Interferon Regulatory Factor-1 Mediates the Proapoptotic but Not Cell Cycle Arrest Effects of the Steroidal Antiestrogen ICI 182,780 (Faslodex, Fulvestrant)

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ABSTRACT

Antiestrogens induce both cytotasis (cell cycle arrest) and apoptosis, but the relationship between these end points and the signaling that regulates their induction are unclear. We have previously implicated the transcription factor and putative tumor suppressor IFN regulatory factor-1 (IRF-1) in acquired antiestrogen resistance (Gu et al., Cancer Res., 62: 3428–3437, 2002). We now show the functional significance of IRF-1 in affecting antiestrogen responsiveness in estrogen receptor-positive antiestrogen-sensitive models (MCF-7, T47D, and ZR-75-1), a model of acquired antiestrogen resistance (MCF7/LCC9; estrogen receptor positive), and a model of de novo antiestrogen resistance (MDA-MB-231; estrogen receptor negative). Basal IRF-1 mRNA expression is lower in MCF7/LCC9 cells when compared with MCF-7, T47D, and ZR-75-1 cells. IRF-1 transcriptional activity in MCF-7/LCC9 cells is 18-fold lower that seen in the parental cells (MCF-7/LCC1) and is comparable with that in MDA-MB-231 cells. Although IRF-1 mRNA expression is induced by ICI 182,780 in sensitive cells, this regulation is lost in MCF-7/LCC9 and is absent in MDA-MB-231 cells. Loss of IRF-1 regulation appears specific to antiestrogen resistance—resistant cells induce IRF-1 mRNA in response to the cytotoxic drug doxorubicin. A dominant-negative IRF-1 eliminates the ICI 182,780-induced apoptotic response (reduced >4-fold) and reduces MCF-7 and T47D cell sensitivity to the antiproliferative effects of ICI 182,780. This effect is not mediated by changes in cell cycle distribution; rather, dominant-negative IRF-1 reduces ICI 182,780-induced apoptosis. These data identify a novel mechanism of antiestrogen resistance and implicate IRF-1 as a key component in signaling some ER-mediated effects on apoptosis/cell survival.

INTRODUCTION

For many women, antiestrogen therapy is the least toxic and most effective means to manage their hormone-dependent breast cancer. The most widely studied antiestrogen has been tamoxifen (TAM), which can increase both disease-free and overall survival in breast cancer patients, reduce the incidence of estrogen receptor-positive (ER+) disease in high-risk women, and reduce the rate of bone loss from osteoporosis (1, 2). Although first line antiestrogen therapy remains the standard of care for these patients (3–5), approximately one-third of all ER breast tumors exhibit de novo antiestrogen resistance, and most initially responsive tumors eventually acquire resistance (6).

The steroidal antiestrogen ICI 182,780 (Faslodex; Fulvestrant) has successfully completed clinical trials and exhibits considerable potential for more widespread clinical use (7). Most currently available antiestrogens show little or no significant activity in TAM-resistant disease, which is often treated with a second-line aromatase inhibitor. However, ICI 182,780 is clearly active in patients who have received TAM treatment and eventually recurred (8). Furthermore, two Phase III clinical trials in TAM-resistant patients have shown ICI 182,780 to be at least as effective as the potent aromatase inhibitor anastrazole (9, 10). Unlike most other antiestrogens, ICI 182,780 is a pure ER antagonist (11) that can induce degradation of ER protein (12) and inhibit receptor dimerization (13). Furthermore, ICI 182,780 is devoid of the uterotropic activity associated with the ability of TAM to increase the risk of developing endometrial cancers (8, 14).

Antiestrogen and estrogen responsiveness are complex phenotypes, and both genomic and nongenomic activities are functionally implicated (6, 15). In sensitive cells, antiestrogens are clearly cytostatic, inducing a G1/G0 cell cycle arrest in vitro. Clinically, the ability of antiestrogens to induce significant reductions in tumor size and increases in overall survival (1, 2) and to inhibit the development of ER+ tumors in the chemopreventive setting (16, 17) strongly suggest that these drugs also may be cytotoxic. Evidence implicates an induction of apoptotic cell death as the major mechanism through which antiestrogens might induce a cytotoxic effect (6). However, the relationship between growth arrest and apoptosis and how antiestrogens functionally affect cell signaling to regulate these two end points remains to be firmly established.

It is becoming apparent that antiestrogen resistance in ER+ tumors is unlikely to be driven by a single gene/signaling pathway. Thus, we have invoked a gene network hypothesis that confers diversity in estrogen/antiestrogen-initiated signaling (15, 18, 19). Ultimately, we envision multiple concurrent signals through this network of integrated and potentially interdependent pathways, some antiapoptotic and some proapoptotic, with cellular response reflecting the dominant signals. In antiestrogen-unresponsive cells, we hypothesize that the endocrine regulation and/or function of key components of this network is changed and that proapoptotic signals are no longer induced and antiapoptotic signals have become dominant. To begin identifying key genes that may make up such a network, we have applied both serial analysis of gene expression and gene expression microarray analyses to a series of antiestrogen-sensitive and –resistant cells. Among the key genes identified is the putative tumor suppressor gene IFN regulatory factor-1 (IRF-1; Ref. 19).

Although initially identified as an IFN-responsive gene, IRF-1 has shown activity as a tumor suppressor in several studies (20–22). For example, IRF-1 is deleted in some cancers (23, 24), and loss of IRF-1 significantly increases tumorigenicity in mouse models driven either by ras or loss of p53 (25). IRF-1 can signal to apoptosis in a p53-dependent or -independent manner (26, 27); with or without induction of p21waf1/cip1 (26) or p27kip1 (28); and through caspase-1 (27), caspase-7 (29), caspase-8 (30), and/or Fas ligand (31). Loss of p53 activity is common in breast cancer (32). Nonetheless, many breast cancers are initially responsive to cytotoxic drugs and hormones (1, 33), implying that drug-induced apoptosis likely occurs through both p53-dependent and -independent mechanisms. TAM-
induced growth arrest can occur independently of p53 (34), but the precise signaling responsible for these effects requires additional study. The primary mechanisms of cell growth arrest and apoptosis for ICI 182,780, and the importance of signaling through p53 are unknown.

In addition to our previous study implicating IRF-1 in affecting antiestrogen responsiveness in MCF-7 cells (19), Harroch et al. (35) observed that interleukin 6 inhibited proliferation and induced IRF-1 mRNA and IRF-1 binding to its target DNA sequence in T47D cells. In an immunohistochemistry study of IRF-1 expression in breast cancer, the authors report less IRF-1 expression in neoplastic compared with normal human breast, consistent with reduced expression of a putative tumor suppressor gene. However, IRF-1 expression was not assessed in association with established prognostic markers or clinical outcome (36).

In this study, we used the ER− MDA-MB-231 cells as a model of de novo antiestrogen resistance (6). To model de novo antiestrogen sensitivity, we used the estrogen-dependent, ER+ MCF-7 (37) and T47D cells (38) and the ER+, antiestrogen-sensitive but estrogen-independent MCF-7 variant MCF7/LCC1 (39). As a model of acquired ICI 182,780 resistance, we studied the MCF7/LCC9 cells, which were derived from MCF7/LCC1 cells and are ER+, estrogen independent, and ICI 182,780 and TAM cross-resistant (40). These studies strongly implicate signaling through IRF-1 and its protein partners as a critical mediator of antiestrogen signaling and as a key gene in a broader gene network (15, 19, 41). Hence, we now show that IRF-1 mRNA expression is induced by ICI 182,780 and repressed by estrogens in antiestrogen-sensitive cells. Hormonal regulation of IRF-1 is absent in ER− cells and is specifically lost in ER+ cells with acquired antiestrogen resistance. Both MCF-7 and T47D breast cancer cells expressing a dominant-negative IRF-1 (dnIRF-1) exhibit a decrease in sensitivity to ICI 182,780. The data separate the proapoptotic activity of ICI 182,780 from its ability to induce cell cycle arrest and are consistent with IRF-1 playing a critical role in those proapoptotic activities of antiestrogens most likely to contribute to their ability to increase overall survival and to reduce the risk of developing ER+ breast cancer (1, 16, 42).

MATERIALS AND METHODS

Cell Culture and Reagents. MCF-7, T47D, ZR-75-1 (ER+, estrogen dependent, antiestrogen sensitive), and MDA-MB-231 (ER−, estrogen independent, antiestrogen unresponsive) cells were routinely grown in improved minimal essential medium (IMEM; Biofluids, Rockville, MD) with phenol red and supplemented with 5% fetal bovine serum. MCF-7 cells were originally cultured in normal growth media for 24 h. To examine the induction of IRF-1 in response to ICI 182,780 or estradiol, cells were seeded in 10 4 cells/well 1 day before treatment with either 1 nM 17β-estradiol or 100 nM ICI 182,780 for 3 days. Cells were lysed in modified radioimmune precipitation assay buffer [150 mM NaCl, 50 mM Tris, 1% Igepal CA-630, and 0.5% deoxycholate (pH 7.5)] supplemented with Complete Mini protease inhibitor mixture tablets (Roche, Mannheim, Germany). Lysates were clarified by centrifugation, and equal volumes were added to 2× Laemmli sample buffer before boiling and loading onto precast 12% acrylamide gels. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (445S; Molecular Dynamics, Sunnyvale, CA).

Cell Lysis and Immunoblotting. For the determination of IRF-1 protein expression, cells were seeded into 6-well dishes at 2×10^5 cells/well and cultured in normal growth media for 24 h. To examine the induction of IRF-1 in response to ICI 182,780 or estradiol, cells were seeded in 10^4 cells/well 1 day before treatment with either 1 nM 17β-estradiol or 100 nM ICI 182,780 for 3 days. Cells were lysed in modified radioimmune precipitation assay buffer [150 mM NaCl, 50 mM Tris, 1% Igepal CA-630, and 0.5% deoxycholate (pH 7.5)] containing 5% nonfat dry milk overnight at 4°C. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature followed by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) and exposure to film (X-OMAT Blue XB-1; Kodak, Rochester, NY). To confirm equal loading, membranes were reprobed as described above using a β-actin monoclonal antibody (1:5000; Sigma, St. Louis, MO).

Generation of dnIRF-1. Although small interfering RNA (siRNA) can be a powerful method to inhibit RNAs, we did not use this method to block IRF-1 because of the recent reports of a marked induction of an IFN response when these molecules are introduced into cells (46, 47). Thus, the best remaining approach was the use of a stably expressed dominant-negative strategy. A wild-type IRF-1 cDNA (kindly provided by Dr. Taniguchi, University of Tokyo, Japan) was subcloned into the Xhol site of the pGEMTZ expression vector (Promega, Madison, WI) and linearized with BstEI. The dnIRF-1 comprises the full-length wild-type IRF-1 DNA with a deletion of bp 647–1173 and contains both the 3′ and 5′ untranslated regions, the DNA-binding domain, and the nuclear localization sequences of IRF-1. We constructed dnIRF-1 by PCR amplification using bp 630–647 (TAGCCAGGGCCCCCTTG) as the forward primers and bp 1173–1187 (ATCAGAGAAGGTATCAGG) as the reverse primers. PCR conditions were 30 cycles of 95°C for 30 s, 55°C for 45 s, and 72°C for 5 min. Integrity of the dnIRF-1 sequence was confirmed by standard dideoxy-mediated chain-termination sequencing. dnIRF-1 was subcloned into both the Xhol site of the pCDNA3 mammalian expression vector (Invitrogen, Carlsbad, CA) and into the Xhol site of the spEGFP-tet vector [a plasmid expressing enhanced green fluorescent protein (EGFP)] under the control of a bidirectional tetracycline-responsive promoter (Clontech, Palo Alto).
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Alto, CA). The IRF-1 riboprobe (above) identifies dnIRF-1, generating a 115-bp protected dnIRF-1 fragment.

**Transient Transfections and Luciferase Reporter Assay.** Cells were transfected using the FuGENE 6 method (Roche Diagnostics, Indianapolis, IN). For reporter assays, 8 × 10^4 cells/well were plated in 12-well plates and allowed to grow for 24 h before transfection. Cells were cotransfected with the pISRE-luc plasmid (contains five copies of the IFN-stimulated response element; ISRE) as provided in the PathDetect kit (Promega) and with either a standard control comprising a pcDNA3 plasmid without the ISRE or a pcDNA3 plasmid containing the cDNA from either IRF-1 or dnIRF-1.

To control for transfection efficiency, a pRL-SV40 plasmid (Promega) containing the Renilla luciferase gene under the control of a constitutive SV40 promoter also was cotransfected into cells. One µg of plasmid DNA was added to serum-free media containing the FuGENE 6 reagent and allowed to incubate for 30 min at room temperature. Where appropriate, cells were maintained in growth media with or without 500 µM /ml IFN-γ for 24–48 h. Subsequently, cells were lysed, and activation of the ISRE-luciferase construct was measured using the Dual Luciferase Assay kit (Promega) according to the manufacturer’s instructions. Luminescence was quantified using a Lumat LB 9501 luminometer (EG&G Berthold, Bundoora, Victoria, Australia).

**Stable Transfection with dnIRF-1**. For stable transfections, cells were plated in T-75 cm² plastic tissue culture flasks at a density of 0.5 × 10⁶ cells/flask and grown for 24 h before transfection. A total of 8 µg of plasmid DNA were transfected into MCF-7 cells stably transfected with the tetR protein (MCF-7/VP16; generously provided by Dr. Susan Conrad, Michigan State University, East Lansing, MI) or T47D cells using the FuGENE6 method (above). Cells were transfected with either an empty pBI-EGFP-tet plasmid containing the EGFP selectable marker (Clontech) or one containing the dnIRF-1 cDNA and the pBabe plasmid (kindly provided by Dr. Matthew Ellis, Washington University, St. Louis, MO) encoding for puromycin resistance. Stably transfected cells were selected for growth in the presence of 1 µg/ml puromycin. Puromycin-resistant colonies expressing EGFP as measured by standard fluorescence activated cell sorting (FACS) were expanded and screened for expression of the dnIRF-1 by RNAse protection. All of the transfectants used in these experiments were from pooled populations. Cells transfected with the empty control vector were designated MCF7/ctrl and T47D/ctrl and those transfected with the dnIRF-1 were designated MCF7/ dnIRF-1 and T47D/dnIRF-1.

**Cell Proliferation.** MCF-7/ctrl, T47D/ctrl, MCF7/dnIRF-1, and T47D/ dnIRF-1 cells were sorted aseptically by FACS in the Lombardi Comprehensive Cancer Center Flow Cytometry Shared Resource for EGFP expression and dnIRF-1 cells were sorted aseptically by FACS in the Lombardi Comprehensive Cancer Center Flow Cytometry Shared Resource for EGFP expression and dnIRF-1 and T47D/dnIRF-1.

**Transfected MCF-7 cells (1 × 10⁵) were seeded in T-75 cm² plastic tissue culture plates at a density of 1.5 × 10⁶ MCF-7 or 2 × 10⁵ T47D cells/well. Twenty-four h post plating, cells were treated with 100 nM or 1 µM ICI 182,780 or vehicle control for 72 h. Cells were then trypsinized, resuspended in PBS, and counted in a Beckman Coulter counter (Beckman Coulter Corp., Fullerton, CA) to assess cellular proliferation.

**Cell Cycle Analyses.** Cells stably transfected with the dnIRF-1 or empty control plasmids were plated in T-75 cm² plastic tissue culture flasks at a concentration of 0.5 × 10⁶ and allowed to grow for 3 days. Cells were then analyzed for alterations in cell cycle via FACS. FACS analysis was conducted by the Lombardi Comprehensive Cancer Center Flow Cytometry Shared Resource, according to the method of Vindelov et al. (48).

**Apoptosis.** Staining for annexin V, an optimal assay for detecting apoptosis in MCF-7 cells (49, 50), was performed according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN). Control- or dnIRF-1-transfected MCF-7 cells (1 × 10⁶) were seeded in T-75 cm² plastic tissue culture dishes and allowed to grow for 24 h. Cells were then treated with ICI 182,780 or vehicle for 72 h, trypsinized, and pelleted by centrifugation. Cell pellets were rinsed twice in ice-cold PBS and stained with 5 µg/ml 7-aminoactino- mycin D for 15 min at room temperature. After staining with 7-aminoactinomycin D, cells were washed and resuspended in annexin V binding buffer [10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/NaOH, 140 mM NaCl, and 2.5 mM CaCl₂ (pH 7.4)]. Cells (1 × 10⁶) were then stained with 0.3 µg of phycoerythrin-conjugated annexin V in the dark, and flow cytometric analysis was performed using a FACStar™ flow cytometer (Becton-Dickinson, Mountain View, CA) to determine the proportion of apoptotic cells in each sample. Apoptosis was measured only in cells expressing the dnIRF-1 transgene or empty vector (as assessed by concurrent EGFP expression).

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**RESULTS**

**IRF-1 Is Differentially Expressed in Antiestrogen-Sensitive and -Resistant Breast Cancer Cells.** We measured basal expression of IRF-1 mRNA by RNase protection analyses in two models of antiestrogen resistance: MCF-7/LCC9 cells (acquired resistance, ER+, ICI 182,780 and TAM cross-resistant) and MDA-MB-231 (de novo resistance, ER−, ICI 182,780 and TAM cross-resistant; Ref. 6). MCF-7/LCC9 cells exhibit a 4.2-fold (P < 0.05) and 2.6-fold (P ≤ 0.01) lower expression of IRF-1 mRNA than the antiestrogen-sensitive MCF-7 and MCF-7/LCC1 cells, respectively (Fig. 1, A and B). IRF-1 mRNA expression in MCF-7/LCC9 cells is not significantly different from that in ER− MDA-MB-231 cells.

The half-life of the IRF-1 protein is less than 30 min, and changes in mRNA levels appear closely associated both with changes in IRF-1 protein expression and IRF-1 transcriptional activity as measured using an ISRE-based promoter-reporter assay (52). To confirm this association, we performed immunoblotting for the cells lines shown in Fig. 1, A and B. The data in Fig. 1C show that, as expected, the mRNA and protein levels are comparable. The MCF-7/LCC1 and MCF-7/ LCC9 cells compared here are derived from the same parental MCF-7 cell line and have similar transfection efficiencies as assessed by transfection with β-galactosidase (not shown). Activity of the cotransfected Renilla construct (constitutively active) was used to correct for any minor differences in transfection efficiency. MCF-7/LCC9 cells exhibit 18-fold lower basal IRSE activity than their immediate parental cells MCF-7/LCC1 (P ≤ 0.01; Fig. 1D). These data reflect the differential IRF-1 mRNA expression that we have previously detected in gene expression microarrays (19) and now confirm by RNase protection analyses (Fig. 1, A and B) and immunoblot (Fig. 1C). These data also confirm that IRF-1 mRNA expression, protein expression, and transcriptional activation are closely related in breast cancer cells. Furthermore, these data show that IRF-1 expression and activity are significantly lower in ER+ and ER− antiestrogen-resistant cells compared with antiestrogen-sensitive cells.

**IRF-1 mRNA Expression Is Regulated through ER in Breast Cancer Cells.** Data on basal expression in the sensitive and resistant cells imply that estrogens and antiestrogens may affect IRF-1 mRNA expression. Fig. 2, A and B, shows the ability of 100 nM ICI 182,780, a clinically relevant concentration (53), to induce IRF-1 mRNA in the three best characterized and most widely used ER+ human breast cancer cell lines (6): MCF-7 (P < 0.001); T47D (P < 0.001); and ZR-75-1 (P < 0.05). This effect appears to be ER mediated, because IRF-1 mRNA induction by ICI 182,780 is blocked in the presence of estradiol in MCF-7 cells (Fig. 2, C and D) and ICI 182,780 is unable to induce IRF-1 in the ER− MDA-MB-231 cells (Fig. 2, A and B). The dose-response relationships for the endocrine regulation of IRF-1 mRNA in MCF-7 and T47D cells were determined. Cells were plated and 24 h later treated with various doses of ICI 182,780 or 0.1% (v/v) ethanol vehicle for 72–96 h before RNA isolation. ICI 182,780 induces IRF-1 mRNA in a dose-dependent manner, with a maximal 2.5-fold induction at a dose of 100 nM in both MCF-7 (Fig. 3, A and B; P ≤ 0.001) and T47D (Fig. 3, C and D; P ≤ 0.01) cells. In contrast, IRF-1 mRNA expression is significantly down-regulated in response to treatment with 1 nM estradiol (Fig. 3, E and F; P ≤ 0.001).

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**Statistical Methods.** Student’s t test was used to compare two groups in which the data are normally distributed; a Wilcoxon t test was used to compare groups in which data are not normally distributed. For multiple comparisons, ANOVA was used with a post hoc test for multiple comparisons. Where several experimental groups were compared with the same control, we used Dunnett’s test (51).
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ER-Mediated Regulation of IRF-1 Is Specifically Lost in Anti-estrogen-Resistant Cells. Although the data in Fig. 2 and Fig. 3 show the regulation of IRF-1 mRNA expression, this would likely be of limited functional relevance if similar patterns of regulation occur in antiestrogen-resistant cells. However, the ability of both estrogen and ICI 182,780 to regulate IRF-1 mRNA expression is lost in the MCF-7/LCC9 cells (Fig. 4, A–D), and ICI 182,780 is unable to regulate IRF-1 expression in the antiestrogen-resistant ER− cell line, MDA-MB-231 (Fig. 2, A and B). In contrast, the estrogen-independent but antiestrogen-sensitive MCF7/LCC1 cells retain the ability of estrogen to inhibit IRF-1 mRNA expression (Fig. 4, A and B). Consistent with the data in Fig. 1, we found similar changes in the hormonal regulation of IRF-1 protein in immunoblots (not shown). MCF7/LCC1 cells do not require estrogens to grow in vitro or in vivo (estrogen independent) but retain some estrogen responsiveness (40, 43). Thus, the apparent loss of ER-mediated regulation of IRF-1 is associated with acquired antiestrogen resistance but not estrogen independence.

We then asked whether the specificity of endocrine regulation of IRF-1 that is lost in antiestrogen-resistant cells also extended to nonhormonal inducers of IRF-1. In mouse embryonic fibroblasts, IRF-1 is induced by treatment with the cytotoxic drug doxorubicin (26), one of the most active single cytotoxic drugs used in the treatment of breast cancer (54, 55), MCF-7 cells induce IRF-1 mRNA in response to treatment with doxorubicin (Fig. 5, A and B). Importantly, MCF-7/LCC9 cells retain their ability to regulate IRF-1 expression in response to 1 μM doxorubicin (increase by over 7-fold; \( P \leq 0.001 \)), a response also shared by MDA-MB-231 cells (increase by over 4-fold; \( P < 0.05 \); Fig. 6, A and B). Thus, antiestrogen resistance is associated with a specific, ER-mediated change in the regulation of IRF-1 expression, rather than a global loss of IRF-1 mRNA regulation. This specificity allows cells to retain the ability to induce IRF-1 and undergo an IRF-1-regulated apoptotic cell death in response to other cytotoxic agents.

ICI 182,780-Induced Inhibition of Cell Proliferation Is Reduced by dnIRF-1. To study the functional relevance of changes in IRF-1 activity in affecting antiestrogen responsiveness, we created dnIRF-1, a mutant form of IRF-1 that lacks the DNA binding domain but retains the protein binding and transcriptional activation domains. Dominant-negative activity of dnIRF-1 was associated with a specific, ER-mediated change in the cellular response to treatment with ICI 182,780. Complete loss of IRF-1 expression was associated with acquired antiestrogen resistance but not estrogen independence.

We then asked whether the specificity of endocrine regulation of IRF-1 that is lost in antiestrogen-resistant cells also extended to nonhormonal inducers of IRF-1. In mouse embryonic fibroblasts, IRF-1 is induced by treatment with the cytotoxic drug doxorubicin (26), one of the most active single cytotoxic drugs used in the treatment of breast cancer (54, 55), MCF-7 cells induce IRF-1 mRNA in response to treatment with doxorubicin (Fig. 5, A and B). Importantly, MCF-7/LCC9 cells retain their ability to regulate IRF-1 expression in response to 1 μM doxorubicin (increase by over 7-fold; \( P \leq 0.001 \)), a response also shared by MDA-MB-231 cells (increase by over 4-fold; \( P < 0.05 \); Fig. 6, A and B). Thus, antiestrogen resistance is associated with a specific, ER-mediated change in the regulation of IRF-1 expression, rather than a global loss of IRF-1 mRNA regulation. This specificity allows cells to retain the ability to induce IRF-1 and undergo an IRF-1-regulated apoptotic cell death in response to other cytotoxic agents.

Fig. 1. Basal IRF-1 mRNA and protein expression and transcriptional activation in breast cancer cell lines. A, representative RNase protection assay. \( \beta \)-actin, loading control. B, IRF-1 mRNA expression measured by RNase protection and presented as mean ± SE of three determinations, in which intensity is expressed as a ratio of IRF-1:36B4 (ANOVA, \( P = 0.009 \); \( \Delta \), \( P < 0.05 \) MCF-7 versus MCF7/LCC9; \( \square \), \( P < 0.01 \) MCF-7/LCC1 versus MCF7/LCC9; \( \diamond \), \( P = 0.528 \) MDA-MB-231 versus MCF7/LCC9). C, representative immunoblot of IRF-1 protein. \( \beta \)-actin, loading control. D, basal transcriptional activity of IRF-1 in breast cancer cell lines (ISRE-luc promoter-reporter assay). Data represent mean ± SE of four determinations and are presented as relative light units. ANOVA, \( P < 0.01 \); \( \Delta \), \( P = 0.001 \) for MCF-7 versus MCF7/LCC1, MCF7/LCC9, and MDA-MB-231. \( \ast \), \( P = NS \) for MCF7/LCC9 versus MDA-MB-231.

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dnIRF-1 Does Not Affect ICI 182,780-Induced Changes in Cell Cycle Distribution. Although antiestrogens can affect both cell cycle distribution and the rate of apoptosis, cell proliferation assays measure the sum of these activities. Hence, we asked directly whether the effects of dnIRF-1 on proliferation reflected an inhibition of the ability of ICI 182,780 to arrest cells in G0-G1. The data in Fig. 9 show that dnIRF-1 does not affect the ICI 182,780-induced cell cycle arrest in G0-G1 in either MCF-7/dnIRF-1 (Fig. 9A) or T47D/dnIRF-1 cells (Fig. 9B). Thus, the residual antiproliferative effects of ICI 182,780 (Fig. 8) in dnIRF-1-expressing cells are those conferred by cell cycle arrest. These data strongly implicate changes in apoptosis as being the primary mechanism through which dnIRF-1 reduces the antiproliferative effects of ICI 182,780 in MCF-7 and T47D cells.

ICI 182,780-Induced Apoptosis Is Reduced by dnIRF-1. To determine whether the effects of dnIRF-1 on cellular sensitivity to ICI 182,780 are mediated by its ability to influence signaling to apoptosis, the ability of dnIRF-1 to affect ICI 182,780-induced apoptosis was assessed directly by measuring annexin V staining (49, 50). Apoptosis was measured only in those cells expressing the dnIRF-1 transgene or empty vector control (as assessed by EGFP expression), to ensure that any effects were likely to be a direct result of the inhibition of IRF-1. When treated with 100 nM ICI 182,780, 30% of MCF-7 control transfectants undergo apoptosis. Expression of dnIRF-1 significantly reduces this ICI 182,780-induced apoptotic response by >4-fold in MCF-7/dnIRF-1 cells (Fig. 10; P < 0.0034). The basal rate of apoptosis, measured in control MCF-7 cells treated with ethanol, is about 5% (Fig. 10). Thus, the full apoptotic response to ICI 182,780 is blocked by dnIRF-1. Studies were also performed in T47D and T47D/dnIRF-1 cells, which unlike MCF-7 cells contain a mutant and nonfunctional p53. T47D/dnIRF-1 cells show a similar 4-fold reduction in the ability of ICI 182,780 to induce apoptosis (not shown). These data with dnIRF-1 in both MCF-7 (wild-type p53) and T47D (mutant p53) strongly suggesting that IRF-1 is a critical mediator of this signal and that its activities are independent of p53.

DISCUSSION

Data from our previous studies demonstrate an association between IRF-1 expression and acquired cross-resistance to antiestrogens (19). We now show that IRF-1 is a key signaling protein involved in mediating the sensitivity of breast cancer cells to ICI 182,780-induced apoptosis. Basal IRF-1 mRNA expression is down-regulated in antiestrogen-resistant cells (both the ER+/MCF7/LCC9 model of acquired resistance and the ER−/MDA-MB-231 model of de novo resistance). Moreover, the ability of antiestrogens to regulate IRF-1 mRNA transcription is absent in the ER− and lost in the ER−/MDA-MB-231 model of de novo resistance). Nonetheless, dnIRF-1 does not eliminate basal IRF-1 activity in these cells. Loss of IRF-1 activity could induce confounding compensatory effects unlikely to occur in breast tumors, which appear to retain detectable IRF-1 protein expression (36). Thus, the data reported herein likely reflect the contribution of only the antiestrogen induced IRF-1.

Specificity of these effects, in the context of the signaling of IRF-1...
in response to ER-mediated events, also is apparent. Estradiol blocks antiestrogen-induced IRF-1 in MCF-7 cells, and no endocrine regulation is seen in ER- cells. Neither the loss of endocrine regulation in MCF7/LCC9 cells nor the absence of its endocrine regulation in MDA-MB-231 cells compromises the ability of doxorubicin to induce IRF-1 in these cells and to inhibit their proliferation (not shown). This latter effect is consistent with patterns of clinical responses to these drugs, because breast cancer patients who are resistant to antiestrogens can respond to cytotoxic chemotherapy.

The ability of dnIRF-1 to block ICI 182,780-induced inhibition of
cell proliferation in MCF-7/dnIRF-1 and T47D/dnIRF-1 cells could reflect changes in the effects of ICI 182,780 on cell cycle and/or apoptosis. However, dnIRF-1 does not affect ICI 182,780-induced cell cycle arrest when expressed in either MCF-7 or T47D cells. In marked contrast, dnIRF-1 effectively eliminates ICI 182,780-induced apoptosis in both MCF-7/dnIRF-1 and T47D/dnIRF-1 cells. Thus, we can separate cell cycle arrest from apoptosis and attribute a significant component of antiestrogen-induced apoptotic signaling to IRF-1. Because dnIRF-1 abrogates ICI 182,780-induced apoptosis (Fig. 10) while enhancing cell growth by approximately 50% (Fig. 8), apoptosis and cell cycle arrest likely contribute equally to the apparent antiproliferative effects of ICI 182,780.

Functionally separating antiestrogen-induced apoptosis from antiestrogen-induced growth arrest has several important implications. Novel therapeutic approaches designed to increase the proapoptotic effects of antiestrogens may be an effective means to improve their ability to increase overall survival in patients because this should increase the proportion of cells undergoing apoptotic cell death. Modalities that increase only the ability of antiestrogens to induce a cell cycle arrest will likely be a less effective strategy. For example, many arrested cells will survive and thereby have more opportunities to adapt, acquire resistance, and generate subsequent disease recurrence. Measuring basal IRF-1 expression and/or the ability of an antiestrogen to induce IRF-1 in the neoadjuvant setting may improve the prediction of endocrine responsiveness. Currently, we incorrectly predict antiestrogen sensitivity in 66% of ER\textsuperscript{–}/H11001/ progesterone receptor-negative, 55% of ER\textsuperscript{–}/H11002/ progesterone receptor-positive, and 25% of ER\textsuperscript{–}/H11001/ progesterone receptor-positive tumors (6).

The ability of IRF-1 to induce growth arrest is associated with the induction of various genes including p53-dependent and -independent events (26, 27) and interactions that may include p21\textsuperscript{waf1/cip1} (26).
Interactions requiring both p21waf1/cip1 and p53 may not be central components in antiestrogen signaling through IRF-1. Nonetheless, preliminary data suggest an increase in p21waf1/cip1 mRNA expression after ICI 182,780 treatment in MCF-7 and T47D cells (2.91 ± 0.89-fold), consistent with both a previous report on p21waf1/cip1 regulation by ICI 182,780 (56) and activation of IRF-1. MCF-7 cells express wild-type p53, whereas T47D cells express a mutant and nonfunctional p53 (57), but both cell lines are responsive to antiestrogen-induced apoptosis in a manner that remains sensitive to the effects of dnIRF-1. However, a role for p53/p21waf1/cip1 signaling in the cell cycle effects of antiestrogens cannot be excluded.

The ability of ICI 182,780 to induce apoptosis through IRF-1 activity is likely mediated through changes in caspase activation. IRF-1 can induce several caspases (27, 29, 30), and inhibition of caspase activity blocks antiestrogen-induced apoptosis (58). A specific requirement for caspase-3 seems unlikely because this caspase is not expressed in MCF-7 cells (59). IRF-1 signaling through caspase-1 (27), caspase-7 (29), and caspase-8 (30) is strongly implicated. For example, IRF-1 induces caspase-1 (60), which can regulate apoptosis in normal mammary epithelial cells (61). Overexpression of caspase-1 is lethal in MCF-7 cells (62). Caspase-7 is expressed in MCF-7 cells and may substitute for the loss of caspase-3 in these cells (63). IFN-γ, which induces IRF-1 activity in MCF-7 cells (Fig. 7), is reported to sensitize both MCF-7 and MDA-MB-231 cells to apoptosis through inducing caspase-8 (64). TAM can induce caspase-8 (58), and consistent with our observations in T47D cells, caspase-8-induced apoptosis occurs independent of p53 (64). Studies to determine which caspases are functionally involved in IRF-1 signaling in breast cancer are currently in progress.

Although data in this study show reduced IRF-1 expression and loss of its endocrine regulation in antiestrogen-resistant cells, the level of IRF-1 activity in cells is also affected by protein-protein interactions with the nucleolar phosphoprotein nucleophosmin (NPM; Ref. 65). Not previously reported in breast cancer cells, we now have preliminary data to suggest that this functional interaction also occurs in T47D cells (not shown). NPM is an estrogen-induced protein in...
MCF-7 cells that is down-regulated by antiestrogens (66), and its expression is increased in MCF-7/LCC9 when compared with MCF-7/LCC1 cells (19). Thus, in addition to down-regulating IRF-1 mRNA expression, antiestrogen-resistant cells have up-regulated expression of an endogenous inhibitor (NPM). Interestingly, we have previously shown that NPM autoantibody levels are lower in TAM-treated patients, suggesting that NPM/IRF-1 interactions also may be clinically relevant (67).

It seems likely that an acquired antiestrogen resistance phenotype is conferred not by the alteration of a single gene or signal transduction pathway but rather through the perturbation of a signaling network of integrated signaling pathways (15). The data presented here are consistent with cell signaling through IRF-1 being a key component or node in such a signaling network. Activity as a signaling node is implied by (a) the potential for diversity/redundancy of signaling to a key end point (apoptosis) downstream of IRF-1 (cooperation with p53, p21waf1/cip1, and regulation of several caspases); (b) the redundancy apparent in regulating IRF-1 activity (down-regulation of basal transcription, loss of ER-mediated transcription, and concurrent up-regulation of the endogenous inhibitor NPM); (c) the apparent specificity for antiestrogen resistance (cytotoxic drugs can induce IRF-1 in antiestrogen-resistant cells); and (d) the altered regulation of IRF-1 activity/expression in models of both de novo and acquired antiestrogen resistance. This node may be important in affecting other signals in breast cancer cells. For example, IRF-1 is downstream of tumor necrosis factor signaling, and both tumor necrosis factor α and its receptor tumor necrosis factor R1 are down-regulated in MCF7/LCC9 cells, implying a cross-resistance to tumor necrosis factor-mediated events (15, 19).

The ability of doxorubicin to induce IRF-1 in antiestrogen-resistant cells and to inhibit proliferation in these cells is a clinically relevant phenotype. We and others (36, 68) have detected IRF-1 expression by immunohistochemistry in breast cancer specimens, this pattern of expression being consistent with a potential tumor suppressor role for IRF-1 (21, 36). Our preliminary data suggest that the pattern of IRF-1 expression in breast cancers, as measured by immunohistochemistry, is consistent with other components of our network. For example, we detect an inverse pattern of expression between IRF-1 and nuclear factor κB as seen in MCF7/LCC1 versus MCF7/LCC9 cells (19, 68). These observations may ultimately lead to a better ability to identify patients that will respond to antiestrogens and to predict which patients will ultimately develop antiestrogen resistance. Interfering with the putative “IRF-1 node” may allow for the development of novel therapeutic strategies in endocrine-resistant breast cancers.

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REFERENCES

IRF-1 AND ANTIESTROGEN RESPONSIVENESS


41. Pratt MAC, Bishop TE, White D, et al. Estrogen withdrawal-induced NF-kB activity and bcl-3 expression in breast cancer cells: roles in growth and hormone independ-
Interferon Regulatory Factor-1 Mediates the Proapoptotic but Not Cell Cycle Arrest Effects of the Steroidal Antiestrogen ICI 182,780 (Faslodex, Fulvestrant)


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