

Sulindac and a cyclooxygenase-2 inhibitor, etodolac, increase APC mRNA in the colon of rats treated with azoxymethane

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Abstract

Background—Non-steroidal anti-inflammatory drugs (NSAIDs) have been reported to protect against the development of colon cancer. However, the mechanism(s) by which NSAIDs exert their effects is not clear.

Aims—The aim of this study was to examine the effects of NSAIDs on mRNA expression of tumour suppressor adenomatous polyposis coli (APC) gene in rat colon mucosa.

Methods—Starting at six weeks of age, three groups of rats (groups 1, 2, and 3) were treated with azoxymethane (AOM), a colon specific carcinogen, and another three groups (groups 4, 5, and 6) were not given AOM. Groups 2 and 3 were given 10 mg/kg of sulindac or etodolac, respectively, three times weekly during the experiment. Groups 4 and 5 were also given sulindac or etodolac, respectively, in the same manner as in groups 2 and 3. Group 6 (untreated control) was not given any agent (AOM or NSAIDs). At 10 weeks of age, preneoplastic lesions (aberrant crypt foci (ACF)) induced by AOM in the colon were counted, and the level of expression of APC mRNA in the colonic mucosa was estimated by the reverse transcription-competitive polymerase chain reaction method and northern blot analysis.

Results—Mean occurrence of ACF in rats in groups 2 and 3 was reduced to approximately 50% of that in group 1. The level of APC mRNA expression in group 1 (AOM alone) was lower than that in group 6 (untreated control) ($p < 0.05$); however, levels of APC mRNA expression in groups 2, 3, 4, and 5, to which NSAIDs had been administered, were significantly increased compared with levels in groups 1 and 6 ($p < 0.01$).

Conclusions—Both sulindac and etodolac reduced the occurrence of ACF and induced an increase in APC mRNA in rat colon mucosa.

(Gut 2000;47:812-819)

Keywords: adenomatous polyposis coli; cyclooxygenase; non-steroidal anti-inflammatory drugs; aberrant crypt foci; colon; azoxymethane

Evidence from numerous human epidemiological studies suggests that administration of non-steroidal anti-inflammatory drugs (NSAIDs) is a potentially viable option in the

chemoprevention of sporadic colon cancer. Animal model studies have also demonstrated that NSAIDs suppress colon carcinogenesis. The most studied molecular target for NSAIDs, first proposed by Vane in 1971,¹ is cyclooxygenase (COX); however, the molecular mechanism(s) by which NSAIDs exert their effects is not clear. A widely used animal model for colon cancer is the azoxymethane (AOM) treated rat in which the chemical carcinogen AOM induces preneoplastic colonic lesions termed aberrant crypt foci (ACF) which later progress to carcinomas.² ACF have been proposed as intermediate biomarkers for colon cancer in many chemopreventive studies.³⁻⁶ Reddy and colleagues⁷ were the first to demonstrate the effects of NSAIDs on tumour formation in this model and found that aspirin reduced the incidence, multiplicity, and size of colonic carcinomas. Other researchers have also reported similar findings for the NSAIDs sulindac and piroxicam.^{8,9} Moreover, Gustafson-Svärd and colleagues¹⁰ reported that the increase in COX-2 mRNA was more pronounced than the increase in COX-1 mRNA in macroscopically normal colonic mucosa, including ACF, in AOM treated rats, and several investigators showed that selective COX-2 inhibitors decreased aberrant crypt formation by 50%.^{11,12}

However, more recently, there have been reports of antineoplastic activities of NSAIDs that are independent of COX.¹³ Schnitzler and colleagues¹⁴ showed that both the sulphide metabolite of sulindac, which inhibits COX, and the sulphone metabolite, which lacks this ability, inhibit the growth of colonic carcinoma cells in vitro and cause an increase in the level of mRNA of the tumour suppressor adenomatous polyposis coli (APC) gene. This indicates that the effect of these agents on colonic carcinogenesis is not mediated entirely by inhibition of COX activity.

Although aberration of the tumour suppressor p53 gene is the most common genetic lesion identified in human carcinomas, no mutations were found in the highly conserved regions of p53 exons 5-8 using the single strand conformation polymorphism method, direct sequencing, or immunohistochemical analysis in either AOM or dimethylhydrazine

Abbreviations used in this paper: ACF, aberrant crypt foci; AOM, azoxymethane; APC, adenomatous polyposis coli; COX, cyclooxygenase; FAP, familial adenomatous polyposis; GSK-3 β , glycogen synthase kinase 3 β ; NSAID, non-steroidal anti-inflammatory drug; PCR, polymerase chain reaction; RT, reverse transcription; Tcf-4, T cell factor 4.

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Accepted for publication
7 June 2000

induced rat colon carcinomas.^{15,16} However, frequent mutations of the β -catenin gene and the consequent abnormal accumulation of cytoplasmic and nuclear β -catenin were reported in AOM induced rat colon carcinomas,¹⁷ and it was suggested that alterations in the stability and localisation of the protein may play an important role in cancer formation (progression) in this colon carcinogenesis model. However, abnormal nuclear and/or cytoplasmic localisation of β -catenin was not observed for ACF,¹⁷ and β -catenin protein was limited to the cell membranes in ACF as in normal colon epithelial cells; thus changes in β -catenin may not be related to aberrant crypt formation.

The tumour suppressor APC gene plays an important role in colon tumour initiation, as shown previously¹⁸; that is, APC is thought to mediate the interaction between β -catenin and glycogen synthase kinase 3 β (GSK-3 β). GSK-3 β phosphorylates β -catenin which targets β -catenin for ubiquitin dependent, proteasome mediated degradation.^{19,20} Furthermore, wild-type APC represses c-Myc oncogene transcription through inhibition of β -catenin/T cell factor 4 (Tcf-4) complex regulated transcription.²¹

In the present study, we examined the effect of two NSAIDs (sulindac and etodolac) on APC mRNA expression in the colon of AOM treated rats. We reasoned that if non-COX biochemical pathways are involved, identification of these pathways should reveal promising new targets for the design of antineoplastic agents.

Materials and methods

ANIMALS

Five week old male F344 rats purchased from SLC (Shizuoka, Japan) were quarantined for one week and allocated randomly to an experimental or control group. All animals were housed 3–4 per wire cage. The holding room was maintained at 23°C, 50% humidity, and with a 12 hour light/12 hour dark cycle. Ethics approval for the study was obtained from the Committee for Animal Experimentation of Tottori University.

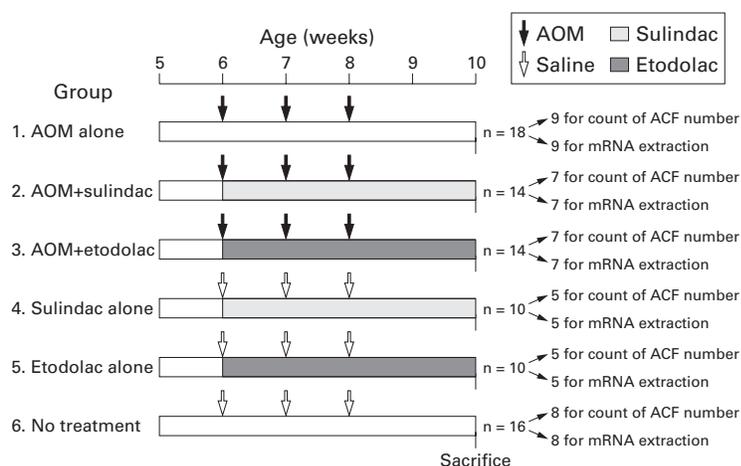


Figure 1 Experimental protocol. Azoxymethane (AOM) was given as 15 mg/kg by subcutaneous injection, saline as 0.3 ml subcutaneously, sulindac as 10 mg/kg in 5% gum arabic aqueous solution three times a week by oral gavage, and etodolac as 10 mg/kg in 5% gum arabic aqueous solution three times a week by oral gavage.

CHEMICALS

AOM (Sigma Chemical Co., St Louis, Missouri, USA) was used to induce ACF in the rat colon. Sulindac (cis-5-fluoro-2-methyl-1-[p-(methyl-sulphonyl) benzylidene] indene-3-acetic acid; Sigma Chemical Co.) inhibits both COX-1 and COX-2 activities with little selectivity.^{22,23} Etodolac ((±)-1,8-diethyl-1,3,4,9,-tetrahydropyrano-[3,4-b] indole-1-acetic acid; generous gift from Wyeth-Ayerst Research Co., Princeton, New Jersey, USA) is a selective COX-2 inhibitor with 10-fold more potent inhibition of COX-2 than of COX-1.²⁴

EXPERIMENTAL PROCEDURES

A total of 82 male rats were divided into six groups (fig 1). Starting at six weeks of age, rats in groups 1, 2, and 3 were treated subcutaneously with AOM in sterile saline at a dose of 15 mg/kg body weight once a week for three weeks. Group 1 received no NSAID. Groups 2 and 3 were given 10 mg/kg body weight sulindac or etodolac, respectively, in 5% gum arabic aqueous solution by oral gavage three times a week (Monday, Wednesday, and Friday) during the experiment. Groups 4 and 5 were given 10 mg/kg body weight sulindac or etodolac, respectively, in the same manner as in groups 2 or 3, without administration of AOM. Group 6 did not receive any agent (AOM or NSAIDs) throughout the study and served as an untreated control. Animals in groups 4, 5, and 6 were given subcutaneous injections of sterile saline instead of AOM. Each group was subdivided into two subgroups: one for counting the number of ACF and the other for mRNA extraction (fig 1). All rats were sacrificed at 10 weeks of age and the colons removed, flushed with saline, and opened from the anus to the caecum. For mRNA extraction, the opened colon was flattened on glass in ice, and the mid distal region was scraped with a surgical knife to collect the mucosa, which was used immediately to extract mRNA. For counting the number of ACF, the opened colon was fixed flat on a paper filter in 10% buffered formalin for 24 hours.

IDENTIFICATION OF ACF

Fixed colons were stained with 0.5% methylene blue in saline. The number of ACF/colon and number of aberrant crypts in each focus were determined on a microscope at $\times 40$ magnification. ACF were recorded as previously described.^{5,6} ACF were distinguished from the surrounding normal crypts by their swelling and discernible pericryptal zone. Crypt multiplicity was defined as the number of aberrant crypts in each focus, categorised as either one, two, three, or ≥ 4 aberrant crypts/focus. The scores were checked by two observers in a double blind manner.

RT-COMPETITIVE PCR TO ESTIMATE COX-1, COX-2, AND APC mRNA EXPRESSION LEVELS

Northern analysis is widely used to quantify specific mRNAs and we tried to detect levels of expression of COX-2, COX-1, and APC mRNA using this method. We were able to detect expression of COX-1 and APC mRNA

using northern blot analysis as mentioned below, but we failed to detect COX-2 mRNA as its expression was very low or below the limit of detection in rat colon in groups 1–6. Therefore, we used the reverse transcription (RT)-competitive polymerase chain reaction (PCR) technique using a competitor DNA in the same PCR reaction to quantitate COX-2, COX-1, and APC mRNA levels in addition to northern blot analysis. The RT-competitive PCR method is more sensitive than northern blotting for quantifying specific mRNAs. The RT-competitive PCR method is explained below.

Colon mucosa was scraped with a surgical knife in the mid distal colon because ACF have been reported to occur more frequently in the mid and distal colons of rats treated with AOM.¹¹ Also, Park and colleagues²⁵ indicated that ACF were marker lesions for colonic neoplasms in the mid and distal colon where tumours were colocalised with adenoma-carcinoma sequences, whereas this was not so for the proximal colon where poorly differentiated, mucin secreting carcinomas were found. Extraction of mRNA from the scraped colon mucosa was performed using a QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions, and cDNA was prepared by RT of mRNA using a First-Strand cDNA Synthesis Kit (Pharmacia Biotech, Uppsala, Sweden). Competitive PCR amplification for quantitation of mRNA levels was performed in a 10 µl reaction volume containing 20 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP) at 200 µM, each primer at 0.5 µM (table 1), 0.25 U of AmpliTaq polymerase (Perkin-Elmer, New Jersey, USA), 1.0 µl of cDNA from the RT reaction (target cDNA with unknown concentration), and 1.0 µl of mimic DNA (10-fold serial dilutions of mimic DNA; ex. 10⁻¹–10⁻⁷ ng/µl) as described in our previous report.²⁶ The conditions for the PCR amplification were: 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for one minute, for 25 cycles. PCR products were separated on 1.8% agarose gels and stained with ethidium bromide.

The amount of target cDNA of a given gene was estimated as follows (fig 2): firstly, we determined the intensity of the bands of target cDNA and mimic DNA present in each lane

Table 1 Primer sequences used for the competitive polymerase chain reaction (PCR) method

Gene	Oligonucleotide	Sequence (5'-3')	PCR products (bp)	
			Target	Mimic
Rat	Sense primer	GAT AAG GAC GAT ATG TCA CG		
APC	Antisense primer	TGA ATG ATG TTG TGG AGG GC	200	400
Rat	Sense primer	TCA AGA CAG ATC AGA AGC GA		
COX-2	Antisense primer	TAC CTG AGT GTC TTT GAT TG	185	424
Rat	Sense primer	CGA GGA TGT CAT CAA GGA G		
COX-1	Antisense primer	TCA GTG AGG CTG TGT TAA CG	350	174
Rat	Sense primer	TAC CAC TGG CAT TGT GAT GG		
β-actin	Antisense primer	TTA ATG TCA CGC ACG ATT TC	198	424

Expected size of product is provided in the right hand column. bp, base pair.

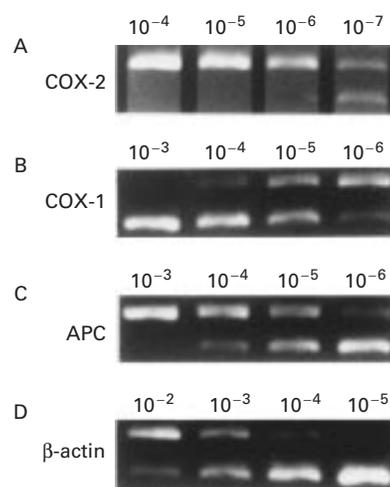


Figure 2 Analysis of cyclooxygenase (COX)-2, COX-1, adenomatous polyposis coli (APC), and β-actin mRNA expression. mRNA extracted from rat colonic tissue was reverse transcribed and amplified in the presence of different amounts of mimic DNA (lanes, from left to right: 10⁻⁴, 10⁻⁵, 10⁻⁶, 10⁻⁷ ng for COX-2; 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶ ng for COX-1 and APC; 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵ ng for β-actin). (A) COX-2 expression in colonic tissue of a representative rat in group 3. The level of COX-2 expression was 10⁻⁷ ng in this case. (B) COX-1 expression in the same rat as in (A). The level of target cDNA of COX-1 was 10⁻³ ng in this case. (C) APC expression in colonic tissue of a representative rat in group 2. The level of target cDNA of APC was 10⁻³ ng in this case. (D) β-actin expression in the same rat as in (C). The level of target cDNA of β-actin was 10⁻³ ng in this case. The level of β-actin expression was used as an internal control.

using a densitometer. Next, the intensity of the target DNA band (the amount of target DNA) was multiplied by the ratio of the length of the mimic DNA to the length of the target cDNA to correct for increased ethidium bromide staining by the larger fragment. The point of equivalence (that is, a 1:1 ratio) is where the value for target cDNA equals that for mimic DNA, and represents the concentration of target cDNA of the gene being examined. The most accurate results are obtained when competitor template and unknown template are amplified at nearly equivalent concentrations, as described above. We generally perform an initial titration in 10-fold increments to determine the approximate concentration of the unknown cDNA. We then perform a finer titration to obtain more accurate results. Two-fold differences in cDNA concentrations can be accurately determined.²⁷ The level of β-actin expression was used as an internal control. Levels of COX-2, COX-1, and APC expression in each case were normalised to that of the corresponding β-actin mRNA (=10⁻³ ng of mimic DNA).

“Mimic DNAs” were DNA fragments used as competitors in PCR amplification for quantitation of mRNA levels of target genes.²⁸ Each mimic DNA consisted of a non-homologous DNA fragment which was recognised by a pair of gene specific primers. Serial dilutions of mimic DNAs were added to the PCR amplification reactions containing constant amounts of the experimental cDNA sample. The mimic and target templates thus competed for the same primers in the reaction. By knowing the amount of mimic DNA that was added to the

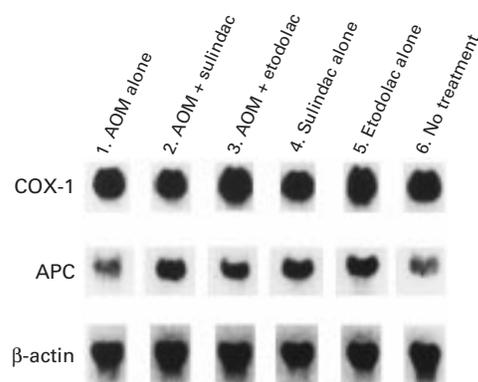


Figure 3 Northern blot analysis of cyclooxygenase (COX)-1 and adenomatous polyposis coli (APC) mRNAs from each group (groups 1, 2, 3, 4, 5, and 6). β -Actin expression was used as an internal control.

reaction, mRNA levels could be determined. Mimic DNAs for the COX-2, COX-1, APC, and β -actin genes were prepared using a PCR MIMIC Construction Kit (Clontech, California, USA) according to the manufacturer's instructions.

NORTHERN BLOT ANALYSIS TO ESTIMATE COX-1 AND APC mRNA EXPRESSION LEVELS

Rat COX-1 and APC cDNA probes were prepared using RT-PCR. Total RNA for amplification of these cDNAs was isolated from the spleen of a rat. The primers used were as follows: 5'-AACCGTGTGTGTGACTTGCTGAA-3' and 5'-AGAAGGAGCCCCTCAGAGCTCAGTG-3' for COX-1 and 5'-GTGGAATGGTGTATTTCATTGTT-3' and 5'-AACTTCCAAAGCACATTCCATCA-3' for APC. The products corresponding to COX-1 (887 base pairs) and APC (804 base pairs) were purified from polyacrylamide gels and used after their sequences had been confirmed to be completely identical to known ones (with reference to the databases of GenBank and EMBL). The cDNA probes were 32 P labelled using the random primer method (Ready-To-Go; Pharmacia Biotec, Uppsala, Sweden). Poly (A)⁺ mRNAs (1 μ g) extracted from rats in each group were separated by electrophoresis on 1.2% agarose gels, transferred to nylon membranes (Gene Screen Plus; New England Nuclear, Boston, Massachusetts, USA), and hybridised with 32 P labelled cDNA probes (fig 3). The level of β -actin expression was used as an internal control. Levels of COX-1 and APC expression in each case were normalised to that

of the corresponding β -actin mRNA level. We tried to detect mRNA expression of COX-2 in the same manner using northern blot analysis but we failed as expression of COX-2 mRNA was very low or undetectable in rat colon tissues in this study.

STATISTICAL ANALYSIS

The number of ACF in each group was expressed as mean (SD), and differences between groups were analysed using the Student's *t* test; $p < 0.05$ was considered significant. The Mann-Whitney test was used to analyse gene expression levels which were examined by the RT-competitive PCR method, and differences were considered significant at $p < 0.05$. mRNA expression levels estimated by northern blot analysis were expressed as mean (SEM), and the differences between groups were analysed using the Student's *t* test; $p < 0.05$ was considered significant.

Results

GENERAL OBSERVATIONS

There were no significant differences in body weight, liver weight, or the ratio of liver weight to body weight among groups at the end of the experiment (data not shown). In this study, stomachs showed no erosive or ulcerative changes, and there were no toxic changes in the liver of any animal.

ABERRANT CRYPTS

Rats in groups 4, 5, and 6 showed no evidence of ACF formation in the colon; animals in these groups were not treated with AOM. In rats in group 1, AOM treatment induced an average of 47.6 ACF/colon and 3.0 foci containing multiple (≥ 4) aberrant crypts/focus (table 2). ACF were predominantly observed in the mid and distal colon. Sulindac (group 2) significantly reduced the total occurrences of ACF/colon and of multicrypt clusters containing 2 crypts/focus; this finding is consistent with a previous study.¹¹ Administration of etodolac (group 3) significantly reduced the total occurrences of ACF/colon and of multicrypt clusters containing 2, 3, or even 4 or more crypts/focus compared with group 1 (table 2). This is the first report showing that etodolac suppresses ACF formation.

COX-2, COX-1, AND APC mRNA EXPRESSION ESTIMATED BY RT-COMPETITIVE PCR

COX-2 mRNA expression was not detected in normal colonic mucosa (groups 4, 5, and 6) by

Table 2 Effects of non-steroidal anti-inflammatory drugs (NSAIDs) on azoxymethane (AOM) induced aberrant crypt foci (ACF) in rat colon

Group	Incidence ^b	No of ACF/colon ^c	No of foci containing			
			1 crypt ^e	2 crypts ^e	3 crypts ^e	≥ 4 crypts ^e
1. AOM alone	9/9	47.6 (5.2)	14.0 (4.9)	24.7 (4.2)	5.9 (1.8)	3.0 (2.0)
2. AOM+sulindac ^d	7/7	23.4 (6.8) ^e	8.4 (4.3)	10.4 (3.6) ^e	3.4 (2.2)	1.1 (0.6)
3. AOM+etodolac ^d	7/7	21.9 (4.3) ^e	4.7 (1.8) ^e	13.0 (3.2) ^e	3.2 (1.0) ^d	0.9 (0.6) ^d
4. Sulindac alone ^d	0/5	0	0	0	0	0
5. Etodolac alone ^d	0/5	0	0	0	0	0
6. No treatment	0/8	0	0	0	0	0

^aEach NSAID was given as 10 mg/kg.

^bNumber of rat colons with ACF/total number of colons scored.

^cMean (SD).

^dSignificantly different from group 1 by Student's *t* test (^e $p < 0.05$ and ^d $p < 0.01$).

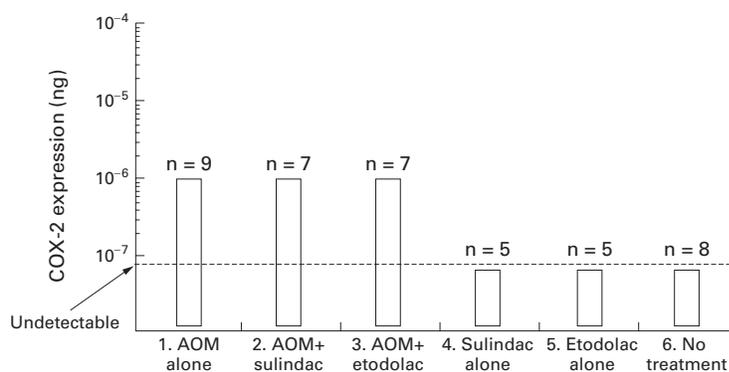


Figure 4 Levels of COX-2 mRNA expression measured by RT-competitive PCR. Data are shown as box plots: the box covers all values (including minimum and maximum values). The broken line indicates the limit of detection for COX-2 mRNA expression by RT-PCR. Levels of COX-2 expression in each case were normalised to that of β -actin mRNA (10^{-3} ng of mimic DNA). There was a tendency towards increased levels of COX-2 expression in the groups administered azoxymethane (AOM). However, levels were not significantly different between groups.

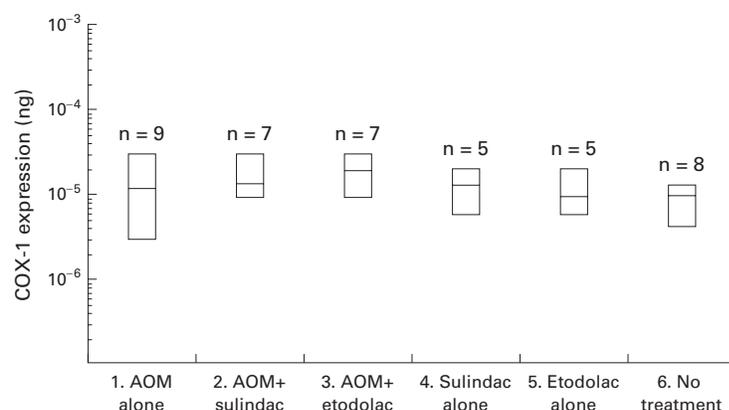


Figure 5 Levels of COX-1 expression measured by RT-competitive PCR. Data are shown as box plots: the box covers all values (including minimum and maximum values), and the central continuous line in each box represents the mean. Levels of COX-1 expression in each case were normalised to that of β -actin mRNA (10^{-3} ng of mimic DNA). There was no difference in COX-1 mRNA expression levels between groups. AOM, azoxymethane.

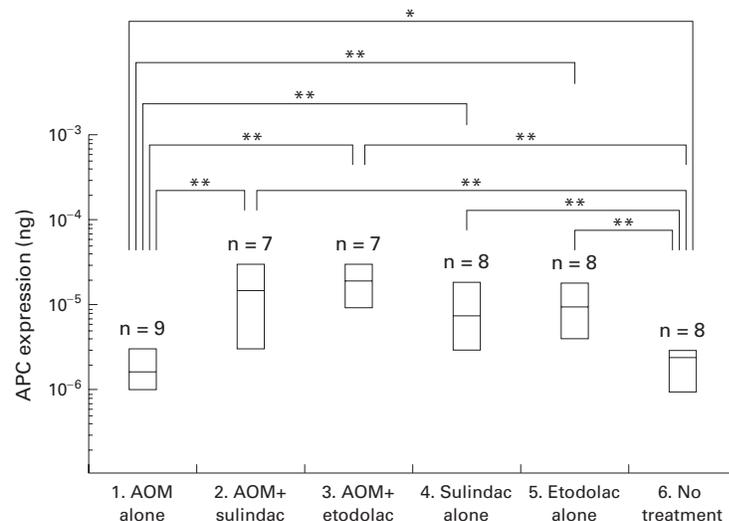


Figure 6 Levels of adenomatous polyposis coli (APC) expression measured by RT-competitive PCR. Data are shown as box plots: the box covers all values (including minimum and maximum values), and the central continuous line in each box represents the mean. Levels of APC expression in each case were normalised to that of β -actin mRNA (10^{-3} ng of mimic DNA). APC mRNA expression level in group 1 (azoxymethane (AOM) alone) was significantly lower than that in group 6 (untreated control) ($p < 0.05$). Both sulindac and etodolac caused an increase in APC mRNA in the rat colonic mucosa (groups 2, 3, 4, and 5) ($p < 0.01$). * $p < 0.05$; ** $p < 0.01$.

RT-PCR (fig 4). There was a tendency towards increased levels of expression of COX-2 in groups 1, 2, and 3, which were treated with AOM, compared with levels in the other groups (groups 4, 5, and 6), presumably due to AOM exposure; however, differences between levels in groups 1, 2, and 3 and levels in the other groups (groups 4, 5, and 6) were not significant (fig 4). There was no difference in levels of COX-1 mRNA expression among the groups (fig 5). The level of APC mRNA expression in group 1 (AOM alone) was significantly lower (about 50%) than that in group 6 (untreated control) ($p < 0.05$). However, levels of expression of APC mRNA in groups 2, 3, 4, and 5 (administered NSAIDs) were significantly increased (about 5–10-fold) compared with those in groups 1 and 6 (fig 6).

COX-1 AND APC mRNA EXPRESSION ESTIMATED BY NORTHERN BLOT ANALYSIS

We tried to detect levels of COX-2 mRNA expression using northern blot but levels of COX-2 mRNA expression were very low or undetectable in this study. We estimated levels of COX-1 and APC mRNA expressions by northern blot analysis. There was no difference in COX-1 mRNA levels among the groups (fig 7). The level of APC mRNA expression in group 1 (AOM alone) was lower (about 66%) than that in group 6 (untreated control) ($p < 0.05$). Levels of expression of APC mRNA in groups 2, 3, 4, and 5 (administered NSAIDs) were significantly increased (about 3–4-fold) compared with those in groups 1 and 6 (fig 7). These results were essentially the same as those shown by RT-competitive PCR.

Discussion

NSAIDs have been shown to have chemopreventive activity in animal models and human trials, especially against colon carcinogenesis.^{29–31} However, the precise mechanism of the activity is not clear. Recently it was reported that COX-2 expression was increased in colon cancers while COX-1 expression was not.^{32–34} The most direct way to determine the role of COX-2 in intestinal neoplasia would be to use a genetic approach. Oshima and colleagues³⁵ used such an approach by assessing the development of intestinal polyposis in *Apc*^{A716} mice, which had both a wild-type *Apc* allele and a mutant allele (*Apc*^{A716}) in cells, in a wild-type and homozygous null COX-2 genetic model. The number and size of polyps were reduced dramatically in the COX-2 null mice compared with COX-2 wild-type mice.³⁵ In addition, treatment of the *Apc*^{A716} mice with a COX-2 inhibitor, MF tricyclic, reduced polyp number more significantly than treatment with the non-COX specific inhibitor sulindac.³⁵ These results provide almost unequivocal support for the argument that COX-2 plays a major role in polyp formation. Furthermore, Takahashi and colleagues¹² recently reported that nimesulide, a selective COX-2 inhibitor, reduced the formation of AOM induced ACF in the rat colon. However, the mechanism of

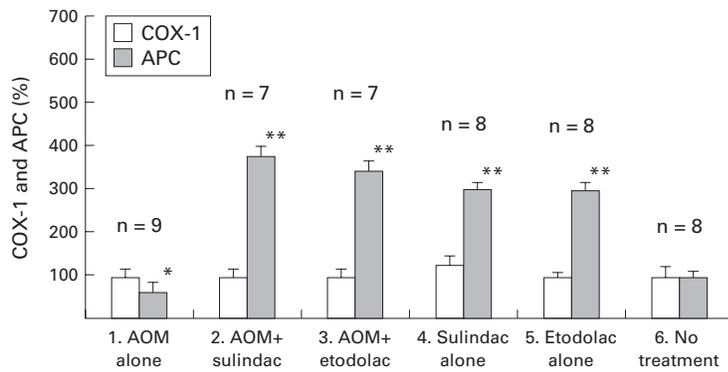


Figure 7 COX-1 and APC mRNA expression measured by northern blot analysis. mRNA levels were measured by northern blot analysis as described in materials and methods. Values are expressed as the ratio (%) to the average of those of group 6 (no treatment). Levels of APC mRNA expression in group 1 were lower than those in group 6 ($p < 0.05$). Levels of APC mRNA expression in groups 2, 3, 4, and 5 were significantly increased compared with those in group 6 ($p < 0.01$). * $p < 0.05$; ** $p < 0.01$.

suppression of ACF formation by NSAIDs, including COX-2 inhibitors, is not well understood. Tsujii and DuBois³⁶ reported that intestinal epithelial cells overexpressing the COX-2 gene showed altered adhesion properties and resistance to apoptosis induced by butyrate. In addition, there are published reports of anti-neoplastic activities of NSAIDs that are independent of COX-1 or COX-2. Such findings are not surprising—many drugs have multiple activities depending on a variety of pharmacokinetic parameters. Schnitzler and colleagues¹⁴ demonstrated that both the sulphide metabolite of sulindac, which can inhibit COX, and the sulphone metabolite, which lacks this ability, can inhibit the growth of a colonic carcinoma cell line and cause an increase in APC mRNA in colonic carcinoma cells. Aspirin has well documented non-COX effects, including inhibition of NF- κ B activation,³⁷ and has been shown to induce apoptosis by activation of p38 kinase in normal human fibroblasts.³⁸ More recently, several NSAIDs were shown to be highly effective activators of the nuclear receptor peroxisome proliferator-activated receptor γ , demonstrating the potential of direct gene transcriptional effects by NSAIDs.³⁹ These results suggest that the effect of these NSAIDs on colonic carcinogenesis may not be mediated entirely by inhibition of COX.

In the present study, we established that the COX-2 inhibitor etodolac reduced the formation of AOM induced ACF in the rat colon. Furthermore, suppression by sulindac of the occurrence of ACF induced by AOM in rat colon was also confirmed. Apart from the suppressive effect on colon carcinogenesis, both sulindac and etodolac induced an increase in expression of APC mRNA. The precise role of the increased APC mRNA in suppressing ACF formation was not clarified in this study. However, we noted that the level of expression of APC mRNA in the group treated with AOM alone (group 1) was lower than that of the untreated control group (group 6) ($p < 0.05$). When the amount of APC is comparatively less than the normal level in colonic mucosa treated with AOM, the

function of APC may not be sufficient to maintain normal cell functions, including the cell cycle control system. It is possible that the findings in this study, namely an increase in expression of APC mRNA in response to the two NSAIDs (sulindac and etodolac) in rat colon mucosa might be related to reduction of ACF development. The finding that these drugs have an effect on APC mRNA is consistent with the evidence from clinical studies indicating an effect of sulindac on polyps in patients with familial adenomatous polyposis (FAP) compared with sporadic polyps in non-FAP patients.^{40–41} In individuals with FAP, the inactivating germline mutation causes a reduction in the amount of wild-type APC protein produced, which may possibly be below a threshold necessary to prevent neoplasia.⁴²

An intriguing interaction between APC and β -catenin was reported recently. β -catenin is a key regulator of the cadherin mediated cell-cell adhesion system and an important element in the Wnt signal transduction pathway. Inomata and colleagues⁴³ showed that β -catenin was localised at the membranes of the cell-cell borders in normal colon epithelial cells but in the nucleus and cytoplasm in both adenoma and cancer cells. It was recently reported that stabilisation and accumulation of cytoplasmic β -catenin, which resulted from mutations in either the β -catenin or APC genes, were causally associated with colon carcinogenesis.^{20–44–45} In addition, Takahashi and colleagues¹⁷ reported frequent mutations of the β -catenin gene in rat colon carcinomas induced by AOM together with altered cellular location of β -catenin (cytoplasmic and nuclear accumulation), but no abnormal cytoplasmic or nuclear accumulation of β -catenin in ACF or the normal appearing background colon mucosa. We also found no abnormal accumulation of β -catenin in ACF and the normal appearing colon mucosa using an immunohistochemical method (data not shown). This suggests that the aberration in β -catenin may not exist in the initiation stage of cancer development. In addition, Filippo and colleagues⁴⁶ reported that APC gene mutations were not observed in AOM induced ACF of F344 rats. However, even normal appearing colon cells might have an abnormal proliferative function in AOM treated rats because the number of argyrophilic nucleolar organiser region/nucleus, one of the biomarkers of cell proliferation, was significantly greater in AOM treated than in non-AOM treated rats.⁶ In the model of AOM induced rat colon carcinogenesis, a comparative decrease in APC mRNA expression in colonic mucosa may be one of the causes of ACF formation, as described above; it is probably not due to APC gene mutations. He and colleagues²¹ reported that wild-type APC directly modulated c-Myc transcription through β -catenin/Tcf-4; namely, wild-type APC prevented β -catenin from forming a complex with Tcf-4 and activating c-Myc oncogene. Induction of APC mRNA by NSAIDs may be one of the causes of a reduction in ACF development. Further

examination is needed to elucidate the mechanism(s) by which NSAIDs induce an increase in APC mRNA.

Several different mechanisms have been proposed to account for the action of NSAIDs in preventing colon neoplasia, including inhibition of COX activity, induction of apoptosis, effects on expression of various genes, etc. It is likely that a combination of different mechanisms, some of which may be interrelated, will ultimately be found to mediate the effects of sulindac and etodolac on the colon epithelium.

Prolonged administration of NSAIDs is known to have side effects, such as gastrointestinal ulceration and renal toxicity.^{47,48} As most NSAIDs inhibit COX-1 rather than COX-2,⁴⁹ the mechanism of these side effects of NSAIDs is considered to be associated with an imbalance in prostaglandins which are produced by constitutive COX-1 in the stomach and/or kidney.^{50,51} Etodolac, a COX-2 inhibitor, can be used for long term treatment without side effects, such as gastrointestinal ulceration and renal toxicity. This study is the first report indicating that etodolac is a COX-2 inhibitor which has potential as a chemopreventive agent against colon cancer.

This work was supported in part by Grant-in-Aid for Scientific Research 11770272 from the Ministry of Education, Science, Sports and Culture, Japan, and by a research grant from Yokoyama Foundation for Clinical Pharmacology.

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