Hematopoietic Prostaglandin D Synthase Suppresses Intestinal Adenomas in \textit{Apc}^{Min/+} Mice

Jae Man Park,\textsuperscript{1} Yoshihide Kanaoka,\textsuperscript{3} Naomi Eguchi,\textsuperscript{4} Kosuke Aritake,\textsuperscript{4} Sava Grujic,\textsuperscript{2} Alicia M. Materi,\textsuperscript{1} Virgilio S. Buslon,\textsuperscript{5} Brigitte L. Tippin,\textsuperscript{1} Alan M. Kwong,\textsuperscript{1} Eduardo Salido,\textsuperscript{5} Samuel W. French,\textsuperscript{7} Yoshihiro Urade,\textsuperscript{1} and Henry J. Lin\textsuperscript{1}

\textsuperscript{1}Division of Medical Genetics, Department of Pediatrics and \textsuperscript{2}Department of Pathology, Los Angeles Biomedical Research Institute, Harbor-University of California-Los Angeles Medical Center, Torrance, California; \textsuperscript{3}Department of Medicine, Harvard Medical School and Brigham and Women’s Hospital, Boston, Massachusetts; \textsuperscript{4}Department of Molecular Behavioral Biology, Osaka Bioscience Institute, Osaka, Japan; \textsuperscript{5}Unidad de Investigacion, Hospital Universitario Canarias, Laguna, Tenerife, Spain

Abstract

Aspirin and other nonsteroidal anti-inflammatory drugs prevent some cases of colon cancer by inhibiting prostaglandin (PG) synthesis. PGE\textsubscript{2} promotes colon neoplasia, as shown by knockout mouse studies on enzymes and receptors in the PG cascade. A few experiments 20 to 30 years ago suggested that PGD\textsubscript{2} synthase in and bronchoconstriction. A few experiments 20 to 30 years ago suggested that PGD\textsubscript{2} synthase in and bronchoconstriction.

Historically, D series prostaglandins (PG) were viewed as by-products of PGE synthesis \textsuperscript{(1)}. When early researchers discovered a PGD synthase, they suspected that it might be part of a pathway for PG removal, instead of an enzyme responsible for a biologically active compound \textsuperscript{(2)}. PGD\textsubscript{2} is now known as a regulator of sleep, platelet aggregation, inflammation, smooth muscle contraction, and bronchoconstriction. Two types of PGD synthase are known \textsuperscript{(3–5)}. The brain type occurs in the nervous system, epididymis, and heart \textsuperscript{(6)}. It resembles lipophilic ligand carrier proteins and is called lipocalin-type PGD synthase (L-PGDS; refs. 7, 8). The enzyme is a homodimer and folds like other glutathione transferases \textsuperscript{(15)}. H-PGDS is a glutathione transferase (sigma type), based on its amino acid sequence and use of glutathione as a cofactor \textsuperscript{(13,14)}. The enzyme is a homodimer and folds like other glutathione transferases \textsuperscript{(15)}.

Materials and Methods

\textbf{Mouse strains.} C57BL/6j, FVB/NJ, and \textit{Apc}^{Min/+} \textsuperscript{(stock no. 002020)} mice came from The Jackson Laboratory (Bar Harbor, ME). \textit{L-Pgds} knockout mice were produced as described \textsuperscript{(9)}. Production of \textit{H-Pgds} knockout and \textit{H-PGDS} transgenic mice at Osaka Bioscience Institute (Osaka, Japan) and the Japan Tobacco, Inc. Pharmaceutical Frontier Research Laboratories (Osaka, Japan) is described below. Animals were fed Purina lab rodent diet (LabDiet 5001, PMI Nutrition International, Richmond, IN) \textit{ad libitum}. All procedures were approved by the institutional Animal Care and Use Review Committee.

\textbf{H-Pgds knockout mice.} The targeting vector for homologous recombination contained a neomycin resistance gene \textit{(neo)} placed between an 8.9-kb \textit{Xho-I} fragment and a lower excretion of a PGE\textsubscript{2} metabolite in transgenic mice to assess the hypothesis that PGD\textsubscript{2} production may influence development of intestinal adenomas. \textit{Apc}^{Min/+} mice deficient in the \textit{H-Pgds} enzyme developed 50% more intestinal adenomas, whereas mice with high expression of \textit{H-PGDS} had \textit{\sim} 80% fewer.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Henry J. Lin, Division of Medical Genetics, Harbor-University of California-Los Angeles Medical Center, 1124 West Carson Street, Torrance, CA 90502. Phone: 310-222-3783; Fax: 310-328-9921; E-mail: hlin@labimed.org.

\textcopyright 2007 American Association for Cancer Research.

doi:10.1158/0008-5472.CAN-05-3767

www.aacrjournals.org 881 Cancer Res 2007; 67: (3). February 1, 2007

Downloaded from cancerres.aacrjournals.org on April 13, 2017. © 2007 American Association for Cancer Research.
H-Pgds yielded homozygous Southern blotting (Fig. 1) that were >50% chimeric with C57BL/6 females and genotyped offspring by pseudopregnant ICR females to generate chimeras. We bred male mice into C57BL/6 blastocysts and transferred the blastocysts to genomic DNA from resistant colonies. We microinjected recombinant ES clones (ES) cells and selected for resistant clones with G418 (200 μg/mL; Invitrogen, Carlsbad, CA) and gancyclovir (2 μg/mL; Sigma, St. Louis, MO). Verification of homologous recombination was by Southern blotting of genomic DNA from resistant colonies. We microinjected the 3.1-kb fragment into pronuclei of fertilized FVB/N mouse eggs. Microinjection was done at DNX (Princeton, NJ). We identified transgenic mice by Southern blotting with a human H-PGDS gene probe.

We bred male H-PGDS transgenic mice (strain FVB/N; line S-55) with C57BL/6 females to produce transgenic mice on a mixed C57BL/6 × FVB/N background. Similarly, we bred C57BL/6 ApcMin/+ males with FVB/N females to produce mixed ApcMin/+ mice. We then intercrossed the progeny to produce ApcMin/+ mice with transgenic H-PGDS (and control ApcMin/+ mice) on a mixed background.

**Genotyping.** We genotyped mice by the use of PCR (Supplementary Table S1). DNA templates were 1-mm punches of dried blood on blotter paper (no. 903; Whatman Schleicher and Schuell, Florham Park, NJ). We collected drops of blood from tail segments at 10 days and when mice were sacrificed. We genotyped both blood specimens. For H-PGDS transgenic mice with ApcMin/+ we also used DNA from 10-μm sections of the paraffin blocks containing coiled intestines. Each PCR was in a total volume of 15 μL. Detection of PCR products was on agarose gels stained with ethidium bromide.

**mRNA isolation and reverse transcription-PCR.** We isolated total RNA from −100 mg colon tissue (RNasey Lipid Tissue Mini kit, Qiagen, Germantown, MD). We did two-step quantitative reverse transcription-PCR (RT-PCR) in triplicate for each sample, by the use of an ABI Prism 7000 Sequence Detector and Taqman Gold RT-PCR reagents (Applied Biosystems, Foster City, CA). Oligonucleotide primers and probes (designed by the use of Primer Express software, Applied Biosystems) spanned introns to prevent any amplification of genomic DNA (Supplementary Table S1). Phragmogene sequences were as follows: 6FAMCGTGGAAAGACAGCGTTGGAGAATG (mouse H-Pgds) and 6FAMCCAAAGCTGGTACCTTAAGAAGATG (human H-PGDS). We amplified endogenous mouse glyceraldehyde-3-phosphate dehydrogenase as a reference in all experiments ( Assays-on-Demand, Applied Biosystems). We estimated copy numbers of mouse H-Pgds or transgenic human H-PGDS transcripts by the use of two standard curves plotted by amplifying a plasmid carrying the human cDNA sequence or a PCR product encoding the mouse cDNA.

**Conditions for reverse transcription (step 1)** were as follows: 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min, with a reaction containing −200 ng total RNA, 1× Taqman RT buffer, 5.5 mmol/L MgCl₂, 500 μmol/L of each deoxynucleotidetriphosphate, 2.5 μmol/L random hexamers, 4 units RNase inhibitor, and 12.5 units MultiScribe reverse transcriptase (Applied Biosystems). PCR conditions for step 2 were as follows: 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min, with a reaction containing 10 μL of the product from step 1, 1× Taqman buffer, 5.5 mmol/L MgCl₂, 200 μmol/L each of dATP, dGTP, and dCTP, 400 μmol/L dUTP, 100 μmol/L of the fluorescent probe, 200 nmol/L of each forward and reverse primer, and 1 unit AmpliTaq Gold DNA polymerase.

**Urinary PGs.** We housed mice individually in metabolic cages to obtain urine. We collected urine during the day and overnight for a 24-h period, spun the sample to pellet the debris, and froze specimens at −80°C until ready for assay. Determinations of 11-hydroxy-11-oxo-2,3,4,5-tetranor-prostan-1,20-dioic acid (PGE-M) were by enzyme immunoassay ( Cayman Chemical, Ann Arbor, MI). The Harbor-University of California-Los Angeles General Clinical Research Center core laboratory measured urine creatinine, by the use of the Jaffe alkaline picrate reaction in an autoanalyzer. We transformed data (in picograms of PG per milligram of creatinine) to their logarithms (base 10) and assessed differences by mixed model ANOVA to account for multiple and varying numbers of urine samples per mouse.

**Intestinal histopathology.** We sacrificed mice at 14 weeks, immediately removed the intestine in one piece, and placed it in a glass dish with Ringer...
solution. We flushed the interior of the intestine with Ringer solution through an 18-gauge needle, then flattened the intestine on Whatman 3MM paper, opened it lengthwise, and fixed it in buffered formalin for 2 to 4 h. We coiled the fixed intestine like a Swiss roll (inside out) around a wooden stick, removed the stick, and placed the coil in an embedding cassette. Specimens were embedded in paraffin, sectioned (4-μm thickness), mounted on slides (Snowcoat X-tra, Surgipath, Richmond, IL), and stained with H&E. We examined sections by the use of a Leica MZ6 stereo microscope (up to ×40 magnification, Leica Microsystems, Wetzlar, Germany) to identify and count adenomas. We used higher magnification (×100–×400) if needed to confirm. We measured adenomas by the use of an eyepiece reticle on the Leica MZ6 microscope. All slides were examined without knowledge of genotypes.

For H-Pgds and L-Pgds knockout mice, we examined 18 sections spaced 240 to 260 μm apart. For H-PGDS transgenic mice, we examined 24 sections spaced ~150 μm apart. We prepared more sections than we did for knockout mice, in case there were substantially fewer adenomas due to the transgene or to the mixed genetic background of the mice (23). Use of sections spaced 150 μm apart allows detection of most small adenomas (see Results for the size range). Numbers of adenomas in our transgenic mice are not directly comparable with numbers in the knockout mice due to these differences in methods.

We used a color-coding procedure to avoid counting adenomas more than once. Each slide had two sections, mounted left to right in the order of cutting, and we examined 9 or 12 consecutive slides. We first marked all adenoma profiles with a red ink dot. We then examined each slide again to compare the left section with the right section. Profiles were marked with a black dot, if the same adenoma was represented on both the left and the right side of the same slide. We then examined all slides a third time to compare adenoma profiles in the right section of each slide with the left section of the next consecutive slide. Profiles were marked with a blue dot, if the adenoma was represented on both the right section of a slide and the left section of the next consecutive slide. Thus, we marked all adenoma profiles with either one red dot, two dots (either red/black or red/blue), or three dots (red/black/blue). We counted only the "red" and "red/black" adenoma profiles on the right section of each slide and the "red" and "red/blue" adenoma profiles on the left section of each slide.

We randomly chose 10% of the intestines for recounting after we examined slides from all 85 mice, again without knowledge of genotypes. Average deviations in counts were 1.4 adenomas for the entire intestine. We also chose slides at random for independent rereview (by S.W.F. and S.G.).

**Statistical analyses of adenoma data.** We transformed numbers of adenomas to their logarithms (base 10) to approximate a normal distribution. We also chose slides at random for independent review (by S.W.F. and S.G.).

**Immunohistochemistry.** For H-Pgds staining, we treated deparaffinized sections with 10 mmol/L Tris-HCl (pH 9.5, 45 min, 80°C) for unmasking followed by 0.3% H2O2 in methanol to inhibit endogenous peroxidases (15 min, room temperature) and 20% normal goat serum in PBS for blocking (30 min, room temperature). Next, we treated sections with 0.1% Triton X-100 (10 min, room temperature). Then, for staining, we used either a rat anti-mouse H-Pgds monoclonal antibody (1:100 dilution) or a rabbit anti-mouse H-Pgds polyclonal antibody (1:200 dilution) in PBS overnight; both antibodies were from Cayman Chemical. Signal detection was with biotinylated antirat or antirabbit IgG, respectively (Vector Laboratories, Burlingame, CA) followed by avidin-biotin-peroxidase complexes and diaminobenzidine as the chromogen. The avidin-biotin-peroxidase reaction was in 0.35 mol/L NaCl to prevent nonspecific avidin binding to mast cells (24).

For macrophage-specific staining, we preincubated deparaffinized and hydrated sections (10-μm thick) with 0.3% H2O2 in methanol followed by PBS with 0.01% Triton X-100. After pretreatment with pepsin for 15 min, we sequentially incubated the sections with Iba-1 antibody, an appropriate biotinylated secondary antibody (rabbit IgG), and avidin-biotin complexes, according to the manufacturer's protocol (Vector Laboratories). We visualized immunoreactivity with 0.03% H2O2 in 50 mmol/L Tris-HCl (pH 7.6) with 0.05% diaminobenzidine.

![Figure 2. Urine PG excretion in WT and H-PGDS transgenic mice. A, urine 11β-PGF2α in WT and H-PGDS transgenic mice, representative of 84 urine collections from 37 mice (one to nine urine collections per mouse). Three points show results of a single measurement, whereas all others represent the average of at least two measurements at different dilutions of urine. Only two mice were ApcMin+ carriers, and both had WT PGD synthase genes [log10 (11β-PGF2α) = 2.56 and 2.50]. The difference in geometric means between WT and H-PGDS transgenic mice was not statistically significant (one-way mixed model ANOVA for these two groups; P = 0.09, two tailed). B, urine PGE-M in WT and H-PGDS transgenic mice, representative of 84 urine collections from 37 mice. Two points show results of a single measurement, whereas all others represent the average of at least two determinations at different urine dilutions. There were no ApcMin− carriers among these mice. The difference in geometric means between WT and H-PGDS transgenic mice was not statistically significant (one-way mixed model ANOVA for these two groups; P = 0.19, two tailed).](https://www.cancerres.aacrjournals.org/content/67/3/883/F2.large.jpg)
Western blot analysis. We harvested bone marrow cells from H-Pgds<sup>+/+</sup> and H-Pgds<sup>−/−</sup> mice and cultured them for 4 weeks in the presence of interleukin (IL)-3. More than 95% of the nonadherent cells were mast cells (by toluidine blue staining). We used SDS-PAGE to separate proteins from 1 × 10<sup>5</sup> bone marrow–derived mast cells and blotted them onto a polyvinylidene fluoride membrane. We incubated the blot with rabbit antimouse H-Pgds antiserum (1:1,000 dilution) and then with horseradish peroxidase–conjugated goat anti-rabbit IgG. After incubation with SuperSignal substrate (Pierce Biotechnology, Inc., Rockford, IL), we visualized immunoreactive proteins with Kodak BioMax film (Molecular Imaging Systems, Eastman Kodak Co., New Haven, CT).

Results

Characterization of H-Pgds knockout mice. Mouse and human H-Pgds genes have six exons, which encode 199 amino acids (21). To produce mice deficient in H-Pgds, we made a targeting vector that disrupted exon 2, which encodes 44 residues, including the NH<sub>2</sub>-terminal methionine and two amino acids important for enzyme function (Tyr<sup>4</sup> and Arg<sup>5</sup>; Fig. 1A; ref. 25). We made clones of mutated ES cells (strain 129/Sv) and used them to generate chimeric mice. Chimeric males bred with C57BL/6 females yielded offspring in expected 1:2:1 ratios: 27.8% transgenic mice (geometric mean, 7.5 × 10<sup>3</sup>mice), 45.6% H-Pgds<sup>+/−</sup> mice (36 of 79), and 26.6% H-Pgds<sup>−/−</sup> mice (21 of 79; χ<sup>2</sup> = 0.65; P = 0.72). Thus, there was no sign of embryonic lethality. H-Pgds null mice developed normally. Average weights at 10 weeks indicated no difference in growth between H-Pgds<sup>−/−</sup> (21.7 g for six mice) and wild-type (WT) mice. H-Pgds<sup>−/−</sup> mice produced no H-Pgds or truncated products detectable by Western blotting of proteins from bone marrow–derived mast cells (Fig. 1D).

High H-PGDS expression in transgenic mice. To generate mice that overproduce H-PGDS, we used a human H-PGDS coding sequence controlled by a chicken β-actin promoter, the rabbit β-globin polyadenylation signal, and the cytomegalovirus immediate early enhancer (22). We microinjected the gene into pronuclei of fertilized FVB/N mouse eggs and identified transgenic founders by Southern blotting. We did not determine copy number or insertion sites. Mice from transgenic lines appeared healthy, had normal growth, and produced offspring, H-PGDS transgenic mice and controls used in experiments below were of a mixed background (C57BL/6 × FVB/N; see Materials and Methods).

Transgenic mice had high expression of human H-PGDS in colon tissue, as shown by reverse transcription and quantitative PCR. We found 998 to 6,090 copies of mouse H-Pgds transcripts per nanogram of total RNA in four mice (geometric mean, 2.0 × 10<sup>3</sup> copies). In contrast, we found 5.97 to 6,090 copies of human H-PGDS transcripts per nanogram of total RNA in two transgenic mice (geometric mean, 7.5 × 10<sup>3</sup> copies). The mean copy numbers correspond to a 375-fold increase in expression of transgenic H-PGDS over endogenous mouse H-Pgds.

Urinary 11β-PGF<sub>2α</sub> and PGE-M in H-PGDS transgenic mice. PGD<sub>2</sub> degrades rapidly in vivo and is removed from the circulation. 11β-PGF<sub>2α</sub> is the first metabolite that appears in the urine and is an indicator of PGD<sub>2</sub> produced in the body (26). Similarly, PGE<sub>2</sub> is quickly catabolized in the lungs, and urinary PGE-M reflects tissue PGE<sub>2</sub> production (27). Excretion of 11β-PGF<sub>2α</sub> varied from 140 to 1,700 pg/mg creatinine for mice with WT H-Pgds genes (geometric mean, 460). Urinary PGE-M varied from 390 to 12,400 pg/mg creatinine for WT controls (geometric mean, 2,520). Compared with controls, H-PGDS transgenic mice had somewhat higher 11β-PGF<sub>2α</sub> excretion [geometric mean, 630; 1.4-fold higher; 95% confidence interval (95% CI), 0.94–2.0; P = 0.09] and lower PGE-M excretion (geometric mean, 1,613; 0.64-fold lower; 95% CI, 0.32–1.26; P = 0.19), but differences were not statistically significant (Fig. 2).

Increased numbers of intestinal adenomas in Apc<sup>Min−/−</sup> mice with H-Pgds gene knockouts. We sacrificed mice at 14 weeks to count adenomas histologically. We prepared the entire intestine like a Swiss roll, embedded it in paraffin, and made slides for

![Figure 3. Observed numbers of adenomas in Apc<sup>Min−/−</sup> mice, with H-Pgds knockout mutations or transgenic H-PGDS, and controls (WT). Plotted points, logarithms of the numbers of adenomas. Horizontal bars, mean. *, P < 0.05, statistically significant difference between the indicated genotype and controls; NS, nonsignificant difference. A, adenomas in the entire intestine of Pgds knockout mice and controls. H-Pgds<sup>+/−</sup>, homozygous H-Pgds knockout mice; H-Pgds<sup>−/−</sup>, heterozygous H-Pgds knockout mice; L-Pgds<sup>+/−</sup>, homozygous L-Pgds knockout mice; WT, control mice (Apc<sup>Min+</sup>). B, colon adenomas in Pgds knockout mice and controls. Labeling is the same as in Fig. 2A above. C, adenomas in the entire intestine (left) and in the colon (right) of H-PGDS transgenic mice and controls. TG, H-PGDS transgenic mice; WT, control mice (Apc<sup>Min+</sup>). We added 0.5 to all numbers of colon adenomas before taking the logarithm because three transgenic mice had zero colon adenomas.]

Cancer Res 2007; 67: (3). February 1, 2007

884

www.aacrjournals.org

Downloaded from cancerres.aacrjournals.org on April 13, 2017. © 2007 American Association for Cancer Research.
The numbers of adenomas in H-PGDS and Intestinal Adenomas

| Table 1. Adenomas in ApcH−/− mice with PGD synthase knockouts or transgenes |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Knockout mice   | Controls         | Transgenic mice | Controls         |
|                 | H-Pgds−/−       | H-Pgds+/−        | H-Pgds−/−       | H-PGDS           |
| No. adenomas in the entire intestine*                          |                 |                 |                 |                 |
| Geometric mean                                              | 224             | 250             | 146             | 155             |
| No. mice                                                   | 29              | 22              | 12              | 22              |
| P, ANOVA †                                                 | <0.05           | <0.05           | NS              | —               |
| P, nonparametric †                                          | 9 × 10−4        | 9 × 10−5        | NS              | —               |
| P, t test †                                                | <0.0001         | 0.002           | —               | —               |
| P, Mann-Whitney                                            |                 |                 |                 |                 |
| No. adenomas in the colon                                   |                 |                 |                 |                 |
| Geometric mean                                              | 8.2             | 8.2             | 3.6             | 4.0             |
| Range                                                      | 2–21            | 1–22            | 1–9             | 1–12            |
| No. mice                                                   | 29              | 22              | 12              | 22              |
| P, ANOVA †                                                 | <0.05           | <0.05           | NS              | —               |
| P, nonparametric †                                          | 0.002           | 0.004           | NS              | —               |
| P, t test †                                                | 0.0001          | 0.0001          | —               | —               |
| P, Mann-Whitney                                            |                 |                 |                 |                 |

Abbreviation: NS, not statistically significant.
*We counted adenomas histologically at 14 weeks of age. For knockout mice, we counted adenomas in 18 sections spaced 250 μm apart. For transgenic mice, we counted adenomas in 24 sections spaced 150 μm apart. Results for knockout and transgenic mice cannot be directly compared with one another due to this difference in methods. In addition, genetic backgrounds of the mice differed: C57BL/6 for knockout mice and C57BL/6 x FVB/N for transgenic mice. We transformed numbers of adenomas to their logarithms before doing ANOVA or t tests.
† P values refer to comparison between the indicated genotype and controls (ApcH−/−) by one-way ANOVA with Dunnett’s method for multiple comparisons (two tailed).
‡ We compared the four groups by use of the Kruskal-Wallis test, obtaining an overall P value of <0.001. P values for comparisons between the indicated genotypes and controls are from Mann-Whitney tests with Bonferroni corrections.
The P value refers to comparison between H-PGDS transgenic mice and controls by the t test (two tailed).
We added 0.5 to numbers of colon adenomas in transgenic mice and controls before transforming data to logarithms (for t tests) because three transgenic mice had zero colon adenomas. The ranges shown are the observed values, however.
For measurement of adenomas in the small bowel, we used three mice for each genotype. Median numbers of adenomas in these nine mice were close to the median number for all mice with the genotype.

microscopic analysis. We identified tumors in 18 sections spaced 240 to 260 μm apart. By this method, we sampled nearly the entire intestine, using the entire paraffin block except for the partial sections at the beginning and end of each block (see Materials and Methods).

ApcH−/− mice that were homozygous or heterozygous for the H-Pgds deletion had 44% to 61% more intestinal adenomas than did ApcH−/− mice with WT H-Pgds (Fig. 3A; Table 1). More than 95% of the adenomas were in the small bowel. Colon adenomas increased 2-fold (Fig. 3B). There was no statistical difference between numbers of adenomas in H-Pgds−/− versus H-Pgds+/− mice. Numbers of adenomas in ApcH−/− mice homozygous for L-Pgds deletions were not different from controls.

We measured sizes of 1,776 adenomas of the small bowel in a total of nine ApcH−/− mice (three mice each with H-Pgds−/−, H-Pgds+/−, and H-Pgds+/− genotypes; Fig. 4; Table 1). We used mice whose numbers of adenomas were very close to the median number for their genotype. Sizes ranged from 0.1 to 2.5 mm, as measured under the microscope. The smallest adenomas were microscopic foci of dysplastic cells. Adenomas in mice with H-Pgds...
knockouts tended to be smaller than adenomas in controls. For example, average sizes for H-Pgds+/− and H-Pgds−/− mice were 0.49 to 0.51 mm compared with 0.68 mm for H-Pgds+/+ mice. However, differences were not assessed statistically.

We measured sizes of 596 colon adenomas in a total of 73 mice (data not shown). We excluded mice with L-Pgds knockouts. Adenoma sizes ranged from 0.1 to 4.6 mm. Most were <1 mm. There appeared to be no difference in sizes of colon adenomas across H-Pgds genotypes, judging from the variation seen.

**Reduced numbers of intestinal adenomas in Apc<sup>Min</sup>/− mice with transgenic human H-PGDS.** We prepared slides as described above, except we examined 24 sections spaced 150 µm apart. We used more sections for transgenic mice than for knockout mice, anticipating potentially fewer adenomas due to the transgene or to the hybrid genetic background of the mice (C57BL/6 × FVB/N). For example, Apc<sup>Min</sup>/− mice on a hybrid C57BL/6 × AKR background had ~80% fewer tumors than did C57BL/6 Apc<sup>Min</sup>/− mice (23). Use of sections spaced 150 µm apart allows detection of a higher proportion of small adenomas (~100 µm in diameter) in the sectioned region. Therefore, results for H-PGDS transgenic and H-Pgds knockout mouse are not directly comparable. Apc<sup>Min</sup>/− mice with H-PGDS transgenes had 70% to 80% fewer adenomas than did controls, in both the small bowel and the colon (Fig. 3C; Table 1). The size range of adenomas in H-PGDS transgenic mice was similar to ranges for H-Pgds knockout mice and controls.

**H-Pgds in macrophages of the intestinal mucosa.** We used immunohistochemistry to detect H-Pgds in the intestinal mucosa. We did avidin-biotin-peroxidase reactions in 0.35 mol/L NaCl (24) to prevent nonspecific staining of mast cells. Intestines from mice with homozygous H-Pgds gene knockouts did not stain with anti-H-Pgds antibodies under these conditions (Fig. 5A).

We detected H-Pgds in macrophages and monocytes in the intestinal mucosa of WT mice (Fig. 5B and D). H-Pgds–positive cells occurred in the same stromal distribution as did macrophages stained with Iba-1 antibodies (Fig. 5C). Mast cells also stained for H-Pgds, but there were only a few mast cells in our sections (data not shown). Peyers patches showed scattered H-Pgds–positive cells rather than uniform staining (data not shown), supporting an interpretation that the stained cells were not lymphocytes. No H-Pgds was detected in fibroblasts, endothelial cells, or epithelium as judged by cell morphology or location.

**Discussion**

Mice prone to intestinal polyposis have been used to study PG effects on adenomas. Disruption of genes in the PGE pathway supports a conclusion that PGE<sub>2</sub> promotes tumors (28–30). Signaling pathways are being elucidated (31, 32). Here, we used knockouts of two types of PGD synthase, as well as transgenic mice with high expression of human H-PGDS, to follow up early work on blocking of cancer by PGD<sub>2</sub> (16–19). These genes represent the only known enzymes in the PGD<sub>2</sub> pathway from PGI<sub>2</sub>.

We found 50% more small bowel adenomas and a 2-fold increase in colon adenomas in Apc<sup>Min</sup>/− mice deficient in H-Pgds (homozygous or heterozygous; Fig. 3; Table 1). Homozygous knockout of L-Pgds did not affect intestinal adenomas, most likely because the "brain" type of the enzyme is nearly absent in the gut. Conversely, there were 70% to 80% fewer adenomas in Apc<sup>Min</sup>/− mice with transgenic H-PGDS (Fig. 3; Table 1). The drop occurred in both the small bowel and the colon. Fewer adenomas in H-PGDS transgenic mice and more adenomas in H-Pgds knockout mice strongly support an interpretation that H-PGDS, PGD<sub>2</sub>, or metabolites can inhibit tumors. Kim et al. (33) reached a similar conclusion by the use of prostate cancer cell lines cultured with prostate stromal cells that produce L-PGDS.

In the gut mucosa, H-Pgds stained in macrophages and monocytes, but generally not in other stromal cells or in the epithelium (Fig. 5). Our results confirm the original rat study, which showed H-Pgds in macrophages in the stomach, small intestine, colon, liver, spleen, and thymus (12). In particular, there were positively stained macrophages in the lamina propria of the small bowel and colon as in our slides. These two studies support an interpretation that macrophages are the major source of H-Pgds in the gastrointestinal tract, the largest macrophase pool in the body (34).

The intestinal mucosa contains cyclooxygenase-2 (Cox-2), mostly in stromal fibroblasts and endothelial cells (35, 36). Colon adenomas and carcinomas also have high levels of inducible

---

Figure 4. Size distribution of adenomas among mice with different H-Pgds genotypes. A, small bowel adenomas in three H-Pgds−/− mice. B, small bowel adenomas in three H-Pgds+/− mice. C, small bowel adenomas in three H-Pgds+/+ mice.

---

K. Aritake and Y. Urade, unpublished observation.
microsomal PGE synthase (mPGES), which produces PGE₂. mPGES stains readily in epithelial cells of colorectal tumors (37). Thus, PG synthesis in at least three cell types, fibroblasts, epithelial cells, and macrophages, influences adenoma growth. Specifically, PGE₂ made in epithelial cells, or imported from stromal cells, seems to promote adenomas. In an opposite effect, H-PGDS in macrophages may suppress adenomas.

Involvement of macrophages in tumorigenesis has been recognized (38, 39). Tumor-associated macrophages have both stimulating and inhibitory effects (40). Stimulating factors produced by macrophages include nitric oxide synthase, vascular endothelial growth factors, matrix enzymes (e.g., metalloproteinases), and other cytokines (e.g., tumor necrosis factor-α and IL-1α; refs. 41, 42).

In an experimental example, Oshima et al. (43) produced transgenic mice with high expression of both Cox-2 and mPGES in stomach epithelium. The mice had heavy macrophage infiltration and large, benign tumors in the stomach. Treating the mice with either a Cox-2 inhibitor or antibiotics reduced macrophage infiltration and tumors. The authors hypothesized that PGE₂ enhances macrophage infiltration, leading to tumors when macrophages are activated by gastric flora.

Ricote et al. (44) found evidence for blunting of macrophage activation by PGD₂ metabolites. Activated peritoneal macrophages have more peroxisome proliferator-activated receptor γ (PPARγ) compared with resting macrophages in bone marrow. But, activated macrophages treated with 15-deoxy-Δ12,14-PGJ₂, a PPARγ ligand and a metabolite of PGD₂, had properties of resting macrophages, such as low inducibility of nitric oxide synthetase and little or no gelatinase B (matrix metalloproteinase 9). The authors concluded that PPARγ may be a negative regulator of macrophage activation in response to PGD₂ metabolites.

The nuclear factor-κB (NF-κB) pathway is a possible target for 15-deoxy-Δ12,14-PGJ₂, both directly and through PPARγ (45). For example, Straus et al. (46) proposed that covalent binding of the compound through its cyclopentenone structure to NF-κB inhibin kinase may block activation of growth factors, cytokines, and other inflammatory proteins. Measurement of such growth-promoting molecules in tumors from ApcMin/+ mice with H-PGDS transgenes may be useful to assess this mechanism (47).

Shibata et al. (48) detected 15-deoxy-Δ12,14-PGJ₂ in foamy macrophages of the human aorta and in RAW264.7 macrophage cells. However, Bell-Parikh et al. (49) found very low levels of this compound in preadipocytes (1 nmol/L in 3T3-L1 fibroblasts or <100 pg/10⁶ cells). The levels were 1,000 times less than needed to cause maturation to adipocytes. Thus, the biological role of 15-deoxy-Δ12,14-PGJ₂ as a PPARγ ligand may be limited (50).

Alternatively, transgenic H-PGPDS may suppress tumors by shifting conversion of PGH₂ away from PGE₂. Similarly, knockout of H-Pgds may increase tumors by routing PGH₂ to PGE₂ (28–32). PGD₂ represents 6% of total PGs in macrophages compared with 63% for PGE₂ (51). We measured urine metabolites to check for rerouting between PGD₂ and PGE₂.
Urine levels of 11β-PGF₂α and PGE-M, the major metabolites of PGD₂ and PGE₂, varied over a 12- to 30-fold range, respectively. For H-PGDS transgenic mice (all non-Apc<sup>Min</sup>-), 11β-PGF₂α excretion was somewhat higher, and PGE-M excretion was somewhat lower than in controls. However, differences were not statistically significant (Fig. 2A and B). A possible explanation is that very high PGD₂ production in transgenic mice requires cell activation, by pain, bacterial lipopolysaccharides, ethanol, or other stimuli (22). For example, Pinzar et al. found that unstimulated L-PGDS transgenic mice had only 1.5-fold more PGD₂ in the brain than did WT controls. Pain stimulation (by tail clipping) led to 17-fold more brain PGD₂ in one of the transgenic lines (B7). It is also possible that 24-h urinary PGs may not closely reflect PGD₂ levels in the intestines. Measurement of PGD₂ in gut or tumor tissue may be needed.

Null mutations of the PGD receptor (DP₁) did not raise numbers of aberrant crypt foci in mice treated with the colon carcinogen, azoxymethane (30). Therefore, DP₁ may not be part of a PGD₂ effect. However, there have been no studies of the DP₁ receptor in Apc<sup>Min</sup>- mice. PGD₂ action through other prostaglandin receptors may be possible.

The exon 2 deletion in our H-Pgds knockout mice could theoretically lead to a truncated protein, unrecognized by the antibody, which might have a dominant-negative effect on the H-Pgds dimer. However, protein translation from the next available, theoretically lead to a truncated protein, unrecognized by the tissue may be needed.

17-fold more brain PGD₂ in one of the transgenic lines (B7). It is also possible that 24-h urinary PGs may not closely reflect PGD₂ levels in the intestines. Measurement of PGD₂ in gut or tumor tissue may be needed.

Null mutations of the PGD receptor (DP₁) did not raise numbers of aberrant crypt foci in mice treated with the colon carcinogen, azoxymethane (30). Therefore, DP₁ may not be part of a PGD₂ effect. However, there have been no studies of the DP₁ receptor in Apc<sup>Min</sup>- mice. PGD₂ action through other prostaglandin receptors may be possible.

The exon 2 deletion in our H-Pgds knockout mice could theoretically lead to a truncated protein, unrecognized by the antibody, which might have a dominant-negative effect on the H-Pgds dimer. However, protein translation from the next available, the theoretic lead to a truncated protein, unrecognized by the tissue may be needed.

17-fold more brain PGD₂ in one of the transgenic lines (B7). It is also possible that 24-h urinary PGs may not closely reflect PGD₂ levels in the intestines. Measurement of PGD₂ in gut or tumor tissue may be needed.
Hematopoietic Prostaglandin D Synthase Suppressed Intestinal Adenomas in $Apc^{\text{Min}/+}$ Mice

Jae Man Park, Yoshihide Kanaoka, Naomi Eguchi, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/67/3/881

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2007/02/08/67.3.881.DC1

Cited articles
This article cites 51 articles, 28 of which you can access for free at:
http://cancerres.aacrjournals.org/content/67/3/881.full.html#ref-list-1

Citing articles
This article has been cited by 3 HighWire-hosted articles. Access the articles at:
/content/67/3/881.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.