15-Hydroxyprostaglandin Dehydrogenase Is a Tumor Suppressor of Human Breast Cancer

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Abstract
Prostaglandin E2 plays a growth-stimulatory role in breast cancer, and the rate-limiting enzyme in its synthesis, cyclooxygenase-2, is often overexpressed in these cancers. Little is known about the role of the key prostaglandin catabolic enzyme 15-hydroxyprostaglandin dehydrogenase (15-PGDH) in breast cancer pathogenesis. Using a pharmacologically based screen for epigenetically silenced genes, we found low levels of 15-PGDH in MDA-MB-231 cells [estrogen receptor (ER) negative] but high levels in MCF-7 cells (ER positive) and observed its up-regulation following demethylation treatment. Further analysis revealed methylation of the 15-PGDH promoter in one breast cancer cell line and 30% of primary tumors. Analysis of 15-PGDH expression revealed low levels in 40% of primary breast tumors and identified a correlation between 15-PGDH and ER expression. Transfection assays showed that transient up-regulation of 15-PGDH levels in MDA-MB-231 cells resulted in a decreased clonal growth, and stable up-regulation significantly decreased the ability of these cells to form tumors in athymic mice. In contrast, transient silencing of 15-PGDH in MCF-7 cells resulted in their enhanced proliferation, and a stable silencing in these cells enhanced cell cycle entry in vitro and tumorigenicity in vivo. Forced expression of 15-PGDH inhibited the ER pathway and silencing of 15-PGDH up-regulated expression of aromatase. In addition, 15-PGDH levels were down-regulated by estrogen but up-regulated by the tumor suppressor gene CAAT/enhancer binding protein α. Our results indicate for the first time that 15-PGDH may be a novel tumor suppressor gene in breast cancer, and suggest that this enzyme can modulate the ER pathway. (Cancer Res 2006; 66(15): 7818-23)

Introduction
Ample data show an important role for prostaglandin E2 (PGE2) in the development of breast cancer. High PGE2 levels within breast tumors are associated with increased proliferation, invasiveness, resistance to apoptosis, and angiogenesis (1, 2), and aberrant expression of cyclooxygenase-2 (COX-2), the rate-limiting enzyme in prostaglandin biosynthesis, can be found in the majority of breast cancers and is associated with an unfavorable outcome (3, 4). PGE2 is a major stimulator of expression of aromatase, thus leading to increased synthesis of estrogen within the breast (5). PGE2 levels are regulated not only by its synthesis but also by its degradation. The key enzyme responsible for the biological inactivation of prostaglandins is NAD+-linked 15-hydroxyprostaglandin dehydrogenase (15-PGDH; ref. 6). Recent studies identified a tumor suppressor activity of 15-PGDH in colon, lung, and bladder cancers (7–10) and suggested epigenetic silencing of the enzyme by DNA methylation and histone modification (11).6 However, the role of 15-PGDH in breast cancer tumorigenesis has not yet been elucidated.

Recently, we used a microarray-based strategy and conducted a global screen for epigenetically silenced tumor suppressor genes in breast cancer.7 We identified differential expression of 15-PGDH: high expression in the well-differentiated, estrogen receptor (ER)-positive MCF-7 cells and low expression in the poorly differentiated, ER-negative MDA-MB-231 cells. In the present study, we identified epigenetic silencing and reduced expression of 15-PGDH in a subset of breast cancer tumors and cell lines and noticed growth-inhibitory activities of 15-PGDH. Our results indicate 15-PGDH as an epigenetically silenced tumor suppressor gene in breast cancer.

Materials and Methods

Chemicals, antibodies, and constructs. 5-Aza-2'-deoxycytidine (5-Aza-dCyd), 17β-estradiol (E2), 4-hydroxytamoxifen (4-Ht), and G418 were obtained from Sigma (St. Louis, MO). Suberoylanilide hydroxamic acid (SAHA) was generously provided by V.M. Richon (Merck, Rockaway, NJ). Anti-15-PGDH antibodies were described previously (9). Anti-COX-2 (H-62), anti-ERα (F-10), anti-CAAT/enhancer binding protein (C/EBP)α, anti-C/EBPβ (H-7), anti-BCL-2 (N-19), anti-p27Kip1 (C-19), and anti-cyclin D1 (H-295) were all from Santa Cruz Biotechnology (Santa Cruz, CA). The pcDNA3-15-PGDH (PGDH-WT), pcDNA3-mutant Y151F 15-PGDH (PGDH-Mut), and the C/EBPα and C/EBPβ expression vectors were described previously (9, 12). The ERα expression vector was a generous gift of P. Chamoun (University of Strasbourg, Strasbourg, France). The estrogen-responsive element (ERE)-luciferase reporter construct was kindly provided by D. Harris [University of California at Los Angeles (UCLA), Los Angeles, CA].

Patient samples. Samples were obtained, after informed consent, from surgically resected primary breast tumors of women diagnosed at Saitama Cancer Center (Saitama, Japan) from 1992 to 2000 and from women diagnosed at Tarzana Medical Center (Tarzana, CA) from 2002 to 2004. All samples were examined histologically for the presence of tumor cells.

Cells and transfections. Breast cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). All transfections used LipofectAMINE 2000 (Invitrogen, Carlsbad, CA). Stable clones were generated by selection in complete culture medium containing 500 mg/L G418.

Real-time reverse transcription-PCR. Total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA) and processed to cDNA with

5 H-H. Tai et al., unpublished data.
7 I. Wolf et al., submitted for publication.
Figure 1. Epigenetic silencing of 15-PGDH in breast cancer. A, 15-PGDH mRNA levels were determined by quantitative real-time RT-PCR in 25 breast tumors (T) and are shown relative to the expression in MCF-7 and MDA-MB-231 cells and in five normal (N) breast samples. B, microarray data and 15-PGDH mRNA expression levels as determined by quantitative real-time RT-PCR. MDA-MB-231 cells were either mock treated (C) or treated with 5-AZA-dCyd (A or AZA; 5 μmol/L, 3 days) combined with SAHA (S or SAHA; 2 μmol/L for the last 24 hours of culture). Columns, mean of three experiments; bars, SD. C, schematic representation of 15-PGDH promoter methylation in breast cancer. Methylation status was determined using bisulfite sequencing. Numbering is relative to the transcriptional start site of exon 1. Bold, underlined, sites of cytosine methylation. D, MDA-MB-231 cells were either mock treated or treated with SAHA (2 μmol/L, 24 hours) and subjected to ChIP analysis using anti-acetyl-histone 3 antibody. 15-PGDH promoter region was amplified from the total DNA extract (input) and immunoprecipitated DNA (ChIP).

15-PGDH activity assay. 15-PGDH activity was assayed by measuring the transfer of tritium from 15(S)-[15-3H]PGE2 to glutamate by coupling 15-PGDH with glutamate dehydrogenase as described previously (9).

Cell cycle assays. Following transfection with the indicated constructs, the cells were harvested, fixed in methanol, and stained with propidium iodide (Abcam, Cambridge, MA). Flow cytometry was done at the Flow Cytometry Core facility of Cedars-Sinai Medical Center (Los Angeles, CA).

Colony assays. Two days following transfection with the indicated plasmids, G418 (500 μg/mL) was added to the culture medium, and at day 14, the cells were stained using gentian violet. Untransfected cells were treated similarly, and all died within 2 weeks of culture in the selection medium. Quantification of the results was done using Alphalumer 2000 (Alpha Innotech, San Leandro, CA).

Luciferase assays. Cells were transfected with the reporter vector and the various constructs at a ratio of 1:1, and transfection efficiency was normalized using pRL-SV40. Luciferase assay was conducted according to the manufacturer’s instructions (Promega, San Luis Obispo, CA).

Animal studies. Animals were maintained, and experiments were done under NIH and institutional guidelines at the Animal Core Facility, Cedars-Sinai Medical Center. Six-week-old female athymic nude mice were injected s.c. in both flanks with stably transfected cells resuspended in Matrigel (BD Biosciences, San Jose, CA), at a density of 1 × 10^6 viable cells/100 μL. Tumor size was measured with a linear caliper, and volume was estimated by using the equation V = (a × b^2) / 2, where a is the larger dimension and b is the perpendicular diameter. After 5 weeks, the tumors were removed and weighed.

Statistical analysis. The study variables were compared between the study groups using Fisher’s exact test for categorical variables. Pearson’s correlation coefficient was used to determine the relationship between continuous variables.

Results

Expression of 15-PGDH in breast cancer. 15-PGDH expression analysis in breast cancer cell lines (MCF-7, T-47D, BT-474, ZR75-1, 213...
MDA-MB-231, MDA-MB-468, SK-BR-3, and BT-20) using real-time reverse transcription-PCR (RT-PCR) and Western blotting revealed low expression in all but MCF-7 cells (Fig. 1A; data not shown). Expression analysis of normal breast and breast cancer samples using real-time RT-PCR revealed low expression of 15-PGDH in 16 (64%) of the tumors (Fig. 1A). In nine (36%) of the samples, the expression was <50% of control average. We then analyzed a set of 28 breast cancer samples and correlated 15-PGDH mRNA expression with various tumor characteristics (Table 1). Higher 15-PGDH mRNA expression was associated with ER expression and lower tumor stage, and borderline significance was noted for the association between low 15-PGDH expression and lymphovascular invasion.

**Methylation of the 15-PGDH promoter in breast cancer.** Using a genome-wide screen for epigenetically silenced genes, we noticed low expression of 15-PGDH in MDA-MB-231 cells and 25-fold increased expression following treatment with the demethylating agent 5-AZA-dCyd and the histone deacetylases inhibitor SAHA.

### Table 1. Distribution of stage and tumor biological characteristics by study group

<table>
<thead>
<tr>
<th>Variable</th>
<th>Category</th>
<th>Low PGDH (n = 14), n (%)</th>
<th>High PGDH (n = 14), n (%)</th>
<th>Overall P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage</td>
<td>1</td>
<td>1 (7)</td>
<td>6 (43)</td>
<td>0.05*</td>
</tr>
<tr>
<td></td>
<td>II/III</td>
<td>11 (79)</td>
<td>8 (57)</td>
<td></td>
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<td></td>
<td>Unknown</td>
<td>2 (14)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>ER</td>
<td>Positive</td>
<td>7 (50)</td>
<td>12 (86)</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>7 (50)</td>
<td>2 (14)</td>
<td></td>
</tr>
<tr>
<td>Grade</td>
<td>1</td>
<td>1 (7)</td>
<td>4 (29)</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>2/3</td>
<td>13 (93)</td>
<td>10 (71)</td>
<td></td>
</tr>
<tr>
<td>Lymphovascular invasion</td>
<td>Present</td>
<td>10 (71)</td>
<td>6 (43)</td>
<td>0.07*</td>
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<tr>
<td></td>
<td>Absent</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Unknown</td>
<td>1 (7)</td>
<td>0 (0)</td>
<td></td>
</tr>
</tbody>
</table>

*Calculated without unknowns.

15-PGDH mRNA expression was associated with ER expression and lower tumor stage, and borderline significance was noted for the association between low 15-PGDH expression and lymphovascular invasion.
MDA-MB-231 cells were then cultured with 5-AZA-dCyd and/or SAHA, and 15-PGDH levels were measured using real-time RT-PCR. An increase of 5.6-fold was noted following 5-AZA-dCyd treatment, 9.8-fold following SAHA treatment, and 27.5-fold following the combined treatment (Fig. 1B). Western blot analysis revealed similar effects (data not shown).

The 15-PGDH promoter contains a CpG island in the region −163 to +140 relative to the start ATG codon. We conducted bisulfite sequencing and analyzed the methylation status of this area in six breast cancer cell lines (MCF-7, MDA-MB-231, MDA-MB-436, BT-474, T-47D, and SK-BR-3) and identified methylation in MDA-MB-436 cells (Fig. 1C). Analysis of 10 primary breast cancers revealed methylation in three tumors. The methylation in primary tumors was less extensive than in MDA-MB-436 cells, but two areas were methylated in all three tumors, one near the TATA box and the other near a site of the methylation-sensitive transcription factor USF (13–15). Importantly, these tumors showed reduced expression of 15-PGDH mRNA compared with either MCF-7 cells (<25%) or the unmethylated tumors.

We investigated the role of histone acetylation in 15-PGDH expression. ChIP analysis of MDA-MB-231 cells revealed an increased interaction of the 15-PGDH promoter with acetylated histone 3 following treatment with SAHA (Fig. 1D). This was associated with an increased expression of the gene (Fig. 1B). These findings suggest a role for histone deacetylation in the regulation of 15-PGDH expression in breast cancer.

15-PGDH induces growth inhibition of MDA-MB-231. MDA-MB-231 cells were transfected with either an empty vector (pcDNA3), wild-type 15-PGDH (WT), or mutated 15-PGDH (Mut) expression vectors (Fig. 2A). Colony formation assays revealed 37% reduction in the number and size of surviving colonies following 15-PGDH expression (P < 0.05; Fig. 2B). No differences in the expression of BCL-2, COX-2, p27, p21, and cyclin D1 levels were noted following 15-PGDH overexpression (Fig. 2A; data not shown).

Three clones of MDA-MB-231 cells were generated, which stably express either the pcDNA3 or WT or Mut expression vectors (Fig. 2C). Western blot analysis revealed expression of the WT and mutated 15-PGDH, and activity analysis revealed increased enzymatic activity only of the WT enzyme. The three clones were injected into the flanks of nude mice, and tumor growth was monitored weekly. 15-PGDH expression resulted in a significant decrease in tumor growth (P < 0.002; Fig. 2C).

We have recently identified tumor-suppressive activities of C/EBPα in breast cancer (12). As the 15-PGDH promoter contains...
several C/EBP consensus sites, the effects of C/EBPα and C/EBPβ overexpression on 15-PGDH levels were investigated in MDA-MB-231 cells. Western blot (Fig. 2D) and real-time RT-PCR analysis (data not shown) revealed up-regulation of 15-PGDH by both C/EBPα; however, the effect of C/EBPβ was more pronounced.

**Down-regulation of 15-PGDH stimulates growth of MCF-7 cells.** 15-PGDH-directed siRNA constructs (siRNA1 and siRNA2) and a control siRNA were prepared and transfected into MCF-7 cells. Analyses of 15-PGDH mRNA levels (data not shown) and protein expression (Fig. 3A) revealed that siRNA1 effectively reduced expression of the gene. Colony formation assays showed that down-regulation of 15-PGDH significantly increased the ability of MCF-7 cells to form colonies (P < 0.05, for siRNA1 compared with the control siRNA and siRNA2; Fig. 3B). Cell cycle analysis using propidium iodide at 24 hours after transfection revealed that siRNA1 significantly increased the number of cells in S phase (39% for siRNA1 compared with 23% for control siRNA and 24% for the siRNA2, P < 0.05; Fig. 3C). Treatment of the cells with 4-HT completely inhibited this growth-stimulatory effect of 15-PGDH down-regulation.

Two clones were generated, which stably expressed the siRNA1 construct and thus have lower levels and activity of 15-PGDH (Fig. 3D). These clones, or a clone that stably express control siRNA construct, were injected on flanks of nude mice, and tumor growth was monitored weekly (Fig. 3D). 15-PGDH down-regulation resulted in a significant increase in tumor growth (P < 0.002). Increased BCL-2 expression was noted in the clones that expressed low 15-PGDH levels, but no effects were observed on the levels of COX-2, p27, p21, and cyclin D1 (data not shown).

**Association between 15-PGDH and the ER pathway.** Inhibition of the ER pathway blocks the pro-proliferative effect of 15-PGDH down-regulation (Fig. 3C). The ERE-luciferase construct was used to analyze further the association between 15-PGDH and the ER pathway activity, and a 3.5-fold increase of the ERE-luciferase activity was noted following 15-PGDH silencing in MCF-7 cells (Fig. 4A). No change was observed in ERα levels (Fig. 3A).

The effect of E2 on 15-PGDH expression was examined in MCF-7 cells. The cells were grown for 3 days in a medium containing charcoal-treated serum and were either mock treated or cultured for 48 hours with either E2, E2 and untreated serum, or E2 and 4-HT. Western blot analysis (Fig. 4B) and real-time RT-PCR studies (data not shown) showed down-regulation of 15-PGDH expression following E2 treatment.

Part of the activities of PGE2 in breast cancer is attributed to its ability to up-regulate aromatase activity (5). Indeed, silencing of 15-PGDH in MCF-7 cells resulted in a 6.7-fold increase of aromatase mRNA levels (Fig. 4C).

**Discussion**

Our observations identify tumor suppressor activities for the key PGE2 catabolic enzyme 15-PGDH in breast cancer. Low 15-PGDH levels were noticed in the poorly differentiated, COX-2-expressing, MDA-MB-231 cells (16) and in primary breast cancers; low levels of 15-PGDH correlated with unfavorable prognostic factors. These results are similar to breast cancer microarray data (17) and suggest that loss of 15-PGDH expression may play a role in the pathogenesis of the less differentiated subtypes of breast cancer.

Recent data identified down-regulation of 15-PGDH expression by epigenetic mechanisms. In prostate cancer, the 15-PGDH promoter was found to be extensively methylated in one cell line, and clusters of DNA methylation were identified in 75% of primary tumors (11); in lung cancer, histone deacetylation was associated with 15-PGDH silencing.6 Our results suggest a role for both mechanisms, methylation of the promoter and histone deacetylation, in the silencing of 15-PGDH expression in breast cancer.

Both models of 15-PGDH manipulation, overexpressing in MDA-MB-231 cells and silencing in MCF-7 cells, suggest a role for this enzyme in breast cancer.
envelope as a tumor suppressor gene in breast cancer. Similarly to studies in lung and colon cell lines, 15-PGDH overexpressing MDA-MB-231 cells showed a reduction of in vivo tumorigenicity (7, 9). As observed in a colon cancer model (7), the in vitro effects of overexpression of 15-PGDH in MDA-MB-231 cells were less impressive. These findings are consistent with the suggestions that PGE2 plays a particularly important role in promoting tumor angiogenesis (2, 7).

Several lines of evidence indicate interactions between PGE2/COX-2 and the ER pathway. For example, up-regulation of COX-2 in the ER-positive MCF-7 cells induced resistance to 4-HT (18), and in clinical samples, COX-2 expression is associated with reduced survival among patients with ER-positive tumors (4). At least part of the effects of PGE2 and COX-2 is mediated by induction of tumor aromatase. In clinical samples, COX-2 expression correlated with aromatase expression (19), and laboratory data revealed that COX-2 activates, and inhibitors of COX-2 suppressed, transcription of aromatase (20). Here, we identified an association between 15-PGDH expression and the ER pathway: increased E2 levels down-regulated expression of 15-PGDH, whereas down-regulation of 15-PGDH increased the ERE activity and aromatase levels. Moreover, inhibition of the ER pathway attenuated the effects of 15-PGDH on the cell cycle. Thus, our results add another dimension to the interactions between PGE2, COX-2, and the ER pathway (Fig. 4D).

Members of the C/EBP family of transcription factors are involved in mammary gland development (21). We have recently identified C/EBPα as a silenced tumor suppressor gene in breast cancer (12). Here, we show that C/EBPα and, to a lesser extent, C/EBPβ up-regulate 15-PGDH expression. These findings suggest a novel mechanism for the growth-inhibitory activities of C/EBPα in breast cancer. Whether the effects of C/EBPα on 15-PGDH expression result from a direct interaction of C/EBPα with the 15-PGDH promoter remains to be elucidated.

In summary, this study identified 15-PGDH as an epigenetically silenced tumor suppressor gene in breast cancer and suggests that its aberrant expression may modulate the activity of the ER pathway.

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