Selective Loss of AKR1C1 and AKR1C2 in Breast Cancer and Their Potential Effect on Progesterone Signaling

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INTRODUCTION

Progesterone plays an essential role in breast development and cancer formation. The local metabolism of progesterone may limit its interactions with the progesterone receptor (PR) and thereby act as a prereceptor regulator. Selective loss of AKR1C1, which encodes a 20α-hydroxysteroid dehydrogenase [20α-HSD (EC 1.1.1.149)], and AKR1C2, which encodes a 3α-hydroxysteroid dehydrogenase [3α-HSD (EC 1.1.1.52)], was found in 24 paired breast cancer samples as compared with paired normal tissues from the same individuals. In contrast, AKR1C3, which shares 84% sequence identity, and 5α-reductase type I (SRD5A1) were minimally affected. Breast cancer cell lines T-47D and MCF-7 also expressed reduced AKR1C1, whereas the breast epithelial cell line MCF-10A expressed AKR1C1 at levels comparable with those of normal breast tissues. Immunohistochemical staining confirmed loss of AKR1C1 expression in breast tumors, AKR1C3 and AKR1C1 were localized on the same myoepithelial and luminal epithelial cell layers. Suppression of AKR1C1 and AKR1C2 by selective small interfering RNAs inhibited production of 20α-dihydropregesterone and was associated with increased progesterone in MCF-10A cells. Suppression of AKR1C1 alone or with AKR1C2 in T-47D cells led to decreased growth in the presence of progesterone. Overexpression of AKR1C1 and, to a lesser extent, AKR1C2 (but not AKR1C3) decreased progesterone-dependent PR activation of a mouse mammary tumor virus promoter in both prostate (PC-3) and breast (T-47D) cancer cell lines. We speculate that loss of AKR1C1 and AKR1C2 in breast cancer results in decreased progesterone catalysis, which, in combination with increased PR expression, may augment progesterone signaling by its nuclear receptors.

ABSTRACT

Progesterone plays an essential role in breast development and cancer formation. The local metabolism of progesterone may limit its interactions with the progesterone receptor (PR) and thereby act as a prereceptor regulator. Selective loss of AKR1C1, which encodes a 20α-hydroxysteroid dehydrogenase [20α-HSD (EC 1.1.1.149)], and AKR1C2, which encodes a 3α-hydroxysteroid dehydrogenase [3α-HSD (EC 1.1.1.52)], was found in 24 paired breast cancer samples as compared with paired normal tissues from the same individuals. In contrast, AKR1C3, which shares 84% sequence identity, and 5α-reductase type I (SRD5A1) were minimally affected. Breast cancer cell lines T-47D and MCF-7 also expressed reduced AKR1C1, whereas the breast epithelial cell line MCF-10A expressed AKR1C1 at levels comparable with those of normal breast tissues. Immunohistochemical staining confirmed loss of AKR1C1 expression in breast tumors, AKR1C3 and AKR1C1 were localized on the same myoepithelial and luminal epithelial cell layers. Suppression of AKR1C1 and AKR1C2 by selective small interfering RNAs inhibited production of 20α-dihydropregesterone and was associated with increased progesterone in MCF-10A cells. Suppression of AKR1C1 alone or with AKR1C2 in T-47D cells led to decreased growth in the presence of progesterone. Overexpression of AKR1C1 and, to a lesser extent, AKR1C2 (but not AKR1C3) decreased progesterone-dependent PR activation of a mouse mammary tumor virus promoter in both prostate (PC-3) and breast (T-47D) cancer cell lines. We speculate that loss of AKR1C1 and AKR1C2 in breast cancer results in decreased progesterone catalysis, which, in combination with increased PR expression, may augment progesterone signaling by its nuclear receptors.

INTRODUCTION

In women, breast cancer is the most common noncutaneous malignancy, and lifetime exposure to ovarian hormones is a well-recognized risk factor for breast cancer development (1, 2). The actions of both estrogen and progesterone are required for normal growth and maturation of breast tissues, and progesterone is required for terminal duct formation required for lactation (3). Estrogen can also regulate expression of the progesterone receptor (PR), linking the action of both these two hormones and suggesting a complex interplay between estrogen and regulation of progesterone-dependent genes. Inconsistent results in breast cancer cell lines and animal studies have made it difficult to assess a role of progesterone in either development or promotion of breast cancer (4–6). However, women receiving progesterin are at greater risk for development of increased mammographic breast density, suggesting (4–6). However, women receiving progesterin are at greater risk for development of increased mammographic breast density, suggesting (4–6). However, women receiving progesterin are at greater risk for development of increased mammographic breast density, suggesting increased PR expression, may augment progesterone signaling by its nuclear receptors.

Received 5/11/04; revised 7/12/04; accepted 8/11/04.

Grant support: University of Southern California/Norris Comprehensive Cancer Center grant 5P30 CA14089–29 from the National Cancer Institute; Robert E. and Mary R. Wright Foundation; Margaret E. Early Medical Research Foundation; and University of Southern California Research Center for Liver Disease DK98-016. This work was presented in part at the 85th ENDO Annual Meeting in Philadelphia, Pennsylvania on June 21, 2003.

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AKR1C2 may act as a prereceptor of PR activity. The significance of these findings has been extended to assess whether changes in expression of AKR1C family members could modify progesterone-dependent transcriptional activity of the mouse mammary tumor virus (MMTV) promoter by PR-B.

**MATERIALS AND METHODS**

**Chemicals and Supplies.** All chemicals were of molecular biology grade or higher and were purchased from Sigma (St. Louis, MO), unless otherwise stated. Molecular biology reagents were purchased from Promega (Madison, WI), Roche Molecular Biochemicals (Indianapolis, IN), and Life Technologies, Inc. (Gaithersburg, MD). Cell culture supplies were purchased from Invitrogen (Carlsbad, CA).

**Breast Tissues and RNA Extraction.** Human breast samples were processed according to our previously described method (24). Twenty-four paired breast tumors and their paired surrounding unaffected tissues were selected from the University of Southern California/Norris Comprehensive Cancer Center (Los Angeles, CA) or Olive View-University of California Los Angeles Medical Center (Sylmar, CA) after institutional review board approval. Samples were fresh frozen in liquid nitrogen, and sections (5 μm) were subsequently reviewed by pathologists for pathological diagnosis along with immunohistochemistry staining for estrogen receptor (ER), PR, Ki-67, and Her2/neu status. Only paired samples in which breast tumors contained >90% tumor cells and normal tissue lacking any tumor cells were used. Clinical information and surgical pathology reports were available without any patient identifiers. The frozen tissues were homogenized with tissue pulverizers (Spectrum Laboratories, Rancho Dominguez, CA), and total RNA was prepared using the RNeasy Mini Kit (Qiagen, Valencia, CA) as described previously (24).

**Quantitative Real-Time Polymerase Chain Reaction Assay.** Real-time PCR for AKR1C1, AKR1C2, AKR1C3, and SRD5A1 was performed as described previously (24). SRD5A1 (forward primer, 5′-ATCTCCCTGGCCTAGTTCTCC-3′; reverse primer, 5′-TGGCACTAGAAGGCCGGTAAT-3′; probe, fluorescein amidite-5′-CGTCACACTACGGGACTGGTGCGC-3′-black hole quencher) was designed using Primer Express version 2.0 software (Applied Biosystems, Foster City, CA). Random primed cDNA libraries were prepared for TaqMan quantitative PCR by using the Omniscript Kit (Qiagen) with random hexamers (Applied Biosystems), and relative expression was calculated as described previously (24).

**Cell Culture.** MCF-7, MCF-10A, T-47D, and PC-3 cell lines were all purchased from American Type Culture Collection (Rockville, MD). Cell lines were cultured in RPMI 1640 with 10% fetal bovine serum (FBS), except for MCF-10A, which was grown in Clonetics Mammary Epithelial Growth Medium (MEGM Bullet Kit, CC-3051, serum-free) supplemented with 100 ng/mL EGF (Life Science, Boston, MA) was added to the media, and cells were then allowed to recover for 24 hours. Nonsilencing fluorescein-labeled siRNA (Qiagen) was used as the control for siRNA transfections.

**Progestosterone Metabolism.** MCF-10A cells (8 × 10^5) were seeded onto 6-well plates and grown for 24 hours to approximately 50% confluence. Small interfering RNAs (4.5 μg) were diluted in 100 μL of suspension buffer, vortexed, and mixed with 15 μL of RNaseFect. Cells were incubated with the samples for 5 to 10 minutes at room temperature, the medium was exchanged with 300 μL of growth medium, and then cells were allowed to recover for 24 hours. Nonsilencing fluorescein-labeled siRNA (Qiagen) was used as the control for siRNA transfections.

**T-47D Proliferation Studies.** T-47D cells were grown in RPMI 1640 without phenol red with 4% charcoal dextran-treated FBS (HyClone, Logan, UT) and treated with different concentrations of progesterone [0.1% in ethanol (v/v)] or 20α-dihydroprogesterone. Cell proliferation assays were performed as described previously (25, 27) with the following modifications: Twenty-four hours before initiation of growth studies, cells were treated with siRNA as described above. A total of 1.5 × 10^5 cells were then seeded into 12-well tissue culture dishes from a common cell culture and grown for an additional 24 hours. Progesterone was then added, and half of the media was replaced on a daily basis. The number of cells in quadruplicate wells was then determined daily with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay using Bio-Rad Microplate Reader Model 3550 (Bio-Rad, Hercules, CA) after a standard curve confirmed a linear relationship between cell number and absorbance.

**PR-B–Dependent Transactivation Assay.** Permanently transfected PC-3 cells lines with varying stable expression levels of AKR1C1, AKR1C2, or AKR1C3 were used for progesterone-dependent transactivation experiments with cotransfection of human PR-B. These PC-3 cell lines were individually transfected with an MMTV-luciferase reporter plasmid provided by Dr. Gerhard A. Coetzez, Keck School of Medicine at University of Southern California, Los Angeles, CA), 0.2 μg of pCMV-HP-B expression plasmid (kindly provided by Dr. Michael R. Stallcup, Keck School of Medicine at University of Southern California), and 5 ng of Renilla luciferase plasmid pRL-SV40 (Promega) to control for transfection efficiency. Cells were transfected with 15 μL of SuperFect (Qiagen) in 600 μL of RPMI 1640 and allowed to recover for 36 hours in RPMI 1640 with 4% charcoal dextran-treated FBS. Cells were then washed with PBS and treated for 16 hours with 100 μm/L progesterone in 4% charcoal dextran-treated fetal calf serum. Luciferase activity was measured using the Luminosas Anset instrument (Labsystems,
DECREASED AKR1C1 AND AKR1C2 IN BREAST CANCER

Franklin, MA) and the Dual Luciferase Activity Kit (Promega). The ratio of firefly to Renilla luciferase activity was normalized to total protein, and luciferase activity of control PC-3 cells was arbitrarily defined as 100 ± SD.

RESULTS

Relative Expression Profile of AKR1C Family Members and SRD5A1 in Paired Breast Tissue Samples. The relative expression profiles of AKR1C1, AKR1C2, AKR1C3, and SRD5A1 were determined in 24 paired breast cancer and normal tissue samples using RNase P as the internal control. RNase P expression was equivalent in tumor and normal tissue (data not shown). Table 1 lists the relative changes in gene expression for AKR1C family members and SRD5A1 in individual pairs of tissue samples, along with clinical features and PR, ER, Her2/neu, and Ki-67 status. A substantial (defined as >5-fold) decrease in AKR1C1 expression was found in 13 of 24 cases, AKR1C2 expression was absent in tumors or reduced in 6 of these 13 cases. AKR1C3 expression was only significantly reduced in one case without a significant decrease in AKR1C1. SRD5A1 expression was significantly decreased in only four cases and increased (5-fold) in three cases, and no apparent relationship existed between reduced expression of SRD5A1 and that of AKR1C1. No consistent pattern was found in the expression profile for AKR1C4 or SRD5A1 and PR, ER, Her2/neu, or Ki-67 status. Thus, reduced gene expression of AKR1C1 appears to be unrelated to PR or ER status.

Development of AKR1C-Specific Antisera and Immunohistochemical Staining in Breast Tissue Samples. Rabbits received injection with synthesized peptides to develop antisera that recognize specific family members, despite the high sequence homology of family members. Lysates harvested from permanently transfected PC-3 cells expressing AKR1C1, AKR1C3, or AKR1C2 (24) were used to assess specificity of rabbit antisera by Western blot. As illustrated in Fig. 1A, α1850 recognized all transfected AKR1C family members expressed in PC-3 cells. We confirmed that α7548, designed according to Pelleiter et al. (25), selectively recognized only AKR1C3, whereas α6621 recognized both AKR1C1 and AKR1C3, but not AKR1C2. As shown in Fig. 1B, both α6621 and α7548, but not their preimmune sera, stained normal myoepithelial and luminal epithelial cells in the alveoli, which was blocked by preincubation with excess peptide, thereby confirming the specificity of IHC staining.

As illustrated in Fig. 1B, IHC staining with both α6621 and α7548 revealed predominant staining on the myoepithelial and luminal epithelial cells. Because α6621 cross-reacts with both AKR1C1 and AKR1C3, selective localization for AKR1C1 was determined in paired BT6 and BN6 tissue samples that lacked AKR1C3 expression. In Fig. 1C, no IHC staining with α7548 was observed, confirming the lack of expression of AKR1C3. IHC staining with α6621, which could only correspond to AKR1C1, was localized on the myoepithelial and luminal epithelial cells. Endothelial cells were also stained with this antisera (data not shown). Decreased IHC staining was observed on the corresponding tumor sample, in close agreement with the 48-fold decrease in relative expression of AKR1C1. Thus, AKR1C1 is normally expressed in the same location as AKR1C3, with staining observed on a majority of myoepithelial and luminal epithelial cells, the latter being the same site of PR expression (3, 29).

Table 1: Clinical features and changes in relative expression of AKR1C family members and SRD5A1 mRNAs in paired samples of breast tumors versus unaffected tissues

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Diagnosis</th>
<th>Age (y)</th>
<th>ER</th>
<th>PR</th>
<th>Ki-67</th>
<th>Her2</th>
<th>AKR1C1 (mean ± SD)</th>
<th>AKR1C2 (mean ± SD)</th>
<th>AKR1C3 (mean ± SD)</th>
<th>SRD5A1 (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTN1</td>
<td>Inv duct</td>
<td>64</td>
<td>+</td>
<td>+</td>
<td>15%</td>
<td>+</td>
<td>12.8 ± 1.1</td>
<td>ND</td>
<td>0.2 ± 0.0</td>
<td>84.5 ± 6.5</td>
</tr>
<tr>
<td>BTN4</td>
<td>Inv duct</td>
<td>52</td>
<td>−</td>
<td>+</td>
<td>NA</td>
<td>+</td>
<td>−3.7 ± 0.4</td>
<td>1.9 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>30.3 ± 2.2</td>
</tr>
<tr>
<td>BTN5</td>
<td>Inv lob</td>
<td>68</td>
<td>−</td>
<td>+</td>
<td>5%</td>
<td>−</td>
<td>5.0 ± 0.5</td>
<td>ND</td>
<td>ND</td>
<td>−24.5 ± 2.1</td>
</tr>
<tr>
<td>BTN6</td>
<td>Inv lob</td>
<td>53</td>
<td>+</td>
<td>+</td>
<td>40%</td>
<td>−</td>
<td>−48.3 ± 3.7</td>
<td>ND</td>
<td>ND</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>BTN7</td>
<td>Inv duct</td>
<td>35</td>
<td>+</td>
<td>+</td>
<td>10%</td>
<td>+/−</td>
<td>−14.7 ± 1.3</td>
<td>ND</td>
<td>ND</td>
<td>1.7 ± 0.1</td>
</tr>
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<td>BTN8</td>
<td>Inv duct</td>
<td>46</td>
<td>−</td>
<td>+</td>
<td>90%</td>
<td>−</td>
<td>−342 ± 25.8</td>
<td>−3.6 ± 0.3</td>
<td>−4.9 ± 0.3</td>
<td>−4.6 ± 0.3</td>
</tr>
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<td>BTN9</td>
<td>Inv duct</td>
<td>33</td>
<td>+</td>
<td>+</td>
<td>20%</td>
<td>+</td>
<td>−285 ± 16.9</td>
<td>−3.5 ± 0.5</td>
<td>−15.0 ± 0.1</td>
<td>−4.6 ± 0.2</td>
</tr>
<tr>
<td>BTN10</td>
<td>Inv duct</td>
<td>50</td>
<td>+</td>
<td>+</td>
<td>5%</td>
<td>+</td>
<td>1.4 ± 0.1</td>
<td>ND</td>
<td>−13.0 ± 1.1</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>BTN11</td>
<td>Inv duct</td>
<td>56</td>
<td>+</td>
<td>+</td>
<td>20%</td>
<td>NA</td>
<td>−67.1 ± 5.8</td>
<td>−2.4 ± 0.2</td>
<td>−3.7 ± 0.2</td>
<td>−6.6 ± 0.4</td>
</tr>
<tr>
<td>BTN15</td>
<td>Muc CA</td>
<td>49</td>
<td>+</td>
<td>+</td>
<td>10%</td>
<td>−</td>
<td>−2.1 ± 0.1</td>
<td>ND</td>
<td>1.7 ± 0.1</td>
<td>−237 ± 29.6</td>
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<tr>
<td>BTN17</td>
<td>ADH</td>
<td>58</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>−3.6 ± 0.2</td>
<td>1.7 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>−237 ± 29.6</td>
</tr>
<tr>
<td>BTN19</td>
<td>Inv lob</td>
<td>58</td>
<td>+</td>
<td>+</td>
<td>10%</td>
<td>−</td>
<td>−330 ± 25.4</td>
<td>−14.1 ± 7.8</td>
<td>−3.6 ± 0.2</td>
<td>−2.1 ± 0.1</td>
</tr>
<tr>
<td>CHTN1</td>
<td>Inv duct</td>
<td>28</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>6.4 ± 0.2</td>
<td>2.4 ± 0.2</td>
<td>−3.6 ± 0.3</td>
<td>4.8 ± 0.2</td>
</tr>
<tr>
<td>CHTN2</td>
<td>Inv duct</td>
<td>37</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>−42.6 ± 3.7</td>
<td>3.6 ± 0.3</td>
<td>2.9 ± 0.3</td>
<td>6.6 ± 0.4</td>
</tr>
<tr>
<td>BTN20</td>
<td>DCIS</td>
<td>50</td>
<td>+</td>
<td>+</td>
<td>15%</td>
<td>+</td>
<td>−7.7 ± 0.5</td>
<td>12.7 ± 0.9</td>
<td>−15.0 ± 1.1</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>BTN21</td>
<td>Inv duct</td>
<td>65</td>
<td>+/−</td>
<td>+/−</td>
<td>20%</td>
<td>+</td>
<td>−6.3 ± 4.4</td>
<td>−15.3 ± 1.1</td>
<td>1.2 ± 0.1</td>
<td>−12.0 ± 1.1</td>
</tr>
<tr>
<td>BTN22</td>
<td>Inv duct</td>
<td>40</td>
<td>+</td>
<td>+</td>
<td>20%</td>
<td>+</td>
<td>−1198 ± 83.8</td>
<td>ND</td>
<td>34.5 ± 2.4</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>BTN23</td>
<td>Inv lob</td>
<td>47</td>
<td>+</td>
<td>+</td>
<td>15%</td>
<td>−</td>
<td>−10.9 ± 0.8</td>
<td>−11.5 ± 0.8</td>
<td>−12.1 ± 0.1</td>
<td>−15.0 ± 1.1</td>
</tr>
<tr>
<td>BTN24</td>
<td>Inv lob</td>
<td>72</td>
<td>+</td>
<td>+</td>
<td>15%</td>
<td>−</td>
<td>−199.2 ± 14.1</td>
<td>−288 ± 20.2</td>
<td>−6.5 ± 0.5</td>
<td>−1.7 ± 0.1</td>
</tr>
<tr>
<td>BTN25</td>
<td>Inv duct</td>
<td>37</td>
<td>+</td>
<td>+</td>
<td>10%</td>
<td>+</td>
<td>−2506 ± 175.4</td>
<td>−24.6 ± 1.7</td>
<td>−1.6 ± 0.1</td>
<td>−2.9 ± 0.2</td>
</tr>
<tr>
<td>BTN26</td>
<td>Inv duct</td>
<td>42</td>
<td>−</td>
<td>−</td>
<td>10%</td>
<td>+</td>
<td>ND in cancer</td>
<td>ND in cancer</td>
<td>−3.6 ± 0.3</td>
<td>−4.6 ± 0.2</td>
</tr>
<tr>
<td>BTN30</td>
<td>Inv duct</td>
<td>62</td>
<td>+</td>
<td>+</td>
<td>25%</td>
<td>+</td>
<td>−1.6 ± 0.1</td>
<td>2.8 ± 0.2</td>
<td>−5.7 ± 0.4</td>
<td>−9.2 ± 0.6</td>
</tr>
<tr>
<td>BTN31</td>
<td>Inv duct</td>
<td>58</td>
<td>+</td>
<td>+</td>
<td>10%</td>
<td>+</td>
<td>24.2 ± 1.7</td>
<td>8.1 ± 0.6</td>
<td>15.5 ± 1.1</td>
<td>−2.3 ± 0.2</td>
</tr>
<tr>
<td>BTN32</td>
<td>Inv duct</td>
<td>51</td>
<td>+</td>
<td>+</td>
<td>20%</td>
<td>+</td>
<td>−222 ± 12.2</td>
<td>−3.9 ± 0.4</td>
<td>−6.6 ± 0.4</td>
<td>−12.0 ± 1.1</td>
</tr>
</tbody>
</table>

NOTE: Numbers listed in the table represent fold changes in mRNA expression in tumor samples compared with paired normal samples relative to RNase P expression. A positive number refers to increased mRNA expression in tumor, whereas a negative number represents decreased expression in tumor as compared with unaffected tissue.

Abbreviations: Inv duct, invasive ductal carcinoma; ND, not detectable in both normal and cancer tissues; NA, not available; Inv lob, invasive lobular carcinoma; Muc CA, mucinous carcinoma; ADH, atypical ductal hyperplasia; DCIS, ductal carcinoma in situ; ND in cancer, not detectable in cancer tissues.
mRNA identified in the tumor samples closely corresponds with loss of protein expression. Due to the lack of AKR1C2-specific antisera, we suspect (but cannot confirm) that decreased AKR1C2 mRNA will be associated with loss of protein expression.

Reduced Relative Expression of AKR1C1 in Human Breast Cancer Cell Lines. Gene and protein levels of AKR1Cs were determined in the established human breast cancer cell lines MCF-7 and T-47D as well as in human breast epithelial cell line MCF-10A (30).

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Changes in AKR1Cs in tumor vs. normal tissue (fold)</th>
<th>α6621 in normal tissue</th>
<th>Changes in AKR1Cs in tumor vs. normal tissue</th>
<th>α7548 in normal tissue</th>
<th>α7548 in cancer tissue</th>
</tr>
</thead>
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<tr>
<td>BTN1</td>
<td>13</td>
<td>1+</td>
<td>2+</td>
<td>7</td>
<td>2+, myoepithelial cell 2+, focally (10%)</td>
</tr>
<tr>
<td>BTN5</td>
<td>5</td>
<td>2+</td>
<td>+/− to 1+</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>BTN6</td>
<td>−48</td>
<td>3+</td>
<td>ND</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>BTN13</td>
<td>−67</td>
<td>2+</td>
<td>−4</td>
<td>2+, myoepi&gt;luminal</td>
<td>Negative</td>
</tr>
<tr>
<td>BTN17</td>
<td>−44</td>
<td>2+</td>
<td>1+</td>
<td>1</td>
<td>2+, myoepi&gt;luminal</td>
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<td>8</td>
<td>2+</td>
<td>−1</td>
<td>1</td>
<td>NA</td>
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<td>BTN21</td>
<td>−63</td>
<td>2+</td>
<td>− to +</td>
<td>1</td>
<td>1+</td>
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<td>BTN22</td>
<td>−199</td>
<td>2+</td>
<td>2+, diffuse cytoplasm</td>
<td>−35</td>
<td>Negative</td>
</tr>
<tr>
<td>BTN23</td>
<td>−11</td>
<td>2+</td>
<td>−1</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>BTN24</td>
<td>−199</td>
<td>2+</td>
<td>2+</td>
<td>1</td>
<td>1+, myoepi&gt;luminal</td>
</tr>
<tr>
<td>BTN28</td>
<td>ND in cancer</td>
<td>3+</td>
<td>+/− to 1+</td>
<td>−4</td>
<td>1+, rare myoepithelium</td>
</tr>
</tbody>
</table>

NOTE. The intensity of the staining was graded as follows on a scale of 1 to 3: negative, lack of staining; 1+, weak staining; 2+, moderate staining; and 3+, strong staining. Distribution of the staining was reported as diffuse (staining present in a majority of the glands) or focal (staining on only some of the glands).

Abbreviations: ND, not detectable; NA, not available; myoepi, myoepithelial cell; luminal, luminal epithelial cell.
As shown in Fig. 2A, significantly less AKR1C1 was found in MCF-7 and T-47D cell lines as compared with MCF-10A, whereas AKR1C3 was equivalently expressed in all cell lines. α7548 Western blot data in Fig. 2B confirmed that AKR1C3 was equally expressed in all cell lines, whereas use of α6621 showed significantly less AKR1C1 expression in MCF-7 and T-47D cell lines as compared with MCF-10A, which paralleled the AKR1C1 gene expression profile.

**Progestosterone Metabolism Catalyzed by AKR1C1 and AKR1C2.**
To assess the role of AKR1C1 and AKR1C2 on progestosterone metabolism, siRNAs were developed. Pilot results showed that Seq-A selectively suppressed AKR1C1, whereas Seq-D suppressed both AKR1C1 and AKR1C2 without affecting expression of AKR1C3, as illustrated in Fig. 2C. The ability of these two siRNAs to alter progestosterone metabolism was then monitored in MCF-10A cells,
which expression levels of AKR1C1 comparable with those found in normal tissue. In Fig. 2D and E, suppression of AKR1C1 in MCF-10A cells significantly reduced the conversion of progesterone to 20α-dihydroprogesterone in media and cell lysates.

Suppression of AKR1C1 and AKR1C2 leads to additional and significant inhibition of 20α-dihydroprogesterone formation. This reduction in 20α-dihydroprogesterone correlated with a significant increase in progesterone in both media and cell lysates.

Suppression of AKR1C1 Alone or with AKR1C2 Affects Progesterone-Mediated Inhibition of Cellular Growth of T-47D. To assess potential loss of AKR1C1 and AKR1C2 on progesterone-dependent growth, siRNA suppression of AKR1C1 and AKR1C1/AKR1C2 in T-47D cells was determined because they endogenously express PRs. In Fig. 2F, suppression of AKR1C1 alone or with AKR1C2 is illustrated. The same cells were then used for proliferation assays with progesterone treatments. As shown in Fig. 2G, no significant changes were found in those cell lines treated with siRNAs in the absence of progesterone. In Fig. 2H and I, progressive inhibition of cellular proliferation was observed with suppression of both AKR1C1 and AKR1C1/AKR1C2 in the presence of 10⁻⁸ or 10⁻¹⁰ mol/L progesterone, suggesting that reduced metabolism of progesterone in T-47D is responsible for progesterone-mediated inhibition of cellular growth.

Inhibition of Progesterone-Dependent Transactivation by AKR1C1 and AKR1C2. To determine whether progesterone catabolism can alter PR-B ligand-dependent activity, the activity of a MMTV luciferase reporter gene was evaluated in the prostate cancer cell line PC-3 stably expressing AKR1C1, AKR1C2, or AKR1C3. These cells, along with PC-3 cells stably transfected with vector alone, were transiently transfected with pCMV-hPR-B, a luciferase reporter driven by the MMTV promoter, as well as a Renilla luciferase to control for transfection efficiency. In the absence of progesterone or PR-B, no activity was detectable (data not shown). In Fig. 3A, two different expression levels of AKR1C1 and AKR1C2, but not AKR1C3, in PC-3 cells were able to inhibit progesterone-dependent activation of the MMTV promoter, indicating that AKR1C1 and AKR1C2 metabolize progesterone to weaker activators of PR-B (17). Despite low levels of AKR1C1 expression, AKR1C1 completely inhibited luciferase activity, confirming that 20α-HSD can effectively block PR-B activation. Despite adequate levels of AKR1C3, AKR1C3 failed to inhibit progesterone-dependent activation, confirming that progesterone is a poor substrate for AKR1C3. These findings were extended to T-47D, which endogenously expresses PRs. As shown in Fig. 3B, different amounts of AKR1C1 plasmid transfected into T-47D cells together with MMTV luciferase reporter plasmid significantly inhibited luciferase activity.

**DISCUSSION**

Catabolism of steroid hormones can modulate availability of critical ligands for their cognate transcription factors and thereby function as effective prereceptor regulators of gene expression. This type of regulation is best characterized for the mineralocorticoid receptor, in which selective expression of 11β-hydroxysteroid dehydrogenase type II in aldosterone target tissue prevents inappropriate activation of the mineralocorticoid receptor by cortisol (31). A similar paradigm is also developing for the prereceptor regulation of progesterone activity by AKR1C1 (17, 31). For example, as the human placenta comes to term, increased 20α-HSD (but not 3α-HSD or 17β-hydroxysteroid dehydrogenase) activity is observed, implicating it as the major catabolic pathway for progesterone elimination (15, 16). Besides the placenta, studies in the human kidney further support this concept because expression of AKR1C1 protects the mineralocorticoid receptor from binding with progesterone, an effective aldosterone antagonist (32, 33). This may be especially important during pregnancy, when serum progesterone levels may reach up to 300 to 700 nmol/L by the end of the third trimester (34). The function of AKR1C1 in the kidney may thus imitate that of 11β-hydroxysteroid dehydrogenase type II to prevent inappropriate occupation of ligand-dependent transcription factor.

We initially assessed the relative expression of AKR1C1 and two highly related family members using a gene-specific real-time PCR. Although mRNA levels of the AKR1C1 family members had been quantified by semi-quantitative reverse transcription-PCR and RNase protection methods (35, 36), real-time PCR provides a reliable and highly accurate method for determining the expression of a gene in a given cell type. During pregnancy, the expression of AKR1C1 was found to be significantly increased in the human kidney further supporting this concept because expression of AKR1C1 protects the mineralocorticoid receptor from binding with progesterone, an effective aldosterone antagonist (32, 33). This may be especially important during pregnancy, when serum progesterone levels may reach up to 300 to 700 nmol/L by the end of the third trimester (34). The function of AKR1C1 in the kidney may thus imitate that of 11β-hydroxysteroid dehydrogenase type II to prevent inappropriate occupation of ligand-dependent transcription factor.
reproducible method to monitor large changes in relative gene expression. On average, we noticed that approximately half of the 24 cases demonstrated a substantial loss of AKR1C1 expression, defined as a >5-fold reduction in tumor as compared with paired unaffected tissue. A similar reduction in AKR1C2 expression was also found in half of these cases, whereas AKR1C3 expression was relatively unchanged. Reduction in AKR1C1 expression was observed in a previous microarray study of human breast cancer, and loss of 20α-HSD activity occurs in 7,12-dimethylbenz(a)anthracene-induced breast tumors in rats (37, 38). Others reported a similar reduction in AKR1C1 and AKR1C2 gene expression by reverse transcription-PCR in nine breast cancer tumors as compared with unaffected surrounding tissue from the same individuals (39). They also noted a significant increase in SRD5A1 expression in tumors as compared with normal breast tissues in contrast to our findings, which may be due to differences in the tissue sample pool sizes, stage of tumor samples, and accuracy of the RNA quantitative techniques. We confirmed that decreased protein expression of AKR1C1 was associated with the previously reported reduction in AKR1C1 gene expression in the MCF-7 and T-47D breast cancer cell lines in contrast to the breast epithelial cell line MCF-10A (35). This pattern differed from that of AKR1C3, which was equally expressed in all cell lines. Furthermore, reduced AKR1C1 and AKR1C2 expression in MCF-10A cells by siRNA suppression leads to decreased production of 20α-hydroxy or 3α-hydroxy progesterone metabolites. Thus, decreased expression of AKR1C1 and AKR1C2 observed in human samples is mimicked in certain breast cancer cell lines, which will prove useful for future studies on the AKR1C gene family.

Cellular localization of specific AKR1C members is difficult to determine due to their high sequence similarity. Immunohistochemistry with AKR1C3-specific antiserum a7548 prominently stained myoepithelial and luminal epithelial cells as observed previously (25), and AKR1C1 was localized to the same epithelial layers in samples that lacked ARK1C3 expression (25). Although a6621 was able to recognize both AKR1C1 and AKR1C3 using the Western blot technique, a6621 IHC staining closely matched AKR1C1 gene expression, and a6621 IHC staining could be used to evaluate relative AKR1C1 expression in pathological samples.

The MCF-10A cell line was used to assess the physiologic function of AKR1C1 because it expresses AKR1C1 at levels comparable with those found in normal breast tissues. Selective reduction of AKR1C1 by siRNA suppression in MCF-10A leads to a decrease in conversion of progesterone to 20α-dihydroprogesterone, present in both cell lysate and media, which was further reduced with loss of AKR1C2. Substantial decrease in 20α-dihydroprogesterone production was associated with a corresponding increase in progesterone levels in MCF-10A cells. Progesterone-mediated inhibition of growth in T-47D cells was also observed with suppression of AKR1C1, which was further enhanced in the absence of AKR1C2 expression. The loss of these catabolic enzymes in breast cancer presumably leads to increased progesterone levels, which, in combination with increased PR, could enhance progesterone-responsive gene expression.

Confounding findings have been reported in the literature on the proliferative effects of progesterone in established human breast cell lines, with a majority of studies reporting growth-inhibitory effects (6, 28, 40–43). However, progesterone was found to cause cells to enter one mitotic cycle with induction of genes associated with cell growth (44, 45). Although progestins are proliferative when administered in a transient or sequential manner, sustained treatment results in growth arrest (46). Our data demonstrated that suppression of AKR1C1 or AKR1C2 and AKR1C2 can significantly inhibit cellular proliferation by progesterone as compared with control cell lines.

The role of progesterone as an antiproliferative agent in cell lines does not mimic its function in vivo. A majority of studies in animals and humans find that progesterin treatment promotes growth in vivo (40, 47, 48). In the recent Women Health Initiative Study, increased incidence of total and invasive breast cancer was observed in the estrogen + progestin group as compared with the placebo group (7). The risks of invasive lobular carcinoma and invasive ductal carcinoma were also significantly increased among women who used combined estrogen + progestin therapy (with continuous or sequential progestin use), but not among those who solely used estrogen therapy (49), strongly implicating progesterone in combination with estrogen as stimulating cellular proliferation.

To explain how loss of AKR1C1 and AKR1C2 expression could modify progesterone-dependent PR-B activation, a progesterone-dependent MMTV reporter activity was monitored in two different cell lines. Prostate cancer PC-3 cells stably expressing either AKR1C1 or AKR1C2, but not AKR1C3, were able to reduce progesterone-dependent transactivation of the MMTV reporter construct cotransfected PR-B. AKR1C1 was able to completely inhibit progesterone-dependent activity, despite its low level of expression. Increasing concentrations of progesterone were able to overcome this kind of inhibition (data not shown), indicating that metabolism, not some nonspecific process, is responsible for reduced PR-B activation. Studies in the T-47D cell line confirmed that AKR1C1 was able to inhibit progesterone-dependent PR-B activation in a breast-derived cell line. In addition to 20α-HSD, the 3α-HSD of AKR1C2 can also inhibit progesterone-dependent activation of PR-B, although not as effectively as AKR1C1.

In normal breast tissues, AKR1C1 is localized predominately in the myoepithelial cells and in the luminal epithelial cells, which also express PR (3, 50). We speculate that expression of AKR1C1 and possibly AKR1C2 in normal tissue may regulate progesterone-dependent gene expression by limiting progesterone’s interactions with nuclear PRs. This observation resembles our recent finding of selective reduction in AKR1C2 expression, but not AKR1C3, in prostate cancer samples (24), suggesting that specific yet highly related AKR1C family members can act as prereceptor regulators for ligand-dependent transcription factors. Thus, AKR1C1 may limit progesterone-dependent signaling of nuclear PRs and thereby indirectly regulate gene expression. This is compatible with the role of AKR1C1 to limit the inhibitory interactions of progesterone with mineralocorticoid receptor.

We hypothesize that loss of key catabolic enzymes in breast cancer tissues in combination with increased expression of PR may enhance progesterone-dependent gene expression. In addition to increased progesterone levels and differences in progesterone metabolites as a result of loss of AKR1C1 or AKR1C2, changes in relative expression of PR-A
and PR-B in breast cancer could also alter the spectrum of progesterone-regulated genes (51). Wiebe et al. (28, 35) have also reported a differential effect of specific progesterone metabolites as either growth promoters or inhibitors of the cell cycle in the MCF-7 cell line, suggesting a specific role for progesterone metabolites. The appearance of specific progesterone metabolite binding sites on MCF-7 membranes further supports a role for progesterone metabolites as signaling molecules (52). Thus, metabolites of progesterone may also have unsuspected effects on cell growth. Fig. 4 illustrates the potential role of AKR1C1 and AKR1C2 functioning as a prereceptor regulator of progesterone interactions with PRs. Loss of AKR1C1 in combination with increased PR in selective breast cancers could therefore provide therapeutic opportunities to block ligand-dependent activation of PRs by using antiprogestational agents.

ACKNOWLEDGMENTS

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