Decreased Androgen Receptor Levels and Receptor Function in Breast Cancer Contribute to the Failure of Response to Medroxyprogesterone Acetate

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Abstract

Previously, we reported that androgen receptor (AR), but not estrogen receptor (ER) or progesterone receptor (PR), is predictive of response to the synthetic progestin, medroxyprogesterone acetate (MPA), in a cohort of 83 patients with metastatic breast cancer. To further investigate the role of AR in determining response to MPA in this cohort, we analyzed AR levels by immunohistochemistry with two discrete antisera directed at either the NH2 or the COOH termini of the receptor. Compared with tumors that responded to MPA (n = 31), there was a significant decrease in the intensity and extent of AR immunoreactivity with both AR antisera in tumors from nonresponders (n = 52). Whereas only a single AR immunostaining pattern was detected in responders to MPA, reflecting concordance of immunoreactivity with the two AR antisera, tumors from nonresponders exhibited four distinct AR immunostaining patterns: (a) concordance with the two antibodies (31%), (b) staining only with the COOH-terminal antibody (33%), (c) staining only with the NH2-terminal antibody (22%), or (d) no immunoreactivity with either NH2- or COOH-terminal antibody (14%). DNA sequencing and functional analysis identified missense gene mutations in the ligand-binding domain of the AR gene (9), an established modulator of AR function (10, 11).

Introduction

Although androgen signaling has been extensively studied in the prostate (1), there is emerging evidence that androgens have inhibitory effects on the growth of breast epithelial cells and play a protective role in the pathogenesis of breast cancer (2–5). The androgen receptor (AR) is expressed in ~70% to 90% of primary breast tumors, a frequency comparable with or higher than that reported for either the estrogen receptor (ER; 70–80%) or the progesterone receptor (PR; 50–70%; refs. 6, 7). Importantly, comprehensive microarray analysis recently determined that mammary tumor cells can be divided into three groups based on steroid receptor activity: luminal (ER+, AR+), basal (ER−, AR−), and apocrine (ER−, AR+; ref. 8). In addition, several case-control studies have identified a relationship between breast cancer risk and length of the polymorphic CAG repeat in exon 1 of the AR gene (9), an established modulator of AR function (10, 11).

Androgens (e.g., fluoxymesterone) have a comparable efficacy with that of current hormonal treatments for advanced breast cancer, but their use as primary therapeutic agents declined with the advent of tamoxifen (12, 13). Nevertheless, the potential for androgens to counterbalance positive growth stimuli in the breast has been pursued in recent in vivo studies in rhesus monkeys, where blocking the action of endogenous androgens resulted in a 2-fold increase in the proliferation of mammary epithelial cells (14). Conversely, low doses of testosterone completely inhibited estrogen-mediated mammary cell proliferation in ovarioctomized animals (14). Similarly, in culture, androgens predominantly exhibit an inhibitory effect on AR-positive breast cancer cell lines (2, 15, 16).

The synthetic progestin, medroxyprogesterone acetate (MPA), was used frequently until the early 1990s as a second-line hormonal therapy for metastatic breast cancer (17). Although the use of MPA declined following the advent of tamoxifen and aromatase inhibitors, recently, there has been renewed interest in alternative hormonal treatments, including MPA, for use both in early disease and in the advanced setting when conventional therapies fail (18–20). Whereas MPA was designed to bind with high affinity to the PR, the androgenic side effects observed in women taking MPA suggested that the AR may contribute to its activity in vivo. This hypothesis is supported by the high binding affinity of MPA to the AR.

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(21) and clinical studies showing the response of breast tumors to high-dose MPA therapy is dependent on expression of the AR but not the PR (22, 23). A recent report that the length of the AR CAG repeat is correlated with breast density in women receiving combined hormone replacement therapy (24) provides additional evidence that AR is a determinant of the effects of MPA in the breast. Furthermore, MPA inhibits the proliferation of AR-positive but not AR-negative breast cancer cell lines, and this inhibition can be reversed by cotreatment with specific AR antagonists (21, 25).

We reported previously that the response of breast cancers to MPA in a cohort 83 women who failed tamoxifen therapy was related to the AR level by radioligand binding in the primary tumor but not to ER or PR. However, 33% of tumors that failed to respond to MPA had detectable AR by radioligand binding. Therefore, in the current study, we used an immunohistochemistry strategy with discrete antisera directed against the NH₂- and COOH-termini of the AR, employed previously in the identification of AR variants in prostate cancer (26, 27), to examine whether failure of MPA therapy is associated with altered AR levels and/or the presence of structural variants of the AR.

Materials and Methods

Tissues and clinical data. This study consisted of a total of 161 postmenopausal women treated for early-stage breast cancer (International Union Against Cancer stage IIa or IIb) between 1984 and 1987 at the Surgical Oncology Unit, Department of Surgery, Flinders Medical Centre (South Australia, Australia). Patients received either a partial (33%) or a total (67%) mastectomy with axillary clearance, and 141 were treated postoperatively with indefinite adjuvant tamoxifen [one 20 mg tablet daily of Nolvadex-D, ICI Pharmaceuticals Pty Ltd., Australia; median (range) duration, 68 (6-110) months]. Of the 89 patients who relapsed on tamoxifen, 83 were subsequently treated with MPA (one 500 mg tablet daily of Farlutal, Farmitalia Pty Ltd., Australia). Clinical examination was done every 3 months, and nuclear bone scans, chest X-rays, and hematologic and biochemical blood analyses were done initially at 3 months and then six monthly or more frequently if clinically indicated. After relapse on MPA, patients were clinically evaluated at least every 3 months until death. Response to MPA was defined by complete disappearance of all known disease, a ≥50% decrease in tumor size below baseline using the sum of the product of perpendicular diameters for bidimensional lesions, or a ≥30% decrease in tumor size from baseline using the sum of the largest diameter for unidimensional lesions. Nonresponders to MPA were defined by stable (no change in lesion size) or progressive (appearance of new lesions or a >25% increase in bidimensional lesions) disease. The cohort of 26 prostate samples is described elsewhere (27).

Radioligand binding. Breast cancer specimens were obtained on ice from theatre immediately after excision and tumor tissue was separated from theatre immediately after excision and tumor tissue was separated from macroscopically normal tissue. AR, ER, and PR status was determined by the South Australian State Diagnostic Hormone Receptor Laboratory at Flinders Medical Centre, with approval of the Committee on Clinical Investigations. Immunohistochemical staining for AR was done on serial 4 μm sections using the AR NH₂-terminal U402 and COOH-terminal R489 antisera directed at the NH₂-terminal 21 and COOH-terminal 20 amino acids of the wild-type AR (wtAR), respectively (28). Visualization of AR was achieved with a standard immunoperoxidase reaction using biotinylated anti-rabbit antibody (1:400, DAKO Corp., Carpenteria, CA), streptavidin-horseradish peroxidase (HRP) complex (1:500, DAKO), and diaminobenzidine tetrahydrochloride to yield an insoluble brown deposit. AR immunoreactivity was assessed in tumor focci at a magnification of ×400. Immunostaining was quantified using color video image analysis (VIA) as described previously (26). Video image measurements included the total nuclear area of tissue examined (i.e., positively and negatively stained nuclear area in pixel units) and the diaminobenzidine tetrahydrochloride– stained area (i.e., positively stained nuclear area in pixel units). These values were used to derive percent AR-positive nuclear area and mean intensity of AR immunoreactivity. VIA data represent image analysis done for at least 20 randomly selected fields in each sample.

Single-strand conformational polymorphism analysis and DNA sequencing. Genomic DNA was isolated from 25 μm sections of breast cancer tissue as described previously (27). The coding sequence of the AR ligand-binding domains (LBD) was amplified by PCR using sets of overlapping primers and a 1:16 mixture of pfu and taq polymerases and analyzed by single-strand conformational polymorphism (SSCP) analysis on non-denaturing polyacrylamide gels (6% polyacrylamide, 5% glycerol gel; either 10°C or 28°C for 16 hours) according to previously published methods (27). Fragments of AR exhibiting a consistent mobility shift in at least two independent SSCP analyses for a single tumor sample were cloned into independent reactions and cloned into the pcRRII TA vector (Invitrogen, San Diego, CA). Sequencing was done using the Sequenase 7-deaza-dGTP DNA Sequencing kit (Amersham) and a 373A PE Biosystems automated DNA sequencer (Applied Biosystems, Foster City, CA). Mutations were scored if they were confirmed by sequencing of both sense and antisense strands in two or more independent clones. Based on a range (10-5,000 copies) of template DNA and the documented error rate of a 1:16 mixture of pfu and taq polymerases (5.6 × 10⁻⁶ per base; ref. 29), <2.5% of final PCR amplifications of 250 bp long (the upper limit in this study) were predicted to contain miscorrection errors, which is below the threshold for detection by SSCP analysis.

Cell culture. COS-1, CV-1, LNCaP, T47D, and MDA-MB-231 cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 supplemented with 5% fetal bovine serum (FBS). The PC-3AR⁻ subline described elsewhere (30) was also used in this study.

Construction of androgen receptor variant expression plasmids. Base substitutions required to generate AR variants were introduced into an expression vector containing the complete human AR gene coding sequence (pCMV-AR; ref 31) by PCR-based megaprimer in vitro mutagenesis as described previously (10, 30). The presence of the required base substitutions and integrity of AR variant plasmid constructs was confirmed by DNA sequencing.

Cell proliferation and viability. T47D and MCF-7 cells were seeded into 24-well tissue culture plates at 50,000 cells per well in phenol red–free RPMI 1640 supplemented with 5% dextran-coated charcoal-stripped FCS. Two days after seeding, cells were washed and treated for 6 days with medium containing the appropriate steroids (replaced each 3 days). Cells were trypsinized and counted using a hemocytometer at day 6, with viability determined using trypan blue exclusion. To knockdown endogenous levels of AR, cells were allowed to adhere for 2 days and then transfected for 5 hours with 850 ng/well of either the specific AR antisense oligonucleotide (5'-CTGCACCTTCCATCT-3’; ref 2) or a scrambled control using Lipofectin (Invitrogen). Cells were subsequently treated and assayed as detailed above.

In vitro transactivation assay. AR and ER transactivation assays were done as described previously (10, 30) in cultured cells (10,000-20,000 cells per well in 96-well plates) cotransfected with 2.5 to 10 ng AR (pCMV-AR; or AR variants) and/or ER (pHEGO) expression vectors and 100 ng of the

Immunohistochemical analysis of androgen receptor. Formalin-fixed, paraffin-embedded breast tumor specimens for the 83 women who received MPA were obtained from the Department of Anatomical Pathology, Flinders Medical Centre, with approval of the Committee on Clinical Investigations. Immunohistochemical staining for AR was done on serial 4 μm sections using the AR NH₂-terminal U402 and COOH-terminal R489 antisera directed at the NH₂-terminal 21 and COOH-terminal 20 amino acids of the wild-type AR (wtAR), respectively (28). Visualization of AR was achieved with a standard immunoperoxidase reaction using biotinylated anti-rabbit antibody (1:400, DAKO Corp., Carpenteria, CA), streptavidin-horseradish peroxidase (HRP) complex (1:500, DAKO), and diaminobenzidine tetrahydrochloride to yield an insoluble brown deposit. AR immunoreactivity was assessed in tumor focci at a magnification of ×400. Immunostaining was quantified using color video image analysis (VIA) as described previously (26). Video image measurements included the total nuclear area of tissue examined (i.e., positively and negatively stained nuclear area in pixel units) and the diaminobenzidine tetrahydrochloride–stained area (i.e., positively stained nuclear area in pixel units). These values were used to derive percent AR-positive nuclear area and mean intensity of AR immunoreactivity. VIA data represent image analysis done for at least 20 randomly selected fields in each sample.

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appropriate androgen [probasin, ARR3tk-luc; mouse mammary tumor virus (MMTV)-Luc] or estrogen (ERE-tk-luc) luciferase reporter constructs using LipofectAMINE 2000 (Life Technologies, Melbourne, Victoria, Australia) according to the manufacturer’s protocol. Luciferase activity was determined using the Luciferase Reporter Gene Assay kit (Promega, Madison, WI) and a plate reading luminometer (Top Count, Packard, Mount Waverley, Australian Capital Territory, Australia) in lysed cells 30 hours after treatment with 0.01 to 1,000 nmol/L steroids and/or specific antagonist in phenol red–free RPMI containing 5% charcoal-stripped FBS. Endogenous ER activity in T47D cells was measured 30 hours after treatment with the appropriate steroids in cells transfected with ERE-tk-luc alone.

Immunoblot analysis. Protein lysates (20 μg) from untransfected LNCaP, T47D, or MCF-7 cells (1 × 10⁶ per 60 mm dish) or COS-1 cells (1 × 10⁶-5 × 10⁶ per 60 mm dish) transfected with 5 μg AR expression vector and grown for 36 hours were electrophoresed on 6% SDS-polyacrylamide gels, transferred to Hybond-C membrane (Amersham), and immunostained using cytokeratin 8 (Sigma, St. Louis, MO), β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), prostate-specific antigen (PSA; DAKO), AR (21), PR (hPRα antibody obtained from Dr. Christine Clarke (Department of Medical Oncology, University of Sydney Westmead Hospital, New South Wales, Australia), or ERα (Santa Cruz Biotechnology) antisera. Immunoreactivity was detected using the appropriate HRP-conjugated IgG and visualized using enhanced chemiluminescence (Amersham).

Affinity of dihydrotestosterone and medroxyprogesterone acetate for the androgen receptor and androgen receptor-ligand stability. Affinity of [3H]dihydrotestosterone (DHT; 126 Ci/mmol; Amersham) and medroxyprogesterone acetate (MPA; Sigma, St. Louis, MO) for the androgen receptor and androgen receptor-ligand stability were determined in LNCaP cells using the radioligand binding assay as previously described.

Figure 1. AR immunoreactivity in human breast tumors. A, comparison of the mean absorbance (MOD; average intensity of AR immunoreactivity) with NH2- and COOH-terminal AR antisera in 83 human breast tumors from women who received MPA therapy. Data for each antibody were generated using quantitative VIA of at least 20 fields of view chosen at random in each sample. Arrows, position of the two examples shown in (B). B, representative AR immunoreactivity in two breast tumors with the anti–NH2-terminal (U402) antibody, raised against the NH2-terminal 21 amino acids, and the anti–COOH-terminal (R489) antibody that was raised against the COOH-terminal 20 amino acids of the receptor. The first tumor shows concordant immunoreactivity with the two antisera in a patient (R29) that responded to MPA treatment. The AR level detected by radioligand binding in this tumor was 221 fmol/mg protein. Insets, separate sections of the same tumor that were not incubated with the primary AR antibody. The second tumor, derived from a patient who did not respond to MPA (NR33), stained strongly with the NH2-terminal but not the COOH-terminal AR antisera. No AR radioligand binding was detected in this tumor.

C, comparison of mean absorbance with NH2- and COOH-terminal AR antisera in 32 breast tumors from women who responded to MPA therapy. There was strong AR immunoreactivity with each antisera in all tumors in this group [32 of 32 (100%), blue-boxed area]. D, comparison of mean absorbance with NH2- and COOH-terminal AR antisera in 51 breast tumors from women who did not respond to MPA therapy. Tumors could be classified into four distinct groups; (a) strong immunoreactivity with both NH2- and COOH-terminal AR antibodies [16 of 51 (31%), blue box with dots], (b) strong immunoreactivity with only the AR COOH-terminal antibody [17 of 51 (33%), green ellipse with stripes on the Y axis], (c) strong immunoreactivity with only the AR NH2-terminal antibody [11 of 51 (22%), pink ellipse with vertical lines on the X axis], and (d) no immunoreactivity with either NH2- or COOH-terminal antibodies [7 of 51 (14%), cross-hatched yellow box].
MPA (46.9 Ci/mmol; Amersham) for AR and stability of AR-ligand complexes were determined in cytosol extracts of COS-1 cells transfected with wtAR or AR variants as described previously (31, 32).

Molecular modeling. Models of MPA and DHT were constructed from molecular sketches drawn in ISIS draw 2.2.1 4 and converted to a three-dimensional representation in Sculpt 3.2. Partial charges and initial energy minimization of ligands were assigned in Vega 1.6.1 (33). The crystallographic structure of the AR LBD (34) was used to generate a model for docking. Water molecules and bound ligand in the structure file were deleted, hydrogen atoms were fixed to polar atoms, and the receptor was placed in a grid (100°C100°C100 units) with a spacing of 0.375 Å centered on the ligand-binding cavity. Ligands were docked into the LBD using Autodock 3.0 (35). The steroid nucleus of the ligands was left rigid, whereas other bonds were allowed to rotate freely during docking simulation. The highest ranking solutions from both MPA and DHT docking simulations were reconfigured in SPDBV 3.7 such that hydrogens were reattached to polar atoms.

In silico receptor mutation was done followed by energy minimization (200 steps of steepest descent with a 10 Å cutoff for nonbonded interactions). Ten independent 100ps molecular dynamic simulations for wild-type or mutant receptors bound to DHT or MPA were carried out allowing atoms within 15 Å of bound ligand to move under constant temperature conditions (300 K bath temperature, 1 fs step time, and 20 equilibration steps). The sets of 10 solutions for each receptor/ligand combination were superimposed using the entire receptor LBD as a reference point. Root mean square (RMS) deviation for side chains within 7 Å was calculated within SPDBV 3.7. Molecular surfaces were constructed using the GRASP implementation within SPDBV and figures were rendered using PovRay.5

Table 1. AR immunostaining and AR mutational analysis in human breast tumors

<table>
<thead>
<tr>
<th>Tumor sample</th>
<th>MOD*</th>
<th>%POS†</th>
<th>Mutation‡</th>
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<tr>
<td></td>
<td>NH₂ terminal</td>
<td>COOH terminal</td>
<td>NH₂ terminal</td>
</tr>
<tr>
<td>All tumors</td>
<td></td>
<td></td>
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<tr>
<td>Mean</td>
<td>0.28</td>
<td>0.29</td>
<td>28</td>
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<tr>
<td>Median (range)</td>
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<td>0.34 (0.00-0.55)</td>
<td>23 (0-75)</td>
</tr>
<tr>
<td>Responders</td>
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<td></td>
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<tr>
<td>Mean</td>
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<td>0.38</td>
<td>44</td>
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<tr>
<td>Median (range)</td>
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<td>0.36 (0.24-0.55)</td>
<td>48 (0-75)</td>
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<tr>
<td>Nonresponders</td>
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<tr>
<td>Mean</td>
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<td>18</td>
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<tr>
<td>Median (range)</td>
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<td>0.30 (0.00-0.55)</td>
<td>5 (0-75)</td>
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Negative for COOH-terminal immunostaining and radioligand binding

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<th>%POS†</th>
<th>Mutation‡</th>
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<tr>
<td>All tumors</td>
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<td>0.00</td>
<td>45</td>
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<tr>
<td>Responders</td>
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<td>0.00</td>
<td>15</td>
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<tr>
<td>Nonresponders</td>
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<td>0.00</td>
<td>5</td>
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<tr>
<td>Mean</td>
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<tr>
<td>Median (range)</td>
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<td>0.00</td>
<td>25</td>
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<td>0.30</td>
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<td>0.52</td>
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<tr>
<td>Median (range)</td>
<td>0.34 (0.30-0.52)</td>
<td>0 (0-0)</td>
<td>15 (5-65)</td>
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*Mean absorbance (average intensity of AR immunoreactivity).
†Percent positive nuclear area (percentage of total nuclear area stained).
‡Numbering is per the AR gene mutations database (47).
§Mann-Whitney U test probability of difference from responders.

Figure 2. AR immunoreactivity in human prostate tumors. Comparison of the mean absorbance with NH₂- and COOH-terminal AR antisera in 26 primary human prostate tumors. Data for each antibody were generated using quantitative VTA of at least 20 fields of view chosen at random in each sample. The concordance between immunoreactivity detected with the NH₂- and COOH-terminal AR antisera is indicated along with the slope from a line of best fit for the data set. Dotted box, extent of positive immunoreactivity detected by both AR antisera in a subset of breast cancer samples.

4 http://www.mdli.com/.
Statistical analysis. Statistical analysis was done using the Statistical Package for the Social Sciences version 11.0 (SPSS, Chicago, IL). The Spearman’s correlation and Mann-Whitney tests were used to compare AR immunostaining measurements with the two AR antibodies. The one-way ANOVA test and the Dunnet post hoc test were used to determine statistical significance between control and treatment groups. Statistical significance was set at \( P < 0.05 \).

Results

Androgen receptor immunostaining and response to medroxyprogesterone acetate therapy. To investigate further the potential role of AR in mediating response to MPA therapy in breast cancer, we conducted immunohistochemistry with two previously characterized antisera specific for the AR NH2- and COOH-termini, respectively (26, 28), on serial paraffin sections of 83 tumor specimens of patients treated with MPA following failure of tamoxifen. Whereas the majority (48 of 83) of breast tumors in this cohort exhibited nuclear AR immunoreactivity with both NH2- and COOH-terminal antisera (Fig. 1 A), a small proportion (7 of 83) did not exhibit staining with either antibody, and approximately one-third (28 of 83) exhibited staining with only one or other of the two AR antisera (Fig. 1 A; see example in Fig. 1 B).

Strong positive nuclear AR immunoreactivity with both NH2- and COOH-terminal antisera (Fig. 1 A) was detected in all 32 samples from responders to MPA (Fig. 1 C), of which 29 of 32 also showed AR radioligand binding. In contrast, tumors from nonresponders exhibited one of four distinct patterns of AR immunoreactivity: (a) strong staining with both antisera (16 of 51; 31%); (b) staining only with the COOH-terminal antibody (17 of 51; 33%); (c) staining only with the NH2-terminal antibody (11 of 51; 22%); or (d) no immunoreactivity with either NH2- or COOH-terminal antisera (7 of 51; 14%; Fig. 1 D).

For both antibodies, the mean intensity of AR immunoreactivity and the total proportion of nuclear area stained for AR were significantly less in nonresponders than in responders (\( P < 0.001 \); Table 1). AR immunoreactivity in nonresponders was also concordant with AR radioligand binding. Of the 16 of 51 samples positive for both AR antisera, 15 were positive for AR radioligand binding. Similarly, of the 17 of 52 nonresponders negative by radioligand binding, 15 were negative for AR immunostaining (\( \chi^2 = 7.7; P = 0.02 \)).

To show that the greater heterogeneity of AR immunostaining with the two antisera in nonresponders to MPA reflects the AR status in those tumors rather than differences in the sensitivity of the AR NH2- and COOH-terminal antisera, we similarly assessed AR immunoreactivity in a cohort of 26 untreated prostate tumors (Fig. 2). All 26 prostate tumor samples exhibited nuclear immunoreactivity with both NH2- and COOH-terminal AR antisera, with a pattern of immunostaining similar to that for breast cancers that responded to MPA but distinct from nonresponders (Fig. 2).
was a linear relationship between immunoreactivity determined with the NH₂- and COOH-terminal antisera in the prostate cancer samples (slope = 0.95; correlation coefficient = 0.609), indicating a high degree of concordance in the capacity to detect AR with the two antisera.

Detection of loss of function androgen receptor mutations in breast cancer samples. A subgroup of nine tumor samples from nonresponders exhibiting immunoreactivity with the NH₂-terminal AR antibody, but neither discernible AR radioligand binding nor immunostaining with the COOH-terminal AR antibody (Table 1), were analyzed for mutations in the AR gene. SSCP analysis and DNA sequencing of the coding region for the AR LBD (residues 670-919) identified mutations (two missense and one silent) in three of the tumor samples (Table 1). Using the NH₂-terminal AR antisera, we were able to specifically detect both endogenous AR (Fig. 3A) and ectopic wild-type and variant AR containing missense mutations (i.e., AR-M780T and AR-M807V) following transient transfection of AR-negative cell lines (Fig. 3B). Compared with wtAR, AR-M780T and AR-M807V were unable to activate the AR-responsive probasin promoter in transiently transfected MDA-MB-231 human breast cancer cells in the presence of either DHT or MPA (Fig. 3B and C). Similar results were obtained with the MMTV promoter in MDA-MB-231 cells and with both MMTV and probasin promoters in human prostate cancer (PC-3) and monkey kidney (COS-1) cells (data not shown).

Molecular basis for loss of medroxyprogesterone acetate function on androgen receptor variants detected in breast cancer. We initially investigated the basis of the defective response of AR-M780T and AR-M807V to DHT and MPA using ligand-binding analysis in transfected COS-1 cells and in silico modeling. The median ± SE of high-affinity binding sites for DHT and MPA in transfected cells was comparable, being 128 ± 32 and 112 ± 16 fmol/mg protein, respectively. This is consistent with our previous observation of comparable binding for MPA and DHT to the endogenous AR in the AR-positive, PR-negative human breast cancer cell line MDA-MB-453 (21). The AR-M780T variant had a moderately reduced relative binding affinity for DHT (Table 2) but exhibited a more markedly reduced stability compared with wtAR (Fig. 4A). AR-M807V had no apparent capacity to bind DHT (Table 2; Fig. 4A).

In contrast to the results for DHT, the binding affinity of MPA for AR-M780T and AR-M807V was not significantly different to wtAR.
(Table 2), and there was no difference in the stability of the AR-MPA complex for the variant receptors compared with wtAR (Fig. 4C). Analysis of the interaction between MPA and the wtAR by in silico docking analysis generated a binding solution that exhibited only a very small deviation (RMS of <0.1 Å) from the published crystal configuration of the DHT-bound receptor (ref. 34; Fig. 4D). In contrast, the predicted positioning of MPA in AR-M780T (Fig. 4D) and AR-M807V (data not shown) was highly variable, suggesting that both of these mutations have a destabilizing effect on receptor configuration in the presence of MPA.

Inverse relationship between progesterone receptor and androgen receptor in breast cancer cells. Although a significantly higher median AR level was determined by radioligand binding in the primary tumors of responders to MPA therapy compared with nonresponders, a similar level of ER was detected in both groups (Table 3A). Considering that the PR is a well-characterized ER target gene, it was surprising that there was a 3-fold higher median level of PR in nonresponders compared with responders, although this was not statistically significant (Table 3A). To investigate a potential relationship between AR and PR expression, we separated the cohort by median AR level (37 fmol/mg protein). Tumors with high AR had significantly lower median levels of PR (30 fmol/mg protein; P < 0.05; Table 3B). Considering that there was no difference in the level of ER between these two groups (Table 3B), these data suggest that the AR may act as a negative regulator of ER function in breast cancer cells.

Inhibition of estrogen receptor signaling by androgen receptor. We analyzed the effect of DHT and MPA on the estradiol-dependent proliferation of the AR-, ER-, and PR-positive T47D human breast cancer cell line. Increasing concentrations of DHT and MPA resulted in a significant (P < 0.01) dose-dependent decrease in T47D cell proliferation (Fig. 5A and B), reaching an inhibition ~50% of that achieved with 1 μmol/L of the ER antagonist, tamoxifen (Fig. 5A). DHT and MPA were also effective in inhibiting the estradiol-dependent growth of MCF-7 breast cancer cells (Fig. 5C), which are strongly positive for ER and AR but have very low levels of PR (insets in Fig. 5B and C). The inhibition of MCF-7 cell growth by DHT and MPA was reversed by treatment with a specific AR antisense oligonucleotide compared with the scrambled control (Fig. 5C). We confirmed for this experiment, as shown previously (2), a >50% reduction in the level of endogenous AR in cells treated with the AR antisense oligonucleotide compared with the scrambled oligonucleotide control (Fig. 5C). Consistent with an effect of AR on ER function, treatment of T47D cells with 10 nmol/L DHT resulted in an ~40% reduction in the capacity of estradiol to induce PR (Fig. 5D). These results are concordant with our analysis of PR mRNA in T47D cells by quantitative real-time PCR, where 10 nmol/L DHT or MPA reduced estradiol (1 nmol/L) induction of PR RNA levels by 71% and 83%, respectively. To determine if the AR exerts a direct inhibitory effect on ER function, as implied by the above experiments, we assessed ER activity in T47D cells transfected with an ER-specific reporter gene. DHT and MPA were able to inhibit ER activity induced by 1 nmol/L estradiol by >50% (Fig. 6A). In the steroid receptor-negative MDA-MB-231 human breast cancer cell line transfected with AR and ER expression vectors, increasing the ratio of AR to ER resulted in a dose-dependent increase in the inhibition of ER activity in the presence, but not the absence, of 1 nmol/L DHT (Fig. 6B).

Discussion

Although MPA has not been used extensively in the management of breast cancer since the advent of tamoxifen and aromatase inhibitors (13), there is considerable interest in its mode of action

Table 3.

A. Steroid receptor levels in breast tumors as determined by radioligand binding

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Median (range) radioligand binding (fmol/mg protein)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Responders†</td>
<td>Nonresponders‡</td>
</tr>
<tr>
<td>AR</td>
<td>107 (0-547)</td>
<td>15 (0-186)</td>
</tr>
<tr>
<td>ER</td>
<td>91 (0-532)</td>
<td>78 (0-1,275)</td>
</tr>
<tr>
<td>PR</td>
<td>36 (0-893)</td>
<td>118 (0-2,322)</td>
</tr>
</tbody>
</table>

B. ER and PR levels in breast tumors by high or low AR protein

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Median (range) radioligand binding (fmol/mg protein)</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low AR† (≤37 fmol/mg protein)</td>
<td>High AR‡ (&gt;37 fmol/mg protein)</td>
</tr>
<tr>
<td>ER</td>
<td>82 (0-1,275)</td>
<td>79 (0-552)</td>
</tr>
<tr>
<td>PR</td>
<td>127 (0-2,221)</td>
<td>30 (0-2,322)</td>
</tr>
</tbody>
</table>

*Two-tailed probability, Mann-Whitney U test.
†Responders to MPA therapy (n = 31).
‡Nonresponders to MPA therapy (n = 52).
Two-tailed probability, Mann-Whitney U test.
Tumors with ≤ median AR level (n = 43).
*Tumors with > median AR level (n = 40).
given the results of the Woman’s Health Initiative and the Million Women Study, among others, which document an increased risk of breast cancer in women taking MPA in the context of combined hormone replacement therapy (36, 37). We reported previously that response of breast tumors to the synthetic progestin, MPA, is dependent on the level of AR measured biochemically by radioligand binding in the primary tumors but not on PR status (23). However, the conundrum raised by that study was detection of AR in a significant proportion of nonresponders. In the present study, we provide evidence that both the level of the AR and its structure and/or function are critical for response to MPA. The loss of AR, implied by a lack of specific radioligand binding and immunoreactivity with antisera specific for the NH2 and COOH termini of the receptor, most likely accounts for the lack of MPA response in a proportion of nonresponders. Strikingly however, >50% of the nonresponders to MPA exhibited strong immunoreactivity with one or other of the two AR antisera. Where immunoreactivity was detected with one or other antisera, the proportion of positive cells and the intensity of immunostaining were comparable with that observed in samples positive with both antisera, suggesting that altered AR structure or function, rather than loss of AR, may be responsible for the failure of response to MPA. Evidence supporting this hypothesis at a structural level was provided by the identification of missense AR gene mutations (i.e., M780T and M807V) in the LBD that prevent activation of the receptor by DHT and MPA. The mutations were identified by focusing on nine tumors that failed to respond to MPA and exhibited positive AR immunoreactivity with the NH2-terminal antibody but negative with the COOH-terminal antisera and lacked specific DHT binding. These findings are consistent with clinical observations of the inherited syndrome of androgen insensitivity, where substitutions at codons 780 and 807 of the AR confer loss of function and result in the complete form of the disease (38). Importantly, our results suggest that the lack of radioligand binding and AR immunostaining does not necessarily reflect either a loss of AR protein or an androgen insensitivity confering mutation in the LBD of the receptor. It is possible that structural changes or mutations elsewhere in the receptor, such as the AR NH2 terminus or DNA-binding domain, could exert an effect on ligand binding and in vivo immunoreactivity with either NH2- or COOH-terminal antisera. For example, assembly of the Hsp90 chaperone hetero-complex on the AR, which is intricately related to the capacity of steroid receptors to interact with their cognate ligands and to undergo cytoplasmic-nuclear translocation (39), involves specific motifs in the AR-NTD (40). Analagous to variation in the two

Figure 5. Inhibition of estrogen signaling in human breast cancer cells. A, effect of DHT on estradiol stimulated growth of human breast cancer T47D cells. Cells were treated as indicated with estradiol (E2) in the presence or absence of and/or the ER antagonist tamoxifen (TAM) or increasing concentrations of DHT or vehicle control. Viable cells were counted 9 days after initiation of treatment using a hemocytometer. Immunoblot analyses for AR, ER, PR-A, PR-B, and β-actin in untreated T47D cells are shown. B, effect of MPA on estradiol stimulated growth of T47D cells done as detailed in (A). C, effect of DHT and MPA on estradiol stimulated growth of MCF-7 human breast cancer cells. The level of AR, ER, PR-A, PR-B, and β-actin proteins in untreated MCF-7 cells are shown (same immunoblot and exposure as for T47D cells in A). Cells were transfected with AR antisense (as) oligonucleotide or a scrambled control (ctrl) and treated with ligands as indicated. Viable cells were counted 6 days after initiation of treatment using a hemocytometer. Inset, AR knockdown by antisense oligonucleotide compared with β-actin in this experiment. D, representative immunoblot analysis of PR in lysates from T47D cells treated as indicated with estradiol in the presence or absence of DHT and BIC for 48 hours. Quantification of immunoblot is presented as the percentage PR in the presence of 1 nmol/L estradiol.

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NH$_2$-terminal LxxLL-like peptides of the AR that interact with the LBD following ligand binding, mutations in regions required for heterocomplex formation could result in a faster dissociation rate of bound ligand from the receptor (41) or change LBD structure in vivo. Moreover, it was recently found that human tumor cells frequently evolve a more sensitized Hsp90 chaperone heterocomplex than present in normal epithelial cells (42). This provides a mechanism through which tumor-specific changes in chaperone expression, as has been observed in breast cancer (43, 44), could alter the conformation of the wild-type apo-AR and its capacity to bind ligand. Alternatively, as the COOH-terminal AR antibody is targeted to a region of the receptor known to form an integral part of the ligand and cofactor binding surface and to undergo significant ligand-dependent conformational change (34, 45), reduced immunoreactivity may occur in tumors where the levels of available ligand or specific cofactors are altered. Indeed, we have shown previously that the COOH-terminal AR antisera has a reduced ability to detect AR in the absence of DHT (28). For the group of tumors that failed to respond to MPA and were positive for the COOH-terminal AR antisera but negative for the NH$_2$-terminal antibody, changes in the length of the polyglutamine repeat in the receptor or predominant expression of the NH$_2$-terminal truncated isoform (AR-A) in lieu of the full-length AR (10, 46) provide alternative explanations for altered function and immunoreactivity. These possibilities are particularly relevant in the context of the current study, as the ability of MPA to activate the AR is critically dependent on a precise receptor conformation, congruent with its atypical agonist activity in the absence of a N/C interaction (47). Subtle changes in AR structure, brought about by alterations in the maturation/chaperone heterocomplex, phosphorylation of the receptor, or interaction with specific cofactors, could prevent MPA activation, ultimately resulting in its failure to inhibit breast cancer cell growth. In effect, structural changes bought about by these mechanisms may have the capacity to switch MPA from an agonist to an antagonist of the AR. The results of our molecular modeling suggest that the two AR variants identified in this study (i.e., M780T, M807V) have a destabilizing effect on receptor configuration in the presence of MPA. This may be sufficient to eliminate their capacity to become activated by MPA despite continued ability to bind the ligand.

Identification in the entire breast cancer cohort of hormone-naive tumors of an inverse relationship between the level of AR and PR, despite similar levels of ER, suggested that AR may directly oppose ER-mediated growth of breast cancer cells. Our in vivo and in vitro data strongly support the hypothesis that the effects of androgens on breast cancer growth are mediated by specific activation of the AR rather than nonspecific effects on estrogen signaling. Importantly, increasing the amount of AR in breast cancer cell lines resulted in greater inhibition of ER function, which suggests that the level of AR in breast epithelial cells could influence estradiol-induced proliferation and the risk of developing cancer. Loss of AR activity or function in a breast tumor would thereby allow unopposed estrogen stimulation of PR expression and cell growth. A balance between estrogen and androgen signaling in the breast may help explain the epidemiologic data linking AR with breast cancer risk and breast density in women who take MPA as a component of HRT and the in vivo effects of androgens on mammary epithelial cell growth (9, 11, 14, 24, 48). Mechanistically, inhibition of ER could occur as a result of activation of AR-responsive gene pathways that negatively impact on ER function or by physical squelching of common coregulators recruited to the receptors following ligand binding (49). Alternatively, a physical interaction between the AR NH$_2$-terminal domain and the ER LBD, which causes a marked reduction in ER activity in yeast and mammalian cells (50), provides a direct mechanism for AR inhibition of ER signaling.

Our finding that immunohistochemistry with AR antisera to distinct epitopes detects discordant levels of AR in a subset of breast tumors, reflecting not only loss of AR protein but also alterations in AR structure, function, or signaling capacity, has important implications for the use of ER and PR immunostaining in the management of breast cancer. Utilization of a similar dual-antibody strategy for ER and PR could assist in the prediction of clinical responsiveness of breast tumors to conventional hormone therapy.

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References

Decreased Androgen Receptor Levels and Receptor Function in Breast Cancer Contribute to the Failure of Response to Medroxyprogesterone Acetate

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