING PROTEIN-1 IN BREAST CANCER

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In studies of acquired Tamoxifen (TAM) and Faslodex (ICI 182,780) resistance, we identified a member of the ATF/CRE family, human X-Box binding protein-1 (hXBP-1), as a potential contributor to endocrine resistance and cell survival in breast cancer. XBP-1 activates genes with cyclic AMP responsive elements (CRE) and is directly implicated in both protein refolding and degradation in endoplasmic reticulum stress responses and as an antiapoptotic factor. Some published gene microarray studies have implicated hXBP-1 as being associated with estrogen receptor (ER)-positive tumors. Thus, we have begun to establish the role of hXBP-1 in breast cancer.

We first introduced the hXBP-1 cDNA into MCF7 cells, which are antiestrogen sensitive and estrogen dependent for growth *in vitro* and *in vivo*. Cells were transfected with a pcDNA 3.1 expression vector (Invitrogen) containing the XBP-1 cDNA (MCF7/hXBP-1). The empty vector (same construct but without the XBP-1 cDNA) also was transfected into MCF-7 cells to generate control cell populations. We assessed whether overexpression of hXBP-1 could confer on MCF-7 cells the ability to grow *in vitro* in the absence of estrogenic supplementation. The data show that hXBP-1 enabled the cells to survive and proliferate in the absence of estrogens, whereas the control cells did not proliferate and began to die between days 3 and 6. In the absence of estrogens, 6% of MCF-7 cells are in S-phase, compared with almost 20% of the MCF7/hXBP-1 cells; showing an effect of hXBP-1 on cell cycle transition. The ability to maintain estrogen-independent growth would be expected to confer resistance to an aromatase-inhibitor, although not necessarily to an antiestrogen. The antiestrogen crossresistance phenotype of MCF7/hXBP-1 cells is reported elsewhere at this meeting. While the precise mechanisms of resistance in these cells are under investigation, consistent with the presence of a CRE in the aromatase gene promoter, MCF7/hXBP-1 cells express increased rates of aromatase mRNA transcription. Thus, hXBP-1 may be a key regulator of this gene in breast cancer. We also have confirmed that hXBP-1 binds to ER.

To determine expression of hXBP-1 in breast tumors, we used immunohistochemical analysis of breast tumor tissue microarrays. We found moderate or strong hXBP-1 in 79% of breast tumors but could not confirm its coexpression with ER as reported in gene microarray studies. However, we did detect a significant coexpression of hXBP-1 with NFκB p65 ($p=0.018$; Spearman's rank correlation analysis). This observation is consistent with evidence from other cell systems implicating NFκB in the transcriptional regulation of hXBP-1 expression. These data provide evidence of an important role for hXBP-1 in breast cancer.