REVIEW

Cyclooxygenase-2 Inhibitors in Tumorigenesis (Part II)

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The rate-limiting step in arachidonate metabolism is mediated by enzymes known as cyclooxygenases (COXs). These enzymes catalyze the biosynthesis of prostaglandin H_2, the precursor of molecules such as prostaglandins, prostacyclin, and thromboxanes. The COX enzyme family consists of the classical COX-1 enzyme, which is constitutively expressed in many tissues, and a second isozyme, i.e., COX-2, which is induced by various stimuli, such as mitogens and cytokines, and is involved in many inflammatory reactions. Because nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit both COX-1 and COX-2, these drugs also cause unwanted side effects, exemplified by gastrointestinal bleeding. Accumulating evidence indicates that NSAIDs can reduce the incidence of colorectal cancers in human and experimental animals and can reduce the number and size of polyps in patients with familial adenomatous polyposis. This Part II (of a two-part review) focuses on the growing clinical and experimental evidence that NSAIDS and COX-2 inhibitors can influence the risk of colon (and possibly of other) cancers. [J Natl Cancer Inst 1998;90:1609–20]

Cyclooxygenase (COX) is the key enzyme in arachidonate metabolism and catalyzes the biosynthesis of prostaglandin H_2, the precursor for prostanoids.[1] In addition to COX-1, which is constitutively expressed in many tissues, another isozyme (COX-2) was identified in 1991. COX-2 is induced in many inflammatory reactions. Because nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit both COX-1 and COX-2, these drugs also cause unwanted side effects exemplified by gastrointestinal bleeding. In Part I of this review (*see* ''Notes'' section), I summarized the biochemistry and pharmacology of COXs and their inhibitors: arachidonate metabolism and COXs, the regulation of prostaglandin synthesis, NSAIDs and the inhibition of COXs, COX-2 selective inhibitors and the inhibition of COX isozymes, and the structural basis of functional differences between COX-1 and COX-2.

In Part II of this review, I will first describe earlier data concerning the effects of NSAIDs on colorectal tumors and will then present an overview of research on the influence of COX-2 and its inhibitors on cancers. I will focus on the role of these molecules in colorectal cancer and its animal models, with some extension to other types of cancer, and discuss the clinical relevance of these compounds: Accumulating evidence indicates that NSAIDs can reduce the incidence of colorectal cancers in human and experimental animals and can reduce the number and size of polyps in patients with familial adenomatous polyposis (FAP). Recently, evidence has been presented that COX-2 is induced in human colorectal cancers and in the polyps of mouse FAP models. When the COX-2 gene is inactivated in FAP-model mice, both the number and the size of polyps are reduced dramatically. In addition, selective inhibitors of COX-2 cause results similar to those caused by COX-2 gene knockout mutations. These genetic and pharmacologic data open up the possibility of effectively treating human FAP and various human cancers with COX-2 selective inhibitors, a new class of NSAIDs.

Colorectal Cancer and Prostaglandins

Colorectal cancer is one of the leading causes of cancer mortality in the United States and other developed countries *(1)*. There is strong evidence suggesting that virtually all colonic adenocarcinomas arise within pre-existing adenomas or areas of dysplasia *(2)*. The risk of cancer increases as an adenoma becomes larger, has a greater villous component, or contains more high-grade dysplasia. In exceptional cases, however, some carcinomas are likely to develop in small and highly dysplastic flat adenomas *(3)*.

As in other systems such as skin cancer *(4)*, colorectal carcinogenesis has been shown to involve many genetics steps *(5–7)*. The triggering events in colorectal carcinogenesis were identified through molecular genetic studies of hereditary forms of the diseases. One such hereditary condition, FAP, was found to be caused by mutations in the adenomatous polyposis coli (APC) gene, whereas hereditary nonpolyposis colorectal cancer (HNPCC) was found to be due to mutations in one of several DNA mismatch repair genes *(7)*. However, details of the molecular processes that occur in the adenoma–carcinoma sequence after biallelic inactivation of APC or the DNA repair genes are yet to be investigated.

On the other hand, circumstantial evidence for possible involvement of COXs in colorectal cancer has been derived from pharmacologic analyses of prostaglandins. Various animal and human tumor tissues, including human colon cancer, have been reported to contain high concentrations of prostaglandins *(8–10)*. This relationship of neoplastic tumors to increased levels of

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prostaglandins provided the rationale for earlier use of NSAIDs as potential chemoprevention agents. These drugs inhibit endogenous prostaglandin synthesis, which plays a role in the control of neoplastic and non-neoplastic cell proliferation and of immune functions *(11–14)*. Three independent lines of research have provided support for this approach in numerous published reports of experiments on animal models, epidemiologic studies, and clinical trials on FAP patients.

Effect of NSAIDs on Colon Cancer: Earlier Studies on Animal Models

In experimental animals transplanted with various tumors [e.g., mast cell ascites tumors *(15,16)*, fibrosarcoma *(17)*, and colon adenocarcinoma *(18)*], indomethacin, aspirin, and piroxicam were shown to reduce tumor growth. With the use of chemical carcinogen-induced rat and mouse tumor models for colorectal cancer, the NSAIDs indomethacin, meclofenamate, piroxicam, sulindac, and aspirin were shown to decrease the incidence, multiplicity, and/or size of tumors in rats or mice (Table 1). Because most such results are also discussed in the reviews by DuBois et al. *(19)* and by Levy *(20)*, I have listed these reports in Table 1 and summarized them only briefly here. These studies used *N*-methyl-*N*-nitrosourea, 1,2-dimethylhydrazine (DMH), azoxymethane, or methylazoxymethanol as carcinogens. All of these carcinogens are metabolically activated to form an active carcinogen *(21,22)*. DMH is converted to azoxymethane, which is further metabolized to methylazoxymethanol and then to methyldiazonium ion, whereas dimethylnitrosamine and related compounds are converted to methyldiazonium ion through a separate pathway. A methyldiazonium ion, once formed, generates a carbonium ion that is responsible for methylation of nucleic acids in animals. In Sprague-Dawley rats, for example, 10 weekly subcutaneous inoculations with methylazoxymethanol acetate at 30 mg/kg of body weight caused about 20 tumors per rat in the intestines of 10 animals after 20 weeks of treatment *(23)*. When indomethacin was given to the rats in drinking water at 2 or 11 weeks after a single dose of methylazoxymethanol, the tumor incidence, multiplicity, and size were reduced substantially *(24)*. In similar experiments with 30 mg/kg azoxymethane, the NSAID piroxicam was given to the rats in their diet at various levels for 40 weeks. Increasing levels of piroxicam in the diet, when fed 1 week or 13 weeks after azoxymethane insult, inhibited the incidence and multiplicity of colon tumors in a dose-dependent manner *(14)*. As a summary of these and other animal experiments listed in Table 1, NSAIDs statistically significantly reduced the incidence and multiplicity of colon tumors, and this effect was sometimes observed even many weeks after carcinogen challenge, although some exceptions were also reported where no effect was observed *(25)* or effects were observed only with the concurrent administration of a carcinogen *(26)*. These reports suggest that NSAIDs act to suppress tumor formation in the rodents during initiation and/or progression.

Sulindac in FAP Patients

On the basis of the results of the early animal experiments described above, sulindac administration studies were initiated in FAP patients. As reported by Waddell and Loughry *(27)* in 1983, three post-subcolectomy patients with FAP and one preoperative patient with Gardner's syndrome (a subtype of FAP with extragastrointestinal tumors) were treated with sulindac for 1 year, and their polyps almost completely disappeared (Table 2). The researchers subsequently confirmed the sulindac-induced polyp regression in a 5-year study of 11 FAP patients, including four preoperative patients. When sulindac was discontinued, however, the polyps recurred; resumption of sulindac

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Table 1. Autochthonous colon tumors effectively suppressed by nonsteroidal anti-inflammatory drugs (NSAIDs)*

Animal and strain (sex)	Carcinogen	NSAID	References and comments
Rat			
SD (m)	DMH	Indomethacin	Pollard and Luckert, 1982 *(108)*
SD (m)	DMH	Indomethacin	Pollard and Luckert, 1983 *(24)*
SD (m)	DMH	Indomethacin or meclofenamate	Metzger et al., 1984 *(109)*
SD (m)	DMH	Sulindac	Skinner et al., 1991 *(110)*
SD (m)	DMH	Aspirin	Craven and DeRubertis, 1992 *(111)* (PGE_2 decreased)
SD (m)	AOM	Piroxicam and DFMO	Nigro et al., 1986 *(112)*
F344 (m)	AOM	Piroxicam	Reddy et al., 1987 *(14)*
F344 (m)	AOM	Piroxicam and DFMO	Rao et al., 1991 *(113)* (8354 or EA, n/e)
F344 (m)	AOM	Aspirin	Reddy et al., 1993 *(114)* (PGE_2 decreased)
F344 (m)	AOM	Sulindac	Rao et al., 1995 *(115)*
SD (m)	DMN	Indomethacin	Pollard and Luckert, 1981 *(116)*
Donryu (m)	MAM	Indomethacin	Kudo et al., 1980 *(117)*
SD (m)	MAM	Indomethacin	Pollard and Luckert, 1983 *(24)*
SD (m)	MAM	Piroxicam	Pollard et al., 1983 *(118)*
F344 (f)	MNU	Indomethacin	Narisawa et al., 1981 *(119)*
F344 (f)	MNU	Indomethacin	Narisawa et al., 1982 *(120)*
F344 (f)	MNU	Indomethacin	Narisawa et al., 1983 *(121)*
SD (m)	MNU	Indomethacin	Narisawa et al., 1984 *(122)*
SD (m)	MNU	Piroxicam	Pollard and Luckert, 1984 *(123)*
Mouse			
BALB/c (f)	DMH	Sulindac	Moorghen et al., 1988 *(26)* Moorghen et al., 1990 *(124)* (after 11 wk of DMH, n/e)

*SD = Sprague-Dawley; m = male; f = female; DMH = 1,2-dimethylhydrazine; AOM = azoxymethane; DMN = dimethylnitrosamine; MAM = methylazoxymethanol; MNU = *N*-methyl-*N*-nitrosourea; DFMO = D,L-α-difluoromethylornithine; 8354 = dehydroepiandrosterone analogue 16α-fluoro-5-androsten-17-one; EA = ellagic acid; n/e = no effects; PGE_2 = prostaglandin E_2.

Table 2. Therapeutic effects of sulindac on patients with familial adenomatous polyposis (FAP)

Authors	Year	No. of patients studied*	Duration dosed†	Effects and comments‡
		Uncontrolled trials		
Waddell and Loughry *(27)*	1983	3 (pst)	1 y	Rectal polyps [−]
		1 (pre)	1 y	Polyps [−]
Waddell et al. *(28)*	1989	7 (pst)	5 y	Most polyps gone
		4 (pre)	5 y	No cancer
Rigau et al. *(29)*	1991	4 (pre)§	6 mo	Polyps [−]
Tonelli and Valanzano *(30)*	1993	13 (pst)	>6 mo	Polyps [−]
Winde et al. *(31)*	1993	15 (pst)	42 wk	10, complete remission
				5, partial remission
Hirota et al. *(125)*	1996	8 (pst)	4–8 wk	6/8 polyps [−]
		Randomized, placebo-controlled, double-blinded trials		
Labayle et al. *(32)*	1991	10 (pst)	4 mo/4 mo—xov	Rectal polyps [−]
Giardiello et al. *(33)*	1993	4 (pst)	9 mo	Rectal polyps [−]
		+18 (pre)‖		
Nugent et al. *(34)*	1993	24 (pst)	6 mo	Rectal polyps [−]
				Duodenal polyps [−]

*pst = after subcolectomy; pre = before surgery.
†xov = crossover study.
‡[−] = decreased.
§Three with non-FAP polyposis.
‖11 + 11 (drug + placebo).

treatment caused tumor regression (Table 2) *(28).* Following these reports, several other studies were published in which similar results were reported, including confirmation of the reversible nature of the polyp regression caused by sulindac (Table 2) *(29–31).*

To evaluate the effects of sulindac on FAP in a more objective way, several randomized, placebo-controlled, double-blinded studies have been conducted and published subsequently (Table 2). Labayle et al. *(32)* employed a 4-month crossover method with a 1-month washout period in 10 post-subcolectomy patients. In contrast, the study conducted by Giardiello et al. *(33)* contained 18 patients seen preoperatively out of 22 subjects. Nugent et al. *(34)* tested 24 post-subcolectomy patients who had rectal and duodenal polyps. In all of the studies, sulindac produced in these patients a statistically significant reduction in both the number and size of the polyps. As in the uncontrolled trials, however, the polyps tended to increase in both number and size during the placebo administration or once sulindac was discontinued. Accordingly, sulindac treatment of preoperative FAP patients was not deemed complete enough to replace colectomy, although it may be useful as an adjunct to surgery in postoperative cases *(33,34).* One of the problems often faced in sulindac treatment of FAP patients appears to be the severe side effects that are common to NSAIDs, e.g., bleeding and ulceration. Although some patients tolerate sulindac without such problems, its side effects can be serious, even fatal, in others. For this reason, there has been a strong desire by researchers to develop new therapeutic agents, such as selective inhibitors of COX-2, that lack these side effects (*see* Part I, published in the previous issue of the Journal [Vol. 90, No. 20, October 21]; *see also below.*)

Epidemiologic Studies

Another line of evidence for the inhibition of colorectal tumorigenesis by NSAIDs was obtained by undertaking epidemiologic studies, which were encouraged by the animal model and FAP studies. Numerous retrospective and prospective studies of NSAID use and colon cancer suppression were conducted, as summarized in Table 3. In most of these studies, the relative risk of developing colon cancer was lower in patients (or the sample population) who took aspirin or other NSAIDs. In elaborate studies in which the effect of dosage and/or duration of NSAID intake was investigated, dose-dependent and/or duration-dependent reductions in the relative risk were often found. In some studies, acetaminophen was used as a control and was found to have no association with colon cancer incidence or mortality. One of the reports *(35)* employed detection of colorectal adenomas, rather than detection of colorectal cancer, as the end point and gave similar results. The exceptions to this conclusion were two studies: one *(36)* on a small retirement community in California, in which aspirin intake was associated with an increased risk of colorectal cancer, and the other *(37)* that used data from the randomized Physician's Health Study. The interpretation of these particular studies remains controversial.

COX-1 and COX-2 Gene Knockout Mice

Taking advantage of homologous recombination, which can be induced in mouse embryonic stem cells, researchers constructed gene knockout mice that have homozygous inactivation of either the COX-1 or the COX-2 gene *(38–40).* Morham et al. *(38)* and Dinchuk et al. *(40)* reported the phenotypes of the COX-2 gene (Ptgs2) knockout mice. While some of the phenotypic characteristics reported are similar in the two reports, there are some distinct differences as well. In both studies, the homozygous Ptgs2 (−/−) mutants showed renal dysplasia and developed severe nephropathy. However, Morham et al. also found suppurative peritonitis in two of three Ptgs2 (−/−) mice, whereas Dinchuk et al. found cardiac fibrosis in 50% of their ''homozygotes.'' Surprisingly, both groups reported that the inflammatory responses of the ear to tetraphorbolacetate and to arachidonic acid were not affected in the homozygous mutants. However,

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Table 3. Nonsteroidal anti-inflammatory drug (NSAID) use and incidence of human colorectal cancer

Authors (study area)	Year of study	No. of patients studied*	NSAID used	RR [95% CI]† (comments)
		Retrospective studies		
Kune et al. *(126)* (Australia)	1988	715	Aspirin	0.57 [0.41–0.79]
Rosenberg et al. *(127)* (Boston, New York City, Philadelphia, and Baltimore)	1991	1326	Aspirin	0.5 [0.4–0.8]
Suh et al. *(128)* (Buffalo, NY)	1993	830	Aspirin	0.44/0.83 (dose dependent) [0.18–1.10]/[0.43–1.61]
Logan et al. *(35)* (Nottingham, U.K.) (*Note*: Outcome of this study is colorectal adenomas rather than colorectal cancer.)	1993	40	Aspirin or other NSAIDs‡	0.49/0.66 (−/+, occult blood tests) [0.3–0.8]/[0.4–1.1]
Peleg et al. *(129)* (Atlanta, GA)	1994	97	Aspirin or other NSAIDs‡	0.52–0.08 (dose dependent) 0.34–0.77 (4-y study)
Peleg et al. *(130)* (Atlanta, GA)	1996	206 (93 carcinomas + 113 adenomas)	Aspirin or other NSAIDs‡	0.31/0.59 (dose dependent) [0.11–0.84]/[0.23–1.48]
Muscat et al. *(131)* (New York City area)	1994	511	NSAIDs‡	0.64 (males) [0.42–0.97] 0.32 (females) [0.18–0.57]
Martinez et al. *(132)* (Houston, TX) (*Note*: Outcome of this study is colorectal adenomatous polyps, rather than colorectal cancer.)	1995	157	Aspirin and/or NSAIDs	0.36 (once a day or more) 0.77 (weekly)
		Prospective studies		
Paganini-Hill et al. *(36)*	1989	68	Aspirin	0.95–1.67
Paganini-Hill et al. *(133)* (California retirement community)	1991	79	Aspirin	0.79–1.46 (no reducing effects)
Thun et al. *(134)* (U.S.; 50 states, DC, and Puerto Rico)	1991	726	Aspirin‡	0.77–0.60 (males) 0.73–0.58 (females) (dose dependent)
Gann et al. *(37)* (U.S. physicians)	1993	63 (of 11 037)	Aspirin	1.15 for carcinoma 0.86 for *in situ* carcinoma and polyps
Schreinemachers and Everson *(135)* (U.S.; National Health and Nutrition Examination Survey I)	1994	10	Aspirin	0.35 (males <65 y old) (lung, 0.68; breast, 0.70)
Giovannucci et al. *(136)* (U.S. health professionals)	1994	67 (1-y use) 8 (3-y use)	Aspirin	0.68 0.35
Giovannucci et al. *(137)* (U.S. female registered nurses)	1995	149 (1-y use) 39 (3-y use)	Aspirin	0.86 0.62

*Excluding the control group patient numbers.

†RR = relative risk; CI = confidence interval; values shown are adjusted for various factors or multivariate estimates.

‡Acetaminophen did not show any significant effects.

Dinchuk et al. found a striking mitigation of endotoxin-induced hepatocellular cytotoxicity in the homozygotes, and their females were sterile. It appears that active Ptgs2 is not essential for these inflammatory responses. However, it should be noted that ear swelling in response to tetraphorbolacetate or arachidonic acid is a complex chain of events involving many mediators of inflammation, and COX-2 induction takes place later than 1–4 hours after application of the chemicals, when both groups assayed the effects *(38,40)*.

In contrast, the homozygous COX-1 gene (Ptgs1) knockout mice survived well, without apparent abnormal phenotypes. Surprisingly, these mice showed no gastric pathology and showed less indomethacin-induced gastric ulceration than did the wild-type mice, despite the fact that their gastric prostaglandin E_2 (PGE_2) levels were about 1% of those of the wild-type mice *(39)*. These animals had reduced platelet aggregation and a decreased inflammatory response to arachidonic acid but not to tetraphorbolacetate. These results suggested that absence of COX-1 is not sufficient to cause stomach ulceration. It is conceivable, however, that lack of COX-1 activity is not equivalent to the inhibition of COX-1 activity by indomethacin. For example, the residual peroxidase activity of the COX-1–indomethacin complex generates peroxidation products of arachidonic acid, which may be responsible for the ulceration *(41)*.

COX-2 Induction in Colorectal Cancer and Effects of NSAIDs: Newer Data

After the discovery of COX-2, studies of the effect of NSAIDs on cancer were focused on their relationship with COX-2 induction. In 1994, Eberhart et al. *(43)* reported that, of 14 human colorectal carcinoma samples, 12 (86%) had marked increases in COX-2 messenger RNA (mRNA), whereas six (43%) of 14 adenomas showed significant levels of COX-2 mRNA induction. In contrast, COX-1 mRNA levels were essentially unchanged in both adenomas and adenocarcinomas. By immunoblot determination, Kargman et al. *(44)* showed that 19 (76%) of 25 human colon cancer tissues had substantially increased levels of induction of COX-2 protein, whereas no such induction was observed in matched normal colonic tissues. However, four premalignant polyps did not show such COX-2 induction. Using an immunohistochemical method, Sano et al. *(45)* demonstrated that 15 human colorectal cancer tissues had marked expression of COX-2 protein in cancer cells, inflammatory cells, vascular endothelium, and fibroblasts when compared with nonlesional and normal colon tissues. In contrast, the ex-

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pression of the COX-1 polypeptide was weak in both normal and cancer specimens. Likewise, Kutchera et al. *(46)* found by *in situ* hybridization that the neoplastic colonocytes had increased expression of COX-2. In addition, five colon cancer cell lines were shown to express high levels of COX-2 mRNA. By transfection experiments with the 5′ regulatory sequence of the COX-2 gene ligated to a luciferase reporter, the researchers found that colon cancer cell line HCT-116 constitutively expressed COX-2, whereas normal control cell lines transcribed the reporter only in response to an exogenous agonist.

In 1995, several groups reported that sulindac and other NSAIDs induce apoptosis in colon cancer cells. By immunohistochemistry, Pasricha et al. *(47)* studied colonic biopsy samples from 22 FAP patients who were enrolled in a sulindac trial. The subdiploid apoptotic fraction was significantly increased to 31%, compared with 10% in the controls, 3 months after treatment with sulindac. Likewise, Bedi et al. *(48)* showed that eight FAP and 10 sporadic adenomas exhibited reduced apoptotic fractions in TdT (terminal deoxynucleotidyltransferase)-mediated deoxyuridine triphosphate-digoxigenin nick-end labeling (TUNEL) and DNA fragmentation assays compared with eight normal colonic epithelial samples. In 11 colorectal carcinoma samples, the reduction of apoptosis was most dramatic, with an abnormal increment in the G_2 fraction.

Using HT-29 human colon adenocarcinoma cells in culture, Shiff et al. *(49)* showed that sulindac and its metabolite sulindac sulfide reduced the cell proliferation rate, changed the cells' morphology, and induced apoptosis in these cells. Because sulindac is a prodrug, it is metabolized to a pharmacologically active sulfide derivative that inhibits prostanoid synthesis *(50)*. Some studies, however, have shown that a sulfone derivative of sulindac, which essentially lacks prostaglandin synthesis inhibitory activity *(50)*, also inhibits chemical carcinogenesis, suggesting an additional mechanism of antineoplastic activity by sulindac and its metabolites *(51)*. Piazza et al. *(52)* found that both sulindac sulfide and sulfone significantly reduced the number of HT-29 cells and of a variety of other tumor cell lines, as well as the number of normal epithelial cells and fibroblasts. It is interesting that both sulindac sulfide and sulfone induced apoptosis in HT-29 cells in a time- and dose-dependent manner. Regarding the apoptosis caused by sulindac and its metabolites in tumor cells, it is worth noting that several other NSAIDs, but not sulindac, cause apoptosis in v-src-transformed chicken embryo fibroblasts. At the same time, NSAIDs induce COX-1 and COX-2 mRNAs. However, the induced COX-2 transcript is in a partially spliced and nonfunctional form *(53)*. Lu et al. *(53)* further showed that expression of bcl-2 is very low in these cells and is not affected by NSAID treatment. In contrast, expression of p20, a protein that may protect against apoptosis when fibroblasts enter the G_0 phase, was strongly repressed, as shown by northern blot analysis.

Tsujii and DuBois *(54)* introduced a rat COX-2 complementary DNA (cDNA) driven by the cytomegalovirus promoter into a nontransformed rat intestinal epithelial (RIE) cell line and established clones that express COX-2 continuously (RIE-S). They also constructed control cell lines in which the cDNA was placed in the antisense orientation (RIE-AS). The RIE-S cells expressed elevated COX-2 protein levels and exhibited increased adhesion to extracellular matrix proteins. The RIE-S cells were resistant to butyrate-induced apoptosis, had elevated BCL2 protein expression, and had reduced levels of the type II receptor for transforming growth factor-β (TGF-β). Such phenotypic changes were reversed by sulindac sulfide. These data, considered together, suggest that overexpression of COX-2 in intestinal epithelial cells may enhance their tumorigenic potential.

Recently, Samaha et al. *(55)* studied the effects of several potential chemopreventive agents on apoptosis in azoxymethane-induced colon tumors in male F344 rats. They found that sulindac, curcumin, and phenylethyl-3-methylcaffeate significantly increased the apoptotic index (percentage of apoptosis) as compared with the control. Ballif et al. *(56)* reported that an autoimmunity- and apoptosis-associated nucleobindin interacts with both COX-1 and COX-2. It remains to be investigated whether nucleobindin is involved in the inhibition of apoptosis by COX-2.

COX-2 and Polyposis: Studies With Apc Knockout Mice as a Model for FAP

Molecular genetic studies of FAP kindreds led to the discovery of the APC gene on human chromosome 5q21 *(57–60)*. Mutations in APC appear to be responsible for not only FAP but also many sporadic cancers of the colorectal axis, stomach, and esophagus *(61–63)*. While most FAP cases have mutations in the upstream half of exon 15 *(64)*, mutations near the 5′ end of the coding region cause an attenuated form of the disease with relatively few colonic polyps *(65)*. Another form of FAP, which is associated with congenital hypertrophy of the retinal pigment epithelium, contains mutations downstream of exon 9 *(66)*. APC consists of 15 coding exons and several 5′ noncoding exons, various combinations of which generate many isoforms by alternative splicing *(59,67,68)*. The gene encodes a huge protein, about 2840 amino acids in length *(57,60)*. The protein contains regions that may form an α-helical, coiled-coil structure; a subdomain of the first 55 amino acids forms a stable, parallel helical dimer *(69)*. Antibody studies showed that the wild-type, but not mutant, Apc protein is associated with the microtubule cytoskeleton *(70,71)*. The predicted structure of Apc, its localization, and its interaction with β-catenin *(72,73)* suggested that it is involved in cell adhesion. In fact, studies have demonstrated that Apc is localized to plasma membrane sites involved in active cell migration *(74)* and in the nucleus as well *(75)*. At the same time, β-catenin interacts with the hTcf-4 and Lef transcription factors. In fact, hTcf-4 transactivates transcription only when associated with β-catenin *(76,77)*.

A dominant mouse mutation, Min (multiple intestinal neoplasia), which was generated by chemical mutagenesis and causes polyposis in the digestive tract, has been located in Apc, the mouse homologue of the human APC gene. It causes truncation of the gene product at codon 850 and multiple polyps in the intestinal tract *(78,79)*. Boolbol et al. *(80)* reported that both the levels of COX-2 protein and PGE_2 production were elevated in the Min mouse intestines, even in the regions where no polyps developed. COX-2 or PGE_2 was not elevated in the intestines of the wild-type littermates. Such increases in COX-2 protein and PGE_2 in Min intestines were reversed when the

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mice were given sulindac in their drinking water, and the polyp number was reduced to 0.1 tumor per mouse compared with 11.9 tumors per mouse in the untreated Min mice. It is interesting that Min mice showed a 27%–47% decrease in enterocyte apoptosis, which was reversed by the sulindac treatment *(80)*.

To investigate the molecular mechanism of polyp formation as a precursor to carcinogenesis in the digestive tract, we earlier constructed gene knockout mice carrying a mutant Apc gene encoding a product truncated at codon 716 ($Apc^{\Delta 716}$) *(81)*. Whereas the homozygous mutant mice die *in utero* before day 8 of gestation, the heterozygotes are viable and develop multiple polyps throughout the intestinal tract, mostly in the small intestine. The earliest polyps arose multifocally during the 3rd week after birth, and new polyps continued to appear thereafter. Surprisingly, every nascent polyp consisted of a microadenoma covered with a layer of the normal villous epithelium. These microadenomas originated from single crypts that formed abnormal outpockets in the inner (lacteal) side of the neighboring villi. We carefully dissected such microadenomas from nascent polyps by peeling off the normal epithelium and determined their genotype by polymerase chain reaction: All microadenomas had already lost the wild-type allele, whereas the mutant allele remained unchanged. These results indicate that loss of heterozygosity (LOH), followed by formation of intravillous microadenomas, is responsible for the microadenoma initiation in $Apc^{\Delta 716}$ intestinal mucosa *(81)*. This mutant mouse strain provided a useful model system for investigation of various carcinogens and for evaluation of anticancer and chemopreventive agents. In fact, we demonstrated that the heterocyclic amines that are generated in overcooked meat stimulate the growth of the intestinal polyps, whereas feeding the $Apc^{\Delta 716}$ mice docosahexaenoic acid substantially reduces the number of polyps *(82,83)*.

To examine the expression of COX-1 and COX-2 in the $Apc^{\Delta 716}$ mice, we first performed immunoblot analyses of polyp proteins by using specific antibodies against COX-1 and COX-2, respectively. The normal intestinal epithelium—as well as the polyps of various sizes—expressed COX-1 protein at similar levels, both in the colon and in the small intestine. In contrast, the normal epithelium of neither the small intestine nor the colon contained any detectable COX-2 protein. However, polyps as small as 2 mm in diameter from either the colon or the small intestine contained substantial levels of COX-2 protein. The results indicate that COX-2 is induced in the polyp tissues at a very early stage of development, long before their malignant transformation *(84)*.

To determine the effect of the absence of COX-2 on $Apc^{\Delta 716}$ polyp formation, we *(84)* introduced a knockout mutation of the COX-2 gene (Ptgs2) *(40)* into the $Apc^{\Delta 716}$ knockout mice by successive crosses and constructed compound mutant mice that carried $Apc^{\Delta 716}$ (+/−) Ptgs2 (+/−) and $Apc^{\Delta 716}$ (+/−) Ptgs2 (–/–) mutations, respectively. The $Apc^{\Delta 716}$ (+/−) Ptgs2 (+/+) littermates were used as positive controls. When the intestinal polyps were scored at the same age, the polyp numbers in the $Apc^{\Delta 716}$ (+/−) Ptgs2 (+/−) and $Apc^{\Delta 716}$ (+/−) Ptgs2 (−/−) mice were reduced to 34% and 14% of the control, respectively (Fig. 1, A). Moreover, the size of the polyps in these mice was statistically significantly smaller than in the controls (Fig. 1, B). To our

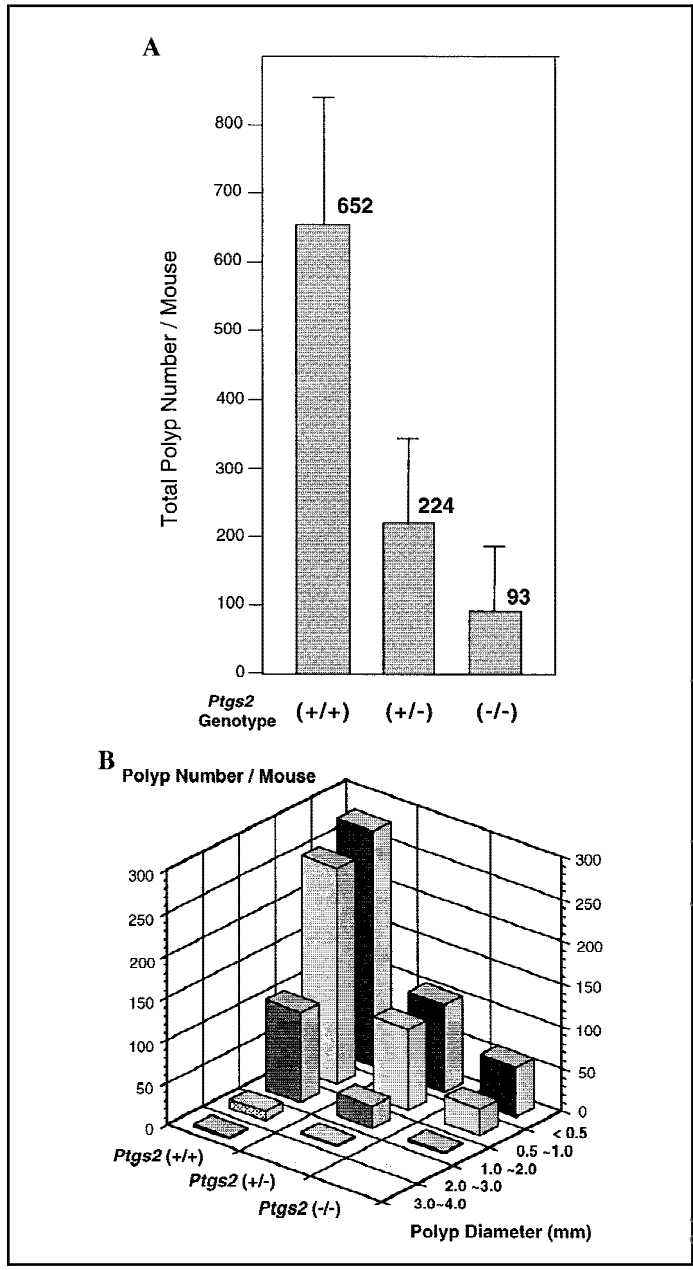

Fig. 1. Effects of Ptgs2 mutations on intestinal polyps in $Apc^{\Delta 716}$ (+/−) Ptgs2 (+/−) and $Apc^{\Delta 716}$ (+/− Ptgs2 (−/−) mice, compared with the $Apc^{\Delta 716}$ (+/−) Ptgs2 (+/+) controls. **A)** Mean numbers of polyps per mouse are shown with standard deviations. **B)** Size distribution of the intestinal polyps. Reproduced with permission from *(84)*.

knowledge, these results are the first direct genetic evidence that COX-2 plays a key role in polyp formation, and they suggest that COX-2 inactivation suppresses polyp growth rather than polyp initiation *(84)*. This is in clear contrast with dietary effects on $Apc^{\Delta 716}$ (+/−) polyps. We *(85)* fed $Apc^{\Delta 716}$ (+/−) mice either a low-fat and high-fiber diet (a low-risk diet) or a high-fat and low-fiber diet (a high-risk diet) for 7 weeks. Although the mice fed a high-risk diet developed polyps in statistically significantly higher numbers than those fed a low-risk diet, both in the small intestine and in the colon, there was essentially no difference in the polyp size distribution between the two groups. It is likely that a high-risk diet increases the frequency of the initial event, i.e., LOH of the Apc gene *(85)*.

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To determine whether we can mimic the Ptgs2 knockout mutation by administering pharmaceutical agents to the $Apc^{\Delta 716}$ mice, we next tested the effects of a novel COX-2 selective inhibitor, MF tricyclic, and a nonselective COX inhibitor, sulindac *(84)*. MF tricyclic is a research compound (Fig. 2, A) that shows more than 100-fold selectivity for COX-2 over COX-1 (i.e., its COX-2/COX-1 IC_{50} ratio) when compared with that of sulindac (Fig. 2, B). (IC_{50} = concentration of the compound that causes half-maximal [50%] inhibition of the enzyme.) When mice were fed MF tricyclic at 14 and 3.5 mg/kg per day, the drug reduced polyp numbers by 62% and 50% of that seen in the control, respectively, compared with only a 26% reduction in the polyp number by sulindac at 12 mg/kg per day (Fig. 2, C).

It is interesting that suppression of COX-2 activity, either by introduction of the knockout mutation or by the COX-2 selective inhibitor MF tricyclic, had a profound effect on the polyp morphology as well. Well-developed polyps in $Apc^{\Delta 716}$ (+/−) Ptgs2 (−/−) mouse intestines appeared to be recessed from the surface of the surrounding villi. This was primarily due to the presence of fewer stromal (or interstitial) cells compared with $Apc^{\Delta 716}$ (+/−) Ptgs2 (+/+) polyps *(40)*. To determine the site of COX-2 expression in the polyps, we constructed another strain of Ptgs2 knockout mice in which one of the Ptgs2 alleles was interrupted by a bacterial β-galactosidase gene (lacZ), placing lacZ under the control of the Ptgs2 promoter. When this mutation was introduced into the $Apc^{\Delta 716}$ (+/−) mice [i.e., $Apc^{\Delta 716}$ (+/−) $Ptgs2^{lacZ}$ (+/−)], the lacZ expression was found almost exclusively in the stromal cells *(84)*. These results strongly suggest that the polyp adenoma grows through interactions between the epithelial and the stromal components *(86)*, reminiscent of many processes of organogenesis in ontogeny.

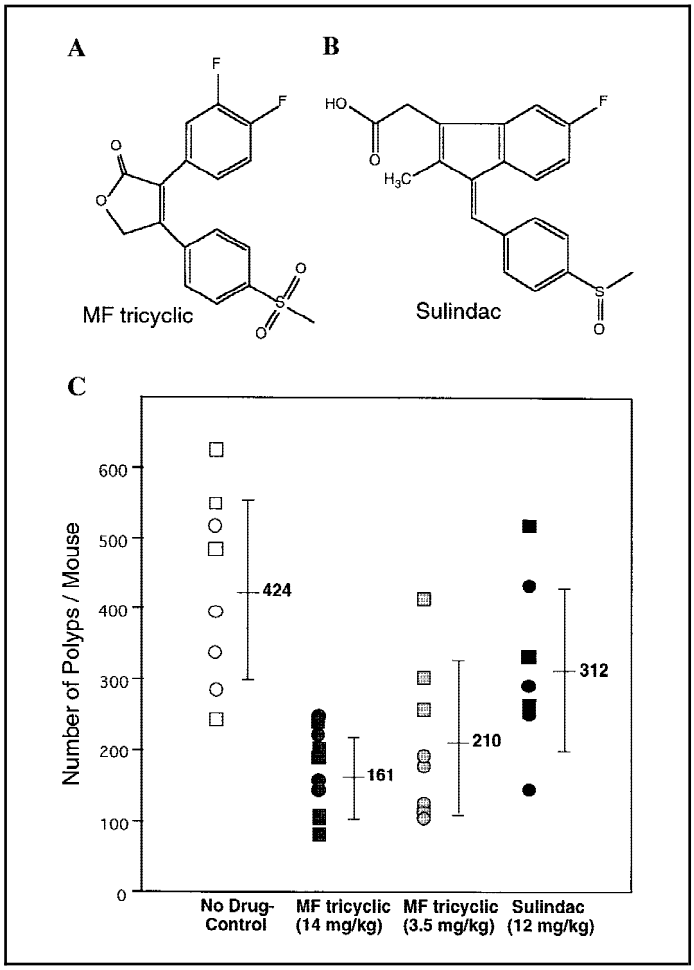

Fig. 2. Effects of a cyclooxygenase 2 (COX-2) inhibitor, MF tricyclic, and sulindac on $Apc^{\Delta 716}$ (+/−) mouse intestinal polyps. **A)** Structure of MF tricyclic. **B)** Structure of sulindac. **C)** Number of polyps per mouse, scored in $Apc^{\Delta 716}$ (+/−) mice fed control diet or diet with MF tricyclic or sulindac. Circles and squares indicate females and males, respectively. Mean numbers of polyps per mouse and standard deviation are shown to the right of each sample group. Reproduced with permission from *(84)*.

It should be noted that Williams et al. *(87)* have also reported that COX-2 levels are elevated in Min mouse adenomas. Northern blot hybridization, reverse transcription–polymerase chain reaction, and immunoblot analyses showed an approximately threefold increase in COX-2 levels in the Min adenomas. Their immunohistochemical staining showed, however, that the immunoreactivity was restricted to dysplastic and neoplastic foci within the intestinal mucosa. From analysis of human colon cancer tissues by use of *in situ* hybridization, Kutchera et al. *(46)* found strong COX-2 mRNA signals in the tumor cell area rather than in the stromal area. In a histochemical analysis, Sano et al. *(45)* reported staining of COX-2 in both cancer epithelium and stromal cells such as inflammatory cells, vascular endothelium, and fibroblasts. The discrepancy between these observations and ours may be explained in two ways: One depends on the stage in the tumor's development, and the other relies on technical details of the immunohistochemistry. We looked at an early stage of polyp development *(84)*, whereas Williams et al. *(87)* looked at much more advanced tumors. In advanced tumors, many secondary reactions take place, such as the proliferation of stromal cells and tissue remodeling, showing a histologic picture very different from that of early tumors. Although several COX-2-specific antibodies have been described, and some are commercially available, many of them show cross-reacting bands upon immunoblot analysis at a high sensitivity. It is also worth noting that the major prostaglandin found in colorectal cancer tissues is PGE_2 *(88,89)*. In contrast, when a rat intestinal epithelium (RIE-1) cell line is stimulated by TGF-α or tissue plasminogen activator, the major prostaglandin secreted into the medium is $6KPGF_{1\alpha}$, the nonenzymatic hydrolysis product of prostacyclin (PGI_2) *(90)*.

These results have several implications and present important questions for future research, as pointed out by Prescott and White *(91)*.

Questions for the laboratory researcher: 1) How does COX-2 expression become dysregulated after loss of APC function? 2) Is the dysregulation transcriptional and, if so, through which factors? 3) Is COX-2 expression alone sufficient to cause colon neoplasia? 4) What are the important metabolites of the COX-2 product and what signaling pathways do they influence? 5) Which cellular responses (e.g., loss of apoptosis) lead to tumors?

Questions for the clinician: 1) Will specific inhibitors of COX-2 be more effective than nonselective NSAIDs? 2) Will inhibition of COX-2 be as effective in patients with sporadic polyps and HNPCC as it is in patients with FAP? 3) What accounts for the residual cases of neoplasia during treatment with NSAIDs—is it a rare event, that occurs only in some early polyps, or will all of the polyps eventually escape the inhibitory

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effect? 4) How should chemoprevention with COX inhibitors be integrated into current surveillance and intervention protocols? *(91)*

Answers to some of these questions are already in hand. Using a human colon cancer cell line, HCA-7, cultured on Transwell filters, Coffey et al. *(92)* succeeded in establishing a polarized cell population. When the cells were stimulated by TGF-α from the basolateral compartment, where the epidermal growth factor receptor (EGFR) resides, a marked secretion of prostaglandins was observed in the basolateral but not in the apical medium, followed by mitogenesis. Two specific COX-2 inhibitors, SC-58125 and NS-398, were found to attenuate COX-2 induction and subsequent mitogenesis. These data indicate that activation of EGFR stimulates COX-2 biosynthesis, vectorial release of prostaglandins, and mitogenesis in polarized HCA-7 cells *(92)*. In addition to the HCA-7 line, which express high levels of COX-2 protein, Sheng et al. *(93)* studied the HCT-116 cell line, which lacks COX-2 expression. Treatment of nude mice implanted with HCA-7 cells with a selective COX-2 inhibitor, SC-58125, reduced tumor formation by 85%–90%. SC-58125 also inhibited colony formation of HCA-7 cells in culture. Conversely, SC-58125 had no effect on HCT-116 implants in nude mice or on HCT-116 colony formation in culture.

The effects of several NSAIDs and another COX-2 inhibitor were evaluated on carcinogen-induced colonic aberrant crypt foci (ACF) in rats. Reddy et al. *(94)* assessed the chemopreventive properties of SC-58635, a COX-2 inhibitor, and of sulindac against azoxymethane-induced colonic ACF in male F344 rats. Administration of 1500 ppm SC-58635 in the diet inhibited total ACF induction and crypt multiplicity by 40%–49%, whereas administration of 330 ppm sulindac in the diet inhibited ACF multiplicity by about 35%. Barnes et al. *(95)* tested various compounds in DMH-treated male Sprague-Dawley rats. Only aspirin, but not sodium salicylate, indomethacin, or nabumetone, reversibly suppressed colonic ACF.

Several reports described the results of NSAID trials on sporadic colonic polyps. Hixson et al. *(96)* studied five sulindac-treated (400 mg/day) and two piroxicam-treated (20 mg/day) patients who completed 6 months of therapy. With the exception of two patients who showed partial response (one treated with sulindac and the other treated with piroxicam), all the patients remained unchanged. Ladenheim et al. *(97,98)* tested 22 patients with sulindac (300 mg/day) for 4 months and an additional group of 22 patients with placebo. Essentially no differences were found between the two groups. In contrast, Matsuhashi et al. *(99)* recently reported the results of a study in which 20 patients were treated with sulindac (300 mg/day) for 4 months. In their study, 13 of 20 polyps shrank or disappeared. However, to evaluate the effects of NSAIDs on sporadic polyps, it would be more meaningful to determine whether these polyps have elevated COX-2 and, if they do, to challenge them with COX-2 selective inhibitors.

NSAIDs and Other Cancers

Studies on animal models showed that the NSAIDs indomethacin, sulindac, ketoprofene, phenylbutazone, and aspirin suppress malignant tumors in experimental animals. As summarized in Table 4, these include both transplanted tumors and autochthonous tumors caused by chemical carcinogens and a retrovirus. It is interesting that NSAIDs suppress not only cancers of epithelial origin but also tumors of mesenchymal origin, such as sarcomas and mast cell tumors.

After the discovery of COX-2, several papers were published on the role of COX-2 in cancers other than colorectal cancer. Subbaramaiah et al. *(100)* studied the expression of COX-2 in mouse mammary epithelial cells transformed by either src or ras oncogenes. Highly tumorigenic cell lines produced markedly increased amounts of PGE_2 and COX-2 mRNA compared with a weakly transformed strain. Tjandrawinata et al. *(101)* treated

Table 4. Other animal cancers that nonsteroidal anti-inflammatory drugs (NSAIDs) were effective in suppressing

Animal	Cancer	Carcinogen*	NSAID	References and comments†
Mouse	Fibrosarcoma (transplanted)	Implant (Millipore filter)	Indomethacin	Tashjian et al., 1974 *(17)* (PGE_2 and carcinomas increased)
	Fibrosarcoma (transplanted)	MCT	Indomethacin and aspirin	Plescia et al., 1975 *(15)* Lynch et al., 1978 *(138)*
	Mast cell tumor (transplanted)	MCT	Aspirin and indomethacin	Hial et al., 1976 *(16)*
	Lewis lung carcinoma (transplanted)	Spontaneous	Indomethacin	Young and Knies, 1984 *(139)*
	Esophageal tumor	DENA	Indomethacin	Rubio, 1984 *(140)*
	Bladder carcinoma	BHBN	Sulindac and ketoprofene	Rao et al., 1996 *(141)* (aspirin, n/e)
Rat	Sarcoma	MSV	Indomethacin	Strausser and Humes, 1975 *(142)* (PGs increased)
	Bladder carcinoma	FANFT	Aspirin	Murasaki et al., 1984 *(143)* (forestomach carcinomas increased)
	Mammary tumor	DMBA	Indomethacin	Carter et al., 1989 *(144)* (carprofen, n/e)
	Bladder carcinoma	BHBN	Aspirin	Klan et al., 1993 *(145)*
Hamster	Pancreatic carcinoma	BOP	Indomethacin and phenylbutazone	Takahashi et al., 1990 *(146)* (aspirin, n/s)

*MCT = 3-methylcholanthrene; DENA = *N*-nitrosodiethylamine or diethylnitrosamine; BHBN = *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine or OH-BBN; MSV = Moloney sarcoma virus; FANFT = *N*-(4-(5-nitro-2-furyl)-2-thiazolyl)-formamide; DMBA = 7,12-dimethylbenz[*a*]anthracene; and BOP = *N*-nitrosobis (2-oxopropyl)amine.

†PGE_2 = prostaglandin E2; n/e = no effect; PGs = prostaglandins; n/s = not significant.

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human prostate cancer cell lines PC-3 and LNCaP, as well as human breast and colorectal cancer cell lines, with dimethylprostaglandin E_2 in culture. This compound increased the COX-2 mRNA level and the cell growth rate, while the NSAID flurbiprofen (5 m*M*) inhibited the up-regulation (increased expression) of COX-2 mRNA and the stimulation of PC-3 cell growth that occurs in the presence of dimethylprostaglandin E_2.

Although PGE_2 has tumor and cell growth-promoting activity, its dehydration products PGA_2 and PGJ_2 have been shown by Fukushima et al. *(102–105)* to inhibit cell growth *in vitro* and to exhibit antitumor activity *in vivo.* Gorospe et al. *(106)* showed in the human breast carcinoma cell line MCF-7 that PGA_2 treatment causes arrest in phase G_1 of the cell cycle and a dramatic decrease in the levels of cell cycle-related proteins cyclin D1 and cyclin-dependent kinase 4, together with an increase in p21 gene and protein expression, independent of p53 status. In the human colorectal carcinoma cell line RKO, PGA_2 treatment fails to induce growth arrest; instead, it results in substantial cell death. These effects are associated with a lack of p21 induction and with enhanced cyclin-dependent kinase 2 activity *(107).*

Conclusion

Genetic and pharmacologic evidence has established that COX-2 is induced in the polyps of $Apc^{\Delta 716}$ and Min mice, two mouse models of human FAP. Selective (or specific) COX-2 inhibitors are much more efficient in suppressing polyposis in these mice or in suppressing ACF induced in rats than are traditional NSAIDs; furthermore, these compounds have the advantage of not causing gastrointestinal side effects. Many additional animal experiments and clinical trials using COX-2 selective inhibitors will be undertaken in coming years to establish the role of these compounds in chemotherapy for polyposis and for various other cancers, as well as in cancer chemoprevention. Before rushing these compounds into clinical trials, however, it would be important for us to determine whether COX-2 is induced and plays a key role in the cancer and/or precancerous condition that is the target of a particular trial. Once this association is established, we can reasonably expect that treatments with COX-2 selective inhibitors will bring us promising chemotherapeutic effects.

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NOTES

[1]Prostanoids is a more accurate term than prostaglandins when all physiologically active metabolites of prostaglandin H_2 are indicated; i.e., prostaglandins A_2, D_2, E_2, $F_{2\alpha}$, I_2 (prostacyclin), J_2, and thromboxane A_2.

Editor's note: Part I of this review, which appears in the Vol. 90, No. 20, October 21, 1998, issue of the Journal, focuses on the discovery of the cyclooxygenases (COXs); their biochemical, molecular, and structural properties; and the discovery of isozyme-specific inhibitors of COX activity.

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