

Cooperation of Cyclooxygenase 1 and Cyclooxygenase 2 in Intestinal Polyposis¹

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ABSTRACT

Membrane arachidonic acid is converted by cyclooxygenase (COX) into prostaglandin (PG) G₂ and then to PGH₂ which is subsequently metabolized to PGE₂ by PGE synthase (PGES). Both COX-1 and COX-2 play critical roles in intestinal polyp formation, whereas COX-2 is also expressed in cancers of a variety of organs. Likewise, inducible microsomal PGES (mPGES-1) is expressed in several types of cancer, although its role in benign polyp formation has not been investigated. We demonstrated recently that most COX-2-expressing cells in the polyps are stromal fibroblasts. Here we show colocalization of COX-1, COX-2 and mPGES in the intestinal polyp stromal fibroblasts of *Apc*^{Δ716} mice, a model for familial adenomatous polyposis. Contrary to COX-2 that was induced only in polyps >1 mm in diameter, COX-1 was found in polyps of any size. In polyps >1 mm, not only COX-2 but also mPGES was induced in the stromal fibroblasts where COX-1 had already been expressed. Although polyp number and size were markedly reduced in COX-1 (–/–) or COX-2 (–/–) compound mutant *Apc* mice, both COX-2 and mPGES were induced in the COX-1 (–/–) polyps, whereas COX-1 was expressed in the COX-2 (–/–) polyps. We found also in human familial adenomatous polyposis polyps that COX-2 and mPGES were induced in the COX-1-expressing fibroblasts. On the basis of these results, we propose that COX-1 expression in the stromal cells secures the basal level of PGE₂ that can support polyp growth to ~1 mm, and that simultaneous inductions of COX-2 and mPGES support the polyp expansion beyond ~1 mm by boosting the stromal PGE₂ production.

INTRODUCTION

Epidemiological and clinical studies indicate that administration of NSAIDs³ lowers the mortality rate of colon cancer (1–3). In addition, numerous animal experiments (*i.e.*, with using chemical carcinogen-induced rat tumors or *Apc* mutant mouse polyps) have shown that NSAIDs prevent intestinal tumor development (reviewed in Refs. 4–6). The chief pharmacological targets of conventional NSAIDs are COX-1 and COX-2, which are rate-limiting isozymes for PG biosynthesis from arachidonic acid (7). Evidence is accumulating that both isoenzymes play critical roles in tumorigenesis. The expression level of COX-2 is elevated in colorectal and other cancer tissues (8, 9). By disruption of the COX-2 gene (*Ptgs2*) in *Apc*^{Δ716} mice, a model for FAP, we demonstrated earlier that COX-2 induction is essential for polyp formation (10). In addition, we showed marked decreases in the intestinal polyp number, and size in *Apc*^{Δ716} and *Apc*^{Min} mice by dosing COX-2 inhibitors (10–12). Subsequently, it has been con-

firmed by a clinical study that a COX-2 inhibitor can significantly reduce the number and size of colonic polyps in FAP patients (13). On the other hand, we have demonstrated that COX-1 also plays an essential role in intestinal polyposis and skin carcinogenesis (14, 15). These results are consistent with earlier epidemiological data that some NSAIDs, such as aspirin, that inhibit only COX-1 at the normal pharmacological doses, can reduce colon cancer incidence and mortality (16).

We have also shown recently that COX-2 is expressed in the polyp stromal cells, rather than in the adenoma epithelial cells in *Apc*^{Δ716} mice, and that ~85% of the COX-2-expressing cells are vimentin-positive fibroblasts (10, 17). However, the cells that express COX-1 and their relationship with the COX-2-expressing cells have not been determined during the intestinal polyp formation.

Regarding the signals downstream of COX-2, we have reported recently that disruption of the gene for the PGE₂ receptor EP2 in the *Apc*^{Δ716} mice causes suppression of intestinal polyposis, indicating that the PGE₂ signal for adenoma cell growth is mediated through the EP2 receptor (18). Whereas COX-1 and COX-2 convert arachidonic acid into an intermediate metabolite PGH₂, it is additionally isomerized to PGE₂ by PGE₂ synthase. Two isoenzymes of PGE₂ synthase, cPGES and mPGES, have been identified and characterized (19–21). Whereas cPGES is expressed constitutively, mPGES is inducible, and at least two enzymes appear to exist for mPGES (22–24). In some cell lines, mPGES is functionally coupled with COX-2, and induced in cancer tissues of the colon and lung, suggesting its role in tumorigenesis (25, 26). However, expression of mPGES in benign polyp tissues has not been investigated thoroughly. In this study, we have analyzed expression of COX-1, COX-2, and mPGES (mPGES-1) in the intestinal polyps of *Apc* mutants and FAP patients, and found that COX-2 and mPGES are induced simultaneously in the stromal fibroblasts in which COX-1 has been expressed constitutively.

MATERIALS AND METHODS

Animals. Constructions of compound mutant mice *Apc*^{Δ716} COX-2 (–/–) and *Apc*^{Min} COX-1 (–/–) were described previously (10, 14). Five age-matched *Apc*^{Δ716} mice were used for scoring of the polyp diameters as described previously (10). Briefly, all of the intestinal polyps excluding those <0.1 mm in diameter were counted by a single examiner under a dissecting microscope.

Quantitative Real-Time RT-PCR. The gene-specific primer sets were designed for COX-2 (forward, 5'-AAT GAG TAC CGC AAA CGC TTC; reverse, 5'-CAG CCA TTT CCT TCT CTC CTG TA) and mPGES (forward, 5'-CAA CGA CAT GGA GAC AAT CTA TCC; reverse, 5'-GGA AAT GTA TCC AGG CGA TCA). More than 5 polyps >1 mm and normal intestines were collected from *Apc*^{Δ716} mice (*n* = 5). Total RNA was prepared using ISOGEN solution (Nippon Gene, Toyama, Japan), and cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time PCR was performed using ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Each reaction mixture contained 100 ng of cDNA as template, 2× master mix solution, 0.3 μM of primers, and 1.25 μM of the gene specific probe. Probes for COX-2 (5'-CCC TGA AGC CGT ACA CAT CAT TTG AAG AAC-3') and mPGES (5'-TTC CTC TTC CTC GGC TTC GTG TAC TCA TTC-3') were labeled with the carboxyfluorescein fluorescent dye.

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³ The abbreviations used are: NSAID, non-steroidal anti-inflammatory drug; *Apc*, adenomatous polyposis coli; COX, cyclooxygenase; cPGES, cytosolic prostaglandin E synthase; FAP, familial adenomatous polyposis; mPGES, microsomal prostaglandin E synthase; PG, prostaglandin; RT-PCR, reverse transcription-PCR.

Western Blotting. The normal intestine and polyp tissues were homogenized and sonicated, respectively, in lysis buffer [50 mM phosphate buffer (pH 7.0), 100 mM NaCl, and 2 mM EDTA] containing a protease inhibitor mixture (Roche). After centrifugation at $10,000 \times g$ at 4°C for 10 min, 40 μg of the supernatant protein was mixed with $5\times$ SDS sample buffer [350 mM Tris HCl (pH 6.8), 36% glycerol, 10% SDS, and 600 mM DTT], and separated in 10% or 10–20% gradient SDS polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes. After blocking with 5% skimmed milk/Tris-buffered saline-Tween 20, membranes were incubated with an antibody for COX-1 (Santa Cruz Biotechnology, Santa Cruz, CA), COX-2, cPGES, or mPGES (Cayman Chemical, Ann Arbor, MI) at 200-, 1,000-, 200-, or 500-fold dilution, respectively. The enhanced chemiluminescence detection system (Amersham Pharmacia, Uppsala, Sweden) was used to detect the specific signals. The same membrane was reprobed with anti- β -actin antibody (Sigma) at 5,000-fold dilution to calibrate the total protein loaded.

Immunohistochemistry and Immunofluorescence Staining. Respective antibodies for COX-1 (Santa Cruz Biotechnology), COX-2, mPGES (Cayman Chemical), vimentin (Sigma), and Ki-67 (MIB-5; DAKO, Copenhagen, Denmark) were used as the primary antibodies for immunostaining. Tissue samples were fixed in 4% paraformaldehyde, embedded in paraffin wax, and sectioned at 4- μm thickness. FAP polyp samples were collected from four FAP patients who underwent operations in 1999 at the Department of Digestive Surgery, Tokyo Medical and Dental University.

For immunohistochemistry, sections were pretreated with 0.3% H_2O_2 for 30 min, blocked with 10% goat serum, 3% BSA in PBS for 1 h, and incubated for 1 h with the primary antibody. Immunostaining signals were visualized using Vectastain Elite kit (Vector Laboratories, Burlingame, CA). For immuno-

staining of Ki-67, sections were pretreated in a microwave oven in 10 mM citrate buffer for 15 min before incubation with the primary antibody. The M.O.M kit (Vector Laboratories) was used to minimize the background stainings caused by mouse IgG used for the primary antibodies. For immunofluorescence staining, paraffin sections and OCT-embedded frozen sections of 7- μm thickness were used. As the secondary antibody, Alexa Fluor antirabbit IgG (Molecular Probes, Eugene, OR) or FITC-conjugated antigoat IgG (Jackson ImmunoResearch, West Grove, PA) was used. To detect COX-2 promoter activity in the COX-2 knockout mouse polyps, an FITC-conjugated antibody for β -galactosidase (Abcam Limited, Cambridge, United Kingdom) was used, because the COX-2 gene coding region was replaced with a bacterial β -galactosidase gene (10).

Statistical Analysis. Data are expressed as mean \pm SD (Fig. 1, B and C), and statistical significance was assessed by Student's *t* test. $P < 0.05$ was considered as statistically significant.

RESULTS

COX-2 and mPGES Are Simultaneously Induced in the Polyps.

We demonstrated earlier that expression of COX-2 is induced in the *Apc* ^{$\Delta 716$} mouse polyps, whereas COX-1 is expressed at a constant level both in the normal intestines and polyps (10). To investigate the downstream enzymes for PGE₂ biosynthesis, we analyzed expression of cPGES and mPGES by Western blotting and compared with that of COX-1 and COX-2 (Fig. 1A). Essentially the same level of cPGES protein was found in both the normal intestines and polyps. In con-

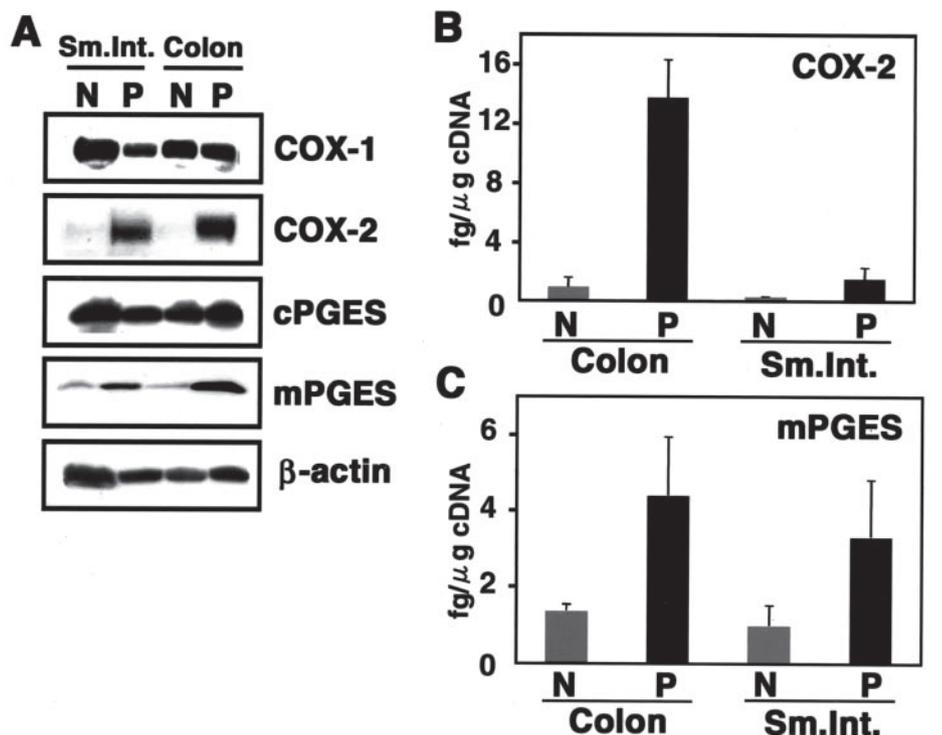
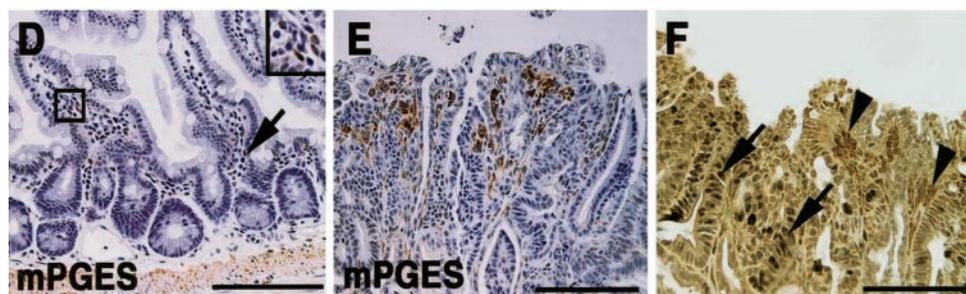


Fig. 1. Expression of COX-1, COX-2, cPGES, and mPGES in the normal intestine and polyp tissues of *Apc* ^{$\Delta 716$} mice. A, Western blotting analysis with internal control β -actin at the bottom. B and C, quantitative real-time RT-PCR of the COX-2 (B) and mPGES (C) mRNAs. The cDNA contents for the respective genes in the total reverse-transcribed cDNA samples are presented as bar graphs. N, normal intestine; P, polyp; and Sm.Int., small intestine; bars, \pm SD. Immunostaining of mPGES-expressing cells in the normal small intestinal villi (D) and a large polyp (E). Inset in D shows an enlarged image of the boxed area containing brown-stained mPGES-expressing cells. Arrow indicates the mPGES-expressing stromal cells (D). F, double immunostaining for mPGES (light brown; arrowheads) and Ki-67 (dark brown; arrows). Bars, 50 μm .



trast, mPGES was expressed at much higher levels in the polyp tissue, although it was expressed at low levels even in the normal intestinal mucosa. These results are consistent with previous reports that cPGES is expressed constitutively, whereas mPGES is induced in some cell lines and macrophages (19–21, 23). To determine the mRNA levels for COX-2 and mPGES precisely in the polyps, we performed a real-time RT-PCR analysis. The polyp COX-2 mRNA was elevated 14 and 6 times in the colon and small intestine, respectively, compared with the normal mucosal levels (Fig. 1B). Interestingly, the COX-2 mRNA level in the colonic polyps was eight times higher than that in the small intestinal polyps, consistent with our previous report on the COX-2 protein levels (10). The difference between the colonic and small intestinal polyps may be explained by the polyp size difference. Polyps in the colon were larger than those in the small intestine, although small intestinal polyps outnumbered colonic polyps (10). Namely, the mean polyp diameters were 0.84 ± 0.12 (SD) mm in the small intestine, and 2.95 ± 1.72 (SD) mm in the colon at 13 weeks of age ($P = 0.026$). In contrast, the mPGES mRNA levels in the polyps were approximately three times higher in both intestines than in the normal mucosa (Fig. 1C).

mPGES Is Also Induced in the Polyp Stromal Cells. To identify the cells that express mPGES, we then investigated the mPGES expression by immunohistochemistry. As shown in Fig. 1D, mPGES was detected in a few stromal cells in the normal intestinal mucosa where COX-2 was not expressed (data not shown). Thus, the low level expression of mPGES may contribute also to the COX-1-dependent PGE₂ synthesis in the normal mucosa (see below). In contrast, plen-

tiful mPGES was expressed in the polyp stromal cells that appeared strikingly similar to the COX-2-expressing cells (Fig. 1E). A double immunostaining with antibodies for mPGES and Ki-67, respectively, showed that mPGES-expressing cells were of the nonproliferating population (Fig. 1F). The data suggest that mPGES was induced in the pre-existing polyp stromal cells rather than that a few mPGES-expressing cells in the normal villi proliferated in the polyp stroma.

COX-2 Is Induced in the COX-1-expressing Polyp Stromal Fibroblasts. Our recent genetic studies have demonstrated that expression of COX-1 is also critical for tumorigenesis (14, 15). To investigate the role of COX-1 in polyp development, we additionally determined the localization of COX-1 in the *Apc*^{Δ716} mouse polyps by immunofluorescence staining and compared with that of COX-2. COX-1 was expressed in the stromal cells even in the small nascent polyps <1 mm in diameter, although COX-2 was not yet detected (Fig. 2, A–C). Because nascent polyps form inside the normal villi (27), most COX-1-positive stromal fibroblasts in the nascent polyps are derived from the normal intestinal villi (Fig. 2, A and B). However, in large polyps >1 mm, both COX-1 and COX-2 were detected in the stromal cells (Fig. 2, D and E). This is consistent with our previous report that expression of COX-2 is induced in polyps >1 mm (28). Importantly, all of the COX-2-expressing cells were also COX-1 positive, although there were some stromal cells that expressed only COX-1 but not COX-2 (Fig. 2F). These data indicate that COX-2 is induced in the COX-1-expressing cells, after polyps expand beyond ~1 mm in diameter. Most COX-1-expressing cells should be fibro-

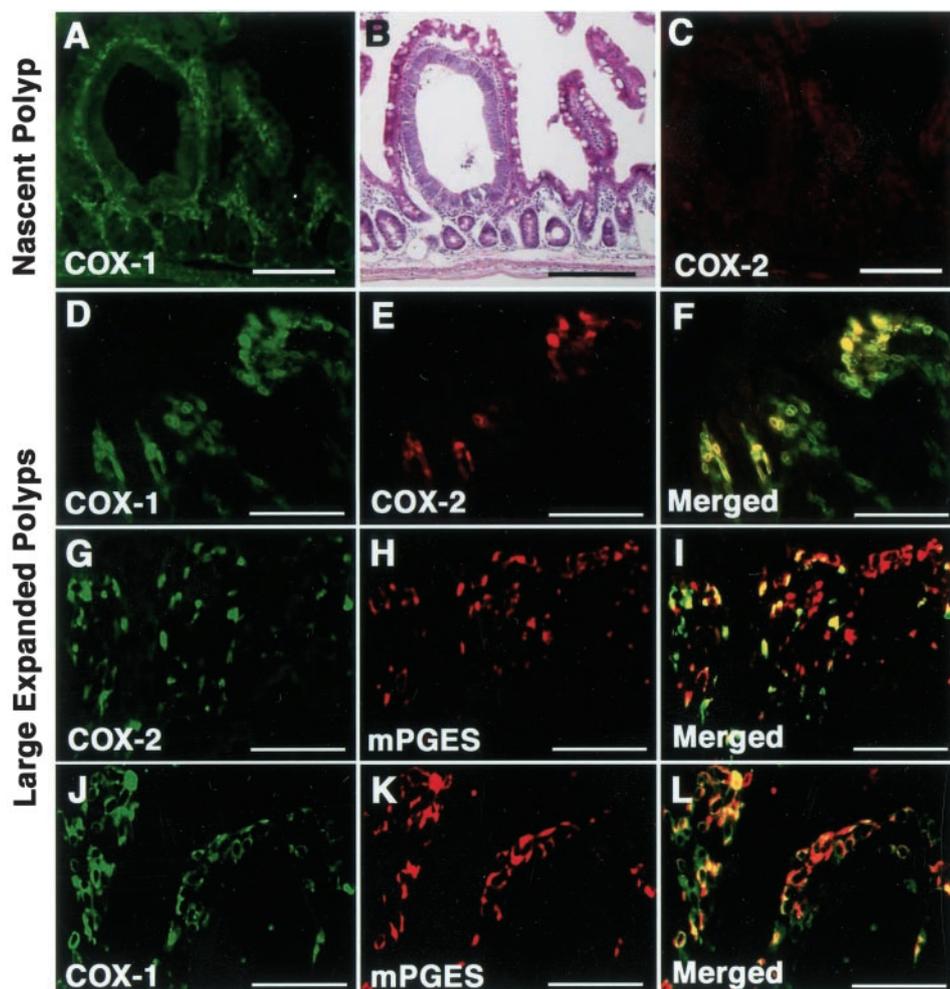


Fig. 2. Immunofluorescent staining of COX-1, COX-2, and mPGES in the intestinal polyps. A small nascent polyp <1 mm stained for COX-1 (A) and COX-2 (C). B, an adjoining section stained with H&E. A large polyp stained for COX-1 (D) and COX-2 (E), with a merged image in F. A large polyp stained for COX-2 (G) and mPGES (H), with a merged image in I. A large polyp stained for COX-1 (J) and mPGES (K), with a merged image in L. Yellow color in the merged images (F, I, and L) indicates the double-positive stromal cells. Bars, A–C, 50 μ m; D–F, 20 μ m; G–I, 50 μ m; and J–L, 30 μ m.

blasts, because 85% of the COX-2-positive cells in the polyps are vimentin-positive fibroblasts (17).

COX-2 and mPGES Are Colocalized with COX-1 in the Polyp Stroma. We next determined the localization of mPGES relative to that of COX-1 and COX-2. Expression of mPGES was detected in approximately half of the COX-2-positive fibroblasts, although there were mPGES-positive cells that did not express COX-2 (Fig. 2, G–I, red cells in merged image Fig. 2I). These data suggest that the production of PGE₂ is increased markedly in the polyp stroma by simultaneous induction of mPGES in approximately at least half of the COX-2-expressing fibroblasts. However, it is worth noting that some mPGES-expressing cells were COX-2 negative. Thus, although mPGES and COX-2 are likely to share common mechanisms of induction, they also appear to contain some separate regulatory components independent of each other. Importantly, all of the mPGES-expressing cells were COX-1 positive, whereas a small population of COX-1-expressing cells was still mPGES negative (Fig. 2, J–L, merged image in L). It has been reported that mPGES can produce PGE₂ from the PGH₂ source supplied by COX-1 when cytosolic phospholipase A₂ is overexpressed in HEK293 kidney cells (21). Therefore, it is possible that mPGES in the polyp stroma synthesizes PGE₂ functionally coupled with COX-1 when COX-2 is not yet induced. We could not determine the precise cPGES localization because of a staining difficulty with the antibody. Collectively, these results strongly suggest that COX-2 and mPGES are induced in the polyp stromal fibroblasts that have already been expressing COX-1 and possibly cPGES.

Both COX-1 and COX-2 Are Involved in the Polyp Development. We demonstrated earlier that both compound mutants *Apc^{Min} COX-1 (-/-)* and *Apc^{Δ716} COX-2 (-/-)* mice show reduced intestinal polyp numbers by ~80% (10, 14), indicating that expression of both COX-1 and COX-2 plays significant roles in the intestinal polyp formation. To investigate whether expression of COX-1, COX-2, or mPGES is affected by disruption of either COX gene, we analyzed the intestinal polyps in the respective compound mutants by immunohistochemistry. The polyp histopathology appeared different between the respective *Apc* compound mutants with COX-1 and COX-2, although the polyp numbers were not large enough for statistical analysis. For example, most polyps were markedly regressed in COX-2 (-/-) *Apc^{Δ716}* mice, whereas such pathology was not found in COX-1 (-/-) *Apc^{Min}* polyps (Fig. 3). Interestingly, COX-2 and mPGES were expressed in the stromal cells of the *Apc^{Min} COX-1 (-/-)* mouse polyps (Fig. 3, A and B), whereas COX-1 and mPGES were detected in the *Apc^{Δ716} COX-2 (-/-)* polyp stromal cells (Fig. 3, C and D). The localization and staining intensities of the respective enzymes were essentially the same as those in the littermate COX (+/+) *Apc* mutants except for the gene-disrupted COX enzymes (data not shown). Therefore, if one of the COX genes is disrupted, the other COX is expressed in the polyp stroma together with mPGES, securing low levels of PGE₂. However, the amount of PGE₂ supplied through a single COX pathway should be insufficient for additional polyp expansion, because essentially no large polyps are found in either COX-1 or COX-2 gene knockout *Apc* mutant mice (10, 14).

COX-2 and mPGES Are Induced in FAP Polyps. Finally, we examined expression of COX-1, COX-2, and mPGES in human FAP polyps. In the normal colonic mucosa adjacent to polyps, COX-1 was expressed in the stromal cells but COX-2 was absent (Fig. 4, A–C). A weak mPGES immunostaining was detected in the normal villous stroma (Fig. 4D). However, in the colonic polyps, COX-1, COX-2, and mPGES were expressed in the stromal fibroblasts that were identified by the vimentin staining (Fig. 4, E–H). These results strongly suggest that COX-2 and mPGES are induced in the COX-1-

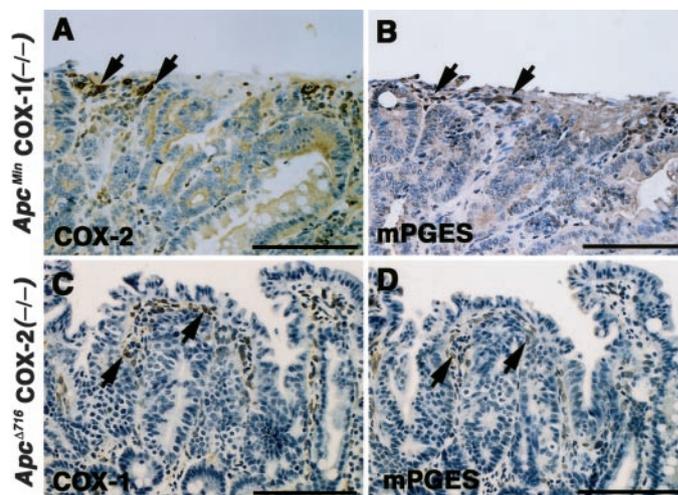


Fig. 3. Immunostaining of COX-1, COX-2, and mPGES in serial sections of the rare small intestinal polyps in the compound mutants of *Apc* with the COX-1 or COX-2 gene. Expression of COX-2 (A) and mPGES (B) in the *Apc^{Min} COX-1 (-/-)* mouse polyp. Expression of COX-1 (C) and mPGES (D) in the *Apc^{Δ716} COX-2 (-/-)* mouse polyp. Arrows indicate representative positively stained stromal cells in the same polyp subregion. Bars, 50 μ m.

expressing fibroblasts also in the human colonic polyps as in the mouse polyps.

DISCUSSION

Accumulating evidence indicates that inducible COX-2 plays a key role in intestinal tumorigenesis (4, 5). At the same time, the importance of constitutive expression of COX-1 has also been demonstrated in epidemiological studies and mutant mouse experiments (1, 16). Regular use of aspirin at low doses that inhibits COX-1, but not COX-2, is associated with a reduced risk of colorectal cancer (16). Moreover, disruption of COX-1 gene in *Apc^{Min}* mice causes suppression of polyposis (14). However, the relationship between the two COXs in intestinal tumorigenesis was not investigated. Using mouse model as well as FAP polyp samples, we have demonstrated here that expression of COX-2 and mPGES are induced simultaneously in the stromal cells where COX-1 has already been expressed constitutively. The cells that express both COX-1 and COX-2 should be fibroblasts, because most COX-2-expressing cells in the intestinal polyp stroma are vimentin-positive fibroblasts (17). Accordingly, the simultaneous induction of COX-2 and mPGES should accelerate polyp growth by boosting the PGE₂ production in the stromal fibroblasts from the basal level secured by constitutive COX-1 (Fig. 4I). Although polyposis is suppressed by disruption of either COX gene, COX-2 and mPGES are expressed even in the COX-1 (-/-) polyps, whereas COX-1 is still expressed constitutively in the COX-2 (-/-) polyps. Thus, it appears to be the total amount of PGE₂ that controls the overall outcome of polyp expansion. However, it is also possible that COX-2 affects polyp formation more significantly than COX-1 at the expansion stage, because a regressive polyp histopathology was found only in *Apc^{Δ716} COX-2 (-/-)* mice.

Whereas COX-2 induction is essential for polyps to expand beyond ~1 mm (28), nascent polyps are likely to develop to ~1 mm with PGE₂ supplied by COX-1 alone. Accordingly, it is possible that suppression of polyposis by COX-1 inhibition is caused by growth arrest before stromal COX-2 is induced. This hypothesis is consistent with the results from some rodent experiments. Low-dose nonselective NSAIDs effectively inhibit the early stages of tumor development when given before or simultaneously with chemical carcinogen chal-

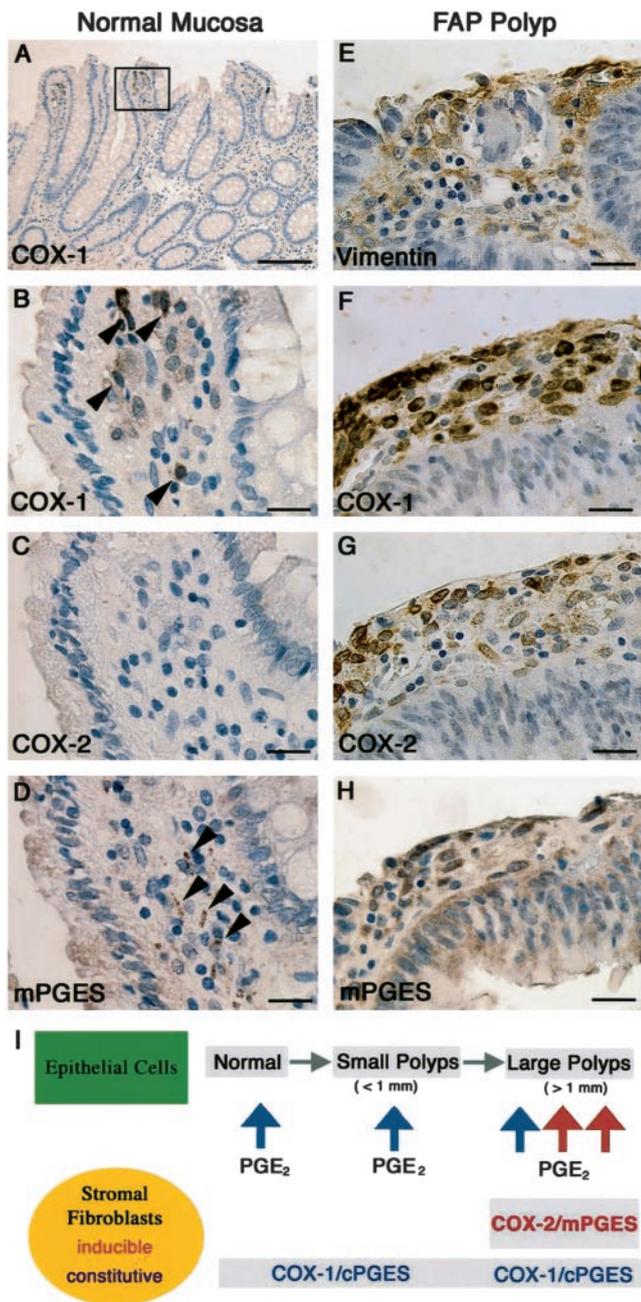


Fig. 4. Immunostaining of COX-1, COX-2, and mPGES in serial sections of colonic samples from a FAP patient. Expression of COX-1 (A and B), COX-2 (C), and mPGES (D) in the normal colonic mucosa. B–D, high magnification of serial sections of the boxed area (A). Expression of vimentin (E) COX-1 (F), COX-2 (G), and mPGES (H) in a colonic polyp. Arrowheads indicate representative positively stained stromal cells for COX-1 (B) and mPGES (D). Bars, A, 100 μ m; and B–H, 10 μ m. I, a schematic diagram showing the roles of COX-1 and COX-2 in intestinal polyp formation.

length (16). If treatment is started after tumor formation, in contrast, only COX-2 selective inhibitors are effective. We have reported recently that microvessel density increases when polyps expand beyond 1 mm in a COX-2-dependent manner (28). At the same time, expression of basement membrane components is controlled by PGE₂ mediated through EP2 receptor (18). These results, taken together, indicate that the stromal PGE₂ secured by constitutive expression of COX-1 and boosted induction of COX-2 is responsible for polyp growth through epithelial-mesenchymal interaction.

It has been reported that COX-2 is induced on activation of COX-1-expressing mast cells (29). The immediate-early phase of PGD₂

production is mediated by constitutively expressed COX-1, whereas the delayed phase is dependent on COX-2 induction in the same cells. It is possible that a similar mechanism is involved in intestinal polyp formation. Such a mechanism may also be involved in skin tumorigenesis, because disruption of either COX gene causes inhibition of chemically induced epidermal carcinogenesis (15). Furthermore, disruption of the COX-2 gene in *Apc*^{1638N} mice results in reduction of the desmoid tumor size but not the number of tumors (30). These data, taken together, strongly suggest that COX-1 plays an important role in the early stage of tumorigenesis, whereas COX-2 is essential in later expansion.

In conclusion, the present results together with reports by others underscore that constitutive expression of COX-1 plays an essential role in tumorigenesis, not to mention COX-2 and mPGES induction.

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