Cyclooxygenase-1 and cyclooxygenase-2 gene expression in human colorectal adenocarcinomas and in azoxymethane induced colonic tumours in rats

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Abstract

Increased prostaglandin E₂ synthesis is considered important in both human and experimental colon carcinogenesis. It is not known, however, which cyclooxygenase isoenzyme is involved. The aim of this study was to compare the content of mRNA for cyclooxygenase-1 and cyclooxygenase-2 in colorectal cancers with the content in normal colonic specimens. Fifteen human colorectal adenocarcinomas, 35 azoxymethane induced colonic tumours from rats, and specimens of normal colon were analysed by reverse transcription and polymerase chain reaction (RT-PCR). It was found that cyclooxygenase-1 and cyclooxygenase-2 mRNA were increased in azoxymethane induced colonic tumours, compared with specimens taken adjacent to the tumours or from the macroscopically normal intestine distant from the tumours. Cyclooxygenase-1 and cyclooxygenase-2 mRNA were increased in specimens from the macroscopically normal intestine of azoxymethane treated animals, compared with colonic specimens from saline treated rats. Cyclooxygenase-2 mRNA, but not cyclooxygenase-1 mRNA, was increased in human colorectal cancers, compared with the adjacent mucosa or macroscopically normal mucosa distant from the tumours. The results suggest that cyclooxygenase-2 is involved in the increased prostaglandin E₂ synthesis in colonic cancers, and that activation of this isoenzyme is an early event in colon carcinogenesis. However, cyclooxygenase-1 may also be involved, at least in experimental colon carcinogenesis.

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Keywords: colorectal cancer, cyclooxygenase, messenger ribonucleic acid.

Human colorectal carcinomas^{1–3} and experimental colonic tumours in rats^{4,5} contain increased amounts of prostaglandin E₂ (PGE₂), and it is generally thought that PGE₂ participates in both human and experimental colon carcinogenesis.^{1–5}

Formation of prostaglandins requires activation of cyclooxygenase (also known as prostaglandin H synthase and prostaglandin endoperoxide synthase), catalysing the formation of endoperoxide PGH₂ from arachidonic

acid.⁶ PGH₂ is then further metabolised by various prostaglandin synthetases to form specific prostaglandins, including PGE₂.⁶ Non-steroidal anti-inflammatory drugs (NSAIDs) are well known inhibitors of cyclooxygenase activity,⁷ and recent epidemiological studies have suggested that regular use of aspirin is associated with a decreased risk for colorectal cancer in humans.^{8–10} Moreover, several NSAIDs have been shown to suppress colon carcinogenesis in rat models.^{5, 11–13} It is probable, therefore, that cyclooxygenase activity is important in both human and experimental colon carcinogenesis.

Cyclooxygenase exists as two genetically different isoforms, cyclooxygenase-1¹⁴ and cyclooxygenase-2.¹⁵ Both cyclooxygenase-1 and cyclooxygenase-2 are constitutively expressed in a large number of cell types and tissues,^{15–18} including the human colon.¹⁹ Nothing is known about the regulation of colonic cyclooxygenase-1 and cyclooxygenase-2 gene expression and activity. In general, however, cyclooxygenase-1 seems to be the isoenzyme most important for the production of prostaglandins during basal conditions, whereas cyclooxygenase-2 seems to be responsible for the increased production in response to various cytokines, mitogens, and growth factors.^{15–18, 20} It is not known which one of the two cyclooxygenase isoenzymes is responsible for the formation of PGE₂ in colorectal cancer tumours, and it is likely that an increased knowledge about the specific roles of cyclooxygenase-1 and cyclooxygenase-2 in colorectal cancer development will bring further insight into the mechanisms of colorectal carcinogenesis.

The specific aim of this study was to compare the content of messenger ribonucleic acid (mRNA) for cyclooxygenase-1 and cyclooxygenase-2 in human colorectal carcinomas and in azoxymethane induced colonic tumours in rats with the content in corresponding normal colonic specimens. The results suggest that cyclooxygenase-2 is involved in the increased production of PGE₂ in both human and azoxymethane induced colonic tumours.

Methods

Animals

Male Fischer-Cooper hybrid rats (ALAB, Stockholm, Sweden) weighing 210–260 g were used. The study was approved by the ethics

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TABLE 1 Sequences of oligonucleotide primers for the polymerase chain reaction (PCR) and of probes for hybridisation of PCR products (expected size of product is given in right column)

Enzyme	Oligonucleotide	Sequence (5'-3')	PCR products (bp)
<i>Rat</i>	Cyclooxygenase-1	Upstream primer Downstream primer Probe	561
	Cyclooxygenase-2	Upstream primer Downstream primer Probe	
<i>Human</i>	Cyclooxygenase-1	Upstream primer Downstream primer Probe	309
	Cyclooxygenase-2	Upstream primer Downstream primer Probe	

bp=base pair.

committee for animal experimentation, Linköping. The animals had free access to standard food pellets and tap water throughout the experiment. The development of tumours during the observation period of six months did not affect the body weight gain. Twenty rats were given azoxymethane (Sigma Chemical, St Louis, USA) subcutaneously as three weekly doses of 16 mg/kg body weight, as we have described previously.²¹ Ten rats were used as controls and were injected with an equal volume of saline. Six months after the first azoxymethane injection, the rats were anaesthetised by an intraperitoneal injection of xylazine (Rompun) 8 mg/kg body weight and ketamine (Ketalar) 80 mg/kg body weight, and the colon was taken out and investigated for tumours.

Experimental design

The tumours were identified macroscopically, and their positions assigned to either the proximal third, the mid-third, or the distal third (including rectum) of the colon. A ruler was used to determine the diameter of the tumours, before they were removed (together with the entire bowel wall), washed in ice cold saline, frozen in liquid nitrogen, and kept at -70°C until analysed.

Of the 20 animals given azoxymethane, one died before the six months was completed. Of the remaining 19 animals, two were without tumours. In total, 35 tumours were found, and sampled, in the 17 tumour bearing rats. In addition, pieces of colon adjacent to 33 of these tumours were sampled. Moreover, one piece of macroscopically normal colon (that is, at least 3 cm from the nearest tumour) was taken from all 19 animals. The median number of tumours in the 17 tumour bearing rats was two (range 1-4). The median diameter of the tumours was 3.0 mm (range 1.2-12). Twenty three tumours were found in the distal third, 11 in the mid-third, and one in the proximal third of the colon. The size of the tumours did not permit both histopathological investigation and mRNA analysis, but we have previously shown that tumours induced by azoxymethane in the same way as in this study includes both adenomas and adenocarcinomas.²¹

Of the 10 rats given only saline, one died before the six months were completed. From eight of the nine remaining animals, one piece

of colonic tissue was taken from the distal and proximal third of the colon, whereas only a piece from the proximal colon was taken from the ninth rat.

Subjects

Fifteen consecutive patients undergoing surgery for colorectal cancer at Linköping University Hospital were studied. Eight were women (average age 72 years; range 51 to 80; median 76) and seven were men (average age 71 years; range 55 to 85; median 73). According to the Dukes's classification, two tumours were stage A, eight stage B, four stage C, and one stage D. Tumours were collected from the right colon (caecum or ascending colon) in six patients, from the transverse colon in one patient, and from the left colon (sigmoid colon or rectum) in eight patients. One of the tumours was well differentiated, nine were moderately well differentiated, and five were poorly differentiated. All tumours were adenocarcinomas.

As soon as the operative specimen was available, we obtained from each patient samples of cancer (about 1 cm² of the outer, non-fibrotic, part of the tumour), mucosa adjacent to the cancer, and macroscopically normal mucosa 10 cm away from the cancer. The specimens were rinsed in ice cold saline and frozen immediately in liquid nitrogen and kept at -70°C until analysed.

The study was approved by the ethics committee of human experimentation, Linköping.

Preparation of total RNA

Total RNA was prepared according to Chomczynski and Sacchi,²² as we have previously described in detail.²³

Reverse transcription and polymerase chain reaction (RT-PCR)

First strand cDNA synthesis and PCR amplification were carried out essentially as we have described previously.²⁴ Briefly, 0.5 (rat) or 1.0 (human) µg total RNA was transcribed to cDNA in a final volume of a 40 µl, and the cDNA sample was then split into aliquots (rat, 3 µl; human, 4 µl) for amplification by specific primers (sequences are given in Table I) for cyclooxygenase-1 and cyclooxygenase-2. A

TABLE II Cyclooxygenase-1 mRNA in colonic specimens from azoxymethane treated rats and saline treated control rats. The specimens from azoxymethane treated rats included tumours, areas adjacent to tumours (Adjacent), and distant from tumours (Distant). Further experimental details are given in Methods section. Values are given as integrated optical density units (IOD), obtained after dot blot analysis of PCR products. Number of samples included are given within parenthesis

Cyclooxygenase-1 (IOD; mean (SEM))					
Specimens from control rats					
Proximal colon (9)	3.0 (0.6)	]	p=0.3865	]	
Distal colon (8)	2.4 (0.6)				
Proximal colon (9) + distal colon (8)	2.7 (0.5)				
Specimens from azoxymethane rats					
Tumour (35)	9.3 (0.8)	]	p<0.0001 p=0.0095		]
Adjacent (33)	3.6 (0.5)				
Distant (19)	6.1 (0.8)				
			p=0.0112	p=0.0012	

repeated three step temperature profile was used, including denaturation of the cDNA for 10 seconds at 94°C, annealing of the primers for 10 seconds at an appropriate temperature (57.7°C for rat cyclooxygenase-1, 54.9°C for rat cyclooxygenase-2, 56.5°C for human cyclooxygenase-1, and 54.9°C for human cyclooxygenase-2), and a primer extension for 10 seconds at 72°C. A single 10 minutes elongation period at 72°C finished the PCR.

Specific primers (sequences are given in Table I) were constructed according to the cDNA sequences of rat cyclooxygenase-1 and cyclooxygenase-2²⁵ and of human cyclooxygenase-1¹⁴ and cyclooxygenase-2¹⁵ synthesised by Scandinavian Gene Synthesis (Köping, Sweden). Sequences are specific as ascertained by computer assisted search of updated versions of GeneBank.

PCR products analysis

PCR products were separated by electrophoresis on 1.6% agarose gel (SeaKem ME, FMC BioProducts, Rockland, ME, USA) and visualised by ethidium bromide staining. All PCR products showed a single band of expected size (number of base pairs are given in Table I) when compared with known molecular weight markers (VI and IX, Boehringer Mannheim, Mannheim, Germany).

Semi-quantitative determination of the PCR products was performed by dot blot analysis, essentially as we have described previously.²⁴ Briefly, PCR products were dot blotted onto a cationised nylon membrane, and the membrane hybridised for three hours in a solution containing a digoxigenine labelled oligonucleotide probe (digoxigenine oligonucleotide tailing system, Boehringer Mannheim,

Mannheim, Germany). The temperatures during hybridisation reaction were adjusted to fit the actual probe (53°C for human and rat cyclooxygenase-1, and 51°C for human and rat cyclooxygenase-2). The hybridised product was detected with digoxigenine luminescent system (Boehringer Mannheim, Mannheim, Germany), and the resulting blots were subjected to autoradiography on Cronex 4 x ray film at room temperature for 1 to 10 minutes before development. The signal intensity was measured with a computerised image system (Bio Image Products, Ann Arbor, MI, USA).

In an attempt to get optimal conditions for comparison between samples, different numbers of amplification cycles were tested for each type of cyclooxygenase. The same batch of reverse transcribed total RNA was divided for use in the analysis of cyclooxygenase-1 and cyclooxygenase-2. Also, the number of cycles used in the amplification of the PCR product and the exposure time of the x ray film, were chosen to give optimal resolution.

Probes were constructed to be complementary to part of one strand of the PCR product and were synthesised by Scandinavian Gene Synthesis (Köping, Sweden). Sequences are given in Table I.

Statistical analysis

Comparative statistics was made with Wilcoxon rank sum test (between the groups), and with Wilcoxon signed rank test (within the groups), with $p < 0.05$ considered significant. In the tests of correlation, Spearman's correlation coefficient was used and $p < 0.05$ considered significant.

Results

Cyclooxygenase gene expression in experimental colonic tumours

The content of cyclooxygenase-1 (Table II) and cyclooxygenase-2 (Table III) mRNA did not differ between the proximal and distal part of the colon in control rats. A higher content of both cyclooxygenase-1 mRNA (Table II) and cyclooxygenase-2 mRNA (Table III) was found, however, in the macroscopically normal colon from azoxymethane treated rats (that is, in samples taken distant from the tumours) than in colonic samples obtained from control rats.

No correlation was found between the size of the 35 tumours investigated and their content of cyclooxygenase-1 ($p = 0.4030$, $r = -0.143$) or cyclooxygenase-2 mRNA ($p = 0.3199$, $r = -0.171$), and the content of cyclooxygenase-1 and cyclooxygenase-2 mRNA did not differ between the 11 tumours from the mid-colon and the 23 tumours from the distal colon (p values were 0.6991 for cyclooxygenase-1 and 0.8540 for cyclooxygenase-2; data not shown).

The content of cyclooxygenase-1 mRNA in the tumours was higher than in the areas adjacent and distant from the tumours, and than in colonic tissues from control rats (Table II).

TABLE III Cyclooxygenase-2 mRNA in colonic specimens from azoxymethane treated rats and saline treated control rats. The specimens from azoxymethane treated rats included tumours, areas adjacent to tumours (Adjacent) and distant from tumours (Distant). Further experimental details are given in Methods section. Values are given as integrated optical density units (IOD), obtained after dot blot analysis of PCR products. Number of samples included are given within parenthesis

Cyclooxygenase-2 (IOD; mean (SEM))							
Specimens from control rats							
Proximal colon (9)	0.3 (0.1)	]	p=0.3606	]	p=0.0046		
Distal colon (8)	0.7 (0.3)						
Proximal colon (9)	0.5 (0.2)						
+ distal colon (8)							
Specimens from azoxymethane rats							
Tumour (35)	14.0 (1.8)	]	p<0.0001			]	p=0.0001
Adjacent (33)	1.9 (0.7)						
Distant (19)	2.5 (0.7)						
		]	p=0.2312	]			

TABLE IV Influence of sex, Dukes's stage, site, and degree of differentiation on the content of mRNA for cyclooxygenase-1 and cyclooxygenase-2 in human colorectal cancers. Values are given as integrated optical density units (IOD), obtained after dot blot analysis of cyclooxygenase-1 and cyclooxygenase-2 PCR products. Further experimental details are given in Methods section. Number of samples included are given within parenthesis

	Cyclooxygenase-1 (IOD; mean (SEM))			Cyclooxygenase-2 (IOD; mean (SEM))		
Sex						
Male (7)	8.1 (1.4)	]	p=0.1649	17.6 (3.6)	]	p=0.2472
Female (8)	6.6 (2.0)			11.8 (3.9)		
Dukes's stage						
A (2)+B (8)	6.6 (1.7)	]	p=0.1984	15.4 (3.8)	]	p=0.8065
C (4)+D (1)	8.7 (1.5)			12.7 (3.3)		
Site						
Right colon (6)	6.5 (1.7)	]	p=0.6514	13.9 (2.8)	]	p=0.1967
Left colon (8)	8.1 (2.0)			12.6 (4.0)		
Degree of differentiation						
Poor (5)	4.5 (2.1)	]	p=0.0500	14.2 (3.4)	]	p>0.9
Moderate (9)+well (1)	8.7 (1.4)			14.6 (3.8)		

The content of cyclooxygenase-1 mRNA was decreased, however, in the area adjacent to the tumours, compared with the macroscopically normal colon distant to the tumours (Table II). A comparison between paired samples with regard to cyclooxygenase-1 mRNA, showed that the content was increased in 30 of 33 (91%) of the tumours compared with the adjacent tissue, and in 26 of 35 (74%) of the tumours compared with the macroscopically normal colon distant from the tumours.

Also the content of cyclooxygenase-2 mRNA was significantly higher in the tumours than in the areas adjacent and distant from the tumours, and than in colonic tissues from control rats (Table III). In contrast with cyclooxygenase-1 mRNA, however, the content of cyclooxygenase-2 mRNA was not significantly decreased in the area adjacent to the tumours, compared with the macroscopically normal colon distant to the tumours (Table III). The increase in cyclooxygenase-2 mRNA (Table III) seemed to be more pronounced than the increase in cyclooxygenase-1 mRNA (Table II); the average content of cyclooxygenase-1 and cyclooxygenase-2 mRNA in tumours was increased about 3 and 28 times respectively, compared with colonic specimens from control rats. A comparison between paired samples with regard to cyclooxygenase-2 mRNA, showed that the content was increased in 28 of 33 (85%) of the tumours compared with the adjacent area, and in 30 of 35 (86%) of the tumours compared with the macroscopically normal colon distant from the tumours.

Cyclooxygenase gene expression in human colonic carcinomas

As Table IV shows, neither sex, Dukes's stage, site, or degree of differentiation significantly affected the content of cyclooxygenase-1 or cyclooxygenase-2 mRNA in the tumours.

The content of cyclooxygenase-1 mRNA in human colorectal cancers was not significantly different from the content in the adjacent mucosa and in the mucosa distant from the tumours (Table V). Although not significant, however, there was a tendency towards a decreased content of cyclooxygenase-1 mRNA in the mucosa adjacent to the tumours (Table V).

The content of cyclooxygenase-2 mRNA in human colonic cancers was significantly higher compared with the mucosa adjacent to the tumours or the mucosa distant from the tumours (Table V). The content of cyclooxygenase-2 mRNA in tumours was increased in 14 of 15 (93%) compared with the adjacent mucosa, and in 14 of 15 (93%) compared with the mucosa distant from the tumours.

Discussion

Previous studies have suggested that cyclooxygenase metabolites, in particular PGE₂, are important in both experimental^{4,5} and human¹⁻³ colon carcinogenesis. It is still unknown, however, which one of the two isoenzymes, cyclooxygenase-1¹⁴ and cyclooxygenase-2,¹⁵ is responsible for the increased prostaglandin production in colorectal cancers.¹⁻⁵ One first step to elucidate this question might be to investigate the gene expression for cyclooxygenase-1 and cyclooxygenase-2 in colorectal tumours and compare it with the normal colonic mucosa. In this study, therefore, we have investigated the level of mRNA for cyclooxygenase-1 and cyclooxygenase-2 in human colorectal cancers and in azoxymethane induced colorectal tumours from rats. Azoxymethane is an organ specific colon carcinogen, widely used in studies on experimental colon carcinogenesis.^{26,27} Human colon carcinogenesis and azoxymethane induced colon carcinogenesis seem to share many similarities,²⁸ such as mutations in ras genes^{27,29,30} and an apparent dependency on cyclooxygenase activity.^{2,3,5,9,10,31-33}

Both the azoxymethane induced tumours and the human colorectal cancers contained significantly increased values of cyclooxygenase-2 mRNA, compared with all other groups of samples investigated. During preparation of this manuscript, Eberhart and coworkers¹⁹ reported increased content of cyclooxygenase-2 mRNA, but not cyclooxygenase-1 mRNA, in human colonic cancer tumours, findings that support the results obtained in this study. This study is the first to show, however, that azoxymethane induced tumours also contain increased expressions of cyclooxygenase-2 mRNA, a finding that supports the use of this experimental model for future studies on cyclooxygenase and colon carcinogenesis. Cyclooxygenase is known to be irreversibly inactivated during catalysis³⁴ and

TABLE V mRNA for cyclooxygenase-1 and cyclooxygenase-2 in human colonic specimens. Values are given as integrated optical density units (IOD), obtained after dot blot analysis of PCR products. Further experimental details are given in Methods section. Means (SEM) of 15 tumours and paired samples of adjacent mucosa (Adjacent) and macroscopically normal mucosa distant from the tumours (Distant)

	IOD (mean (SEM))			
Cyclooxygenase-1				
Tumour	7.2 (1.2)	]	p=0.0962 p=0.1398	p=0.8871
Adjacent	4.7 (0.9)			
Distant	7.3 (1.0)			
Cyclooxygenase-2				
Tumour	14.5 (2.7)	]	p=0.0008 p=0.3942	p=0.0008
Adjacent	3.0 (0.7)			
Distant	4.0 (1.0)			

the rate of formation of PGH_2 from arachidonic acid thus dependent on the rate at which cyclooxygenase can be resynthesised.³⁴ It seems probable, therefore, that an increased cyclooxygenase-2 mRNA content is closely correlated with an increased cyclooxygenase-2 enzymatic activity. It can be speculated, therefore, that cyclooxygenase-2 is responsible for the increased PGE_2 formation found in colonic cancer tumours, and that activation of this particular isoenzyme might be an important step in colon carcinogenesis.

We found that the content of cyclooxygenase-2 mRNA was increased in the macroscopically normal colon of azoxymethane treated rats, compared with the samples from control animals. Previous findings by others have shown that the content of PGE_2 is increased in the macroscopically normal colon of azoxymethane treated animals,^{4 5 26 32} and in non-cancerous colonic polyps from humans.^{19 33} Taken together, these findings suggest that cyclooxygenase-2 activation and PGE_2 formation might be important events in the early steps of colon carcinogenesis. Our findings that neither Dukes's stage or the size of the azoxymethane induced tumours were related to the cyclooxygenase mRNA expression further support the hypothesis of an early role for cyclooxygenase-2 in colon carcinogenesis.

The presence of mRNA for cyclooxygenase-1 and cyclooxygenase-2 in the non-malignant human colon has recently been reported,¹⁹ but to our knowledge our study is the first to show the presence of mRNA for cyclooxygenase-1 and cyclooxygenase-2 in the normal colon of rats. However, the cellular origin of the two cyclooxygenase isoenzymes and their relative contribution to the basal colonic PGE_2 production remains to be investigated, in both humans and rats. The cellular origin of colonic PGE_2 has not been established with certainty, but results from both human³⁵ and rat^{36 37} studies show that the basal colonic prostaglandin production originates from the submucosal layers, in particular from the muscle layers. In this study, the azoxymethane induced tumours (for technical reasons) were sampled together with the whole bowel wall, whereas only the outer part of the human cancers were collected. As cyclooxygenase-1 is generally thought to be the isoenzyme most important in basal prostaglandin production,¹⁵⁻¹⁸ it may be speculated that cyclooxygenase-1 is present at particularly high amounts in the submucosa, and that a proportionately high number of submucosal cells in the preparations of azoxymethane induced tumours explains why cyclooxygenase-1 mRNA was increased in these preparations but not in preparations of human tumours. Whatever the cellular origin of cyclooxygenase-1, the increased expression of cyclooxygenase-1 mRNA in the azoxymethane induced tumours, and also in the macroscopically normal colon of azoxymethane treated rats, may suggest that cyclooxygenase-1 plays a part in the increased synthesis of PGE_2 in colorectal cancers. It cannot be excluded, however, that

the difference between the azoxymethane induced tumours and the human cancers with regard to cyclooxygenase-1 mRNA resulted from differences between human and experimental colon carcinogenesis.

The mucosa adjacent to a colonic carcinoma is of special interest, because adjacent to regions of carcinoma a field of genetically abnormal high risk mucosa may exist from which the carcinoma have arisen. The PGE_2 production in this zone has never been investigated, however, and although this study suggests that cyclooxygenase-1 mRNA might be decreased in this region, further studies are needed to clarify if this is a finding relevant to colonic PGE_2 production and colon carcinogenesis.

PGE_2 cannot be formed unless free arachidonic acid is available for cyclooxygenase. This generation of free arachidonic acid is mediated by phospholipase A_2 , cleaving the esterified arachidonic acid from various cellular phospholipids.³⁸ Colonic tumours from humans and rats contain increased amounts of arachidonic acid,^{3 39 40} and thus increased amounts of substrate for cyclooxygenase. Recent research points to the possibility that an increased phospholipase A_2 activity might play a part in carcinogenesis in various organs,⁴¹⁻⁴³ including the colon.⁴⁴ It may be speculated, therefore, that an increased phospholipase A_2 activity in the colonic tumours, in combination with an increased arachidonic acid content, may support colon carcinogenesis by providing cyclooxygenase with increased amounts of substrate for PGE_2 production. Nothing is known, however, about the presence and activity of the three known isoforms of phospholipase A_2 (for references see²⁴) in colorectal cancer tumours. We are therefore, in ongoing studies, investigating the presence and activity of different phospholipase A_2 isoenzymes in colorectal tumours from rats and humans.

Taken together, the results of this study strongly suggest that cyclooxygenase-2 activity is important for the production of PGE_2 in colonic tumours of humans and azoxymethane treated rats, but does not exclude a role for cyclooxygenase-1, at least not in experimental colon carcinogenesis. Further studies are needed, however, to elucidate the regulation, cellular origin, and precise role of the cyclooxygenase isoenzymes in the normal colonic mucosa and in colon carcinogenesis.

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