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PREVENTION OF COLORECTAL CANCER USING COX-2 INHIBITORS: BASIC SCIENCE AND CLINICAL APPLICATIONS

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1. ABSTRACT

Cyclooxygenase-2 (COX-2), an inducible prostaglandin G/H synthase, is overexpressed in pre-neoplastic tissues and several human cancers including colorectal cancer. Evidence linking COX-2 activity to carcinogenesis was derived from epidemiologic studies and animal models with defect adenomatous polyposis coli (APC) gene. PGE_2 induced by COX-2 exerts several biological properties that may be advantageous for tumorigenesis: 1) Promoting angiogenesis (increased VEGF, bFGF, and PDGF production), 2) Anti-apoptosis mechanism (via increased bcl-2 and Akt activity), 3) Stimulating tumor metastasis (by increasing matrix metalloproteinases) and 4) Decreased immune surveillance (decreased cytokine production and NK activity). In addition, COX-2 reaction can cause DNA oxidation and induce mutations. Chemoprevention of colorectal cancer has attracted great attention in recent years. Epidemiologic data showed that chronic intake of traditional nonsteroidal anti-inflammatory drugs (NSAIDs) could reduce the incidence of colorectal cancer. Recent clinical trial studies showed that celecoxib, a selective COX-2 inhibitor, is equally effective in reducing colorectal adenomas in animal models and patients with familial adenomatous polyposis (FAP), yet with superior GI safety. Two COX-2 inhibitors (celecoxib and refocoxib) have been approved by FDA as adjuncts to usual care in FPA patients, and are currently being studied in patients with sporadic adenomas and other types of cancers. These studies are expected to generate evidence in favor of targeting COX-2 and its gene products as chemopreventive strategies, which may provide an alternative in current approach to reducing the morbidity and mortality of this disease.

2. INTRODUCTION

Colorectal cancer is the third most common cancer diagnosed in both men and women in the United States. The American Cancer Society estimates that 105,500 new cases of colon cancer (49,000 men and 56,500 women) and 42,000 new cases of rectal cancer (23,800 men and 18,200 women) will be diagnosed in the year of 2003 (1). Colorectal cancer develops through a multistep process that involves the transition from normal mucosa to adenomatous polyps (adenoma) and to invasive carcinoma. The great majorities of colorectal cancer cases are sporadic and exhibit no obvious heritable tendency or clear causes.

Somatic mutation of the adenomatous polyposis coli (APC) gene is a characteristic of approximately 80% of these patients with nonhereditary sporadic adenomatous polyposis (SAP). Mutation of the APC gene is thought to be an early step in the development of colorectal cancer. Following initial genetic change, the process of carcinogenesis involves a complex series of genetic, phenotypic and pathologic changes, including the loss of p53 tumor suppressor gene in the process toward invasive colorectal carcinoma (2).

In a small group of patients (0.5%) with familial adenomatous polyposis (FAP), the disease is hereditary. Genetic evidence indicates that patients with FAP inherit a germ line mutation of the APC gene and the lifetime incidence of colorectal cancer with untreated FAP is almost 100%. They are the most at risk group; if untreated, most of the subjects will develop colorectal cancer in their lifetime. The progress from adenoma to carcinoma pathway in FAP is very similar to that of sporadic colon cancer patients. Because the penetrance of the APC mutation is essentially 100%, this small group of subjects has become very important in the study of chemopreventive agents. The second group of heritable colorectal cancer consists of those diagnosed with hereditary nonpolyposis colorectal cancer (HNPCC). The genetic defects in HNPCC patients are less specific and may involve the mutations of a number of DNA mismatch repair genes including MLH1, MSH2, PMS1 and PMS2 genes. The penetrance of HNPCC is significantly less than that of FAP although it is larger in size (2% of all colorectal cancer)(3). A much larger third group of "heritable" colorectal cancer is those with a family history of colorectal cancer but is distinct from either of the first two groups. The genetic mutations associated with this group of patients appear to be unique to each individual.

Advances in the etiology and pathology of colorectal cancer have made it easier to identify patients with high-risk diseases. The treatment of colorectal cancers without evidence of metastasis is surgical resection of the affected bowel. Recurrence rates of cancer are high after resection and may be treated by further resection followed by radiotherapy and chemotherapy. Chemotherapy is also given as an adjuvant therapy to reduce the potential of metastases following surgery. More recent development is the use of neoadjuvant treatment, when appropriate. Once the metastases occur, however, the survival rate for colorectal cancer is often very low. Colorectal cancer is the second-leading cause of cancer death in the U.S., yet, it is considered to be a highly preventable disease. Chemoprevention is defined as the use of specific natural or synthetic chemical agents to reverse, suppress or prevent the progress from adenoma to invasive colorectal cancers. Given the high frequencies of occurrence and recurrence of the disease, chemoprevention of colorectal cancer has attracted great attention in recent years. Effective chemopreventive strategies may provide an attractive alternative in the current approach to reducing the morbidity and mortality from this disease. Recently, Gwyn and Sinicrope (4) reviewed a number of pharmacological agents that have been studied for chemoprevention of colorectal cancer. This review focuses on recent progress of PGE_2 production and selective COX-2 inhibitors as potential chemoprevention agents. COX enzymes catalyze the synthesis of prostaglandins (PGs) from arachidonic acid (AA). Two major COX isoforms have been identified: COX-1, an enzyme constitutively expressed in most mammalian tissues, and COX-2, an enzyme inducible by a variety of stimuli, including cytokines, growth factors and tumor promoters.

COX-2 was first described as an enzyme induced during the transformation of cells by the viral oncogene, v-src in the early '90s (2). Subsequent studies showed that COX-2 is inducible by a variety of mitogenic and inflammatory stimuli, resulting in enhanced synthesis of PGs in various cell types (4-6). With the exception of select tissues, i.e., reproductive organs, kidney and brain, COX-2 is not expressed in normal nonmalignant tissues. Since the finding that COX-2 is linked to carcinogenesis in several human cancers, inhibition of COX-2 with specific inhibitors has attracted considerable attention as an alternative therapeutic target for cancer treatment and chemoprevention.

3. BIOSYNTHETIC PATHWAY OF PGE_2 PRODUCTION

Figure 1 depicts AA metabolism for the synthesis of PGs and related derivatives. Upon cell activation, AA, mainly from membrane phospholipids (PL) undergoes catalytic hydrolysis by phospholipase A_2 (PLA_2). AA is a common precursor to leukotrienes, isoprostanes, epoxyeicosatrienoic acids (EET), and various PGs. Lipoxygenases (LOX) convert AA into leukotrienes involved in the process of asthma, and tumor cell adhesion, invasion, metastasis and angiogenesis. Following a nonenzymatic free radical mechanism, isoprostanes are formed serving as markers for *in vivo* peroxidation. After the catalysis by epoxygenases via cytochrome P450s, AA is converted to EETs that act as endothelial derived hyperpolarizing factor (EDHF) to regulate hypertension in addition to EET roles in renal electrolyte transport.

In this review, pertinent to the COX-2 process, we will focus on PGE_2 production that is preferentially carried out by ER lumenal surface PHGS-2 enzyme complex consisting of (from N- to C-terminus) a signal domain, a EGF-like domain, a membrane binding domain, and a globular catalytic domain with COX-2 and peroxidase sites. Initially, COX-2 removes hydrogen from AA at C-13 (S) position followed by the insertion of oxygen into AA at C-11, 15 positions yielding PGG_2- a 15-hydroperoxide. The resulting PGG_2 undergoes a reduction by peroxidase to form PGH_2, a common intermediate for various prostanoids following the catalyses of specific synthases. In the case of PGE_2 production, prostaglandin E synthase (PGES) is responsible for the conversion of PGH_2 to PGE_2.

4. REGULATION ON PGE_2 PRODUCTION

PGE_2 production is susceptible to various conditions (Table 1). LPS (7), TNF-alpha (8), INF-gamma

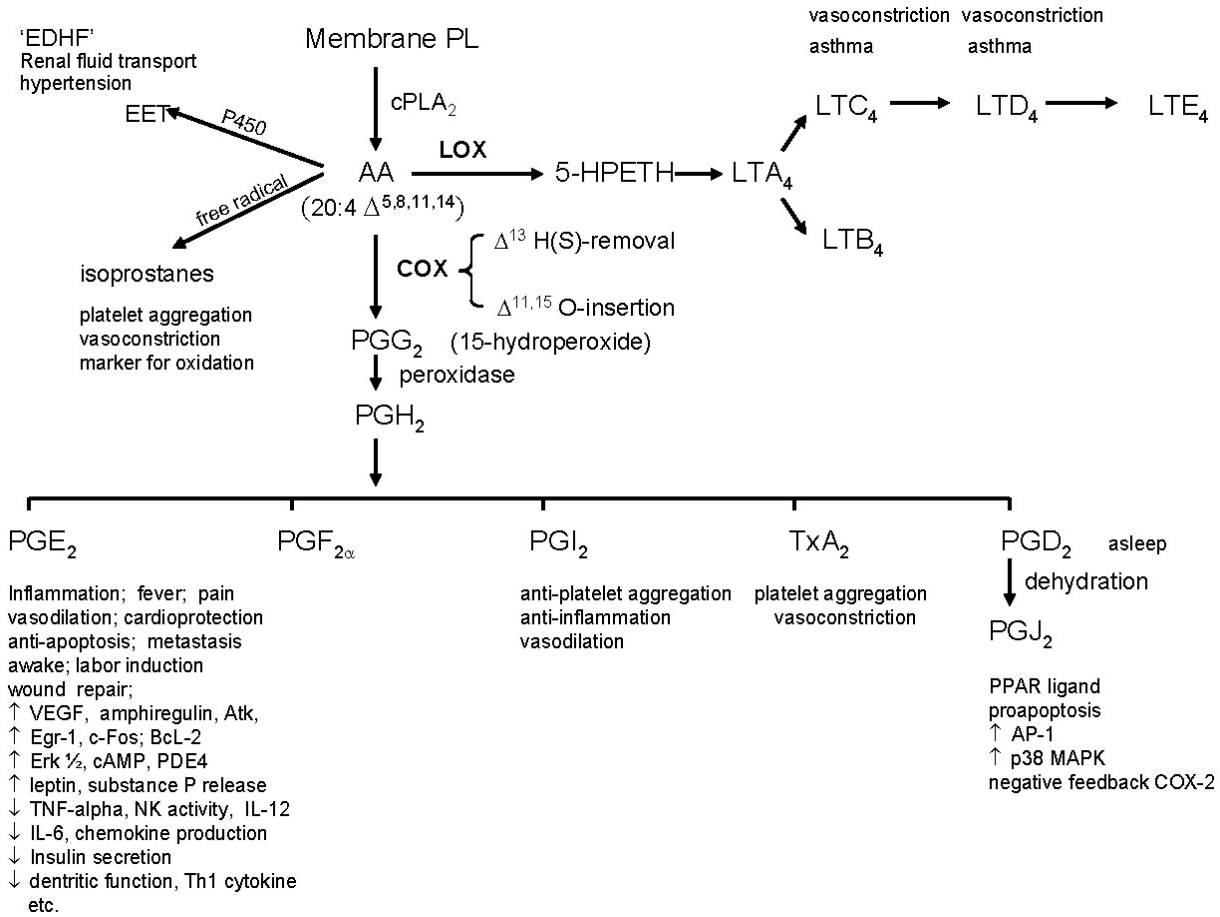

Figure 1. Biosynthetic pathway for PGE_2. The major provision of arachidonic acid (AA) results from membrane phospholipids (PL) that are subjected to the agonist activation of $cPLA_2$. AA is a common precursor to leukotrienes, isoprostanes, epoxyeicosatrienoic acids (EET), and various PGs. Apart from PGE_2 functions; the major actions of AA metabolites are also included. ↑or↓ denotes activation or inhibition, respectively.

(9), IL-1beta (10), TGF-beta (11), VEGF (12), bradykinin (BK) (13), vasoconstrictor endothelin (ET-1) (14), GM-CSF (15), platelet-activating factor (PAF) (16), PDGF (17), NO (18), thrombin (19), S-1-P (20), H_2O_2 (21), adenosine (22), cAMP (22), CD40L (23) and 12-O-tetradecanoylphorbol 13-acetate (TPA) activates PGE_2 production in many cell types. The elevated production is also in response to infection such as Candida (24), C. pneumoniae (25), Salmonella (26) and HHV-6 (27). Some environmental factors including heat shock (Hsp90) (28), hypoxia (29), shear stress (30) and ozone stress (31) also have shown to upregulate PGE_2 production.

PGE_2 production is inhibited by antioxidant flavonoid (32), gamma-tocopherol (33), vitamin D (34), estrogen (35), dexamethason (36), cortisol (37), or chitosan (38). Food components such as conjugated linoleic acid (39), oleic acid (40), soybean isoflavone (41), tea polyphenols (42), or caffeic acid (43) also depresses PGE_2 production.

4.1. COX-2 regulation

4.1.1. COX-2 expression

Unlike COX-1 that is constitutive under developmental regulation, COX-2 is inducible in response to various upregulating factors and processes (Table 2). COX-2 expression elevates upon the activation of various intracellular signaling involving MAPK, PKC, transcription factors and/or cAMP system. IL-1beta (10), LPS (7), INF-gamma, TGF-beta (11), TNF-alpha, TPA, ET-1 (14) activates MAPK (Erk1/2 & p38) to enhance COX-2 expression. The activation of PKC mediates the upregulated COX-2 expression by INF-gamma (9), BK (13), IL-1beta (44), LPS (9), TGF-beta (11), TNF-alpha (8) and TPA (45). The activation of AP-1, NF-kappaB or c-Jun by IL-1 (10), TPA (45), C. pneumoniae (25), arsenite (46), LPS (7, 47) and INF mediates the induction. Adenosine (22), human herpesvirus 6 (HHV-6) (27), BK (48) or LPS (49) elevates cAMP level to up regulate COX-2 expression. OxLDL (50) activates PPAR to induce COX-2 expression. Others such as hypoxia (29), UV (51), reactive oxygen species (52) and NO (18) enhanace COX-2 expression with undefined mechanism(s).

The down regulation of COX-2 expression is largely mediated by the inhibition of MAPK, PKC, AP-1, NF-kappaB and many other intracellular signaling. Caffine (43), alpha-tocopherol (50), ceramide (53), PGJ_2 (54), cortisol (37), dexathmasone (55), PPAR-gamma ligand

Table 1. Regulation of PGE_2 production

Up regulation	Down regulation
LPS; TNF-alpha; IFN-gamma; IL-1beta; TGF-beta; VEGF	Conjugated linoleic acid; oleic acid
BK; ET-1; AT-II; GM-CSF; PAF; PDGF	estrogen; dexmathason; cortisol
NO; dopamine	gamma-tocopherol; vitamin D
adenosine, cAMP	Chitosan
thrombin; PAF; S-1-P	Flavonoid
H_2O_2; bile acid	Statin
Hsp90; CD40L	taurine chlorium
Candida; C. pneumoniae; Salmonella	Antioxidant N-acetyl cysteine
HHV-6; CpG DNA	PGJ_2 with COX-2 inducer
tetracycline	honey; ginger; soybean isoflavone
gamma-glucocorticoid	tea polyphenols
TPA; arsenite	fructose-1, 6-diphosphate
Hypoxia; ozone	chronic lithium
ultrasound; shear stress	caffeic acid

The up or down regulation of PGE_2 synthesis includes inflammation, infection, hormones, environmental factors, vitamins, food components, etc.

Table 2. Regulation of COX-2 expression

	Mechanism of Action
(1) Up regulation	
IL-1beta; LPS; INF-gamma; TGF-beta; TPA	↑ Erk ½; p38 MAPK; PKC-alpha; NFkappaB; IkappaK
PMA	↑ p38 ; PKC; AP-1; c-Jun; cAMP
PDGF; PAF; AT II; VEGF; IL-6	-
Thrombin; S-1-P	-
Bradykinin	↑ cAMP; PKC-epsilon
Adenosine	↑ cAMP;
TNF-alpha; arsenite	↑ NFkappaB; IkappaB
LPS; HHV-6	↑ cAMP; AP-1
UV; Hypoxia; reactive O^-	-
PGJ_2 without COX-2 inducer	-
PGE_2 & PGE_3	↑ cAMP;
Vomitoxin; ET-1; AT II	↑ Erk ½; p38 MAPK
Gastrin	↑ Erk-5 kinase; ELK-1; c-Fos; c-Jun; AP-1
OxLDL	↑ PPAR
Salmonella; Chlora toxin; CpG DNA	-
C. pneumoniae	↑ NFkappaB
NO; benzo[a]pyrene (smoke)	-
Aging; parathyroid, luteinising hormone	-
Dihydroxy bile acid	↑ PKC; AP-1
Tetracycline	-
(2) Down regulation	
alpha-tocopherol	↓ NFkappaB; PKC; Erk ½; ↑ PP2A
bee venom	-
PGJ_2 with COX-2 inducer	↓ NFkappaB via PPAR
chronic lithium	-
fructose-1,6-diphosphate	-
cortisol; dexathmasone	↓ NFkappaB
Polyhydroxybenzene	↓ NFkappaB; IkappaK
PPAR-gamma ligand	↓ AP-1; CREB binding
Retinoic acid	-
Ursolic acid	↓ p38; AP-1; c-Jun; cAMP
Estradiol; ceramide	-
Vitamin D	-

Accompanying with the up or down regulation of COX-2 expression, the major mechanism(s) and intracellular signaling components involved are provided. ↑ or ↓ denotes activation or inhibition, respectively

(56), retinoic acid (57), estradiol (58) or vitamin D (34) depresses COX-2 expression.

4.1.2. Molecular structure and COX-2 inhibition

COX-2 shares nearly 60% sequence homology with COX-1, while a specific COX-1 variant is referred to as COX-3. The binding specificity of COX-3 is similar to that of COX-1. The inhibitory response of COX-1 and COX-2, however, are distinct. Crystallographic studies predict that COX-2 active site is 17% larger than its counterpart COX-1. Arg-120 is the primary site for interacting –COOH of AA, while Try-385 interacts with (S)-hydrogen at C-13 position responsible for the formation of peroxide. Try radical is the key to reaction. As a result, COX-2 is not sensitive to the acidic inhibitor such as aspirin. A growing number of COX-2 selective inhibitors are developed. The application of COX-2 inhibitors on colorectal cancers is summarized in the following sections.

4.2. $cPLA_2$ regulation

In addition to hormonal stimulation including vitamin D, $cPLA_2$ is susceptible to up regulation by inflammatory signals such as LPS, IL-1 (59) and TNF-alpha. BK, OxLDL, thrombin (19), or ET-1 (14) also activates $cPLA_2$. In contrast, TGF-beta1, IL-4, or glucocorticoid inhibits $cPLA_2$. Inhibition on $cPLA_2$ diminshes substrate AA bioavailability, thereby contributing to the down regulation of PGE_2 production.

4.3. Peroxidase regulation

Little is known about the regulation on peroxidase; aspirin or COX-2 inhibitors show no effect on peroxidase.

4.4. PGES regulation

PGES isomerizes PGH_2 into PGE_2. Several forms of this isomerase enzyme including cytosolic (cPGES), microsomal membrane-bound (mPGES), and Mu class have recently been found to express ubiquitously in different cell types. Essentially, PGES is the terminal enzyme for PGE_2 synthesis at the downstream of COX and they share similar regulatory responses. mPGES expression is coordinately up regulated with COX-2 under various inflammatory conditions, which is reversed by glucocorticoid. mPGES activity is inhibited by COX-2 inhibitor NS-398. Statin suppresses COX-2/PGES coupled regulatory system (60). cPGES specifically converts COX-1 derived PGH_2 to PGE_2; its expression is drastically up regulated by LPS by several-fold. It is likely that the existence of cPGES-COX-1 and COX-2-mPGES pathways are to ensure the regulation of PGE_2 production under various pathophysiological conditions. It remains to be established whether the Mu class contributes to PGE_2 production with respect to its coupling with COX subclass.

5. PGE_2 FUNCTIONS

Functioning as a local hormone, PGE_2 plays diverse roles in cell signaling. PGE_2 expression in inflammatory process is associated with pain and fever. Its vasodilation property offers cardioprotection. The major functions reported in recent studies involve awake, labor induction (61), wound healing (62), and the activation of Akt (63), Egr-1(64), c-Fos (65), Erk ½ (66), cAMP (67), Jun (65). PGE_2 suppresses dentritic cell function (68), insulin secretion (69), leptin (70), substance P release (71) TNF-alpha (72), IL-12 (73), IL-6 (74), chemokine (75) and Th1 cytokine (76) productions. Those cellular responses are mainly transmitted by G-protein coupled PGE_2 receptors. One could find in-depth reviews elsewhere.

Possible links between COX-2 expression and cancer development are evident. The inhibition of apoptosis (77) and NK activity (78) accompanied by the promotion of metastasis (77) and the expression of BcL-2 expression (78), VEGF (79), amphiregulin (80), or MMP-2 (81) particularly makes PGE_2 closely in relation to cancer cell proliferation, which is consistent with the positive correlations of PGE_2 level and COX-2 expression with cancer risks.

6. COX-2 EXPRESSION AND COLORECTAL CANCER

6.1. Epidemiologic Studies

The potential for COX-2 inhibition using selective agents in cancer chemoprevention is derived primarily from epidemiology with studies in patients using "low-dosage" traditional NSAIDs such as aspirin as prophylaxis against cardiovascular problems. These nonselective NSAIDs, which include aspirin, indomethacin, sulindac and ibuprofen, are inhibitors of both COX isoforms. Case-controlled and cohort-controlled studies examining the association between NSAIDs ingestion and the risk of colorectal cancer show that chronic use of nonselective NSAIDs could lower the incidence of colorectal adenomas and carcinomas (82). Chronic use of aspirin also has been linked to possible reduction of colorectal cancer death (83). One key finding common to these studies is that prolonged and consistent use of NSAIDs are essential to lowering the incidence of colorectal cancer (4, 5, 82, 84). In the Health Professional Follow-Up Study, Giovannucci *et al*. reported that a statistically significant reduction in risk of colorectal cancer with regular aspirin use was identified for both men and women reporting over 20 years of consistent aspirin use (85, 86). In contrast, Sturmer *et al*. noted that there is no association found between the use of aspirin and the incidence of colorectal cancer in a 12-year follow-up study (87). The lack of effectiveness in this study is not clear, but it seems possible that the short treatment period with aspirin may account for the null findings. Results from epidemiologic studies provided the basis for thorough preclinical testing and prospective clinical studies examining the effect of COX inhibition on colorectal cancer.

6.2. Clinical trials with NSAIDs

Waddell and Loughry first reported that sulindac could reduce the number of adenomatous polys in four patients with Gardner's syndrome, a variant of FAP (88). Subsequent studies reported a similar reduced risk of colorectal cancer with regular use of NSAIDs. Trials using the NSAID, sulindac, reduced the number of polyps in

patients with familiar adenomatous polyposis (FAP) (89). Consistent with data obtained from epidemiologic studies, prolonged and consistent use of NSAIDs is crucial in reducing the number of polyps in patients. Giardiello *et al.* reported that 3 months after the termination of sulindac treatment, regrowth of polyps were detected in patients with FAP (90). In a phase III clinical trials, treatments with indomethacin and prednisolone also have been shown to significantly prolong survival rate and offer palliative support to terminal cancer patients with advanced solid tumors compared to placebo treatment (91).

In addition, the protective effect of NSAIDs against colon cancer development has been confirmed in numerous studies using induced colon cancer animal models. In these studies, treatment of animals with traditional NSAIDs such as aspirin, indomethacin and sulindac could significantly prevent chemically induced colon carcinogenesis and the incidence of adenocarcinomas. Results from the animal studies are in line with the epidemiologic evidence and clinical trials in colorectal cancer patients indicating that ingestion of NSAIDs can prevent colon carcinogenesis (92-95).

7. CANCER PREVENTION WITH COX-2 INHIBITORS

7.1. COX-2 expression and Cancer

The finding that COX-2 expression is linked to cancer development is significant. The expression of COX-2 has been detected in neoplastic tissue from colorectal cancer patients but is absent from adjacent histologically normal intestinal tissue. In contrast, expression of COX-1 is detected in both normal and neoplastic tissue. Increased COX-2 protein and RNA expression also have been detected in premalignant and malignant polyp tissue samples from adenomas and carcinomas in patients with either FAP or sporadic adenoma (96-101). Hao *et al*. reported that COX-2 protein was highly expressed in hyperplastic tissue versus nonmalignant mucosal tissue (97). Masferrer *et al*. reported the expression of COX-2 protein in endothelial cells of small blood vessels located within the polyps of some sporadic adenoma patients whereas COX-1 was broadly distributed in normal and in neoplastic tissues (100). In addition, slight-to-moderate COX-2 protein expression was detected in neuroendocrine cells of colonic crypts, infiltrating macrophages and neutrophils, and lymphocytes in gut-associated lymphoid tissue (99). The fact that COX-2, not COX-1, is markedly elevated in neoplasm suggests that COX-2, rather than COX-1, may contribute to colorectal carcinogenesis and promoting colon cancer formation.

In addition to colorectal cancer, enhanced expression of COX-2 has been detected in various forms of human cancers and premalignant lesions such as gastric cancer (102), esophageal cancer (102), hepatocellular cancer (103, 104), pancreatic cancer (105), head and neck cancer (106), non-small cell lung cancer (101, 107), breast cancer (100, 101), prostate cancer (100, 108), bladder dysplasia and bladder cancer (109), cervical cancer (110) and endometrial cancer (111). In spite of the correlations, there is no evidence to indicate that COX-2 gene is mutated during carcinogenesis or that COX-2 itself is oncogenic. Yet, overexpression of COX-2 is frequently associated with reduced cancer prognosis as reported in colorectal, NSCLC, breast, cervical and esophageal cancers (96, 112-115). The widespread expression of COX-2 in neoplastic and pre-neoplastic tissues raises the possibility of using COX-2 inhibitors as chemopreventive agents in treating not only colorectal cancer but also other forms of neoplastic diseases.

An important question is why COX-2 overexpression leads to tumor formation. *In vitro* studies showed that COX-2 can induce various biological responses that may be advantageous for tumorigenesis: 1) Anti-apoptotic mechanisms mediated through Bcl-2 upregulation and protein kinase B (Akt) signaling pathway (77, 116), 2) Modulating metastasis through the activation of matrix metalloproteinases (117), 3) Regulation of angiogenesis through increased production of vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and PGs (79, 118, 119) and 4) Conversion of precarcinogens to carcinogens by COX-2 peroxidase activity. Conceivably, these biological properties associated with COX overexpression are all crucial in promoting tumor formation. Detailed mechanisms of the biological responses associated with COX-2 will be further discussed in Section 8 of this review.

7.2. Preclinical Studies (Animal models) and COX-2 inhibitor

Animal models contribute significantly to elucidating the role of COX-2 in colon cancer development. Several rodent models that spontaneously develop intestinal adenomatous polyposis have been developed. These animals (Min mice) lack functional APC genes, which are thought to be the same genetic defect in the pathogenesis of most human colorectal tumors (120-122). Preclinical studies using these rodent models established a direct link between the expression and activity of COX-2 and the development of intestinal adenomatous polyposis. These APC defect animals also provide an invaluable *in vivo* model to examine the chemopreventive effect of selective COX-2 inhibitors.

Using the APC mutant Min [C57BL/6J$^{Min/+}$] mouse model, Jacoby *et al*. (120) showed that early treatment of the animal (before the development of intestinal adenomatous polyposis) with celecoxib significantly decreased the total tumor load (tumor multiplicity and volume) by 85% relative to control ($p<0.01$). Late treatment (after the development of intestinal adenomatous polyposis) with celecoxib decreased total tumor multiplicity to 48% of that of the control group. Nonselective NSAID, piroxicam, also reduced tumor multiplicity in these animals. Whereas piroxicam caused numerous gastrointestinal ulcerations and bleeding, celecoxib did not cause any apparent toxicity. In another study using double-mutant mice lacking both APC protein (APC$^{\Delta 716}$ knockout rodent) and COX-2 protein (Ptgs2$^{-/-}$) Oshima *et al*. (122) showed APC knockout double mutant animals developed significantly fewer gastrointestinal

adenomatous polyps due to the lack of functional COX-2 proteins. Treatment of single-mutant $APC^{\Delta 716}$ knockout mice with a selective COX-2 inhibitor (MF tricyclic) reduced the number of adenomatous polyps in a manner similar to that of double-mutant mice. In a similar study, Sunayama *et al*. reported that treatment of $APC^{\Delta 474}$ knockout mice with a selective COX-2 inhibitor, JTE-522, significantly reduced the incidence of large adenomas with increased apoptosis index in adenomas (121). In addition, JTE-522 inhibits the expression of VEGF by the infiltrating macrophages in the submucosal layer of adenomas, resulting in a decrease in vascularity in the area. They concluded that selective COX-2 inhibition reduced adenoma growth and the inhibition may be mediated through its inhibitory effect on angiogenesis.

Results from these animal studies showed that selective inhibitors of COX-2 are safe and effective for the prevention and regression of adenomas. These studies also provide specific data that firmly establish a cause-effect connection between COX-2 expression and colorectal cancer development.

7.3. FAP Clinical Studies with selective COX-2 inhibitor

Since 1999, FDA has approved two selective COX-2 inhibitors (celecoxib and rofecoxib) for FAP patients as an adjuvant to other regimens (82). The approval was based on earlier studies with sulindac and two small trial studies (123, 124). Between these two agents, only celecoxib has received extensive clinical studies (125, 126). The first clinical trial to evaluate the effect of celecoxib was carried out by Steinbach *et al*. in FAP patients (126). This double blind, placebo-controlled trial showed a significant reduction ($p=0.003$) in the number of colorectal polyps in 83 patients with FAP treated with celecoxib (400 mg twice daily for six months) compared with placebo. Celecoxib 100 mg twice daily numerically reduced the polyps burden but not significant statistically. A second endpoint of this trial study was to evaluate the effect of celecoxib on duodenal disease (125). Again, treatment with celecoxib 400 mg twice daily for six months reduced polyp formation in 30 FAP patients, significantly involved areas of duodenal polyposis ($p=0.049$) relative to placebo (15 patients). The results of this study appears to be consistent with a recent epidemiological study in which the effect of rofecoxib and celecoxib on colorectal cancer and colorectal adenoma was investigated using data from a government insurance database on patients (127). Results of that study showed that exposure to rofecoxib, celecoxib and traditional NASIDs reduces the risk of colorectal adenoma and are protective against both neoplasias.

Selective COX-2 inhibitors are as effective as traditional NSAIDs such as sulindac or aspirin in reducing colorectal polyps. Yet, celecoxib appears to have much less GI mucosal toxicity than traditional COX inhibitors. Celecoxib 400 mg twice daily is well tolerated in patients. The most common adverse events were diarrhea and abdominal pain but there were no significant differences in side effects between subjects receiving celecoxib and those receiving placebo (126). Much lower potential of the selective COX-2 inhibitors for gastro-intestinal side effects also have been demonstrated previously in patients with rheumatoid arthritis and osteoarthritis undergoing treatment with selective COX-2 inhibitors and/or nonselective NSAIDs (128-131). This fact suggests that COX-2 is the desired therapeutic target for chemoprevention of colorectal cancer.

Besides FAP patients, data about the chemopreventive effect of COX-2 inhibitors in patients with other forms of colorectal cancer such as sporadic adenoma and in average-risk individuals in the general population is scanty. Despite this limitation, data obtained from FAP patients treated with COX-2 inhibitor and from preclinical findings justify additional clinical trials to evaluate COX-2 inhibitors for the potential chemoprevention of colorectal cancer. Currently, two multicenter studies are under way to assess the efficacy of adding celecoxib to an IFL chemotherapy regimen (5-FU/leucovorin/Camptosar) in patients with advanced colorectal cancer (132, 133). It is pertinent to mention that FDA has not approved celecoxib for the prevention of colorectal cancer. As present, celecoxib has been indicated for: 1) the relief of the signs and symptoms of osteoarthritis; 2) the relief of the signs and symptoms of rheumatoid arthritis, 3) the management of acute pain and 4) the treatment of primary dysmenorrheal.

8. POSSIBLE MECHANISM OF ACTION OF SELECTIVE COX-2 INHIBITORS

The exact mechanisms of chemoprevention by COX-2 inhibition are not fully understood. Based on data obtained from both *in vitro* and *in vivo* experiments, at least five potential pathways induced by COX-2 inhibitors have been proposed: 1) Increased sensitivity to apoptosis, 2) Inhibition of angiogenesis, 3) Modulation of inflammation/immunosuppression, 4) Decreased metastasis and 5) Inhibition of endogenous carcinogen formation (5, 6, 82, 134).

8.1. Increased sensitivity to apoptosis

Decreased apoptosis of intestinal epithelial cells is often associated with the genesis of colorectal cancer. In Min mice, the intestinal epithelium showed a decreased apoptotic rate due to the absence of a functional APC gene (135). Earlier studies by Piazza *et al*. showed that inhibition of COX activity with sulindac could inhibit cell proliferation and induce apoptosis in cultured colon cancer cell lines (136). Tsujii and DuBois showed that overexpression of COX-2 in rat intestinal epithelial cells resulted in a decreased sensitivity to butyrate-induced apoptosis (137). Decreased sensitivity to apoptosis was associated with increased levels of PGE_2 and Bcl-2 protein. Treatment of these cells with NSAIDs could reverse the levels of Bcl-2 protein induced by COX-2 overexpression (77, 138). Furthermore, the effect is detected only in COX-2 expressing cells but not those lack COX-2 expression, indicating that the anti-proliferative effect of COX-2 inhibitor was mediated through COX-2 pathway (116). However, recent studies suggested that the anti-proliferative effect of COX-2 inhibitor might act through COX-independent pathways. For example, Waskewich *et al*. reported that the anti-proliferative effect of celecoxib

and rofecoxib was detected in both COX-2 positive and negative cell lines (139). Further, celecoxib has been found to have a much more potent anti-proliferative activity than rofecoxib. Therefore, it is possible that COX-2 inhibitor(s) may act on additional cellular targets other than COX-2.

8.2. Inhibition of Angiogenesis

Solid tumor growth and metastases depend on the acquisition of an adequate blood supply (140). Folkman was the first to suggest that inhibiting angiogenesis might facilitate tumor growth and metastases. Tumor cells ensure their own growth by the release of angiogenic growth factors, such as VEGF, bFGF and PDGF that promote angiogenesis (140, 141). Histological studies showed that COX-2 is expressed in both tumors and the surrounding neovasculature. PGE_2 is potent inducers of VEGF (79). Uefuji *et al*. showed that there is a correlation between the microvessel density in gastric cancers and COX-2 overexpression, particularly around the periphery of tumors (142). Tsujii *et al*. showed that COX-2 expressing tumor cells were larger and more angiogenic than those lacked COX-2 expression. Treatment with NS-398, a selective COX-2 inhibitor, markedly inhibits the production of angiogenic factors by colon cancer cells, concomitantly with reduced tumor growth (119). Sunayama *et al*. reported that JTE-522, a COX-2 inhibitor, inhibited the expression of VEGF by the infiltrating macrophages in the submucosal layer of the adenomas, resulting in a decrease in vascular area and reduced adenoma growth (121).

The anti-angiogenesis effect of COX-2 inhibitors also has been demonstrated in various non-tumor models. Masferrer *et al*. investigated the anti-angiogenic activity of celecoxib using rat corneal pocket model of angiogenesis stimulated by fibroblast growth factor-2 (FGF-2) (100). They showed that celecoxib, but not a COX-1 inhibitor, could block angiogenesis in this model. Their results suggest that PGs produced by FGF-2 induction of COX-2 expression are essential to neovascularization. Using the same rat corneal model, Leahy *et al*. showed that celecoxib (30 mg/kg/day p.o.) inhibited angiogenesis and PG (PGE_2, TXB_2) production (143). Decreased PG production in corneas was associated with increased cellular apoptosis and decrease in cell proliferation. In an animal model involving COX-2 overexpressing cancer xenograft, Sawaoka *et al*. showed that COX-2 inhibitors suppress angiogenesis and tumor growth by inhibiting expression of angiogenic factors and vascular endothelial cell growth (144). COX-2 also has been implicated in the release of angiogenic proteins and production of various eicosanoids (TxA_2, PGI_2, PGE_2) that directly stimulate the migration of endothelial cells and angiogenesis *in vivo,* and possibly the survival of vascular endothelial cells via the upregulation of Bcl-2 proteins and Akt kinase (145). Taken together, these studies clearly show that the antitumor activity of COX-2 inhibitors may be attributable, at least in part, to a direct effect on host stromal elements, such as the angiogenic vasculature (143).

8.3. Modulation of inflammation/immunosuppression

Chronic inflammation is a major risk factor for epithelial carcinogenesis. It has been documented that patients with ulcerative colitis, the most serious form of symptomatic chronic inflammation, for more than 35 years can carry an absolute risk of developing colorectal cancer (134, 146). Increased COX-2 protein and RNA expression were detected frequently in these inflamed tissues including both premalignant and malignant polyp specimens from patients with FAP and sporadic adenoma. Inflammation is associated with increased PG synthesis due to inflammatory cytokine-mediated COX-2 induction. This process suggests that there may be a role for COX-2 inhibition as a modulator of carcinogenesis secondary to reduction in inflammation (5). Thus, general inhibition in local inducible anti-inflammatory responses with selective COX-2 inhibitor may be responsible for reduced incidence of "inflammation-mediated" colorectal cancers in the patients. In addition, COX-2 induction and increased PG synthesis haven been shown to cause an "immunosuppressed" states in colorectal patients. Although the mechanisms are complex and not well understood, chronic immunesuppression is known to cause a significantly increased appearance of tumors. PGE_2 released by activated macrophages can inhibit the production of immune regulatory lymphokines, chemokines, Th1 cytokines (72-76) and the cytotoxic activity of natural killer cells (78). Increased amounts of PGs have been detected in adenomas and colorectal cancers and this is primarily associated with increased expression of COX-2 but not COX-1 (137, 138). In theory, inhibition of COX-2 could potentially reduce the levels of PGs and promote the host's immune system against tumorigenesis.

8.4. Decreased Metastasis

Several lines of evidence suggest that COX-2-derived PGs plays an important role in tumor growth and metastasis. The ability of malignant cells to break loose from their own tissue is dependent of the invasiveness of the tumor and the ability to digest biological membranes by matrix-metalloproteinases (MMP), which reduce the intercellular anchorage. Human colon cancer cells stably transfected with a COX-2 expression vector had an increased production of PGs and become more invasive compared with control cells without COX-2 expression vector. Tsujii *et al*. reported that increased invasiveness was associated with elevated levels of PGE_2 and MMP mRNA and activation of MMP and was reversed by treatment with sulindac sulfide, a known COX inhibitor (147). Enhanced expression of MMP-2 by PGE_2 also has been reported in cultures of rat mesangial cells (81). In a mouse colorectal caner liver metastasis model, Yao *et al*. showed that rofecoxib could significantly decrease the levels of COX-2 protein, cyclin D1, cytosolic beta-catenin, MMP-2 and MMP-9, and VEGF. Their results showed that rofecoxib decreases the growth and metastatic potential of colorectal cancer in mice through multiple mechanisms (148). In a similar study, Nagatsuka *et al*. reported that JTE-522 downregulated PGE_2 and PDGF production and inhibited MMP-2 secretion by LM-H3 cells. These inhibitory effects on the production of PDGF and MMP-2 may contribute to inhibition of liver metastasis of colon cancer (149). More recently, Yamauchi *et al*. showed that selective JTE-522 could reduce tumor growth and liver metastasis of a highly-metastatic colon cancer cell line,

HT-29, in a human tumor xenografts/severe combined immune-deficient (SCID) mouse model. Since JTE-522 is a potent inhibitor of VEGF production, their findings suggest that JTE-522-induced inhibition of liver metastasis is mediated through the suppression of VEGF expression (150). It is pertinent to mention that many of the anti-metastatic property associated with selective COX-2 inhibitors may be linked to their anti-angiogenesis property described above (100).

8.5. Inhibition of endogenous carcinogen formation

COX-2 reactions involve production of reactive oxygen radicals that can potentially damage biological macromolecules. COX-2 is a bifunctional enzyme. In addition to arachidonate, COX-2 can co-oxidise compounds such as benzo pyrene, generating highly carcinogenic derivatives (151, 152). COX-2 inhibitors can decrease COX-2-mediated oxidation that can transform certain xenobiotic into reactive carcinogenic moieties. This might be an important phenomenon for the colon because of its exposure to xenobiotics (134). Additionally, COX-2-mediated oxidation could be particularly relevant for tissues exposed to tobacco-related carcinogens, such as those of the lung, head and neck and bladder cancers (151, 152). COX-2 reaction also has been shown to cause oxidation-mediated DNA damage, a well-recognized mutagenic event (153). The damage was enzyme-dependent and could be prevented by COX-2 inhibitor or antioxidants (154). This finding is consistent with the fact that COX-2 in inflammatory disease contributes to additional DNA oxidation and the induction of mutations. Thus, COX inhibition could in theory reduce the generation of endogenous carcinogens and oxidation-induced DNA damage.

9. SUMMARY AND PERSPECTIVES

In the views of many investigators, there are ample evidences to show that selective COX-2 inhibitors are potentially very effective chemopreventive agents against colorectal cancer. This conclusion is derived from various studies including epidemiological studies, animal model studies, *in vitro* experiments and randomized trials in FAP patients. Results from the FAP clinical trials with celecoxib are significant even though FAP patients represent only a small sub-group of the colorectal cancer cases. In FAP study, the efficacy of celecoxib as a chemopreventive agent is at least equivalent to that of traditional NSAIDs such as sulindac and aspirin in every aspect. Unlike traditional NSAIDs, celecoxib is well tolerated and appears to be safe for long-term use. The low level of side effects associated with selective COX-2 inhibitors is crucial to further studying their long-term effectiveness for colon cancer prevention in general population. At present, FDA has approved two selective COX-2 inhibitors (celecoxib and rofecoxib) to reduce the number of adenomatous colorectal polyps in FAP patients, as an adjunct to usual care (e.g., endoscopic surveillance, surgery). The FDA has not approved neither one of them for the treatment of colorectal cancer. At present, the chemopreventive effect of COX-2 inhibitors in sporadic colorectal neoplasia and average-risk individuals in general population has not been investigated. Given that the adenoma to carcinoma pathway in sporadic colon cancer is similar to that of FAP, a medication that prevents adenomas in FAP may eventually prove to be effective in sporadic adenomas. Another important issue is how long the effects of COX-2 inhibitors will persist after the agents are discontinued. Cost-effectiveness will be a major concern if the patients will have to take the agent for a prolonged period in order to achieve its chemopreventive effect. Both FDA approved COX-2 inhibitors are relatively expensive compared with traditional nonselective NSAIDs. Ongoing colorectal neoplasia prevention trials using COX-2 inhibitors are in progress (82). In addition, two multicenter studies are under way to evaluate the efficacy of adding celecoxib to an IFL chemotherapy regimen in patients with advanced colorectal cancer (132, 133). These studies, when completed, will produce considerable more information about the long-term efficacy and safety of celecoxib as a possible chemopreventive agent.

To further understand the mechanism of the PG pathway and tumor progression and to develop future generation of agents in chemoprevention, a number of studies are now in progress. PGE_2 mediates its physiological effects by interactions with a subfamily of G-protein-coupled receptors known as EP receptors. Up to date, four sub-types of E prostanoid receptors, EP_1R, EP_2R, EP_3R and EP_4R have been identified (68). The exact roles of these receptors in mediating the biological effect of PGs and tumor development are not fully established yet. A number of studies showed that the action of PGE_2 is mediated primarily through the EP_4 receptors, which utilize a PI3K/ERK-dependent signaling pathway (64, 73). These finding suggests EP_4 receptor, rather than EP_2 receptor, is involved in inflammation and cancer development (64). Using LPS-stimulated human macrophages, Takayama *et al*. showed that EP_4 antagonist could completely reversed PGE_2-mediated chemokine production by inflammatory macrophages (75). Still others, using animal model that lacks EP_2 receptor, $EP_2^{(-/-)}$, showed that these animals exhibited significantly attenuated tumor growth and longer survival times as compared with their wild-type littermates. Furthermore, EP_2 receptor-null mice were refractory to the PGE_2 induced inhibition of DC cell differentiation. Thus, EP_2 receptor may have an important role in inhibiting the host reaction to tumor growth through diminished immune responses (155). Development of selective EP_2 antagonists may provide a better alternative to improve the host antitumor immunity than currently used COX-2 inhibitor. These EP_2 receptor antagonists may be more specific that the use of COX-2 inhibitors and avoid inhibition of desirable products such as PGI.

In addition to COX-2 inhibitor, a number of pharmacological and nonpharmacological agents have been proposed as potential chemopreventive agents for colorectal cancer (4). These include 1) difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase, 2) hepatic hydroxymethylglutaryl coenzyme A reductase inhibitor, 3) ursodexoycholic acid, the 7-B epimer of chenodeoxycholic acid, 4) calcium, 5) folate, 6) vitamin E, vitamin C, and beta-carotene, 7)

selenium and its derivatives, and 8) dietary fiber. The mechanism of action of each agent appears to be unique and different in lowering the incidence of colorectal cancer. These agents, either alone or in combination with other agents including COX-2 inhibitors, are being studied in various preclinical settings of colorectal cancer. The success of these studies will have far-reaching impact on our current understanding and approach to treatment and prevention of colorectal cancer. The field of chemoprevention is still in an early stage of development. Advances in molecular epidemiologic risk models and genetic studies may yield novel drug targets suitable for chemoprevention and cancer treatment. Before a practical and safe protocol can be developed, however, a proper combination of healthy diets, vitamin supplements and exercise plus prolonged use of COX inhibitors may prove to be the right approach for colorectal cancer prevention for now.

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Abbreviations: activating factor-1, AP-1; angiotensin-II, AT-II; arachidonic acid, AA; adenomatous polyposis coli, APC; bradykinin, BK; cyclooxygenase-2, COX-2; early response factor-1, Egr-1; endothelin-1, ET-1; familial adenomatous polyposis, FAP; hereditary nonpolyposis colorectal cancer, HNPCC; interferon, IFN; lipopolysaccharide, LPS; Lipoxygenases, LOX; matrix metalloproteinases, MMP; mitogenic activating protein kinase, MAPK; nitric oxide synthase, NOS; nonsteroidal anti-inflammatory drugs, NSAIDs; oxidized low density lipoprotein, OxLDL; phospholipase A_2, PLA_2; platelet activating factor, PAF; platelet derived growth factor, PDGF; prostaglandins, PGs; proxisomal proliferating activated receptor, PPAR; sphingosine-1-phosphate, S-1-P; sporadic adenomatous polyposis, SAP; transforming growth factor, TGF; tumor necrosis factor-alpha, TNF-alpha; vascular endothelial growth factor, VEGF

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