Regulation of Prostaglandin Transporters in Colorectal Neoplasia

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Abstract

Prostaglandin E2 (PGE2) promotes cancer progression by affecting cell proliferation, apoptosis, angiogenesis, and the immune response. It has been reported that PGE2 is transported or passes through the cell membrane via prostaglandin-specific transporters including the prostaglandin transporter (PGT, an influx transporter) and the multidrug resistance-associated protein 4 (an efflux transporter). PGT can facilitate the removal of PGE2 from the extracellular milieu by transporting it into the cell, where 15-hydroxyprostaglandin dehydrogenase (15-PGDH) then oxidizes PGE2 into 15-keto PGE2. We previously reported that 15-PGDH expression is reduced in most colorectal cancers, indicating the tumor suppressor role of this gene. In the present study, we show that PGT expression is also decreased (whereas multidrug resistance-associated protein 4 expression is elevated) in human colorectal cancer specimens (compared with expression in normal mucosa) and in colorectal cancer cell lines. Furthermore, we found that PGT expression decreased in premalignant adenomas in Apcmin mice and was partially restored (in human colorectal cancer cell lines) by treatment with a DNA demethylating agent or histone deacetylase inhibitor. Forced PGT overexpression in vitro reduced extracellular PGE2 levels and increased intracellular levels of its catabolic product 15-keto PGE2. Our data suggest that the existing model to explain increased PGE2 in colorectal neoplasia should be modified to include the novel mechanism of coordinated up- and down-regulation of genes involved in PGE2 transport.
Materials and Methods

Reagents
PGT antibody was obtained from Cayman Chemical. Monoclonal β-actin antibody, 5-Aza-2′-deoxycytidine (Aza-dC) and trichostatin A were obtained from Sigma.

Cell culture
Colon cancer cell lines were maintained in McCoy’s 5A medium containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin in a 5% CO₂ atmosphere.

Quantitative real-time PCR
Total cellular RNA was isolated from cells using TRI reagent (Molecular Research Center) according to the manufacturer’s protocol. cDNA for each RNA sample was synthesized in 20-μL reactions using the SuperScript First Strand synthesis system for reverse transcription-PCR (Invitrogen) following the manufacturer’s protocol. PCR reaction contained iQ SYBR Green Supermix (Bio-Rad), 50 ng of each primer, and 5 μL of 1:500 diluted reverse transcriptase template in a 25 μL reaction volume. Primers used for PCR were as follows: human PGT forward, 5′-TTCGGCAACATTAAGGTG-3′ and reverse, 5′-GATGAAGAACCTGAGAGG-3′; mouse PGT forward, 5′-TCGCGCTGTATATCTCCATC-3′ and reverse, 5′-GTAGCCGTTCCACCTCTCG-3′; human multidrug resistance-associated protein 4 (MRP4) forward, 5′-CTGTCAGATGTAAGAC-3′ and reverse, 5′-GTGATGAAACACTCCTCC-3′; mouse MRP4 forward, 5′-TCTGGTTATTCTCTCCTGG-3′ and reverse, 5′-CGGAGTCATATGGGT-3′; and β-actin forward, 5′-AGAAAATCTTCCACCCACC-3′ and reverse, 5′-AGAGGCGTCAGG-GATAGCA-3′.

Western blot analysis
Cells were washed with PBS and lysed with radioimmunoprecipitation assay buffer [50 mmol/L Tris-Cl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% Nonidet P-40].

Fig. 1. PGT and MRP4 expression in human cancer. A and B, total RNA was isolated from 38 human colorectal carcinomas and corresponding normal mucosa. PGT and MRP4 expression was examined by real-time PCR analysis.
SDS, and protease inhibitors from Roche Diagnostics]. Proteins were then separated on precast SDS-polyacrylamide gels and electrotransferred onto nitrocellulose membranes. Membranes were blocked in 5% milk in TBST [10 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, and 0.1% Tween 20] and incubated with primary antibody overnight at 4°C. The membranes were then treated with horseradish peroxidase-conjugated secondary antibody and developed using an enhanced chemiluminescence kit (Amersham Biosciences).

**Northern blot analysis**

Nylon membrane containing RNA samples derived from various matched tumor and normal tissues on Cancer Profiling Array II (BD Biosciences) were hybridized in Hybrisol I (Intergen Company) with a 3²P-labeled 533-bp human PGT cDNA in the coding region. The blot was washed and subjected to autoradiography.

**Human colorectal tissue samples**

Human colorectal tumor specimens were obtained from surgical resections with Vanderbilt University Internal Review Board approval. For each tumor sample, matched adjacent normal mucosa was collected for comparison. All samples were snap frozen and stored in liquid nitrogen until use. RNA preparation from tissues was done using TRI reagent as described above. All of these experiments were completed at Vanderbilt University.

**Aza-dC and trichostatin A treatment**

Cell lines (LS174, HT-29) were seeded at a density of 1 × 10⁶ cells/100-mm plate. The cells were serum starved for 24 h before treating with Aza-dC for 72 h or trichostatin A for 48 h. The cells were harvested as described above.

**PGT transfection and prostaglandin profiles**

HCA-7 cells (5 × 10⁵ in six-well plates) were transiently cotransfected with 0 to 5 μg of PGT expression construct (full-length PGT cDNA in pCMV-SPORT6 vector from Open Biosystems). Four hours after transfection, the medium was replaced with medium containing 10% serum. After 48 h, the supernatants and cells were collected and analyzed by mass spectrometry as previously described (22, 23).

**Statistical analysis**

Each experiment was done at least three times, and data were expressed as the means ± SE. Statistical significance was determined by paired Student’s t test. P values <0.05 were considered statistically significant.

**Results**

**Reciprocal regulation of PGT and MRP4 expression in human colorectal cancers and in Apc<sup>Min/+</sup> mouse polyps**

PGT transports PGE₂ to the cytoplasm where 15-PGDH catalyzes its inactivation, thus reducing effective PGE₂ levels in the tumor microenvironment. To examine this process more carefully, we measured the expression levels of PGT in colorectal cancers compared with that of MRP4, an

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**Fig. 1 Continued. C, direct comparison of PGT and MRP4 from the above experiment A and B. *, P < 0.0001; #, P = 0.011. D, a Cancer Profiling Array II was hybridized with a PGT cDNA probe and examined for differential expression in paired normal and tumor samples from the following tissues: colon (n = 38), stomach (n = 28), ovary (n = 16), lung (n = 21), kidney (n = 20), and rectum (n = 19).**
efflux prostaglandin transporter. The relative expression of *PGT* mRNA by quantitative reverse transcription-PCR in fresh-frozen human colorectal tumor specimens from 38 individuals was significantly lower than that observed in matched normal mucosa (Fig. 1A). In contrast, *MRP4* expression was significantly increased in the 38 colorectal tumor specimens compared with normal tissue (Fig. 1B). Direct comparison reveals that expression of *PGT* and *MRP4* are inversely correlated (Fig. 1C). A Northern blot analysis in commercially available specimens showed that PGT expression was also reduced (versus matched normal-tissue samples) in colon or rectum tumors (n = 57 matched specimens) and in other human cancers, including stomach, ovary, lung, and kidney cancers (Fig. 1D).

Next, we analyzed *Pgt* and *Mrp4* expression in polyps (pre-malignant lesions) and matched normal mucosa from Apc^{Min/+} mice. As shown in Fig. 2A, the expression of *Pgt* was significantly decreased in polyps taken from 12-week-old Apc^{Min/+} mice compared with normal mucosa (P = 0.003), whereas *Mrp4* expression is significantly increased (P = 0.002; Fig. 2B). Direct comparison of PGT and MRP4 showed a similar inverse relationship as we previously observed in human tissue (Fig. 2C). Taken together, our *in vivo* data suggest that decreased expression of PGT and increased expression of MRP4 can occur early in the sequence of events leading from an adenoma to a carcinoma.

**Repression of PGT in colorectal cancer cell lines**

Examination of PGT expression in multiple colorectal cancer cell lines by quantitative real-time PCR and Western blot analysis revealed that PGT expression was below the limits of detection in half of the colorectal cancer cell lines (four of eight) we examined. Only LoVo cells displayed significant PGT expression at both the mRNA (Fig. 3A) and protein (Fig. 3B) levels. In comparison, high levels of MRP4 expression were observed in most of the colorectal cancer cell lines, with HCT-116 cells showing the highest level of expression of the carcinoma cells we examined (Fig. 3C).

**PGT repression is mediated by epigenetic silencing**

To begin to examine the mechanism(s) responsible for down-regulation of PGT, we treated colorectal cancer cells with reagents that alter methylation status or modify histone acetylation. As shown in Fig. 4, treatment of multiple colorectal cancer cells with a demethylating agent, Aza-dC, resulted in restoration of PGT expression as evidenced by increased mRNA (Fig. 4A and B) and protein (Fig. 4C and D) expression. Furthermore, treatment of colorectal cancer cells with the HDAC inhibitor trichostatin A also resulted in similar increase in PGT expression (Fig. 5A and B).

**PGT overexpression in HCA-7 cells reduces extracellular PGE₂**

To understand the biological role of PGT, we transiently transfected a PGT overexpression vector into colorectal cancer cells and examined the effects on prostaglandin levels. HCA-7 cells express high levels of COX-2, are known to produce large amounts of PGE₂ that can be measured in the extracellular medium (24), and have detectable levels of 15-PGDH (17). Forced PGT overexpression reduced extracellular PGE₂, increased intracellular levels of its inactive metabolite 15-keto PGE₂, and PGF₂α, in a dose-dependent manner (Fig. 6).

**Discussion**

The current study presents the first evidence we know of indicating that PGT (an influx PGE₂ transporter) is down-regulated

![Fig. 2. PGT and MRP4 expression in mouse model of human colon cancer. A and B, total RNA from polyps and normal adjacent tissue of six Apc^{Min/+} mice were isolated and examined by real-time PCR analysis.](http://cancerpreventionresearch.aacrjournals.org)
in colorectal neoplasia. PGT expression was decreased in human colorectal cancer tissues, mouse adenoma samples, and colorectal cancer cell lines. We also show that MRP4, which is reported to transport PGE2 out of the cell, is increased in colorectal cancers and adenomas. The down-regulation of PGT seems to be mediated, in part, through epigenetic silencing.

Intracellular PGE2 can cross through the membrane by simple diffusion or via a prostaglandin efflux transporter,

**Fig. 3.** PGT and MRP4 expression in human colorectal cancer cell lines. **A** and **B**, total RNA from multiple colorectal cancer cell lines was isolated and examined by real-time PCR analysis. **C**, protein lysates from multiple colorectal cancer cell lines were isolated and equal amounts of proteins were separated by SDS-PAGE. The gels were exposed to PGT antibody.

**Fig. 4.** Restoration of PGT expression by Aza-dC. LS174 (**A and C**) and HT-29 (**B and D**) cells were treated with different concentrations of Aza-dC for 72 h. Total RNA was isolated and examined by real-time PCR analysis for PGT expression. Protein lysates were isolated and equal amounts of proteins were separated by SDS-PAGE. The gels were exposed to PGT antibody.
such as MRP4, which has been shown to efficiently transport PGE2 in an ATP-dependent manner (25). Extracellular PGE2 exerts its actions via cell surface G protein–coupled receptors (EP receptors) that activate a number of signaling cascades. Extracellular PGE2 can be transported into the cell, where it can be inactivated or act indirectly via specific nuclear receptors (26). We previously reported that expression of 15-PGDH, which regulates the enzymatic degradation of PGE2, is repressed in most colorectal cancers, resulting in increased PGE2 levels. Our current observation of inversely related expression of MRP4 and PGT in colorectal cancer, adenomas, and cell lines could help explain other mechanisms responsible for increased local PGE2 levels in the tumor microenvironment.

Tumor-suppressor genes can be down-regulated by histone modification of the chromatin and hypermethylation of CpG-rich sequences in the promoter region (27, 28). Recent data illustrate that 15-PGDH expression is regulated by several epigenetic mechanisms. The 15-PGDH promoter DNA was 75% methylated in primary prostate tumors and extensively methylated in one cell line (29); 30% of primary breast tumors and one breast cancer cell line were associated with hypermethylation and histone deacetylation of the 15-PGDH promoter (20). Our results here suggest that another component (besides 15-PGDH) of the PGE2 catabolism pathway, namely PGT, is also down-regulated by epigenetic mechanisms.

In summary, our present results reveal novel mechanisms that help explain increased PGE2 in the tumor microenvironment. We have previously shown that two key enzymes involved in prostaglandin metabolism are inversely regulated in colorectal adenomas and cancer—COX-2, which synthesizes prostaglandins, is increased and 15-PGDH, which converts PGE2 to an inactive keto compound, is repressed. In the present study, we show that PGT and MRP4 mRNA levels are inversely regulated in human colorectal cancer relative to normal mucosa (Fig. 7). A similar relationship between PGT and MRP4 is found in intestinal adenomas taken from ApcMin/+ mice. Taken together, our data provide evidence that delivery of PGE2 to the cytoplasm as substrate for 15-PGDH could be an important step in regulating local levels of PGE2 in invasive or intraepithelial neoplasia. Increased PGE2 in neoplasia results in part from reduced

Fig. 5. Restoration of PGT expression by HDAC inhibitor. LS174 (A and C) and HT-29 (B and D) cells were treated with different concentrations of trichostatin A (TSA) for 48 h. Total RNA was isolated and examined by real-time PCR analysis for PGT expression. Protein lysates were isolated and equal amounts of proteins were separated by SDS-PAGE. The gels were exposed to PGT antibody.

Fig. 6. PGT overexpression affects prostaglandin levels in HCA-7 cells. HCA-7 cells were transfected with different concentrations of PGT expression vector. Forty-eight hours after transfection, the culture medium and cells were assessed for prostaglandin profile by mass spectrometry. Various prostaglandins, including PGE2, 15-keto PGE2, and PGF2α, were analyzed.
expression of genes involved in PGE2 inactivation. Future studies of the regulation of prostaglandin influx and efflux carrier genes and their effects on epithelial biology will provide a greater understanding of this very important prostaglandin pathway in cancer.

References


Fig. 7. The roles of prostaglandin transporters PGT and MRP4 in determining the fate of PGE2 in colorectal adenoma cells. AA, Arachidonic acid; PGE2, Prostaglandin E2; PGT, Prostaglandin transporter; MRP4, Multidrug resistance-associated protein 4; COX-2, Cyclooxygenase-2; 15-PGDH, 15-PGDH2. COX-2/PGT/MRP4mediated degradation of PGE2.

Disclosure of Potential Conflicts of Interest


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