Resistance to different antiestrogens is caused by different multi-factorial changes and is associated with reduced expression of IGF receptor Iα

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Abstract

Development of antiestrogen resistance is a major clinical problem, and therefore it is crucial to elucidate the mechanisms involved. To investigate whether gain-of-function or loss-of-function mechanisms was most likely to be involved, cell fusion between the antiestrogen-sensitive MCF-7 and the ICI 164384- and ICI 182780-resistant MCF-7/164R-5 cell lines was performed. Furthermore, a fusion cell line between the tamoxifen-resistant MCF-7/TAMR-1 and the MCF-7/164R-5 cell line was established. A thorough investigation of growth parameters and expression of selected proteins (estrogen receptor-α (ERα), progesterone receptor (PR), Bcl-2, IGF-binding protein-2 (IGFBP2) and IGF receptor Iα (IGF-IRα)) in the fusion partners and fusion cells revealed that both gain- and loss-of-function changes occurred, and that the mechanisms resulting in resistance to the two antiestrogens were different. This multi-factoriality of antiestrogen resistance is promising in relation to sequential treatment of breast cancer patients with different types of endocrine therapy. Furthermore, we found an association between antiestrogen resistance and reduced IGF-IRα expression. Overall, the data presented in this report support the usefulness of cell fusion to clarify the mechanisms involved in development of resistance to the pure antiestrogens ICI 182780 and ICI 164384 and the selective ER modulator tamoxifen and suggest IGF-IRα as a new sensitive marker for response to antiestrogen treatment.

Endocrine-Related Cancer (2003) 10 579–590

Introduction

Antiestrogens are the primary agents for treatment of advanced breast cancer. However, patients generally develop resistance to the treatment over time. To fully understand the cause of antiestrogen resistance, much work has focused on factors known to be involved in cell growth and differentiation of the mammary gland (King 1993). Recently, techniques such as: differential display (Liang & Pardee 1992, Liang et al. 1995) and micro-array technology (Brown & Botstein 1999) have been used to compare antiestrogen-resistant and antiestrogen-sensitive cell lines (Hilsenbeck et al. 1999), as well as to predict the clinical outcome in breast cancer patients (Gruvberger et al. 2001, Bertucci et al. 2002, van’t Veer et al. 2002). Several mechanisms may be responsible for the development of antiestrogen resistance, among them: loss of estrogen receptor (ER), altered expression of co-factors, up-regulation of growth factor production and/or receptors (Katzenellenbogen et al. 1997), increased oncogenic kinase signaling, deregulated cell proliferation (Blume-Jensen & Hunter 2001) and suppressed cell death (Evan & Vousden 2001). Our research over the last decade has focused on: differential expression of estrogen-regulated gene products (Lykkesfeldt & Sørensen 1992, Larsen et al. 1997, Jensen et al. 1999), ERα splice variants (Madsen et al. 1995, 1997, Ohlsson et al. 1998), expression and regulation of the ERα (Larsen et al. 1997, Jensen et al. 1999), altered expression of growth factors (Larsen et al. 1999), and differential expression of genes in general (Jensen et al. 1999, Brockdorff et al. 2000). However, it still remains unclear which mechanisms are involved in progression of breast cancer from an antiestrogen-responsive to an antiestrogen-resistant type.

Previously, conflicting data on this issue have been presented. First, Zajchowski et al. (1997) presented evidence
that the tumor-forming ability of breast cancer cells, exemplified by MCF-7, could be suppressed by somatic cell fusion with normal immortalized human breast epithelial cells, indicating involvement of a recessive mechanism. In contrast to this, Safarians et al. (1996) found that human breast cancer progression was regulated by dominant transacting factors, by studying the effect of fusing MCF-7 with a highly metastatic cell line (C8161). Paik et al. (1994) reported that antiestrogen resistance (to LY 117018) in an ERα-positive cell line was a recessive trait when the resistant cells were fused with MCF-7, whereas, van Agthoven et al. (1998) reported that antiestrogen resistance was due to a dominant mechanism and could be induced in sensitive cells either by transfection with a breast cancer antiestrogen resistance gene-3 (BCAR3)-containing expression vector or by fusing sensitive cells with BCAR3-expressing cells. Clearly, the cell context as well as the method used for the different studies may have affected the results.

In order to clarify whether antiestrogen resistance in fact involves gain-of-function and/or loss-of-function mechanisms, we performed cell fusion and have taken advantage of having several antiestrogen-resistant cell lines derived from the same parental MCF-7 cell line via long-term treatment with high concentrations of tamoxifen, ICI 164384 or ICI 182780 (Lykkesfeldt & Briand 1986, Lykkesfeldt et al. 1995). In this study, antiestrogen sensitivity, response to long-term antiestrogen treatment, DNA index (DI) and cell cycle distribution were determined for both the fusion partners and the fusion cell lines described below. First, a stable fusion cell line was established by polyethylene glycol (PEG)-induced cell fusion of MCF-7/neo and the ICI 164384- and ICI 182780-resistant cell line MCF-7/164R-5/neo cell line. Based on our initial investigations, it seemed possible that antiestrogen resistance was a recessive trait in these cells (164:MCF). However, when fusing two different antiestrogen-resistant cell lines, MCF-7/TAMR-1/neo and MCF-7/164R-5/neo and MCF-7/TAMR-1/neo and MCF-7/164R-5/hygB, we found that the response to antiestrogen treatment represented an intermediate between the sensitivity of the two fusion partners. However, the doubling time of the 164:TAM fusion cell line only increased slightly upon treatment with ICI 164384 compared with untreated cultures whereas the doubling time of the 164:MCF cell line increased more dramatically. Looking at the expression of proteins known to be differentially expressed between antiestrogen-sensitive and antiestrogen-resistant cell lines, we found that resistance is neither an exclusively recessive nor a dominant characteristic in either fusion cell line. However, we identified severely reduced levels of insulin-like growth factor (IGF) receptor Iα (IGF-IRα) as a general marker for antiestrogen resistance. Overall, based on the present results and in accord with our previous findings of several transcripts being differentially expressed in antiestrogen-sensitive and -resistant cell lines (Jensen et al. 1999), we conclude that development of resistance to antiestrogens is multi-factorial and resistance to different types of antiestrogens occurs through distinct pathways. For investigation of the mechanisms involved in resistance to the selective ER modulator (SERM) tamoxifen and the pure antiestrogens ICI 164384 and ICI 182780, we found cell fusion to be a useful method.

Materials and methods

Cell lines and growth experiments

The MCF-7 cell line was obtained from the Breast Cancer Task Force Cell Culture Bank, Mason Research Institute (Worcester, MA, USA). It has been adapted to grow in standard growth medium consisting of DME/F12 medium supplemented with 1% heat-inactivated fetal calf serum (FCS) (Gibco BRL, Life Technologies), 6 ng/ml bovine insulin (Novo-Nordic, Copenhagen, Denmark), and 2.5 mM Glutamax (Life Technologies) (Briand & Lykkesfeldt 1984). The MCF-7/164R-5 cell line, which is resistant to the pure antiestrogens ICI 164384 and ICI 182780 (Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK), has been established in this laboratory by long-term treatment of MCF-7 cells with 10⁻⁷ M ICI 164384. It is maintained in DME/F12 medium supplemented with 1% FCS, 6 ng/ml insulin, 2.5 mM Glutamax, and 10⁻⁷ M ICI 164384 (Lykkesfeldt et al. 1995). The tamoxifen-resistant cell line has been produced by long-term treatment with 10⁻⁶ M tamoxifen (Lykkesfeldt & Briand 1986, Lykkesfeldt et al. 1994). Growth medium was changed every second or third day, and the cultures were subcultivated by trypsinization once a week (split ratio about 20). The fusion partners (MCF-7/neo, MCF-7/TAMR-1/neo and MCF-7/164R-5/hygB) and the fusion cell lines (164:MCF and 164:TAM), which are described below, were maintained in DME/F12 medium supplemented with 1% FCS, 6 ng/ml insulin and 2.5 mM Glutaxam along with 800 µg/ml G418 (Life Technologies) and/or 140 µg/ml hygromycin B (Calbiochem). For dose–response experiments, cells were seeded at a density of 2×10⁴ cells/well in 24-well multidishtes in this control medium (Nunc, Roskilde, Denmark). Medium containing concentrations of antiestrogen ranging from 10⁻¹⁰ M to 10⁻⁶ M (ICI 164384) or 10⁻⁷ M to 10⁻³ M (tamoxifen), was added after cells were allowed to plate for 2 days. Cell number was determined indirectly by crystal violet staining after 6 days in experimental medium. The cells were stained for 10 min with crystal violet (0.5% crystal violet and 25% methanol in distilled water) prior to thorough washing in water, drying, re-dissolution in citrate buffer (0.1 M sodium citrate and 50% ethanol in distilled water), and measurement of diluted samples at 570 nm. For determination of the population doubling time of the fusion cells, cells were seeded in 24-well dishes at a density of 2×10⁴ cells/well in control medium. After about 48 h, when the cells had plated, the medium was changed to experimental medium which was either control medium or medium
supplemented with 10⁻⁶ M tamoxifen or 10⁻⁷ M ICI 164384. Cell number was determined directly in quadruplicate samples by manual counting in a Bürker–Türk chamber every second or third day. Experimental medium was renewed in the remaining wells after each cell count. The doubling time was determined from the exponential part of the growth curve, between days 0 and 4 or 5.

**Fusion partners**

To enable establishment of the fusions, stable transfectants of the MCF-7, MCF-7/TAM⁰-1 and MCF-7/164⁴-5 cell lines with antibiotic markers were developed. Cells were seeded in six-well multidishes (Nunc) at a density of 2×10⁶ cells/well. The cells were seeded in the standard growth medium; MCF-7/164⁴-5 and MCF-7/TAM⁰-1 cells were supplemented with 10⁻⁷ M ICI 164384 or 10⁻⁸ M tamoxifen respectively. At a confluency of between 30 and 60%, transfection with either the pSV2neo (gift from Dr S Stacy, Danish Cancer Society) or pY3 vector (gift from Dr P Chambon, CNRS/INSERM/ULP, Strasbourg, France), was carried out using the FuGENE6 Transfection Reagent (Boehringer Mannheim/Roche) or pPc2c (gift from Dr P Chambon, CNRS/INSERM/ULP, Strasbourg, France), was carried out using the FuGENE6 Transfection Reagent (Boehringer Mannheim/Roche) or the Profection Mammalian Transfection System (Promega). Twenty-four hours after transfection, cells from one six-well dish were split to three T-25 flasks (Nunc), and selection with the relevant antibiotic was initiated. Colonies of stable transfectants usually emerged after 1 month of selection and the antibiotic-resistant cell lines were a pool of many colonies to avoid clonal differences. Resistance to the respective antibiotic was ensured by dose–response experiments prior to PEG-1500-induced cell fusion.

**Cell fusion of breast cancer cell lines**

The three cell lines described above were used for the fusion experiments. MCF-7/164⁴-5/hygB cells were fused with MCF-7/neom in one experiment, and with MCF-7/TAM⁰-1/neom in another, giving rise to the two fusion cell lines denoted 164:MCF (MCF-7/164⁴-5/hygB fused with MCF-7/neom) and 164:TAM (MCF-7/164⁴-5/hygB fused with MCF-7/TAM⁰-1/neom) respectively. Cells (10⁶) of each fusion partner were mixed and seeded in a 60 mm Petri dish in standard growth medium. The medium was removed the next day and 3 ml PEG-1500 (Boehringer Mannheim/Roche) were added. After 2 min incubation, the PEG-1500 was removed and the cells were washed twice in DMEM/F12 without serum. Then fresh growth medium was added and the cells were left for 48 h, before they were transferred to 10 mm Petri dishes (approximately 10⁶ cells per dish) and selection with 800 μg/ml G418 and 140 μg/ml hygromycin B was initiated (method modified from Davidson & Gerald (1976) and Safarians et al. (1996)). Colonies of resistant cells emerged after about 1 month. A pooled population of resistant cells, which could be maintained in culture and split every week, was obtained after approximately 2 months of selection.

**Flow cytometric analysis**

Fusion cells were treated with or without 10⁻⁷ M ICI 164384 for 48 h prior to harvest in trypsin–EDTA (0.25% trypsin in PBS without Ca²⁺ and Mg²⁺ supplemented with 100 mM EDTA, pH 7.6). The cells were resuspended in citrate buffer (205 mM sucrose, 40 mM trisodium citrate dihydrate, and 5% DMSO (Merck, Whitehouse Station, NY, USA)) at a concentration of 10⁶ cells/200 μl, before staining with propidium iodide according to the Vindeløv method (Vindeløv et al. 1983), and analyzed on a FACSort (Becton Dickinson, Boston, USA) fluorescence-activated cell sorter (FACS) flow cytometer. Cell cycle distribution and DNA Index (DI) were determined by the use of internal chicken and trout erythrocyte controls as described by Vindeløv & Christensen (1990).

**Western analysis**

Fusion cells and fusion partners were propagated in T25 flasks (Nunc) in medium, as described above. Cells were treated with 10⁻⁷ M ICI 164384, 10⁻⁶ M tamoxifen or 10⁻⁹ M estradiol for 6 or 48 h prior to harvest in RIPA buffer (100 mM sodium chloride, 20 mM Trizma-base, 1% Triton X-100, 0.5% sodium desoxycholate, 0.1% SDS and 1 mM sodium-EDTA, pH 8.0) at a confluency of approximately 70%. Protein determination (BioRad, Hercules, CA, USA) was carried out, 20 μg total protein of each sample were run on a 15% SDS-PAGE, and immunoblotting onto Immobilon-P membranes (Millipore, Billerica, NA, USA) was performed. The immunoreactions were carried out using either a primary mouse monoclonal Bcl-2 antibody (B46620; Transduction Laboratories, Woburn, MA, USA), a primary mouse monoclonal anti-human ERα antibody (ID5; DAKO, Glostrup, Denmark), a primary goat polyclonal IGF-binding protein-2 (IGFBP-2) antibody (sc-6001; Santa Cruz, CA, USA), a primary rabbit polyclonal IGF-IR antibody (sc-712; Santa Cruz, CA, USA), a primary mouse monoclonal anti-human ERTβ antibody (ID5; DAKO, Glostrup, Denmark), a primary goat polyclonal IGF-binding protein-2 (IGFBP-2) antibody (sc-6001; Santa Cruz, CA, USA), a primary rabbit polyclonal IGF-IR antibody (sc-712; Santa Cruz, CA, USA), a primary mouse monoclonal keratin 7 antibody (K7) (gift from Dr J Bartek, Danish Cancer Society), or a primary mouse monoclonal progesterone receptor (PR) antibody (NCL-PR; Novo Castra, Newcastle-upon-Tyne, UK). The secondary antibodies used were rabbit anti-mouse IgG peroxidase-conjugated antibody (P0260; DAKO), rabbit anti-goat IgG peroxidase-conjugated antibody (P0449; DAKO) or goat anti-rabbit IgG peroxidase-conjugated antibody (P0448; DAKO). The immunocomplexes were visualized by using ECL plus (Amersham Pharmacia Biotech) and Hyperfilm ECL films (Amersham Pharmacia Biotech), and quantified using a Fujifilm LAS-1000 detection system (Fujifilm, Sweden) and ImageGauge software.
Results and discussion

Fusion of human breast cancer cell lines

Three human breast cancer cell lines were included in this study. The well-characterized cell line MCF-7 was used along with one tamoxifen-resistant cell line (MCF-7/TAM<sup>®</sup>-1) and one ICI 164384- and ICI 182780-resistant cell line (MCF-7/164<sup>®</sup>-5). Prior to PEG-1500-induced cell fusion, the cells were stably transfected with either pSV2neo or pY3hygB to enable selection of the fusion cell lines with a combination of G-418 and hygromycin B. MCF-7/1neo was fused with MCF-7/164<sup>®</sup>-5/hygB whereby the 164-MCF cell line was produced, and MCF-7/164<sup>®</sup>-5/hygB was fused with the MCF-7/TAM<sup>®</sup>-1/neo producing the 164:TAM cell line. Both fusion cell lines were maintained in standard growth medium continuously supplemented with both antibiotics.

DI and cell morphology

The DI of the fusion partners and the fusion cell lines was investigated by FACS analysis. The DI for MCF-7/1neo of 1.78 (Table 1) is similar to the index of 1.76 which has previously been determined for several passages of the untransfected MCF-7 cell line (data not shown). These observations demonstrate that the transfection and selection procedure had no major effect on the DNA content. The DIs of the antiestrogen-resistant cell lines were determined to be 1.74 (Table 1), which is similar to the DIs of untransfected antiestrogen-resistant cell lines which vary between 1.74 and 1.77 (data not shown), indicating that no major changes in DNA content occurred during development of acquired resistance and during the transfection and selection of these antibiotic-resistant cell lines. The detected DIs of 3.43 and 3.36 respectively for the two fusion cell lines were very close to the expected DIs of 3.42. The DIs of the fusion cell lines were determined in several passages (from passage four to nine) and were found to remain constant during the passages used for these experiments. In Fig. 1, a microscopic view of the different cell lines is shown. The figure shows that the morphology of the fusion cells and fusion partners are quite different. The fusion cells tend to be bigger, more granulated and have more nucleoli than the fusion partners. Comparison of the nuclear size (measured in arbitrary units using an Olympus DP-3030 camera and Olympus IX-70 software [Olympus, Denmark]) also demonstrated a significant difference ($P < 0.01$) as the size of the nucleus in the fusion cells was found to be approximately 1.5 times the size in the fusion partners (Table 1).

These findings demonstrate that PEG-induced cell fusion gives rise to fusion cells which maintain the DNA of both parent cell lines for several passages. We found cell fusion to be a gentle method where viable cells are easily produced, thus enabling the study of the influence of the genetic input from both fusion partners in relation to cell growth and gene expression.

Cell cycle kinetics and growth characteristics

Generally, antiestrogen-sensitive cell lines are G<sub>1</sub>-arrested upon treatment with tamoxifen or the pure antiestrogens ICI 164384 and ICI 182780 (Osborne et al. 1983, Sutherland et al. 1983, Lykkesfeldt et al. 1984, Wakeling et al. 1989, Cariou et al. 2000). In order to investigate whether the fusion cell lines resembled the antiestrogen-sensitive or -resistant fusion partners in regard to G<sub>1</sub> accumulation, cell cycle phase distribution analysis of fusion partners and fusion cell lines was performed. The percentage of cells in G<sub>1</sub> phase after 48 h of treatment with ICI 164384 is presented in Table 1. MCF-7/1neo and MCF-7/TAM<sup>®</sup>-1/neo displayed G<sub>1</sub> accumulation upon treatment with ICI 164384 in accord with the

Table 1 DI, G<sub>1</sub> phase fraction, doubling time and nuclear size of fusion partners and fusion cells. All cell lines were maintained in control medium for at least 1 week before onset of the experiment. At a cell density of about 40–50%, cells were either changed to control medium or medium supplemented with 10<sup>-7</sup> M ICI 164384. After 48 h, cells were harvested and analyzed on a Becton Dickinson FACSsort flow cytometer. DI and percent of cells in G<sub>1</sub> phase of control and ICI 164384-treated cultures are shown. For determination of the population doubling time, cells were seeded in 24-well dishes and left to plate for about 48 h before the experiment was started (day 0). Then the medium was changed to either control medium, or control medium supplemented with 10<sup>-7</sup> M ICI 164384. Cell number was determined by counting quadruplicate samples in a Buerker–Turk chamber every second or third day. The population doubling time was determined from the exponential part of the growth curve, between day 0 and day 4 or 5. Most values are shown as means ± s.d. Number in parenthesis is the number of individual experiments or for nuclear size the number of measured nuclei

<table>
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<th>Cell lines</th>
<th>DI</th>
<th>G&lt;sub&gt;1&lt;/sub&gt; cells (%)</th>
<th>Doubling time (h)</th>
<th>Nuclear size (arbitrary units)</th>
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<td></td>
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<td>Control + ICI 164384</td>
<td>Control + ICI 164384</td>
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<tr>
<td>MCF-7/1neo</td>
<td>1.78</td>
<td>64 ± 1 (2) 84 ± 8 (2)</td>
<td>ND ND</td>
<td>52 ± 6 (10)</td>
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<tr>
<td>MCF-7/TAM&lt;sup&gt;®&lt;/sup&gt;-1/neo</td>
<td>1.74</td>
<td>64 87</td>
<td>ND ND</td>
<td>50 ± 5 (10)</td>
</tr>
<tr>
<td>MCF-7/164&lt;sup&gt;®&lt;/sup&gt;-5/hygB</td>
<td>1.74 ± 0.01 (2)</td>
<td>73 ± 5 (3) 75 ± 5 (3)</td>
<td>ND ND</td>
<td>51 ± 5 (10)</td>
</tr>
<tr>
<td>164-MCF</td>
<td>3.43 ± 0.00 (5) 68 ± 1 (3) 86 ± 2 (3)</td>
<td>31 60</td>
<td>78 ± 14 (10)</td>
<td></td>
</tr>
<tr>
<td>164:TAM</td>
<td>3.36 ± 0.00 (3) 74 ± 1 (3) 82 ± 2 (3)</td>
<td>35 41</td>
<td>66 ± 6 (10)</td>
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ND, not done.
Figure 1 Phase contrast microscopy pictures of the fusion partners (MCF-7/neo, MCF-7/164R-5/hygB and MCF-7/TAMR-1/neo) and fusion cell lines (164:MCF and 164:TAM). Images produced by the Olympus DP-3030 camera and Olympus IX-70 software.

significant growth inhibition reported for the MCF-7 and MCF-7/TAMR-1 cells (Lykkesfeldt et al. 1994). As expected, the ICI 164384-resistant MCF-7/164R-5/hygB cells did not exhibit significant G1 accumulation upon treatment with ICI 164384 ($P = 0.51$). The fusion cell line 164:MCF, which was produced to elucidate if antiestrogen resistance was a recessive or dominant feature, displayed a significant G1 accumulation during treatment with ICI 164384 ($P = 0.0002$). These data indicate that resistance to ICI 164384 is a recessive trait, thus suggesting that the resistant phenotype in the MCF-7/164R-5/hygB cell line probably can have occurred as a consequence of a loss-of-function mechanism. Interestingly, the other fusion cell line, 164:TAM, also showed a significant G1 accumulation upon ICI 164384 treatment ($P = 0.005$). This indicates that the molecular changes responsible for development of resistance to tamoxifen and to ICI 164384 are not identical.

Even though presence or absence of G1 accumulation is a good indicator of cells being sensitive or resistant to antiestrogen treatment, long-term exposure of the cells to antiestrogen is the ultimate test. Thus, we define resistance as the ability of cells to grow and to be continuously passaged in antiestrogen-containing medium. To test this, long-term exposure of the fusion cell lines with their respective antiestrogen was carried out. We observed that 164:MCF could be maintained in ICI 164384-containing medium for one passage only, whereas the 164:TAM cell line could be passaged once or twice in the presence of ICI 164384 before the cells ultimately died. When the 164:TAM cells were maintained in medium with tamoxifen, rapid cell loss occurred and the cells could not be passaged. Consequently, neither of these fusion cell lines can be classified as resistant. These observations suggest that different recessive changes are involved in development of resistance to different antiestrogens, giving the net result that the fusion cells are sensitive to long-term treatment with both tamoxifen and ICI 164384.

The population doubling time of the fusion cell lines in control medium and medium containing $10^{-7}$ M ICI 164384 was also determined (Table 1). The doubling time of 164: MCF under control conditions was found to be approximately 31 h, which is quite similar to the growth rate of MCF-7 under the same conditions (Lykkesfeldt et al. 1995). Treatment of the fusion cells with $10^{-7}$ M ICI 164384 increased the doubling time to about 60 h, which also is very similar to the response observed in the antiestrogen-sensitive MCF-7 cells (Lykkesfeldt et al. 1995), but different from the response observed in MCF-7/164R-5 cells, which were only slightly growth inhibited by addition of ICI 164384 (Lykkesfeldt et al. 1995). These data are in support of the findings presented above concluding that the changes resulting in development of the antiestrogen-resistant phenotype of the MCF-7/164R-5/hygB cell line may be recessive.
Antiestrogen resistance is caused by multi-factorial changes

The other fusion cell line, 164:TAM, which has been produced in order to clarify if resistance to pure antiestrogens and the SERM tamoxifen occurs through the same molecular changes, responded to ICI 164384 treatment by an increase in population doubling time from about 35 to 41 h. This response is much weaker than the response seen in MCF-7/TAM\(^{\text{R}-1}\) cells, which are totally growth arrested upon treated with ICI 164384 (Lykkesfeldt et al. 1994). However, the response is very similar to the response observed for the MCF-7/164\(^{-5}\) cell line (Lykkesfeldt et al. 1995), which is resistant to treatment with ICI 164384, although the growth rate is slightly reduced. These growth studies indicate that 164:TAM is more similar to MCF-7/164\(^{-5}\) than to MCF-7/TAM\(^{\text{R}-1}\).

Taken together, cell cycle kinetic analyses indicate that resistance to ICI 164384 is a recessive trait, and furthermore, it seems that the mechanisms leading to resistance to pure antiestrogens and to tamoxifen occur through different molecular pathways. To clarify these hypotheses further, dose–response experiments and protein expression analyses of the fusion partners and fusion cell lines were performed.

**Dose–response experiments with ICI 164384 and tamoxifen**

Several dose–response experiments were carried out in growth medium supplemented with antibiotic(s) and different concentrations of either ICI 164384 or tamoxifen to further investigate whether recessive or dominant traits were involved in development of acquired resistance to antiestrogens. The concentration giving rise to 50% reduction in growth (IC\(_{50}\) values) for each cell line was determined and the median of each data set was found (noted on the x-axis in Fig. 2A and B). As seen in Fig. 2A, the 164:MCF fusion cell line appears to display intermediate sensitivity to ICI 164384 treatment, when compared with the fusion partners. The statistical analysis revealed that the IC\(_{50}\) values of 164:MCF were found to be significantly lower than the values for the MCF-7/164\(^{-5}\)/hyg\(_{B}\) cell line (\(P = 0.002\)), and significantly higher than MCF-7/neo (\(P = 0.004\)) (Mann–Whitney two-tailed U test). However, the observed decreased sensitivity of 164:MCF cells to ICI 164384 treatment, compared with MCF-7/neo, is not concurrent with development of a resistant phenotype defined by the ability to grow continuously in the presence of antiestrogen. These findings can be taken to suggest that resistance to ICI 164384 and ICI 182780 is a recessive feature due to a single gene alteration, or more likely that both recessive and dominant genes are involved in the process.

Data on the other fusion cell line, 164:TAM, treated with either ICI 164384 (Fig. 2A) or tamoxifen (Fig. 2B), revealed that the fusion cells exhibited similar IC\(_{50}\) values to the fusion partners, but without being resistant (as mentioned above). When the IC\(_{50}\) values were compared with the
values of the parental cell lines MCF-7/164R-5/hygB and MCF-7/TAMR-1/neo using the Mann–Whitney U test, no significant difference between the fusion cell line and the fusion partners could be detected, either when cells were treated with ICI 164384 or tamoxifen.

Overall, the findings from this part of the study demonstrate that antiestrogen resistance is not a completely recessive or dominant feature. However, our data indicate that development of antiestrogen resistance is a multi-factorial process, which involves different pathways in the different cell lines. To explore this further, the expression and regulation of proteins known to be differentially expressed between antiestrogen-sensitive and -resistant cell lines were examined by Western analysis.

**Regulation of ERα in fusion partners and fusion cells**

Regulation of the ERα protein in the different cell lines treated with estradiol (10^{-9} M), tamoxifen (10^{-6} M) or ICI 164384 (10^{-7} M) for either 6 or 48 h was investigated to explore whether the fusion partners and fusion cell lines had retained normal regulation of ERα (Fig. 3). Normal expression and regulation of ERα is a prerequisite both for the effectiveness of an antiestrogen and also for the expression of genes directly under the control of an estrogen-responsive element (ERE), or genes affected indirectly by estradiol, e.g. through a paracrine mechanism or cross-talk. As seen in Fig. 3, a striking similarity was observed both in the fusion partners and in the fusion cell lines with respect to hormonal regulation of ERα. Estradiol and ICI 164384 down-regulated the ERα protein, as reported earlier for both the parental MCF-7 cells and an ICI 182780-resistant cell line (Jensen et al. 1999). Treatment with tamoxifen resulted in stabilization of the ERα protein in both fusion partners and fusion cell lines as previously described for other ERα-positive human breast cancer cell lines (Kiang et al. 1989, Pink & Jordan 1996, Legros et al. 1997, McDougal et al. 2001).

These data demonstrate that the selection procedure for generation of antibiotic-resistant cells did not affect the ho-
monal regulation of ERα in the fusion partners. Furthermore, we observed that the hormonal regulation was also retained after cell fusion, indicating normal function of the ERα protein in the fusion cell lines.

**Western analysis of ERα, PR, Bcl-2, IGF-IRα and IGFBP-2**

ERα (Jensen et al. 1999), PR (May et al. 1989, Kastner et al. 1990), Bcl-2 (Kumar et al. 2000) and IGF-IRα (Oesterreich et al. 2001) are classical estradiol-influenced gene products. The expression of ERα along with the PR, Bcl-2, IGFBP-2 and IGF-IRα was determined in the fusion partners and fusion cell lines, in order to elucidate whether dominant or recessive mechanisms were involved in the expression of these proteins (Fig. 4). These particular proteins were chosen because they are known to be differentially expressed between the parental MCF-7 and the antiestrogen-resistant cell lines (Lykkefsfeldt et al. 1995, van den Berg et al. 1996). The expression of the total intracellular level of IGFBP-2 protein was included in the analysis because both the mRNA and the intracellular level of this secreted protein is increased in most of the antiestrogen-resistant cell lines in our laboratory (data not shown).

**ERα expression**

As seen in Fig. 4A and B, the expression of ERα is decreased in all investigated cell lines compared with MCF-7/neo. When using the Fujifilm LAS-1000 detection system and the ImageGauge software, setting MCF-7/neo to 100%, we find a reduction of between 40 and 60% in the other cell lines (Fig. 4B). In general, low expression of ERα is correlated with reduced sensitivity towards antiestrogen treatment in our parental cell lines (Lykkefsfeldt et al. 1995) and this was also seen to be the case in the resistant fusion partners and in the fusion cell lines. The similar low ERα expression in the 164:MCF cell line and the MCF-7/164*-5/hygB could indicate the involvement of a gain-of-function mechanism in the resistant cell line, one possibility being increased expression of a repressor or co-repressor, as ERα is regulated through its own receptor (Jensen et al. 1999).

**PR expression**

The MCF-7/neo cell line expresses both PR-A (94 kDa) and PR-B (114 kDa), whereas no expression was detected in any of the other cell lines. The decreased expression of ERα in the resistant cell lines and the fusion cell lines may explain the absent expression of PR protein, as van Agthoven et al. (1994) have earlier suggested that a certain threshold of ERα is required to obtain the expression of genes under control of an ERE. Since ERα controls expression of many genes, decreased expression of ERα may be the driving force for (some of) the observed changes in expression of PR, Bcl-2 and IGF-IRα. We believe that the decreased ERα expression level may represent one of the initial genetic changes in the complex of alterations ultimately leading to acquired resistance towards antiestrogens. However, the exact mechanism for and the biological significance of reduced or absent PR protein expression both in vivo and in vitro remains to be elucidated.

**Bcl-2 expression**

Low expression of Bcl-2 in antiestrogen-resistant cell lines have earlier been reported (Larsen et al. 2001), and are also observed here in the MCF-7/TAM*-1/neo (15% of the level in MCF-7/neo) and MCF-7/164*-5/hygB (34% of the level in MCF-7/neo) cell lines (Fig. 4A and B). In 164:MCF, the Bcl-2 level was similar to the level in the fusion partner MCF-7/164*-5/hygB (41% of the level in MCF-7/neo), demonstrating dominance of MCF-7/164*-5/hygB over MCF-7/neo in respect to Bcl-2 expression in this cell line. The 164:TAM cell line, on the other hand, exhibited an expression level of 56% of control (higher than both fusion partners). These data indicate that the mechanisms leading to a low Bcl-2 expression in the two resistant cell lines are different and that both gain- and loss-of-function changes are likely to be involved in the process. The decreased expression of Bcl-2 in resistant cell lines may represent an altered balance between the pro-apoptotic and anti-apoptotic signals in the cells. More investigations of the Bcl-2 family members are needed before the biological significance of this observation can be elucidated.

**IGFBP-2 expression**

The IGFBP-2 protein expression was found to be at about the same level in all fusion partners. This finding is rather similar to observations made with the untransfected parental cell lines (unpublished observations, BL Brockdorff), where MCF-7/TAM*-1 and the MCF-7/164*-5 cell lines were found to express similar or slightly increased levels of IGFBP-2 compared with MCF-7. In the fusion cell lines, however, the expression level is significantly reduced to about 35% of the level in MCF-7/neo. This finding of ‘compensatory expression’, when a fusion between two ‘high expressers’ leads to the formation of a ‘low expresser’, is in support of the hypothesis that development of antiestrogen resistance is a multi-factorial process, which occurs through different mechanisms in different resistant cell lines. Overall, our data suggest that IGFBP-2 is probably not causally involved in development of antiestrogen resistance in MCF-7/TAM*-1 and MCF-7/164*-5, since no significant change in expression is observed compared with MCF-7.

**IGF-IRα expression**

It has been reported that expression of IGF-IRα is decreased in ER-negative cell lines (Bartucci et al. 2001), which may
Figure 4  (A) Expression of ERα (65 kDa), PR (PR-A 94 kDa and PR-B 114 kDa), Bcl-2 (26 kDa), IGFBP-2 (38 kDa) and IGF-IRα (206 and 135 kDa) protein in fusion partners and fusion cells; 20 µg total protein were loaded in each lane on a 15% SDS-PAGE gel. The proteins were visualized using antibodies as described in the Materials and methods section, using the enhanced chemiluminescence detection system (ECL plus; Amersham Pharmacia Biotech). Equal loading and transfer was ensured by the K7 loading control. The results were expressed relative to K7 and shown as percent of MCF-7/neo control. One of two individual experiments, giving similar results, is shown. (B) Expression of ERα, Bcl-2, IGFBP-2 and IGF-IRα was analyzed and calculated using the Fujifilm LAS-1000 detection system and ImageGauge software. Data from two individual experiments using cells from two different passages are shown.
be expected since IGF-IRα is an ER-regulated protein. Furthermore, it has also been reported that IGF-IRα is reduced in an antiestrogen-resistant cell line (van den Berg et al. 1996) and we have found significantly reduced levels of IGF-IRα in six different antiestrogen-resistant cell lines (data not shown). In the present study, we found that the antioestrogen-resistant cell lines expressed about 25% of the level in MCF-7/neo, whereas both 164:MCF and 164:TAM showed higher expression levels (Fig. 4). In the 164:MCF cell line, the IGF-IRα expression was found to be 68% of the level in MCF-7/neo, showing an intermediary expression level in this fusion cell line compared with the fusion partners (Fig. 4). In the 164:TAM cell line, however, the IGF-IRα expression was found to be 62% of the level in MCF-7/neo, which is significantly above the expression level in both fusion partners (Fig. 4). In the 164:TAM cell line, however, the IGF-IRα expression was found to be 62% of the level in MCF-7/neo, which is significantly above the expression level in both fusion partners, thus indicating that different loss-of-function mechanisms are accountable for the loss of IGF-IRα expression in the two antioestrogen-resistant fusion partners (Fig. 4). This finding supports the hypothesis of antioestrogen resistance being caused by different multi-factorial changes within the individual antioestrogen-resistant cell line. Noteworthy, reappearance of IGF-IRα is associated with regained sensitivity to antioestrogen treatment. Based on these observations and unpublished observations from our laboratory showing that both ICI 182780 and tamoxifen-resistant cell lines have either lost IGF-IRα expression or have extremely low IGF-IRα expression (SS Larson), we suggest that IGF-IRα may be used as a very sensitive marker for predicting response to treatment with antioestrogens.

### Table 2 Parameters studied in the fusion partners and fusion cell lines. The values or levels of the measured parameters are listed for fusion partners and 164:MCF (A) or 164:TAM (B). For the fusion cell lines, the mechanisms involved are indicated as recessive, dominant, intermediate or compensatory (right column)

<table>
<thead>
<tr>
<th>(A) Parameter</th>
<th>MCF-7/neo</th>
<th>MCF-7/164R-5/hygB</th>
<th>164:MCF</th>
</tr>
</thead>
<tbody>
<tr>
<td>DI</td>
<td>1.78</td>
<td>1.74</td>
<td>3.43</td>
</tr>
<tr>
<td>Resistant to ICI 164384</td>
<td>No</td>
<td>Yes</td>
<td>No, MCF-7/164R-5 recessive</td>
</tr>
<tr>
<td>Growth inhibition by ICI 164384</td>
<td>Strong</td>
<td>Weak</td>
<td>Intermediate</td>
</tr>
<tr>
<td>ER protein expression</td>
<td>High</td>
<td>Low</td>
<td>Low, MCF-7/164R-5 dominant</td>
</tr>
<tr>
<td>PR protein expression</td>
<td>High</td>
<td>Low</td>
<td>Low, MCF-7/164R-5 dominant</td>
</tr>
<tr>
<td>Bcl-2 protein expression</td>
<td>High</td>
<td>Low</td>
<td>Weak</td>
</tr>
<tr>
<td>IGFBP-2 protein expression</td>
<td>High</td>
<td>Low</td>
<td>Low, compensatory</td>
</tr>
<tr>
<td>IGF-IRα protein expression</td>
<td>High</td>
<td>Low</td>
<td>Intermediate</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(B) Parameter</th>
<th>MCF-7/TAM1-1/neo</th>
<th>MCF-7/164R-5/hygB</th>
<th>164:TAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>DI</td>
<td>1.74</td>
<td>1.74</td>
<td>3.36</td>
</tr>
<tr>
<td>Resistant to ICI 164384</td>
<td>No</td>
<td>Yes</td>
<td>No, MCF-7/164R-5 recessive</td>
</tr>
<tr>
<td>Resistant to tamoxifen</td>
<td>Yes</td>
<td>No</td>
<td>No, MCF-7/TAM1-1 recessive</td>
</tr>
<tr>
<td>Growth inhibition by ICI 164384</td>
<td>Weak</td>
<td>Weak</td>
<td>Weak</td>
</tr>
<tr>
<td>Growth inhibition by tamoxifen</td>
<td>Weak</td>
<td>Weak</td>
<td>Weak</td>
</tr>
<tr>
<td>ER protein expression</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>PR protein expression</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Bcl-2 protein expression</td>
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<td>Low</td>
<td>Low, MCF-7/TAM1-1 recessive</td>
</tr>
<tr>
<td>IGFBP-2 protein expression</td>
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<td>Low</td>
<td>Low, compensatory</td>
</tr>
<tr>
<td>IGF-IRα protein expression</td>
<td>Low</td>
<td>Low</td>
<td>Intermediate, compensatory</td>
</tr>
</tbody>
</table>

## Conclusion

In this report, we show that cell fusion is a useful method for studying whether similar or different genetic changes have occurred in human breast cancer cell lines resistant to different types of antioestrogens. The importance of elucidating the sequence of changes leading to antioestrogen resistance and determining whether these changes are recessive or dominant is crucial in relation to planning the optimal treatment strategy for breast cancer patients in the future. In this study, several parameters have been compared in the fusion partners and the fusion cell lines. A comprehensive list of the parameters is presented in Table 2. As summarized in the table, antioestrogen resistance is neither a completely recessive nor a completely dominant characteristic as suggested by others (Zajchowski et al. 1990, Paik et al. 1994, Safarians et al. 1996, van Agthoven et al. 1998).

Apart from the dominant or recessive type of mechanism observed for some of the parameters, a compensatory type of mechanism indicating that more genes are involved also occurs. In the case of IGFBP-2, we find a lower level in 164:MCF and 164:TAM than in any of the fusion partners. In contrast, IGF-IRα is expressed at a higher level in 164:TAM than in either fusion partner. We find these results, with the fusion between the two antioestrogen-resistant cell lines, particularly interesting as they indicate that different mechanisms are involved in development of resistance to ICI 164384 and tamoxifen. This possibility of different mechanisms being responsible for acquired resistance to different
antiestrogens is very promising in relation to sequential treatment of patients with different types of antiestrogens. The observation that IGF-IRα, but not PR, is re-expressed in the 164:TAM fusion cells which have regained sensitivity to ICI 164384 and tamoxifen treatment indicates that IGF-IRα expression may be a more sensitive marker for response to endocrine therapy with antiestrogens than PR expression. However, further research in this field is needed in order to determine the precise biological significance of the gene expression changes observed in the different cell lines. For this purpose, cell fusion is one method that might contribute to the understanding. We propose that it will be relevant to fuse cells resistant to the same antiestrogen in order to determine whether the sequence of events leading to resistance is similar. This can, for example, be done by PEG-1500-induced cell fusion, as described here, followed by gene expression screening using the DNA chip technology. Also, further cross-resistance growth studies on “un-fused” cells, using both antiestrogens and the novel aromatase inhibitor, will be tremendously important for elucidating the optimal sequence of treatment with endocrine therapy. Such studies are currently underway utilizing our large collection of antiestrogen- and aromatase inhibitor-resistant cell lines.

Acknowledgements

Excellent technical assistance was provided by Birgit E Reiter, Jette Larsen, Ib J Christensen and Jørgen K Larsen, from the Finsen Laboratory, have been very helpful in regard to the FACS analysis. The ICI 164384 was kindly supplied by AstraZeneca. The presented work has been financially supported by grants from the Danish Cancer Society, the A V Lykfeldt Foundation, the Astrid Thaysen Foundation, and the John and Birthe Meyer Foundation.

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