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Loss of 15-hydroxyprostaglandin dehydrogenase expression disrupts urothelial differentiation

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Abstract

Objectives—Urothelial differentiation is essential for the maintenance of urinary bladder function. We explored expression and function of 15-hydroxyprostaglandin dehydrogenase (PGDH) during urothelial differentiation.

Methods—Expression of PGDH was evaluated by Northern and Western blotting and immunostaining in human urothelial cultures, cell lines and tissues. Enzymatic function was determined using enzyme-linked immunosorbent assay. Small inhibitory ribonucleic acids were used to inhibit PGDH expression in human bladder cancer cells.

Results—PGDH messenger ribonucleic acid was increased in an *in vitro* model of human urothelial differentiation by Northern blotting. Western blotting of human bladder cancer cell lines showed expression in the well-differentiated RT4 cells, and no expression in poorly-differentiated UC3 cells. Immunostaining showed that PGDH expression increased with differentiation in normal bladder urothelium. The enzyme is functional in the well-differentiated RT4 human bladder cancer cell line. Inhibition of PGDH expression results in disruption of E-cadherin expression at cell-cell contacts in well-differentiated RT4 bladder cancer cells.

Conclusions—These studies indicate that PGDH expression is associated with urothelial differentiation, and loss of PGDH expression results in disruption of urothelial differentiation.

Introduction

After differentiation, urothelial cells acquire their mature structures and functions, including barrier function and storage; immune surveillance; mechanoreceptor function; cell-cell communication and others¹. Maintenance of differentiation is essential for continued functioning of urothelial cells. Disrupted urothelial differentiation, which occurs in interstitial cystitis², partial bladder outlet obstruction³, and bladder cancer⁴, can lead to irritative voiding, pain and bleeding.

Prostaglandins are involved in a number of biologic processes, including angiogenesis, proliferation, motility, and inhibition of immune responses and apoptosis⁵. Recent studies of

prostaglandin metabolism in the urinary tract have focused on the inducible form of prostaglandin synthase, cyclooxygenase-2⁶ (COX-2). COX-2 is induced by stretch in the urinary bladder⁷ and is overexpressed in higher stage, more aggressive bladder cancers⁸

However, prostaglandin levels are also regulated by inactivation via 15-hydroxyprostaglandin dehydrogenase (PGDH) which catalyzes the inactivation of bioactive prostaglandin E2 (PGE2) to a less active keto form⁹. Proteomic studies have shown that PGDH is expressed in tissue extracts from normal bladder but expression is reduced or lost in invasive bladder cancers¹⁰. Further, PGDH expression is reduced during ureteral stretching¹¹.

The goal of this report was to evaluate PGDH expression and function in urothelial cells and bladder cancer cell lines, as well as ureteral and bladder tissues, and to determine the impact of PGDH expression on urothelial differentiation. This study extends previous findings demonstrating that PGDH is expressed in urothelial tissues, and provides new information on the relationship between urothelial differentiation and PGDH expression.

Materials and Methods

Tissues, Cell Lines and Culture

Normal urothelial cells were cultured from human ureter, obtained under IRB-approved protocol, as previously described¹². Briefly, cells were isolated from the ureter obtained from patients undergoing nephrectomy for living-related renal transplantation or renal cell carcinoma, and grown in low calcium serum-free medium (SF-KGM, Invitrogen). These cells were differentiated by changing the medium to high calcium Minimal Essential Medium (MEM; Invitrogen) containing 10% fetal calf serum, as previously described¹². Human bladder cancer cell lines RT4 and UC3 were cultured in Dulbecco's Modified Eagle Medium, containing 10% fetal calf serum and 1% penicillin-streptomycin.

Northern Blotting

Total RNA was prepared from cells using RNeasy Lysis Buffer (Qiagen). Equal amounts of RNA (15 µg/lane) from each source were separated by electrophoresis on formaldehyde-agarose gels and transferred onto Zeta-probe blotting membranes (Bio-Rad). A 0.5 kb cDNA probe containing PGDH coding sequence and a control probe for GAPDH were labeled by random priming using alpha ³²P dCTP (Rediprime, Amersham). The blots were hybridized with the probes, washed and developed by autoradiography.

Western Blotting

Antibodies to PGDH were prepared by immunization of rabbits with KLH-conjugated peptide TSKGIHFQDYDTTPFQAKTQ corresponding to the 21-amino acid peptide sequence of the carboxy terminus of human PGDH. Cells and tissues were extracted in lysis buffer and equal amounts of protein were separated on SDS-polyacrylamide gels, blotted, and probed with PGDH antibody and actin antibody (Santa Cruz) as a control to demonstrate equal protein loading of the lanes. Absorption to demonstrate specificity was performed using recombinant human PGDH protein expressed in *E. coli* (Cayman Chemical). Horseradish peroxidase - conjugated donkey anti-rabbit antibody (Cell Signaling) was incubated with the membrane. After washing, the membrane was developed using a chemiluminescent detection system at room temperature (ECL Plus, Amersham Bioscience) and exposure to X-ray film.

Immunostaining

Human tissues from patients undergoing cystectomy for bladder cancer were obtained under an IRB-approved protocol. Paraffin-embedded sections were deparaffinized, rehydrated, and subjected to antigen retrieval using Citra solution (Biogenex), blocked and stained overnight

at 4°C with rabbit antibody to PGDH or control rabbit immunoglobulin (Zymed). In additional experiments, the PGDH antibody was pre-incubated with recombinant PGDH (Cayman Chemical). The tissues were washed with phosphate buffered saline (PBS) and developed using biotinylated anti-rabbit antibody and avidin-biotin peroxidase complex reaction (Vector Laboratories). The reaction was completed by incubation with 3, 3'-diaminobenzidine tetrahydrochloride and peroxide, resulting in a brown stain. Tissues were counterstained with hematoxylin. For fluorescent staining, cultured cells were grown on 8-chamber slides. The slides were fixed in ice-cold acetone, air-dried, blocked with blocking buffer, and incubated with mouse anti-human E-cadherin antibody (Zymed). After washing with PBS, the slides were incubated with fluorescein-isothiocyanate conjugated anti-mouse antibody (Zymed). After washing with PBS, the slides were mounted in Vectashield (Zymed) and evaluated.

PGDH Activity

Confluent RT4 and UC3 cells were tested using the PGE2 metabolite enzyme immunoassay (EIA) kit (Cayman Chemical). Briefly, cells were grown on 6-well plates to approximately 90–95 % confluence. The cells were washed twice with iced-cold Waymouth buffer (135 mM NaCl, 13 mM HEPES, pH 7.2, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 0.8 mM MgSO₄, 5 mM KCl, and 28 mM Glucose) and incubated with 1 ml of Waymouth buffer per well with or without 10 nM PGE2 (dissolved in phosphate buffered saline; Cayman Chemical) at room temperature for 10 min. Supernatant was then collected separately and centrifuged at 1500 rpm for 5 min at 4°C and tested for PGE metabolite using PGEM EIA kit (Cayman Chemical), following the manufacturer's instructions. Results were confirmed using [³H] PGE2 followed by thin layer chromatography separate of metabolic products (data not shown).

Small inhibitory RNA and RT-PCR

Diced siRNA pool was synthesized using the BLOCK-iT complete Dicer RNAi kit (Invitrogen). Briefly, RT4 total RNA was used to synthesize an 841 bp long PGDH cDNA fragment using oligo-d(T) primer for first strand cDNA. PGDH specific primers were used for the subsequent PCR. The cDNA was ligated to T7 promoter sequences at both ends and subjected to PCR amplification using T7-specific primers. The resulting T7-PGDH DNA fragment served as the template in the following T7 in vitro transcription to yield the PGDH RNA fragment, which was then subjected to a dicer reaction to produce PGDH siRNA pools.

RT4 cells were transfected with PGDH and/or control siRNA using lipofectamine 2000 (Invitrogen) in 6-well plates. Other PGDH siRNA 8933 and 9027, along with the control ("scramble" control) were purchased (Ambion). To examine if the transfection of siRNA resulted in activation of interferon stimulated genes (ISGs), the Interferon Response Detection kit (System Biosciences, Mountain View, CA) was used. RNA was isolated 24 hours post-transfection and RT-PCR was performed to check the expression of ISGs, including OAS1, OAS2, MX1, IFITM1, and ISGF3γ by RT-PCR following the manufacturer's instructions.

Statistical Analysis

Statistical analysis was performed with a computer program (SigmaStat 3.5, Systat Software, Inc, Port Richmond, CA) using the non-parametric Mann-Whitney Rank Sum test. The difference was considered significant at a level of $p < .05$. All assays were repeated at least three times, and representative results are presented.

Results

PGDH Expression in Cells and Tissues

Using an *in vitro* method of urothelial differentiation, PGDH mRNA expression was detected by Northern blotting in some differentiated, but not undifferentiated, normal human urothelial cultured cells (Figure 1A). To verify and extend these findings, expression of PGDH was examined by immunoperoxidase staining of bladder tissue, which showed that PGDH expression is increased with increasing urothelial differentiation, with the greatest staining on the most differentiated cells at the luminal surface (Figure 1B). Tissues treated with a negative control antibody showed no staining, and staining was abrogated by preincubation of the antibody with recombinant PGDH protein (data not shown). Additionally, as shown in Figure 1C, PDGH protein expression was found in human bladder tissue and in the well-differentiated RT4 bladder cancer cell line by Western blotting. Poorly-differentiated UC3 cells showed no PGDH expression. Immunoblotting performed with antibody pre-incubated with recombinant PGDH protein failed to develop the 29kD band, demonstrating specificity of the antibody (data not shown).

PGDH Enzymatic Function

PGDH activity was performed using a commercially-available EIA kit. As shown in Figure 1D, the well-differentiated RT4 cells metabolize PGE₂, while the less differentiated UC3 cells fail to generate PGE₂ metabolites. The difference was statistically significant ($p=.002$, Mann-Whitney Rank Sum test). These results were confirmed using analysis with metabolism of radioactive PGE₂ (data not shown).

Effects of PGDH inhibition on E-cadherin expression

PGDH protein expression in the well-differentiated RT4 human bladder cancer cell line was inhibited by siRNA as shown in Figure 2A. Two transfections were required to obtain significant inhibition. Similar inhibition of PGDH mRNA was also observed (data not shown). PGDH protein levels were reduced using the Dicer pool siRNA (HM-PGDH) compared to the Dicer pool non-specific control (HM-LacZ), as well as commercially available (Ambion) PGDH 9027 siRNA compared to the 8933 siRNA or the negative non-specific control ("scramble"). Differentiation of these cells was evaluated by E-cadherin staining. As shown in Figures 2B and C, RT4 cells treated with the HM-LacZ non-specific negative control siRNA show normal E-cadherin staining at the cell junctions, while HM-PGDH siRNA treated RT4 cells demonstrated cytoplasmic E-cadherin staining with less staining at the cell junctions. Levels of E-cadherin protein and mRNA were modestly reduced but not totally inhibited based on RT-PCR evaluation and Western blotting (data not shown).

Discussion

PGDH was identified in our search for genes expressed during urothelial differentiation. Our studies confirmed that PGDH was expressed in urothelial cells and tissues in a manner consistent with an association with urothelial differentiation. These data are consistent with previous proteomic studies¹⁰. The well-differentiated PGDH-positive RT4 cell line demonstrated PGDH enzymatic activity, while the poorly-differentiation PGDH-negative UC3 cell line did not. Finally, inhibition of PGDH expression using siRNA showed disruption of E cadherin expression at the cell surface in the well-differentiated RT4 cell line.

The siRNA approach used in this study involves transfection with double stranded RNA. Earlier studies showed that this procedure could result in interferon signaling, mimicking a viral infection¹³. We are confident that our observations are specific for PGDH inhibition. First, the effect was observed using both the HM-PGDH and the commercially-available 9027

siRNAs. Second, no disruption of E-cadherin expression was observed in any of the control siRNA transfected cells. Finally, we did not detect increased activation of interferon pathways in PGDH siRNA treated cells as compared to controls using a commercially-available RT-PCR kit (data not shown).

PGDH expression has been linked to differentiation in other studies. Lennon and co-workers demonstrated that PGDH expression was increased during trophoblast differentiation *in vitro*¹⁴. Pichaud *et al.* found that 1,25-dihydroxyvitamin D induction of differentiation of neonatal monocytes into preosteoclastic cells lead to increased expression of PGDH¹⁵. Phorbol myristate acetate differentiation of HL60 cells into monocyte/macrophage lineage resulted in increased expression of PGDH¹⁶. Therefore, our observations are consistent with these studies which also show an association between differentiation and PGDH expression.

What is the mechanism of E-cadherin disruption in our studies? Recent work has shown that treatment of colon cancer cells with epidermal growth factor results in transcriptional repression of both PGDH and E-cadherin expression¹⁷. However, these studies demonstrated a complete loss of E-cadherin protein. In contrast, our studies showed levels of E-cadherin protein are reduced but not completely lost in PGDH-inhibited RT4 cells. To be functional, E-cadherin is assembled into a complex with beta-catenin, plakoglobin and alpha catenin¹⁸. Our observation that E-cadherin is expressed but maintained in the cytoplasm in the PGDH-inhibited RT4 cells is more consistent with a loss of E-cadherin complex assembly. Additional studies to determine the mechanism of action are in progress.

The link between PGDH expression and urothelial differentiation may present therapeutic opportunities. Previous work has shown that PGDH protein expression is lost in bladder cancer¹⁰. Furthermore, a recent study of interstitial cystitis demonstrated an increased cytoplasmic expression of E-cadherin with less of the total E-cadherin expressed at cell junctions². Induction of PGDH expression would protect the urothelium from continued stimulation by PGE2 and may promote maintenance of urothelial differentiation in bladder cancer or in interstitial cystitis. Additional studies are needed to determine if this approach will be useful.

Conclusion

PGDH expression is associated with urothelial differentiation, and is functional in urothelial cells. Loss of PGDH results in disruption of E-cadherin expression at cell junctions, reflecting a loss of urothelial differentiation. Treatments increasing PGDH expression may protect the urothelium from PGE2 stimulation and may present a therapeutic opportunity in disorders such as bladder cancer and interstitial cystitis.

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Figure 1.

A. Northern blotting of in vitro differentiated culture normal urothelial cells for PGDH (top lanes). Lane 1, NU undif are undifferentiated cultured normal urothelial cells maintained in SF-KGM; Lane 2, NU dif are cells differentiated by transfer to high-calcium medium containing 10% fetal calf serum. GADPH expression (lower lanes) is a control.

B. Immunostaining of normal bladder tissue for PGDH expression. Brown color indicates PGDH antibody binding. No staining was observed in antibody absorbed with recombinant PGDH (data not shown). Arrow indicates increased staining at the apical (most differentiated) cells.

C. Western blotting of normal bladder tissue and bladder cancer cell line extracts for PGDH expression. Lane 1, normal bladder mucosa from tissue; Lane 2, RT4 human bladder cancer cells; Lane 3, UC 3 human bladder cancer cells. Top row, probed with PGDH antibody; bottom row, probed with actin antibody as a control for equal sample loading.

D. PGDH Enzymatic Activity. Activity was measured using an EIA kit. Results shown $\pm$ 1 standard deviation. RT4 cells showed significantly more PGE2 metabolism than UC3 cells ($p=.002$, Mann-Whitney Rank Sum test).

Figure 2.

A. siRNA Inhibition of PGDH Expression. Levels of PGDH protein expression by Western blotting after Treatment with siRNA. Labels indicate the source of siRNA: Negative, untreated RT4 cells; HM-LacZ, treated with the Dicer non-specific control siRNA; HM-PGDH, treated with the Dicer PGDH siRNA; scramble, treated with the Ambion non-specific control siRNA; 8993, treated with the Ambion 8993 PGDH siRNA; 9027, treated with the Ambion 9027 PGDH siRNA. Blot top row: probed with PGDH antibody. Bottom row: probed with actin antibody as a control for equal sample loading.

B. E-cadherin staining of RT4 Cells after siRNA treatment using nonspecific control HM-LacZ siRNA. Strong staining at the cell junctions results in the normal “honeycomb” pattern.

C. E-cadherin staining of RT4 cells after siRNA treatment using HM-PGDH siRNA. Staining is observed in the cytoplasm, but little cell junctional staining is observed.