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Possible Involvement of Hyperlipidemia in Increasing Risk of Colorectal Tumor Development in Human Familial Adenomatous Polyposis

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Background: Familial adenomatous polyposis (FAP) results from germline *adenomatous polyposis coli (APC)* gene mutations and many affected patients die from colorectal cancers which arise from colorectal polyps. We previously reported that two strains of *Apc* gene-deficient mice developing multiple intestinal polyps exhibit a hyperlipidemic state. The triglyceride (TG) levels were ~10-fold higher than the levels observed in wild-type mice.

Methods: To examine whether a positive relationship might exist between hyperlipidemia and colorectal tumor development in FAP patients, as with *Apc* gene-deficient mice, a pilot experiment was performed using readily available clinical data such as ages, serum lipid levels, number of colorectal polyps and cancer development in 28 FAP patients from the National Cancer Center Hospital, Japan.

Results: The overall prevalence of hyperlipidemia in FAP cases was 58%. Average TG levels in the 40–60 year age groups of FAP patients were ≥ 150 mg/dl (the defined threshold level of hyperlipidemia). Moreover, there was a tendency for higher serum TG levels in patients who developed colorectal cancer, as compared with those without colorectal cancer.

Conclusions: These results show that a hyperlipidemic state occurs in FAP patients. Although it is weaker than that in *Apc* gene-deficient mice, it may be linked to colon tumor development. These data warrant further studies for wider populations of FAP patients.

Key words: APC gene – colorectal cancer – familial adenomatous polyposis – hyperlipidemia

INTRODUCTION

Familial adenomatous polyposis (FAP) is characterized by the appearance of hundreds or thousands of adenomatous polyps in the colon and rectum. The polyps are caused by germline mutations of the *adenomatous polyposis coli (APC)* gene located on chromosome 5q21. Patients face increased mortality due to inevitable colorectal cancer developing from intestinal polyps. Thus, prophylactic colectomy is performed usually

before 25 years of age (1). In such individuals at extremely elevated risk of colorectal cancer, it is mandatory that any promoting factors be elucidated and appropriate preventive measures be devised.

There are several mouse models for FAP with different germline *Apc* mutation sites such as codons 716, 850, 1309 and 1638 (2–4). We previously reported that two strains of *Apc* gene-deficient mice, Min (*Apc* gene mutation at codon 850) and *Apc*¹³⁰⁹ (mutated at codon 1309) mice, show particularly large numbers of intestinal polyps and a hyperlipidemic state (5,6). In these mice, serum triglyceride (TG) levels increase with age, to ~500–800 mg/dl when 12–15 weeks old, associated with low mRNA expression levels of lipoprotein lipase (LPL) in the liver and small intestine. These TG levels are ~10-fold higher than the 70 mg/dl typically observed in wild-type mice. Serum total cholesterol (TC) levels are slightly elevated. Moreover, we have reported that a peroxisome proliferator-activated receptor (PPAR) α agonist, bezafibrate, and a PPAR γ agonist, pioglitazone,

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Abbreviations: BMI, body mass index; ChE, cholinesterase; COX, cyclooxygenase; FAP, familial adenomatous polyposis; LPL, lipoprotein lipase; NSAIDs, non-steroidal anti-inflammatory drugs; PPAR, peroxisome proliferator-activated receptor; TC, total cholesterol; TG, triglyceride

concomitantly suppress hyperlipidemia and intestinal polyp formation in the mice, with induction of LPL mRNA. Indeed, an LPL inducer, NO-1886, also suppresses hyperlipidemia and intestinal polyp formation in the mice (7).

In the present study, we performed a pilot study to examine whether hyperlipidemia might also be a complication in FAP patients, similar to FAP model mice, and possible associations with colon tumor development are discussed.

PATIENTS AND METHODS

FAP PATIENTS

Twenty-eight Japanese FAP patients, presenting at the National Cancer Center Hospital from 1999 to 2004 for follow-up of their health conditions, were reviewed. FAP was diagnosed by observing >100 intestinal polyps and all 28 patients underwent prophylactic colectomy. In addition, pathological studies were performed on these polyps. Germline mutations of the *APC* gene were investigated in 24 of the 28 cases, but the other 4 patients did not give written informed consent. The following data were collected: age; sex; body mass index (BMI = kg/m²); history of previous surgery; serum levels of albumin, total protein, cholinesterase (ChE), TC and TG; and presence or absence of colon and gastric tumors. Patients who did not give written informed consent for collecting samples and clinical information were excluded. The use of each individual's material was approved by the ethics review committee of the National Cancer Center.

DETECTION OF HYPERLIPIDEMIA IN FAP PATIENTS

The criteria for hyperlipidemia (hypertriglyceridemia and/or hypercholesterolemia) were based on the Japan Atherosclerosis Society Guideline (8): a fasting serum TG level ≥ 150 mg/dl and a fasting serum TC level ≥ 220 mg/dl. Decisions were made using data from more than two blood samples collected independently, with at least a month's interval. Values obtained within a month of receiving abdominal surgery were disregarded. The data for TG levels at different ages were those from 2002.

DETECTION OF RECTAL POLYPS IN FAP PATIENTS

Rectal polyps were examined by front-viewing endoscopy and recorded as digital photographs. The numbers were counted and the rectal polyps were classified into two types as follows: confluent (jammed together) and scattered (isolated from each other). The extent of rectal polyp development was further classified into five groups: no polyps/field of view (-), 1-5 polyps/field of view (\pm), 6-10 polyps/field of view (+), 11-20 polyps/field of view (++) and >20 polyps/field of view (+++).

IDENTIFICATION OF GERMLINE MUTATIONS IN THE *APC* GENE

Germline *APC* gene mutations were analysed in colon cancer samples or genomic DNA and cDNA samples prepared from the peripheral lymphocytes. Mutations were first screened by a protein truncation test (9) followed by confirmation by direct sequencing of the PCR-amplified genomic sequences.

RESULTS

THE OVERALL PREVALENCE OF HYPERLIPIDEMIA IN FAP PATIENTS

Data for history of colorectal cancer development and numbers of rectal polyps in the 28 FAP patients presenting at the National Cancer Center Hospital from 1999 to 2004 are summarized in Table 1. Serum lipid levels for the patients are given in Table 2. Twenty-seven FAP patients had undergone prophylactic colectomy and none received medical treatment and/or nutritional management for hyperlipidemia. Serum lipid data for Patients 7 and 8 were not informative enough and were excluded from the analysis.

Table 1 provides general data for ages and genders, the positions of *APC* germline mutation, existing rectal polyps in 2004 and past history of colorectal cancer. In Table 2, the data for minimum, maximum and average TG levels, and also the maximum levels of TC are shown. Patients in a hyperlipidemic state with serum TG levels ≥ 150 mg/dl and/or TC levels ≥ 220 mg/dl, determined at least two times independently, are underlined. In addition, frequencies of blood examination and number of occasions on which TG was ≥ 150 mg/dl are shown. The overall prevalence of hyperlipidemia in the patients was 57.7% (15/26): the prevalence of hypertriglyceridemia (150-429 mg/dl) was 73.3% (11/15) and the prevalence of hypercholesterolemia (220-296 mg/dl) was 53.3% (8/15). Differences in other clinical data such as BMI, serum albumin levels, serum total protein levels and serum ChE levels were not observed between patients with and without hyperlipidemia (data not shown).

CHANGE OF SERUM TG AND TC LEVELS DURING AGING

Regarding the changes in average serum TG levels during aging, age-dependent increases of TG levels were observed in patients in their thirties and maximum levels were observed in those in their sixties (Fig. 1A). Reported average serum TG levels in each 10 year cohort of the Japanese are highest in those in their forties [129 mg/dl, total number = 12,839, ref. (10)]. Meanwhile, the average serum TC levels in FAP patients were <220 mg/dl except for two FAP patients in their seventies (253 mg/dl, Fig. 1B). Reported average serum TC levels in each 10 year cohort of the Japanese are highest in those in their sixties [211 mg/dl, ref. (10)]. Sex difference is apparent in the serum lipid levels: males tend to have high TG levels (150 mg/dl for men in their forties) and females to have TC levels (218 mg/dl for women in their fifties and sixties). In the

Table 1. General data, DNA mutations and colorectal tumors in FAP patients

| Patient | Age* (age at colectomy) | Sex | Codon | Mutation | Colorectal polyps [#] | Colorectal cancer |
|---------|-------------------------|-----|-----------|-------------|--------------------------------|-------------------|
| 1 | 18 (16) | M | 836 | TCA→TGA | ± | - |
| 2 | 24 (22) | F | 693 | AAA→TAA | +++ | + |
| 3 | 32 (31) | F | 564 | CGA→TGA | ++ | - |
| 4 | 32 (26) | M | 1061-1063 | AAACA del | ++ | - |
| 5 | 33 (27) | F | | L | + | - |
| 6 | 36 (23) | F | N | N | ++ | - |
| 7 | 37 (n) | M | 1342 | TTA→TAA | + | - |
| 8 | 39 (39) | F | | D | c | - |
| 9 | 42 (30) | M | N | N | c | + |
| 10 | 46 (19) | M | N | N | c | + |
| 11 | 46 (35) | M | 723 | T del | +++ | + |
| 12 | 46 (31) | M | n | n | c | + |
| 13 | 46 (34) | M | n | n | +++ | + |
| 14 | 47 (43) | M | 558 | A del | ± | + |
| 15 | 47 (39) | M | 159 | A insertion | ++ | - |
| 16 | 49 (27) | M | N | N | c | - |
| 17 | 49 (47) | M | N | N | ± | - |
| 18 | 49 (30) | M | 1166-1167 | TATAA del | + | - |
| 19 | 52 (26) | F | 1309-1311 | GAAAA del | c | - |
| 20 | 54 (41) | F | n | n | c | + |
| 21 | 60 (31) | M | N | N | +++ | - |
| 22 | 60 (37) | M | 367 | TC del | c | + |
| 23 | 64 (58) | F | n | n | + | + |
| 24 | 65 (60) | M | 1594-1595 | CCAG del | + | + |
| 25 | 66 (36) | M | 283 | CGA→TGA | ± | + |
| 26 | 73 (71) | M | N | N | ± | - |
| 27 | 75 (43) | F | N | N | n | - |
| 28 | 88 (85) | M | N | N | c | + |

n, not informative; N, not detected; L, large deletion from exon 6 to 15; D, duplication exon 2 + exon 3.

*Age in 2004.

[#](-), no polyps/field; (±), 1-5 polyps/field; (+), 6-10 polyps/field; (++) , 11-20 polyps/field; (+++), >20 polyps/field; c, confluent type.

present study, the number of male patients was twice the number of female patients, but the average TG levels exceeded 150 mg/dl between the ages of 40 and 60 years.

OVERALL PREVALENCE OF COLORECTAL POLYPS AND CANCER IN FAP PATIENTS

Among FAP patients who had >20 polyps in an endoscopic field or had polyps of the confluent type, hyperlipidemia was observed in eight patients and normal serum lipid levels in five patients (Table 1). Fifteen patients had colorectal cancer, all diagnosed as adenocarcinomas, five had gastric cancers (Patients 8, 12, 13, 16 and 21) and two had both. The percentage of hyperlipidemic patients with colorectal cancer was

53.8% (7/13) and with hypertriglyceridemia was 46.2% (6/13). Interestingly, when counting levels of serum TG ≥ 150 mg/dl and/or serum TC ≥ 220 mg/dl occurring even once as hyperlipidemic, 93.3% of the patients who had colorectal cancer demonstrated hyperlipidemic states. There were four patients who had gastric cancer with hyperlipidemia.

Statistically significant differences were not observed in serum lipid levels between the patients with colorectal cancer and those without colorectal cancer. However, the average maximum serum TG and TC values in FAP cases with colorectal cancer tended to be higher than in those without colorectal cancer, 222.7 versus 158.9 and 232.6 versus 192.5 mg/dl, respectively (Table 2 and Fig. 2).

DISCUSSION

In the present pilot study, we found hyperlipidemia to be a relatively frequent complication in FAP patients, suggesting its possible link to colorectal cancer development. There is standard serum lipid level data for the Japanese ($n = 12\ 839$, aged 4 through 99 years) collected in 36 institutes from various districts around Japan in 2000 (10). The mean serum TG and TC levels in each 10 year group were <150 and <220 mg/dl, respectively (mean TG levels for Japanese in their thirties, forties, fifties and sixties were 118, 129, 129 and 123 mg/dl; TC levels for Japanese in their thirties, forties, fifties and sixties were 195, 201, 211 and 209 mg/dl, respectively). Although males tend to have higher TG levels than females, the population ratio of hyperlipidemia did not show any difference. Thus, our pilot study suggested the need for a larger number study to confirm high TG levels in female FAP patients. Extracolonic and serious complications in FAP include adenocarcinomas in the duodenum and pancreas, and desmoid tumors developing from operation scars. Reported benign lesions are osteomas, odontomas, epidermoid cysts, stomach and thyroid adenocarcinomas, congenital hypertrophy of the retinal pigment epithelium and fundic gland polyposis (11-14), but a hyperlipidemic state has hitherto not received attention as a potentially important aspect. Three points can be raised as explanations for the lack of any focus on blood lipids: (i) myocardial infarction and stroke are not major causes of death in FAP [1.9 and 1.5% in Japanese FAP patients, respectively, ref. (15)]; (ii) hyperlipidemia may not develop at an early age [the mean ages at death of FAP patients were 44.1 years for males and 40.5 years for females before 1990, ref. (15)]; and (iii) no correlation between the APC gene and hyperlipidemia has hitherto been reported. Since we found only a tendency for serum TG levels to be associated with colorectal tumor development, we are now planning to investigate a large number of FAP patients for confirmation.

Prophylactic colectomy may weaken gastrointestinal function with a disorder of liver bile circulation including the lipid absorbing function of the small intestine, and if hyperlipidemia is caused by APC germline mutations, it is assumed that much more severe hyperlipidemia may be observed in FAP patients before prophylactic colectomy. The position of the APC

Table 2. Serum lipids levels in FAP patients

| Patient | Minimum triglyceride level (mg/dl) | Maximum triglyceride level (mg/dl) | Average triglyceride level \pm SD | No. of detected TG \geq 150 mg/dl/blood examinations | Maximum cholesterol level [†] (mg/dl) |
|-----------|------------------------------------|------------------------------------|-------------------------------------|--------------------------------------------------------|------------------------------------------------|
| 1 | 51 | 160 | 93.7 \pm 47.5 | 1/3 | 130 |
| 2 | 31 | 95 | 56.3 \pm 22.8 | 0/6 | 236 |
| 3 | 43 | 129 | 82.3 \pm 35.5 | 0/3 | 200 |
| <u>4</u> | 31 | 95 | 68.2 \pm 22.8 | 0/6 | <u>271</u> |
| 5 | 43 | 126 | 67.2 \pm 27.8 | 0/6 | 183 |
| 6 | 29 | 44 | 36.7 \pm 6.0 | 0/7 | 153 |
| 7 | | | | | |
| 8 | n | n | n | | 181 |
| 9 | 40 | 148 | 67.9 \pm 26.1 | 0/12 | 241 |
| 10 | 35 | 125 | 66.9 \pm 21.6 | 0/34 | 220 |
| <u>11</u> | 68 | 278 | 108.2 \pm 59.8 | 1/10 | <u>298</u> |
| <u>12</u> | 140 | <u>372</u> | 239.0 \pm 71.4 | 9/10 | <u>261</u> |
| <u>13</u> | 124 | <u>425</u> | 236.4 \pm 80.3 | 12/13 | <u>250</u> |
| 14 | 79 | 181 | 120.3 \pm 43.8 | 1/3 | 228 |
| <u>15</u> | 120 | <u>223</u> | 185.3 \pm 46.4 | 2/3 | 214 |
| 16 | 86 | 173 | 114.2 \pm 29.7 | 2/10 | 182 |
| 17 | 133 | 214 | 173.5 \pm 40.5 | 1/2 | 215 |
| 18 | 41 | 55 | 49.0 \pm 5.9 | 0/3 | 185 |
| <u>19</u> | 153 | <u>203</u> | 179.0 \pm 16.3 | 7/7 | 202 |
| <u>20</u> | 162 | <u>275</u> | 208.1 \pm 35.0 | 7/7 | <u>277</u> |
| <u>21</u> | 167 | <u>429</u> | 274.9 \pm 80.1 | 9/9 | 181 |
| 22 | 168 | 168 | 168 | 0/1 | 150 |
| <u>23</u> | 57 | <u>290</u> | 143.9 \pm 78.0 | 5/14 | <u>237</u> |
| <u>24</u> | 67 | <u>250</u> | 111.8 \pm 54.4 | 2/11 | 212 |
| <u>25</u> | 66 | <u>186</u> | 142.5 \pm 48.3 | 2/4 | 169 |
| <u>26</u> | 72 | <u>215</u> | 138.5 \pm 37.3 | 6/16 | 206 |
| <u>27</u> | 107 | 118 | 112.5 \pm 5.5 | 0/2 | <u>265</u> |
| <u>28</u> | 84 | 102 | 93.6 \pm 6.2 | 0/6 | <u>247</u> |

n, not informative.

[†]Abnormally high serum levels detected more than two times are underlined.

germline mutation may affect the severity of FAP (16). The weak dominant negative effects on the wild-type APC protein by formation of unstable heterodimers result in small numbers of polyps (17). Since codons 1014–1210 and 1263–2013 are suggested to be the binding sites of β -catenin, their mutation could play an important role in formation of intestinal polyps and hyperlipidemic states (16). An FAP patient with mutations at codon 1309 (Patient 19) showed a similar hyperlipidemic state to *Apc*¹³⁰⁹ mice. Clearly, serum lipid levels may be more readily affected by environmental factors or aging than the mutated position of the APC gene, and other genetic factors such as *LPL* and *angiopoietin-like protein 3* may also influence the extent of hyperlipidemia (18). Therefore, our pilot study suggests the need for further studies comparing the patient

before and after colectomy and also for studies to elucidate possible mechanisms linking functional genetic alteration, hyperlipidemia and colorectal cancer development.

It is of interest to point out that an agent reducing polyp formation without affecting serum lipid levels in *Apc*-deficient mice may clarify the relationship between polyps and hyperlipidemia. We are in favor of the hypothesis that hyperlipidemia is not causative at least for the onset of adenoma, but may promote intestinal polyp development. If so, an antihyperlipidemic agent is justified to be a candidate for chemoprevention. Early prophylactic colectomy is considered to be the most effective way to prevent colorectal cancer development in FAP patients, although chemopreventive agents such as selective cyclooxygenase-1 (COX-1)

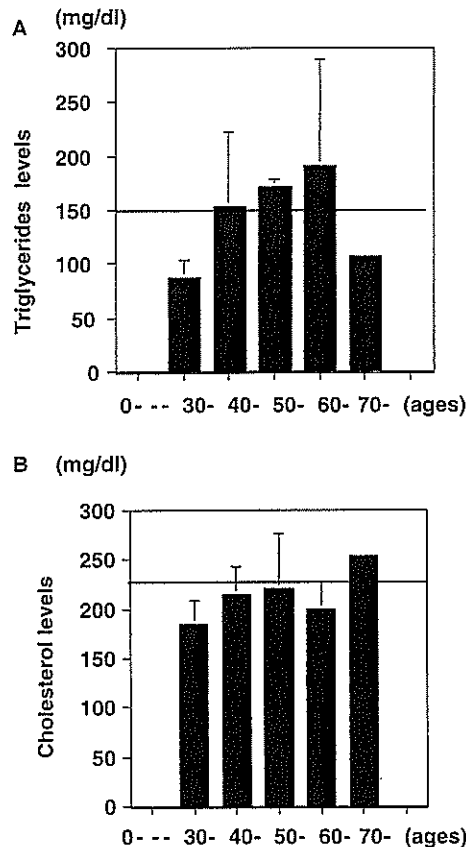


Figure 1. Serum TG (A) and TC (B) levels in FAP patients in 2002. Overall averages are arranged chronologically by age. Dotted lines are at the concentrations of 150 mg/dl for TG and 220 mg/dl for TC.

and -2 inhibitors, prostaglandin receptor EP₁ and EP₄ selective antagonists, PPAR α agonist and PPAR γ agonist can reduce intestinal polyps in *Apc*-deficient mice (5,6,19–22). Several clinical studies have already been performed with non-steroidal anti-inflammatory drugs (NSAIDs) for prophylactic purposes in FAP patients (23,24). From our present results, not only NSAIDs and/or COX-2 inhibitors but also PPAR α/γ agonists might warrant further attention and clinical trials.

In conclusion, our data lead us to hypothesize that both hyperlipidemia and polyp formation may be caused by *APC* mutation, where a hyperlipidemic state may contribute to the development of polyps. This encourages us to investigate a large number of FAP patients with matched controls. Hyperlipidemia may be observed even after prophylactic colectomy, and improving a hyperlipidemic state might be of benefit for protection against neoplasia or other adverse outcomes.

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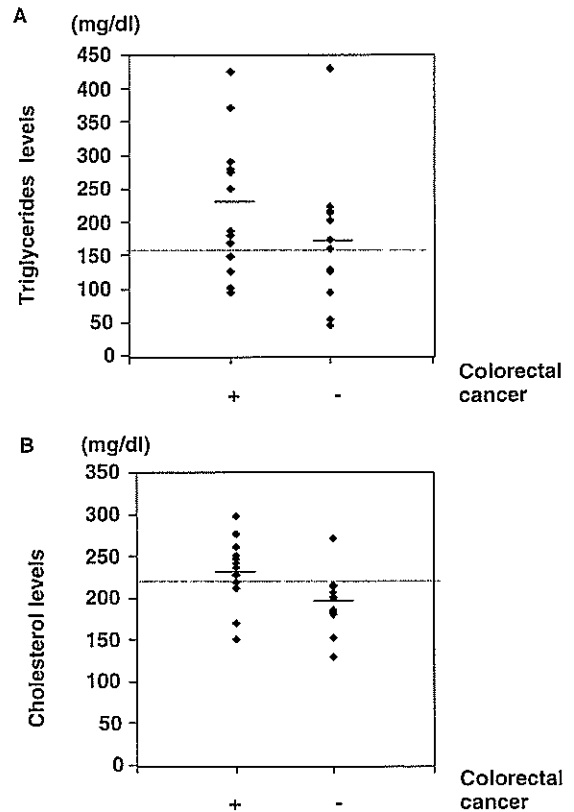


Figure 2. Serum lipid levels in FAP patients with or without a past history of colorectal cancer. The maximum serum TG (A) and TC (B) levels detected in each FAP patient from 1999 to 2004 are shown with or without past history of colorectal cancer presented as + and -, respectively. Dotted lines are at the concentrations of 150 mg/dl for TG and 220 mg/dl for TC.

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Minireview

Concomitant suppression of hyperlipidemia and intestinal polyp formation by increasing lipoprotein lipase activity in *Apc*-deficient mice

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Abstract

Epidemiologically, a high-fat diet is associated with the risk of colon cancer. In addition, serum levels of triglycerides (TGs) and cholesterol have been demonstrated to be positively associated with colon carcinogenesis. We recently found that an age-dependent hyperlipidemic state (high serum TG levels) exists in *Apc*-deficient mice, an animal model for human familial adenomatous polyposis. The mRNA levels of lipoprotein lipase (LPL), which catalyzes TG hydrolysis, were shown to be downregulated in the liver and intestines of mice. Moreover, treatment with a peroxisome proliferator-activated receptor (PPAR) α agonist, bezafibrate, or a PPAR γ agonist, pioglitazone, suppressed both hyperlipidemia and intestinal polyp formation in the mice, with induction of LPL mRNA. PPAR α and PPAR γ agonists are reported to exert anti-proliferative and pro-apoptotic effects in cancer cells. One compound that also increases LPL expression levels but does not possess PPAR agonistic activity is NO-1886. When given at 400 or 800 ppm in the diet, it suppresses both hyperlipidemia and intestinal polyp formation in *Apc*-deficient mice, with elevation of LPL mRNA. In conclusion, a decrease in serum lipid levels by increasing LPL activity may contribute to a reduction in intestinal polyp formation with *Apc* deficiency. PPAR α and PPAR γ agonists, as well as NO-1886, could be useful as chemopreventive agents for colon cancer.

Keywords: *Apc*-deficient mice; hyperlipidemia; intestinal polyp; lipoprotein lipase.

Introduction

Colorectal cancer is one of the most common cancers in developed countries, and several epidemiological studies have suggested a correlation with obesity, a high-fat diet and hyperlipidemia (Le Marchand et al., 1997; Bruce et al., 2000), with clear links to high levels of serum triglycerides (TGs) and cholesterol (McKeown-Eyssen, 1994; Jarvinen et al., 2001). Thus, it is conceivable that not only

a reduction in cholesterol levels by HMG-CoA reductase inhibitors (Agarwal et al., 1999) but also a decrease in TG levels by inhibitors may reduce colon carcinogenesis.

TGs are enriched in lipoproteins such as chylomicrons and very low-density lipoprotein (VLDL), and are hydrolyzed by lipoprotein lipase (LPL) to free fatty acids (FFA) and monoacylglycerol (Schoonjans et al., 1996a). LPL mRNA is expressed ubiquitously in the whole body, i.e., in adipose tissue, skeletal muscle, liver and intestine. Once synthesized, LPL is transferred to the surface of endothelial cells to become bound to membrane-anchored heparan sulfate proteoglycans (Semenkovich et al., 1989; Goldberg, 1996). Physiologically, a decrease in or deficiency of LPL is associated with hyperlipidemia (Gehrisch, 1999; Mead et al., 2002). However, no direct evidence of links between LPL and colorectal carcinogenesis has been reported.

Age-dependent increase in TGs in *Apc* gene-deficient mice with low LPL mRNA levels

The *Apc*¹³⁰⁹ (C57BL/6J*Apc*¹³⁰⁹) mouse, an animal model of human familial adenomatous polyposis (FAP), develops large numbers of intestinal polyps because of a truncation mutation in the *adenomatous polyposis coli* (*Apc*) gene (*Apc*¹³⁰⁹; Quesada et al., 1998). It is considered to have advantages for evaluation of chemopreventive agents, as with other FAP model mice, such as *Apc*^{Min} (Min), *Apc*^{J716} and *Apc*¹⁶³⁸ mice (Moser et al., 1990; Fodde et al., 1994; Oshima et al., 1995). During experiments to evaluate chemopreventive agents, we found that the serum of the *Apc*¹³⁰⁹ mouse is very pale in color and compared lipid levels with similarly aged wild-type mice. Although no significant differences were observed at 6 weeks of age, TG levels were obviously increased in *Apc*¹³⁰⁹ mice with aging; the average value at 12 weeks was almost 10-fold higher than that at 6 weeks (Niho et al., 2003a). Such an increase was not observed in the wild-type counterparts. Total cholesterol levels in *Apc*¹³⁰⁹ mice were also increased between 6 and 12 weeks of age, from 87.0 \pm 3.2 to 162.4 \pm 33.0 mg/dl. Moreover, FFA levels were increased with age. Differences in serum lipid levels were not observed between male and female *Apc*¹³⁰⁹ mice aged 12 weeks. An age-dependent hyperlipidemic state was also observed in Min mice (Niho et al., 2003a). TG levels in female Min mice at 15 weeks of age were elevated to levels almost 10-fold higher than those at 8 weeks of age. Values for total cholesterol at 8 and 15 weeks of age were 83.7 \pm 6.3 and 107.8 \pm 15.6 mg/dl, and those for FFA were 1.0 \pm 0.1 and 3.1 \pm 0.4 mEQ/l, respectively.

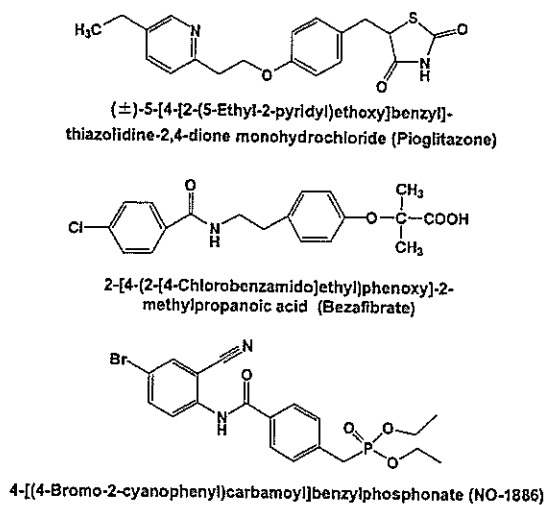


Figure 1 Structures of LPL inducers.

To cast light on mechanisms underlying the increased levels of serum lipids, especially TGs, in *Apc*-deficient mice with age, we investigated expression levels of mRNAs encoding metabolic enzymes (Niho et al., 2003a). In the liver and small intestine of *Apc*¹³⁰⁹ and wild-type mice at 6, 8 and 12 weeks of age, there were no obvious differences in mRNA levels for fatty acid synthase (FAS), stearoyl-CoA desaturase-1, acyl-CoA oxidase, carnitine palmitoyl transferase-1 and phosphoenolpyruvate carboxykinase, enzymes involved in the hydrolysis of TGs, lipogenesis, β -oxidation and glucose homeostasis. Similar expression levels for these genes were also observed in Min and wild-type mice at all ages. However, LPL mRNA levels in the liver were clearly lowered in *Apc*¹³⁰⁹ mice at 6, 8 and 12 weeks, and in Min mice at 8, 11 and 15 weeks, proportional to aging. A similar decrease was also observed for small intestinal mRNA levels. These data provide evidence that the expression levels of LPL, which catalyzes TG hydrolysis, correlate with hypertriglyceridemia in *Apc*¹³⁰⁹ and Min mice.

Reduction in serum TG levels in *Apc*-deficient mice by administration of the PPAR γ ligand, pioglitazone, or the PPAR α ligand, bezafibrate

Pioglitazone, ((±)-5-[4-[2-(5-ethyl-2-pyridyl)ethoxy]benzyl]thiazolidine-2,4-dione monohydrochloride), is a potent PPAR α ligand that also demonstrates weak binding to PPAR γ ; bezafibrate, {2-[4-(2-[4-chlorobenzamido]ethyl)phenoxy]-2-methylpropanoic acid}, is a specific PPAR α ligand (Sakamoto et al., 2000) (Figure 1). PPARs are predominantly expressed in the liver, heart, kidney, intestinal mucosa and brown adipose tissue, all with high catabolic rates for fatty acids and peroxisomal metabolism (Schoonjans et al., 1996b). Thus, these ligands are used clinically to improve hypertriglyceridemia and hypercholesterolemia through induction of lipid metabolism-related genes such as *LPL* (Schoonjans et al., 1996a). Transcriptional regulation of the *LPL* gene has been reported to be mediated through binding of PPAR-

retinoid X receptor heterodimers to the functional responsible element sequence in the *LPL* gene promoter lesion (Schoonjans et al., 1996a).

Administration of each ligand for 6 weeks did not affect food intake or behavior of male *Apc*¹³⁰⁹ mice. However, final body weights in the groups treated with 100 and 200 ppm pioglitazone were increased to 113–115% of those in the AIN-76A basal diet group, and those in the bezafibrate-treated groups increased to 118–122% (Niho et al., 2003a). Serum lipid levels are summarized in Table 1. Serum TG levels at 12 weeks of age were reduced by 44% and 50% by 100 and 200 ppm pioglitazone, respectively. The respective levels of total cholesterol were also decreased by 15% and 28%. Administration of pioglitazone caused a 27% decrease in FFA levels at both concentrations, but significance was not attained. Similar results were obtained in male Min mice (Niho et al., 2003b). Serum TG levels in the basal diet group were elevated 13–15-fold relative to those in wild-type counterparts at 20 weeks of age. They were reduced dose-dependently by treatment with 100, 200, 400 and 1600 ppm pioglitazone from 6 to 20 weeks of age, with suppression to almost the wild-type level at 1600 ppm (Niho et al., 2003b). Although the values for total cholesterol in Min mice were not changed by pioglitazone treatment, the balance of HDL-C, LDL-C, and VLDL-C in the total cholesterol of Min mice was improved to almost the wild-type level. The levels of FFA in Min mice were decreased by pioglitazone to 44% of the untreated control value.

Administration of bezafibrate to *Apc*¹³⁰⁹ mice reduced serum TG levels dose-dependently by 30% and 55% ($p < 0.05$) at 100 and 200 ppm, respectively (Niho et al., 2003a). The levels of total cholesterol and FFA showed a tendency to decrease by 6–18%.

Suppression of intestinal polyp formation in *Apc*-deficient mice by pioglitazone or bezafibrate

In *Apc*-deficient mice, almost all polyps developed in the small intestine, with only a few in the colon (Niho et al., 2003a,b, 2005). The total numbers of polyps in the groups treated with pioglitazone at 100 and 200 ppm in *Apc*¹³⁰⁹ mice were reduced to 67% ($p < 0.05$) of the value in the untreated control group (Table 1). The numbers of polyps in the proximal and middle parts of the small intestine in *Apc*¹³⁰⁹ mice treated with 200 ppm pioglitazone were 58% ($p < 0.05$) and 61% ($p < 0.01$) of the untreated control values, respectively. Dietary administration of 100 and 200 ppm bezafibrate reduced the total numbers of polyps by 13% and 25% ($p < 0.05$), respectively (Table 1). The numbers of polyps in the proximal, middle, and distal parts of the small intestine in *Apc*¹³⁰⁹ mice treated with 100 and 200 ppm bezafibrate were also reduced by 4–27%, albeit without statistical significance.

The size distribution of intestinal polyps in groups on a basal diet and treated with pioglitazone or bezafibrate was also investigated (Niho et al., 2003a,b). Treatment of *Apc*¹³⁰⁹ mice with 100 and 200 ppm pioglitazone reduced the numbers of polyps measuring more than 1.0 and more than 0.5 mm in diameter, respectively. On the other

Table 1 Serum lipid levels and total number of polyps in *Apc*-deficient mice.

| Mice (age) | Agent | Dose (ppm) | Triglycerides (mg/dl) | Cholesterol (mg/dl) | Total number of polyps/mouse |
|---------------------------------------|--------------|------------|-----------------------|---------------------|------------------------------|
| <i>Apc</i> ¹³⁰⁹ (12 weeks) | Pioglitazone | 0 | 710±131 | 133±15 | 36.7±2.7 |
| | | 100 | 396±116 | 113±15 | 24.6±4.4 |
| | | 200 | 355±101 | 96±15 | 24.5±4.2 |
| | Bezafibrate | 0 | 682±119 | 141±17 | 37.7±2.9 |
| | | 100 | 480±111 | 132±10 | 32.7±1.7 |
| | | 200 | 309±70 | 119±14 | 28.3±2.7 |
| Min (20 weeks) | Pioglitazone | 0 | 512±127 | 113±11 | 71.9±6.7 |
| | | 100 | 324±117 | 113±12 | 45.5±10.2 |
| | | 200 | 171±69 | 104±8 | 32.7±11.3 |
| | | 400 | 86±39 | 104±14 | 33.5±19.0 |
| | | 1600 | 21±3 | 121±9 | 6.2±5.1 |
| | NO-1886 | 0 | 607±120 | 147±13 | 121.7±26.0 |
| | | 400 | 238±49 | 101±9 | 58.0±10.8 |
| | | 800 | 186±81 | 113±10 | 50.5±7.8 |

Data are mean±SEM.

hand, 100 and 200 ppm bezafibrate reduced the numbers of polyps, especially those of 0.5–1.5 mm in diameter. Min mice given 100–1600 ppm pioglitazone for 14 weeks demonstrated decreased numbers of intestinal polyps to 9–63% of the control value (Table 1). The numbers of polyps in the proximal, middle, and distal parts in Min mice treated with 200 ppm pioglitazone were reduced by 50–60%, with particularly marked effects on polyps measuring less than 1.0 and 3.0–4.0 mm in diameter.

Administration of 100 and 200 ppm pioglitazone or bezafibrate raised hepatic LPL mRNA levels in *Apc*¹³⁰⁹ mice. Similar upregulation was also evident for small intestinal mRNA levels, although the degree of elevation was small.

Concomitant suppression of serum lipid levels and intestinal polyp formation in Min mice by a LPL selective inducer, NO-1886

It is well known that PPAR γ and PPAR α agonists induce cell growth arrest and apoptosis in various types of cancer cells, including colon cancer cells (Rosen and Spiegelman, 2001). Thus, the decreases in polyp numbers in *Apc*¹³⁰⁹ and Min mice by pioglitazone or bezafibrate in our study might have resulted from such actions. LPL selective inducers are necessary for determining the relationship between hyperlipidemia and intestinal carcinogenesis, and NO-1886, 4-[[4-bromo-2-cyanophenyl]carbamoyl]benzylphosphonate, chemically synthesized at Otsuka Pharmaceutical Factory (Tsutsumi et al., 1993) (Figure 1), is thus a useful tool. Using a reporter gene assay, NO-1886 was revealed not to possess PPAR γ and PPAR α agonistic activity, unlike bezafibrate and pioglitazone (Doi et al., 2003). In the next study, we therefore examined the effects of 400 and 800 ppm NO-1886 in the diet on both hyperlipidemia and intestinal polyp formation in female Min mice (Niho et al., 2005).

Administration for 13 weeks did not affect body weights or clinical signs of Min mice throughout the experimental period and amounts of daily food intake did not differ among the groups. In addition, there were no changes observed in any organ weights that could be

attributable to toxicity. Administration of 400 and 800 ppm NO-1886 clearly decreased serum TG levels to 39% and 31% of the untreated control value, respectively. The levels of total cholesterol were also decreased by 31% and 23%. Moreover, levels of both TG-rich lipoproteins, VLDL-C and LDL-C, were dramatically decreased by NO-1886 treatment to 15% and 32% of the untreated control values, respectively. In contrast, HDL-C levels were increased to the wild-type value at 800 ppm. Overall, administration of NO-1886 improved the balance of HDL-C, LDL-C, and VLDL-C in the total cholesterol of Min mice. LPL mRNA levels in the liver and the small intestine were markedly increased by treatment with NO-1886.

The data for numbers of intestinal polyps in the AIN-76A basal diet and NO-1886-treated groups are also shown in Table 1 (Niho et al., 2005). The total number of polyps were significantly decreased by administration of 400 and 800 ppm NO-1886 to 48% and 42% of the untreated control value, respectively, with reduction in the proximal, middle, and distal parts by 63%, 57% and 45% with 400 ppm, and by 74%, 63% and 49% with 800 ppm. Treatment with NO-1886 also significantly decreased the numbers of colon polyps. Administration of NO-1886 reduced the numbers of polyps of all sizes (0.5–3.0 mm in diameter) observed in the basal diet groups.

Down-regulation of COX-2 expression levels by NO-1886

To elucidate the mechanisms of the NO-1886 effects on colon carcinogenesis, we also investigated expression levels of mRNAs for inflammation-associated enzymes, cyclooxygenase-1 (COX-1), COX-2 and inducible nitric oxide synthase (iNOS), in DLD-1 human colon cancer cells (Niho et al., 2005). RT-PCR analysis revealed that the TGF α -stimulated COX-2 mRNA levels were reduced to non-stimulated levels by NO-1886 at 5 and 10 μ M. On the other hand, there was no obvious change in mRNA levels for COX-1 and iNOS. The results were also confirmed by a β -gal reporter gene assay in DLD-1 cells. COX-2 promoter transcriptional activity was normalized

to total protein as measured by colorimetric assay. Treatment of cells with 100 ng/ml TGF α for 48 h increased COX-2 promoter transcriptional activity to 1.6-fold of the control value, whereas NO-1886 at 5 and 10 μ M suppressed TGF α -stimulated COX-2 promoter transcriptional activity to only 1.2-fold of the control value, with no significant cytotoxicity. Consistent with the *in vitro* data, administration of NO-1886 at 400 and 800 ppm reduced mRNA levels of COX-2 in normal parts of the small intestine of Min mice at 20 weeks of age.

It is well known that expression of COX-2 is markedly elevated in colon cancers of humans and AOM-treated rats and in intestinal polyps of *Apc*-deficient mice (Sano et al., 1995; DuBois et al., 1996; Williams et al., 1996), playing an important role in cancer cell proliferation and angiogenesis (Tsujii et al., 1998). Therefore, suppression of COX-2 by NO-1886 is one possible mechanism underlying the suppression of intestinal polyp development.

Conclusions

Our studies have demonstrated that a hyperlipidemic state exists in two strains of FAP model mice. The levels of serum lipids, especially TGs, are thus dramatically increased with age in both *Apc*¹³⁰⁹ and Min mice. Possible involvement of hyperlipidemia in human FAP and in sporadic colorectal tumor patients is now under investigation.

It is interesting that LPL mRNA levels in the livers and small intestines of *Apc*-deficient mice were markedly lower than those of wild-type mice. At present, the biological relationship between *Apc* deficiency and severe hyperlipidemia is uncertain, but it has been reported that Wnt signaling inhibits the transcription factors CCAAT/enhancer binding protein and PPAR, and maintains preadipocytes in an undifferentiated state (Ross et al., 2000), suggesting the involvement of Wnt signaling in lipogenesis.

Although it still cannot be stated with certainty whether hyperlipidemia is a leading cause of intestinal polyp formation, our study demonstrated that LPL inducers, such as the PPAR ligands pioglitazone and bezafibrate, and NO-1886 have the potential to suppress both hyperlipidemia and polyp formation in *Apc*-deficient mice. It is therefore speculated that LPL activity itself may play an important causative role in tumor induction.

A large number of chemopreventive agents have been examined in light of their effects on colon carcinogenesis in *Apc*-deficient mice models, including enzyme inhibitors (ornithine decarboxylase and iNOS), non-steroidal anti-inflammatory drugs, micronutrients (selenium, vitamins, etc.) and PPAR activators (Jackson et al., 2003). LPL inducers have an advantage as novel targeting chemopreventive reagents and could be useful as new strategies. In addition, PPAR agonists in chemoprevention show a limitation in comparison with the above-mentioned agents because of their controversial effect on colon carcinogenesis. Previous animal studies have suggested that activation of PPAR α reduces intestinal polyp formation (Lefebvre et al., 1998), whereas PPAR β and PPAR γ activation by specific agonists results in either

increased (Tanaka et al., 2001) or reduced formation of neoplastic lesions (Corpet and Pierre, 2003). We demonstrated that both PPAR α and PPAR γ agonists suppressed intestinal polyp formation. The number of polyps was reduced to a greater extent by pioglitazone than by bezafibrate. Moreover, pioglitazone reduced the number of polyps of all sizes, whereas bezafibrate reduced only small polyp numbers. It can be speculated that the differential effects of PPAR subtype specific agonists may be associated with their effects on lipid metabolism and may also be affected by their chemical structure. For instance, troglitazone, which has a quinone structure, causes lethal liver toxicity and may increase polyp formation (Tetty et al., 2001).

Clearly, we need to elucidate the mechanisms underlying the hypertriglyceridemia in FAP model mice and the roles of LPL in intestinal polyp development, in which COX-2 suppression may be partly involved. For the present, however, we can conclude that PPAR ligands and NO-1886 could be useful as chemopreventive agents for colon cancer.

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Roles of Prostanoids in Colon Carcinogenesis and their Potential Targeting for Cancer Chemoprevention

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Abstract: Prostanoids are produced in response to numerous growth factors and environmental stimuli. Their synthesis is dependent on two cyclooxygenase (COX) enzymes, COX-1 and COX-2, which are rate-limiting for the production of prostaglandins (PGs) and thromboxanes from free arachidonic acid. Selective inhibitors of both COX forms have the potential to inhibit colon tumorigenesis, and there is abundant documented evidence of elevated expression of COX-2 in colon tumors and a variety of other malignancies. The resultant high level PGE₂ production may play an important role in cell proliferation, modulation of apoptosis, angiogenesis, inflammation and immune surveillance. Prostanoids exert their biological actions through binding to eight specific membrane receptors; the four subtypes EP₁ to EP₄ for PGE₂; DP for PGD₂; FP for PGF₂; IP for PGI₂; and TP for thromboxane A₂. Recently, genetic and pharmacologic experiments have suggested that all PGE₂ receptors can contribute to colon tumorigenesis. Moreover, it is suggested that EP₁ and EP₄ play roles in polyp formation independently. It is important to determine details of the down-stream signaling pathways of prostanoid receptors for further understanding of the mechanisms of cancer development. Furthermore, it is anticipated that development of specific receptor antagonists will provide new advantageous tools for chemoprevention.

Key Words: Chemoprevention, colon carcinogenesis, EP receptors, Prostanoids.

EPIDEMIOLOGICAL FINDINGS AND CLINICAL EVIDENCE OF COLON CANCER CHEMOPREVENTION

A) Epidemiological Findings

Several epidemiological studies have indicated that non-steroidal anti-inflammatory drugs (NSAIDs) reduce the risk of colon cancer. For example, it has been reported that individuals taking aspirin demonstrate at most 40% reduction in the relative risk of colorectal cancer and associated mortality [1]. Although the molecular mechanisms by which NSAIDs reduce colorectal cancer and other neoplasms remain to be determined in detail, the most likely possibility is due to their inhibition of cyclooxygenase (COX).

COX involvement in prostanoid synthesis is schematically illustrated in Fig. (1). PGs are synthesized by human tissues ubiquitously and are involved in diverse biological processes such as blood clotting, maintaining blood vessel tone, bone metabolism, immune responses, implantation, ovulation, initiation of labor, kidney function, nerve growth, inflammation and wound healing [2]. The release of arachidonic acid (AA), a 20-carbon polyunsaturated fatty acid, from cell membrane phospholipids is mainly mediated *via* the action of phospholipase A₂ (PLA₂) [3]. COX catalyzes the conversion of AA to PGG₂ and PGH₂. Addition of molecular oxygen produces the unstable product, PGG₂,

which is rapidly converted to PGH₂ by the peroxidase activity of the enzyme. It is well established that there are two isoforms of COX, the constitutive enzyme COX-1, present in many cells and tissues, and the inducible enzyme, COX-2, produced in response to growth factors, mitogens, pro-inflammatory cytokines and mucins [4, 5]. PGH₂ is additionally isomerized to PGE₂ by PGE₂ synthase [6] and converted to a variety of other PGs such as PGD₂, PGF₂, PGI₂, and thromboxane A₂ (TXA₂) by their respective PG synthases. Nonenzymatic dehydration of PGD₂ results in generation of PGJ₂, 12-PGJ₂, and 15-deoxy-Δ^{12,14}-PGJ₂ (15-Δ-PGJ₂) [7].

B) Clinical Evidence

Enhanced COX-2 expression and increased PLA₂ activity have been observed in human colon cancer tissues and pre-malignant polyps compared with the non-lesional and/or normal colon tissues, localized in epithelial cancer cells, inflammatory cells, vascular endothelium, and fibroblasts [8-11]. Furthermore, the major prostanoid found in colorectal cancers appears to be PGE₂ [12]. A COX-2 selective inhibitor, celecoxib, and conventional NSAIDs which inhibit both COX-1 and COX2, indomethacin and sulindac, (Fig. (2)) have actually caused regression of existing colorectal polyps in patients with familial adenomatous polyposis (FAP) [13-15]. FAP patients, affected by a rare hereditary condition resulting from germline inactivation of one allele of the *adenomatous polyposis coli* (APC) gene, develop tens to thousands of adenomatous polyps. Recently, it was found that sporadic colorectal cancers also acquire somatic mutations in the APC gene with defects in APC-dependent signaling [16].

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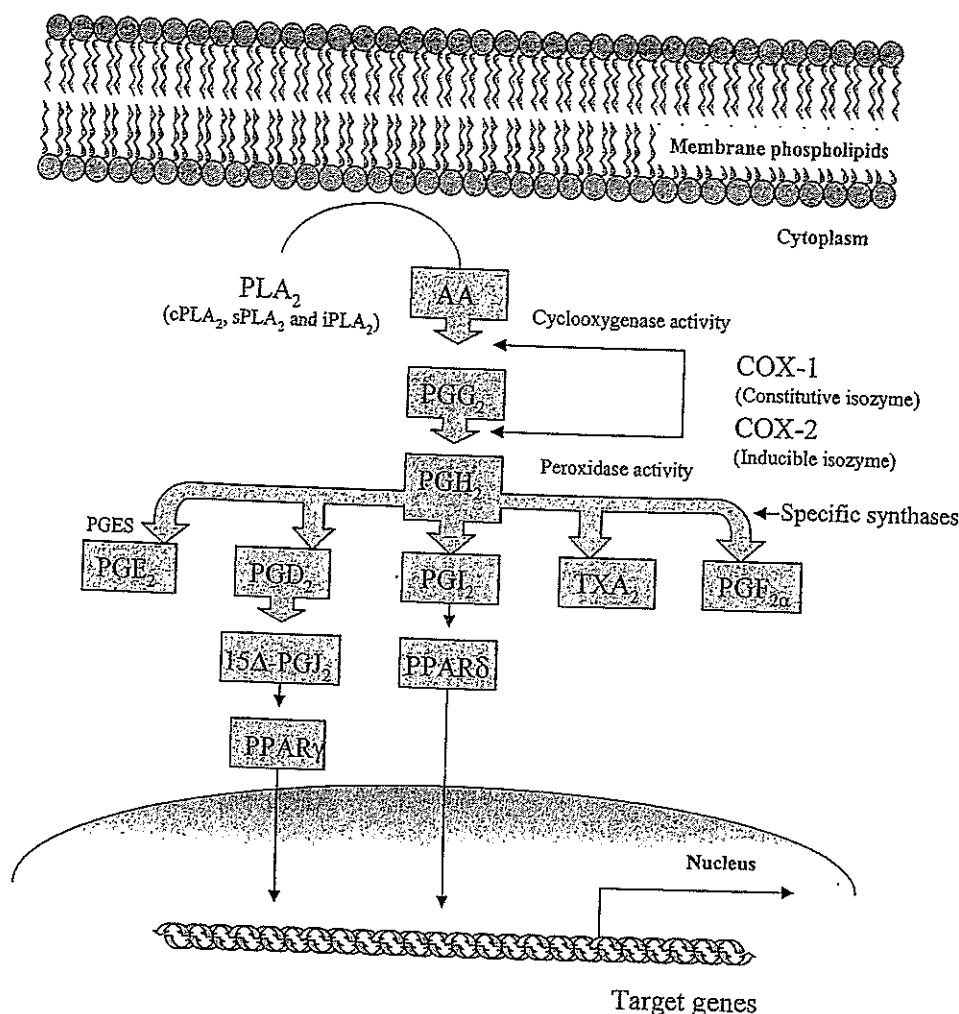


Fig. (1). Schematic illustration of the pathways involved in prostanoid synthesis. AA = Arachidonic acid; COX = Cyclooxygenase; PG = Prostaglandin; PGES = PGE₂ synthase; PLA₂ = Phospholipase A₂; PPAR = peroxisome proliferator-activated receptor; TXA₂ = Thromboxane A₂.

CYCLOOXYGENASE INVOLVEMENT IN COLON CARCINOGENESIS

Conventional NSAIDs, including indomethacin, sulindac and aspirin have been shown to inhibit the development of colon cancers in animal models [17-19]. Treatment with COX-2 selective inhibitors alone or combined with COX-2 gene knockout results in reduction of polyp development in *Apc^{Δ716}* mice [20, 21] and there is abundant evidence from animal models that COX-2 plays an important role in colon carcinogenesis. In addition, both pharmacologic and genetic studies have indicated that COX-1 also makes a key contribution to intestinal tumorigenesis. Dietary administration of 1200 ppm mofezolac, a COX-1 selective inhibitor (Fig. (2)), was thus found to reduce the number of aberrant crypt foci (ACFs), putative preneoplastic lesions, per rat, and the 5-bromodeoxyuridine (BrdU) labeling index of the crypt epithelium. Treatment with the same dose of mofezolac reduced the number of intestinal polyps in APC1309 mice to 59% of that in the control diet group [22]. Homologous genetic disruption of either COX-1 or COX-2 furthermore markedly

reduced polyp formation in Min mice with a nonsense mutation in the *APC* gene [23]. Combined treatment with 600 ppm mofezolac and 400 ppm nimesulide, a COX-2 selective inhibitor (Fig. (2)), in the APC1309 female mice resulted in inhibition of polyp development that was almost equal to the sum of the effects of each agent alone [24]. The results indicate that COX-1 and -2 may contribute to polyp formation independently and support the idea that prostanoid production by either of the COX isoforms plays an important role in colon carcinogenesis. Structures of some COX-1 and COX-2 selective inhibitors are shown in Fig. (2).

ROLE OF PROSTAGLANDIN RECEPTORS

A) Effect of PGE₂ Receptor Deficiency and Treatment with Antagonists

We have conducted a series of experiments to determine effects of prostanoid receptors on colon carcinogenesis with a genetic approach. Examination of the induction of ACFs by AOM in knockout mice deficient in EP₁, EP₂, EP₃, EP₄, DP,

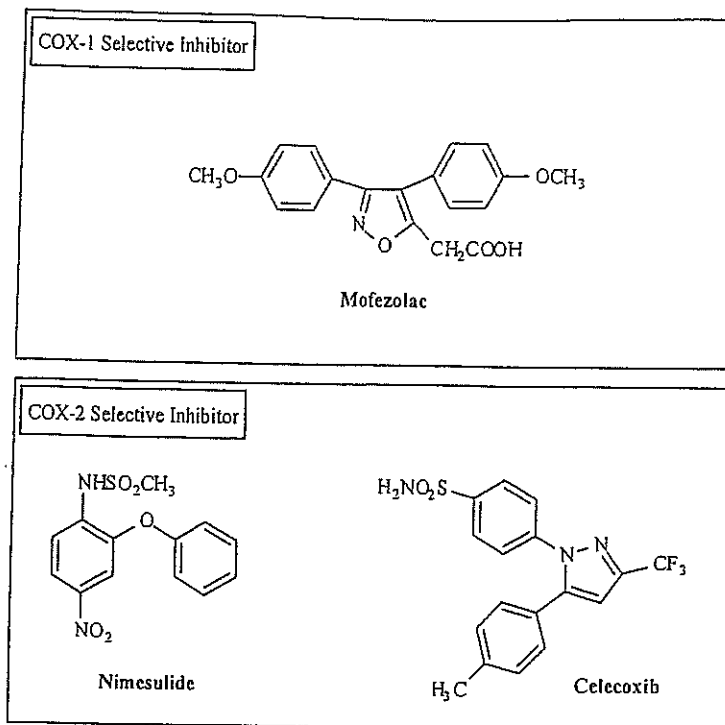


Fig. (2). Structures of COX-1 and COX-2 inhibitors.

FP, IP or TP, revealed decrease only in the EP₁ and EP₄-knockout cases, to approximately 60% and 56% of the level in wild-type mice, respectively [25, 26]. A pharmacological approach with selective antagonists for EP₁ and EP₄ receptors was then adopted to confirm involvement of the two receptors in colon carcinogenesis using two animal models, the AOM-induced ACF model and the Min mouse model. Structures of the EP₁ receptor selective antagonists, ONO-8711 and ONO-8713, and the EP₄ receptor selective antagonist, ONO-AE2-227, are shown in Fig. (3). Both ONO-8711 and ONO-8713 inhibited development of AOM-induced ACFs in male C57BL/6J mice. Moreover, when Min mice were given 500 ppm ONO-8711 in the diet, the number of intestinal polyps was significantly reduced to 57% of that in the basal diet group [25]. Administration of ONO-AE2-227 to AOM-treated wild mice and Min mice decreased ACFs and intestinal polyp formation, respectively. Interestingly, in the latter case the number of polyps ≥ 1.0 mm in diameter, but not those < 1.0 mm in diameter, were reduced, suggesting reduction in tumor growth [26]. In order to determine the contribution of EP₁ and EP₄ receptors to intestinal tumorigenesis, further experiments were designed to investigate the combined effects of EP₁ and EP₄ antagonists, ONO-8711 and ONO-AE2-227, on polyp formation in APC1309 mice. A summative tendency for suppression was also observed with respect to the size and numbers of polyps in the intestine. In this experiment, polyp size reduction was more remarkable with the EP₄ antagonist, while reduction in the number was more pronounced with the EP₁ antagonist [27].

Regarding the other receptor types, it was reported that homozygous deletion of the EP₂ receptor gene also resulted

in decrease of intestinal polyp formation in the APC knockout mice [28]. Moreover, EP₃ appears to play an opposing important role in protecting the colon from tumor development induced by AOM [29]. Long-term colon carcinogenesis experiments with EP₁, EP₂ and EP₄ antagonists are now needed to decide which are significant with respect to involvement in cancer progression.

B) Effect of PGE₂ Treatment

It is known that the PGE₂ levels are elevated in the colon tumor as compared with surrounding normal tissue [12]. Intraperitoneal injection of 7.7 μg PGE₂ once a week for 25 weeks significantly increased the AOM-induced rat colon tumor incidence (percentages rats with tumors, 92 versus 53), especially for adenocarcinomas (92 versus 47%), and multiplicity (number of tumors per rat, 2.8 versus 1.0) in comparison with animals treated with the vehicle alone [30]. There are reports suggesting that PGE₂ is important in the maintenance of tumor integrity and may be adequate to promote colon cancer development. Administration of the PGE₂ analogues 16,16-dimethyl-PGE₂ and 17-phenyl-trinor-PGE₂ of 10 μg each 3 times daily *via* gavage or intraperitoneal injection to Min mice counteracted the reduction in number of polyps caused by NSAIDs (piroxicam and sulindac) treatment, while elevating the intracellular Ca²⁺ concentration [31]. However, not all the data are consistent and Min mice treated with a stable PGE₂ analogue 16,16-dimethyl-PGE₂ from 6-18 weeks of age demonstrated an approximately 50-70% decrease in tumor incidence, with a 20-50% reduction in the number of lesions and a 10-70% reduction in their size [32].

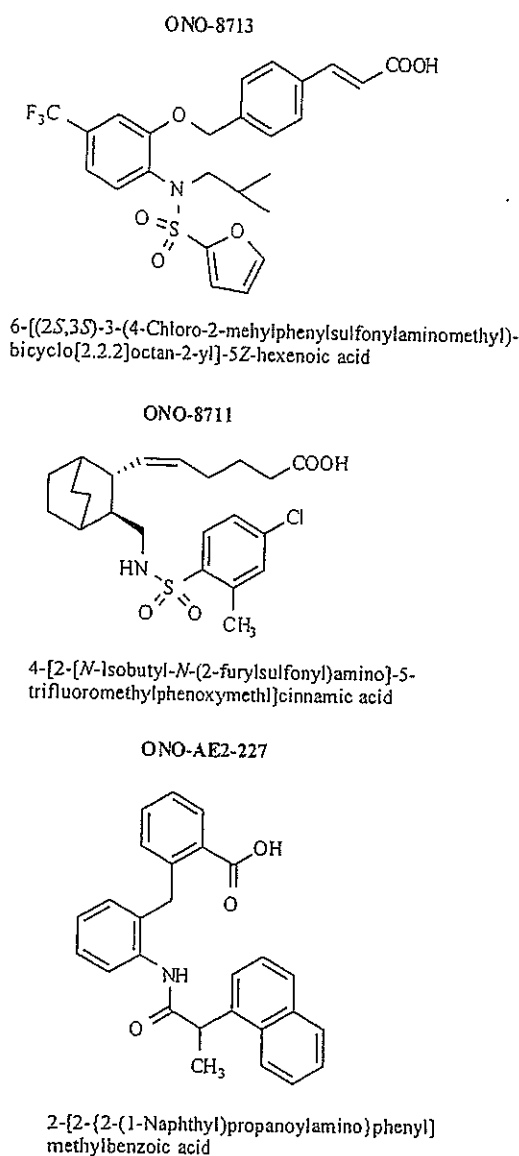


Fig. (3). Structures of EP₁ and EP₄ antagonist.

DOWNSTREAM SIGNALING PATHWAYS FROM ACTIVATED PROSTANOID RECEPTORS

Two classes of prostanoid receptors exist which can transduce signals from prostanoids and other ligands: the G protein-coupled cytoplasmic receptor class [33] and the nuclear peroxisome proliferator-activated receptor (PPAR) class [34]. As noted above, the prostanoids such as PGE₂, PGD₂, PGF₂, PGI₂, and TXA₂ exert their biological actions through binding to the eight specific membrane receptors; the four subtypes EP₁ to EP₄ for PGE₂; DP for PGD₂; FP for PGF₂; IP for PGI₂; and TP for TXA₂ [35, 36]. 15-Δ-PGJ₂ and PGI₂ are the ligands of PPARγ and PPARδ, respectively (Fig. (4)).

A) EP₁ and EP₃ Variants

The EP₁ receptor is a transmembrane G protein-coupled receptor, similar to other PGE₂ receptors, and its rat cDNA

clone encodes 405 amino acid residues with seven transmembrane-spanning domains. EP₁ signals transmitted by increased intracellular Ca²⁺ concentrations are known to activate protein kinase C (PKC) [35, 36] but the actual signal transduction mechanisms are not known in detail.

There are multiple receptor isoforms of EP₁, EP₃, FP and TP, modified by RNA splicing. The rat EP₁-variant receptor is translated from mRNA which is not spliced at nucleotide position 952 in the segment VI transmembrane region and retains the ligand binding activity with affinity and specificity similar to rat EP₁ receptor, but without the ability to couple with signal transduction systems by itself. When rat EP₁-variant receptor was stably co-expressed with rat EP₁ or rat EP₄ receptor in CHO cells, the Ca²⁺ mobilization mediated by the EP₁ receptor and the cAMP formation due to activation of the endogenous EP₄ receptor were significantly suppressed [37].

B) EP₂ and EP₄

Activation of EP₂ and EP₄ receptors in both cases involves coupling with stimulatory G protein (Gs protein), leading to up-regulation of adenylate cyclase (AC) activity. In the AC pathway, increased cAMP levels result in activation of cAMP-dependent protein kinase (PKA) and increase in a transcriptional factor that binds to cAMP-responsive elements (CRE), transactivating the transcription of specific primary response genes [37]. However there are clear differences between EP₂ and EP₄ in their structure and functions. cDNAs encoding EP₂ and EP₄ receptors share less than 30% amino acid homology and are no more related to each other than to other prostanoid receptor subtypes [38]. In fact, the EP₂ receptor shows a closer phylogenetic relationship to the DP and IP receptors than it does to the EP₄ receptor. Largely due to differences in the carboxyl (C)-terminal domain, the human EP₄ receptor is considerably larger than the human EP₂ receptor (488 versus 358 amino acids).

Both EP₂ and EP₄ receptors can activate T-cell factor (Tcf) signaling (Fig. (4)). However, EP₂ receptors do this primarily through a PKA-dependent pathway, whereas EP₄ receptors primarily utilize a phosphatidylinositol 3-kinase (PI3K)-dependent pathway [39]. PGE₂ stimulation of EP₄, but not EP₂, leads to phosphorylation of the extracellular signal-regulated kinases (ERKs) through a PI3K-dependent mechanism [40]. EP₂ is responsible for inducing the COX-2 gene through a positive feedback mechanism by PGE₂ [28] because of CRE stimulation in its promoter region [41].

C) EP₃ and EP₃ Variants

The activated EP₃ receptor couples with Gi protein, leading to inhibition of AC activity and resulting in a decrease of cAMP concentration. Known to have multiple splice variants in the human (9 variants), mouse (3 variants), rat (4 variants) and rabbit (4 variants), in the rat case, the EP₃ splice variants differ in the sequence of the intracellular C-terminus [42,43]. Interestingly, rabbit EP₃ receptor splice variants can stimulate CRE/β-galactosidase-mediated activity, and this appears to be independent of cAMP generation [44].

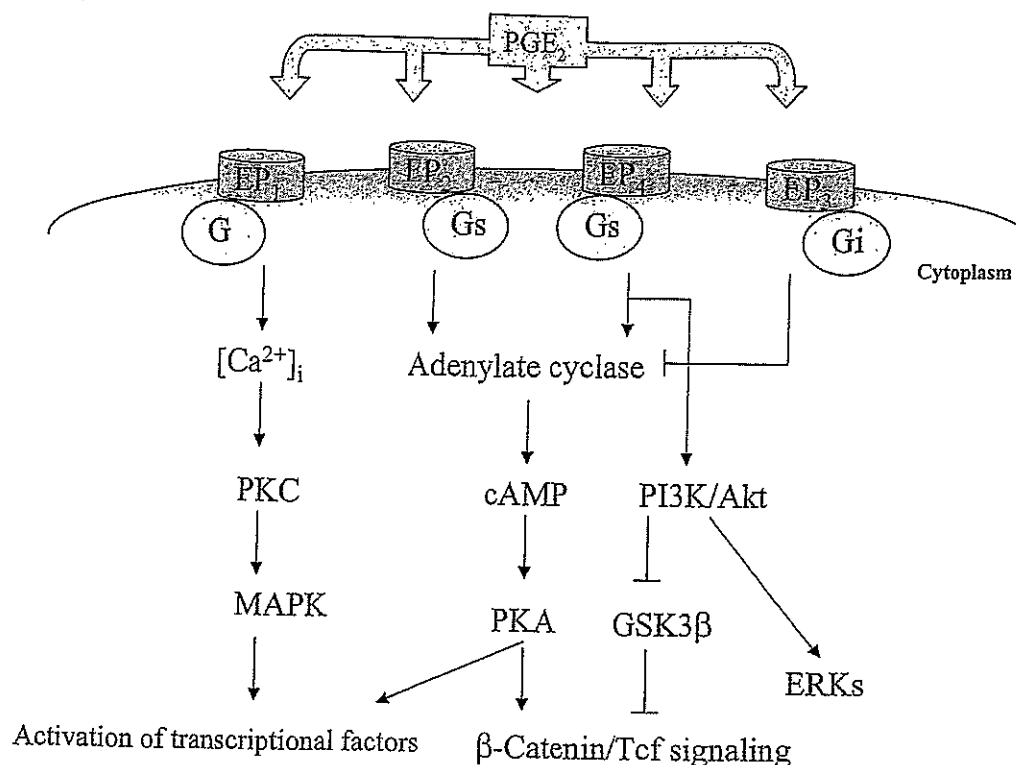


Fig. (4). Down stream signaling pathways from PGE₂. ERK=Extracellular signal-regulated kinase; GSK=glycogen synthase kinase-3; MAPK=Mitogen-activated protein kinase; PKC=Protein kinase C; PI3K=Phosphatidylinositol 3-kinase.

MECHANISMS BY WHICH PROSTANOIDS CONTRIBUTE TO CARCINOGENESIS

Many lines of evidence suggest that modulation of prostanoid synthesis and its functions are promising targets for the prevention or treatment of colon cancer [45]. Although the question of how prostanoids promote colon carcinogenesis has not been fully elucidated to date, several mechanisms can be speculated.

A) Cell Proliferation

PGE₂ may stimulate cell proliferation by production of growth factor [46, 47], activation of PI3K/Akt signaling and activation of Src, a downstream effector of the epithelial growth factor (EGF) receptor [48]. This involves activation of transcriptional factors such as c-fos, an induced response gene, and the early growth response factor-1 (EGR-1). The PI3K/Akt pathway promotes growth factor-mediated cell survival, inhibits apoptosis [49] and modulations cell cycle progression *via* modifications to cyclin D1 and p27^{kip1} [50, 51].

Treatment of LS-174 human colon cancer cells, seeded in Matrigel[®], with PGE₂ resulted in a dose-dependent increase in colony diameter through actions on the EP₄ receptor [52]. Consistent with this, an EP₄-selective agonist, 16-(3-methoxymethyl)phenyl- ω -tetranor-3,7-dithia-PGE₂ (ONO-AE1-329), was found to increase colony formation of HCA-7 cells to a similar extent as PGE₂ [26]. ONO-AE1-329 and PGE₂ but not EP₂ and EP₃ agonists, up-regulate the expression of c-fos and increased colony formation in a gallbladder

adenocarcinoma cell line, Mz-ChA-2, in which COX-2 protein and mRNA are hardly detectable [53]. Furthermore, this activation of PI3K/Akt signaling by the EP₄ receptor induces functional expression of EGR-1 [40], a member of the zinc finger family of transcription factors which plays a key role in cell growth and differentiation by direct regulation of the expression of cyclin D1 [54].

While PGE₂ has been reported to inhibit the proliferation of certain colorectal cancer cell lines [55], the apparently anomalous effects could depend on characteristics of the cells such as different activation of prostanoid receptors and their cross talk or dependence on prostanoid stimulus. For instance, the growth of primary adult human keratinocytes is stimulated by activation of EP₂ receptors and is inhibited by activation of EP₃ receptors *via* an AC independent mechanism [56]. In a human colon cell line, an EP₃-selective agonist has been shown to inhibit cell growth of EP₃ receptor expressing but not of EP₃ receptor undetectable cells [29].

B) Apoptosis

During carcinogenesis, apoptosis is decreased with substantial induction of antiapoptotic proteins. Several reports which addressed the possible causal linkage between expression of COX-2 and inhibition of apoptosis suggest that enzyme-promoted antiapoptosis is mediated by release of prostanoids, especially PGE₂. It has also been reported that the antiapoptotic protein, Bcl-2, is involved in antiapoptotic effects of COX-2 [57-59]. Furthermore, PGE₂ promotes induction of a cAMP-dependent cellular inhibitor of apoptosis,

c-IAP-2. Three cAMP agonists, PGE₂, cholera toxin and a membrane-permeable cAMP analog, 8-CPT-cAMP, all protect RIE-1, T84 and/or HCA-7 cells from Fas and staurosporine-induced apoptosis by induction of c-IAP-2 and delayed induction of LIVIN, another member of the IAP family [60]. PI3K/Akt is known to inhibit pro-apoptotic signaling through BAD, caspase-9 and Fas, while activating anti-apoptotic signaling through NFκB, an up-stream mediator of IAP [61].

C) Inflammation and Immune Surveillance

In inflamed tissues, up-regulation of COX-2 and increased synthesis of PGE₂ (elevation 3-fold or more) can be observed. It is suggested that a high level of PGE₂ production in tumor tissue could mediate a profound alteration in the cytokine balance in the cancer microenvironment. For instance, PGE₂ may reduce tumor necrosis factor (TNF) production in lipopolysaccharide-treated murine macrophages [62]. Lung tumor-derived PGE₂ promotes induction of lymphocyte and macrophage IL-10, an immunosuppressive cytokine, while simultaneously inhibiting macrophage IL-12 production [63]. Furthermore, liver cells and macrophages isolated from EP₄ knockout mice have been documented to produce significantly less IL-1β and IL-6 than control samples [64].

Recent evidence suggests that change in expression of prostanoid receptors also correlates with several chronic inflammation diseases. EP₄-deficient mice, but not DP, EP₁, EP₂, EP₃, FP, IP, or TP deficient mice, develop severe dextran sodium sulfate-induced (DSS-induced) colitis with aggregation of neutrophils and lymphocytes in the colon. Also administration of AE3-208, an EP₄-selective antagonist, mimicks DSS-induced colitis in wild-type mice [65]. In a rheumatoid arthritis model, EP₄ receptor-deficient mice, but not their EP_{1,3} counterparts, have shown decreased incidence and severity [64]. The apparently conflicting effects on inflammation reflect the complicated immune system and various functions of prostanoids. However, as immune suppression is well established to favor tumor growth, the facts provide a basis for a cause-and-effect link between chronic inflammation and carcinogenesis.

D) Angiogenesis

Without vascular supply of nutrients and oxygen, tumors can not increase their mass. Hypoxia induces microvascular endothelial COX-2 expression [66] which in turn stimulates production of angiogenic factors. Several reports suggest that prostanoids can mediate tumor angiogenesis and, recently, PGE₂ was reported to mediate angiogenesis *via* stimulation of EP₂, EP₄ and TP receptors [67-69].

CONCLUSIONS AND FUTURE DIRECTIONS

Increasingly, attention has become focused on studies of the significance of prostanoid receptors for carcinogenesis over the last several years. The present review aims to provide an up date of publications in this field of research, with particular attention to the possible mechanisms of prostanoid action and potential application of prostanoid receptor inhibitors/agonists for colon cancer prevention. Indeed, since COX-2 may be involved in cancer development in sites as wide-

spread as the breast, stomach, head and neck, lung and pancreas [45], these might also be targets for chemoprevention by selective prostanoid receptor inhibitors. Further clarification of prostanoid receptor function is now a high priority and development of selective inhibitors needs to be further addressed.

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