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Inhibitory Effect of NS-398, a Selective Cyclooxygenase-2 Inhibitor, on Azoxymethane-induced Aberrant Crypt Foci in Colon Carcinogenesis of F344 Rats

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Prostaglandin E_2, which is produced by cyclooxygenase (COX) during arachidonic acid metabolism, is considered to be related to colon carcinogenesis. Therefore, the effect of NS-398 (*N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide), a COX-2 inhibitor, was examined in azoxymethane (AOM)-induced colon carcinogenesis in rats in this study. In the first experiment, groups 1–3 were treated with AOM (15 mg/kg, s.c.) 3 times at intervals of a week from 5 weeks of age. Groups 2 and 3 were respectively given 1 mg/kg and 10 mg/kg of NS-398 in 5% gum arabic aqueous solution 3 times per week by oral gavage during the experiment. Six weeks after the first exposure to AOM, aberrant crypt foci (ACF) were counted in the colonic mucosa of all rats. The mean occurrence of ACF per length in rats given 1 mg/kg b.w. or 10 mg/kg b.w. of NS-398 was reduced to 65.7% or 52.8%, respectively, of that in rats treated with only AOM. Levels of COX-2 mRNA expression in groups treated with AOM, regardless of NS-398, were slightly higher than that in the group treated with NS-398 alone as judged from reverse transcription-polymerase chain reaction analysis. In the second experiment, the effect of NS-398 at different times, i.e., during initiation and post-initiation, was examined. Treatment with NS-398 in both phases significantly inhibited the appearance of ACF. The results imply that NS-398 might have a chemopreventive potential.

Key words: Cyclooxygenase-2 — Chemoprevention — Rat colon carcinogenesis

Since colorectal cancer is an increasingly important cause of cancer deaths worldwide, including Japan,[1] the feasibility of a chemopreventive approach is of interest. Based on epidemiological studies, NSAIDs such as aspirin have been reported to reduce colorectal cancer risk.[2,3] In patients with familial adenomatous polyposis, the administration of sulindac, another NSAID, decreased the size and number of adenomas.[4,5] Moreover, in animal models, several groups have demonstrated chemopreventive effects of NSAIDs, such as aspirin, piroxicam, sulindac and indomethacin, in chemically induced colon carcinogenesis.[6–9]

At present, the mechanism by which NSAIDs inhibit colon carcinogenesis is unclear. However, since NSAIDs are COX inhibitors and the PGs, especially PGE_2, are modulators of cell proliferation,[10] one possible mechanism is for NSAIDs to inhibit PG synthesis from arachidonic acid by COX. In fact, the PGE_2 level in cancerous tissues is elevated when compared with that in the corresponding normal-appearing tissues in humans and rats.[11,12]

COX is one of the rate-limiting enzymes in PG synthesis.[13] Recently, two isozymes of COX have been identified in the rat, constitutive COX-1 and inducible COX-2.[14] While COX-1 exists in most tissues and is involved in the physiological production of PGs under normal homeostasis,[13,15] COX-2 is induced by mitogens, cytokines and growth factors, and is responsible for production of PGs in inflammation.[16–18] In human colon cancers, COX-2 expression has been shown to be increased rather than COX-1 expression.[19–21] We also observed the overexpression of COX-2 mRNA in rat colon carcinogenesis.[22]

NS-398, synthesized by Taisho Pharmaceutical Co. in Japan, has been reported to have high selectivity for COX-2.[23–25] Recently, other COX-2 inhibitors have been shown to inhibit the development of azoxymethane-induced ACF.[26,27] Therefore, in the present study, we examined the effect of NS-398 on the formation of azoxymethane-induced ACF and on expression of COX mRNA in male F344 rats.

MATERIALS AND METHODS

Chemicals AOM was purchased from Sigma (St. Louis, MO) as a colon carcinogenesis. NS-398, *N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide, was supplied

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Abbreviations: NSAIDs, non-steroidal anti-inflammatory drugs; PG, prostaglandin; COX, cyclooxygenase; ACF, aberrant crypt foci; AOM, azoxymethane; PCNA, proliferative cell nuclear antigen; RT-PCR, reverse transcription-polymerase chain reaction; s.c., subcutaneous injection; b.w., body weight.

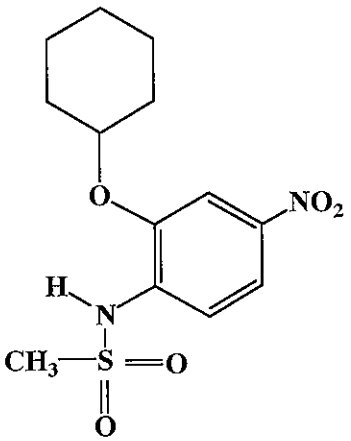

Fig. 1. Structure of NS-398, *N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide. The structure is similar to that of nimesulide.

by Taisho Pharmaceutical Co. (Tokyo) through Dr. N. Futaki (Research Center, Taisho Pharmaceutical Co., Saitama). The molecular structure of NS-398 is shown in Fig. 1.

Animal treatment We employed the two different protocols, as illustrated in Fig. 2.

Experiment I: Forty male F344 rats, 4 weeks old, purchased from Japan SLC Inc. (Hamamatsu), were divided into five groups, and kept in a room controlled at 23± 2°C and 50%±10% humidity on a 12 h light/dark cycle. Groups 1–3 (10 rats each) were treated with AOM, 15 mg/kg b.w., s.c., at 6, 7 and 8 weeks of age. Group 2 was given NS-398, 1 mg/kg b.w., in 5% gum arabic aqueous solution, by oral gavage, 3 times (Monday, Wednesday and Friday) per a week during the experiment. Groups 3 and 4 were treated with 10 mg/kg b.w. of NS-398 in the same manner as group 2. Groups 1 and 5 were treated with 5% gum arabic alone, without NS-398. At 12 weeks of age, all rats were killed, and the colons were removed, flushed with saline and opened from anus to cecum. The opened colon was flattened on glass in ice, and the middle region (approximately 5 cm) was scraped with a surgical knife to collect the mucosa, which was stored at −80°C until RNA extraction. The remaining colon was fixed flat on a paper filter in 10% buffered formalin for 24 h.

Experiment II: We performed the second experiment to examine the effects of NS-398 in the initiation and post-initiation phases. Thirty rats were purchased, treated in the same manner as in experiment I, and divided into three groups. The administration of NS-398 was done in the two different phases as shown in Fig. 2. As in experiment I, groups 6–8 were treated with AOM, 15 mg/kg b.w., s.c., at 6, 7 and 8 weeks of age. Group 7 was treated

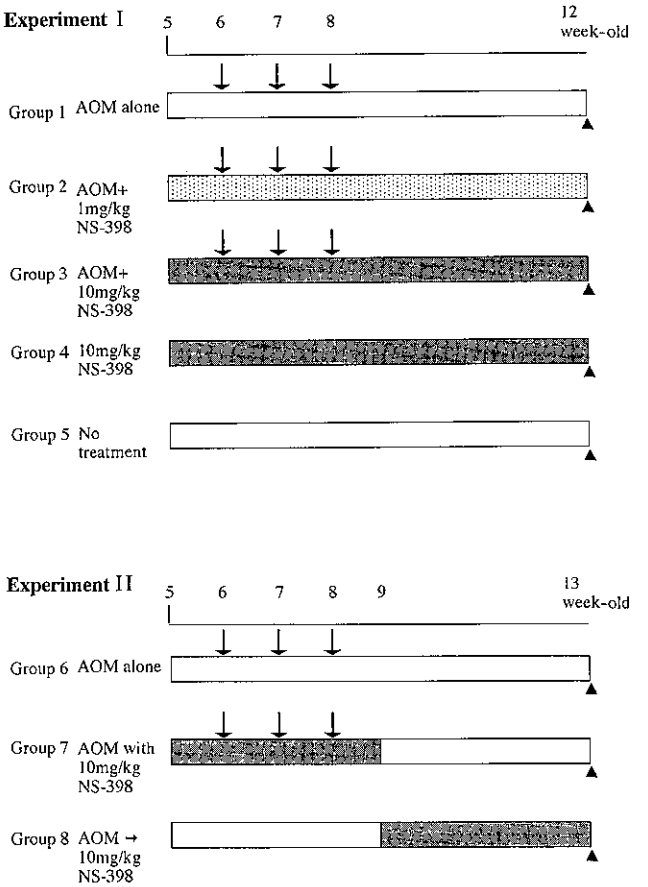

Fig. 2. The experimental protocols. ↓ AOM, 15 mg/kg, s.c. injection; ▲ killed; □ basal diet (CE-2) and 5% gum arabic aqueous solution, oral gavage; ▨ 1 mg/kg of NS-398 in 5% gum arabic aqueous solution, 3 times a week by oral gavage; ■ 10 mg/kg of NS-398 in 5% gum arabic aqueous solution, 3 times (Monday, Wednesday and Friday) a week in experiment I and every morning, except Saturday and Sunday, in experiment II, by oral gavage.

with NS-398, 10 mg/kg b.w., in 5% gum arabic aqueous solution, by oral gavage daily in the morning, except Saturday and Sunday, for 4 weeks, from 5 weeks to 9 weeks of age. Group 8 was treated in the same manner as group 7 from 9 weeks to 13 weeks of age. All rats were killed at 13 weeks of age, and the colons were removed, flushed with saline and opened from anus to cecum. The opened colon was fixed flat on a paper filter in 10% buffered formalin for 24 h to observe ACF in the whole colon without scraping the mucosa.

Identification of ACF The fixed colons were stained with 0.5% methylene blue in saline. ACF were recorded according to the procedure of Bird[28] and our laboratory.[29] ACF were distinguished from the surrounding normal

crypts by their swelling and discernible pericryptal zone. In this study, we observed the mucosa of distal colon (approximately 6 cm from the anus) in experiment I (because the mucosa of the middle colon was scraped off for RNA extraction) and the mucosa of the whole colon in experiment II. The occurrence and the multiplicity of ACF were assessed. The crypt multiplicity means the number of aberrant crypts in each focus, categorized as up to three, or four or more aberrant crypts/focus. The scores were checked by two observers in a double-blind manner.

PCNA immunohistochemistry The distal colon tissues which had been observed for ACF in experiment I were embedded in paraffin for PCNA immunohistochemical analysis. The immunohistochemical staining was performed according to the method in our previous paper.[30] The embedded tissues were sectioned at 4 μm, then stained by using PCNA antibody (Novocastra Lab., Newcastle, UK) and an ABC kit (Vector Lab., Burlingame, CA). The number of PCNA-positive nuclei in crypts per section was counted as described in previous papers.[30, 31]

mRNA of COX-1 and -2 The total RNA was obtained from the stored colon mucosa in experiment I by the rapid method.[22, 32, 33] The mRNA expression levels of COX-1 and -2 were examined by RT-PCR.[22, 33] Briefly, the total RNA (1 μg) was reverse-transcribed into cDNA and then 5 μl of the cDNA solution was amplified in 50 μl of PCR mix; 1× PCR buffer, 2.5 U AmpliTaq DNA polymerase (Perkin Elmer Cetus, Emeryville, CA), 0.2 m*M* 4dNTPs, and 0.4 μM each of 5′ and 3′ primers for rat COX-1, COX-2, or β-actin. The PCR was run in a DNA Thermal Cycler (Perkin Elmer Cetus) for 20 (β-actin), 28 (COX-1) or 30 cycles (COX-2) (94°C for 45 s, then 54°C for COX-2 or 56°C for COX-1 and β-actin for 45 s, then 72°C for 2 min), followed by 5 min at 72°C. The optimal PCR cycle number was confirmed to be within the region of linear amplification by preliminary PCR.[22, 33] The primers for rat COX-1 and -2, and β-actin were designed in our laboratory (COX-1, 5′-ACCCATTTCCTGCTGACACA-3′ (sense), 5′-TGGTGGGTGAAGTGTTGTGC-3′ (antisense); COX-2, 5′-CAGCCCACCAACTTACAATG-3′ (sense), 5′-TACACCTCTCCACCGATGAC-3′ (antisense); β-actin, 5′-GAGGCCCAGAGCAAGAGAGG-3′ (sense), 5′-GCATACAGGGACAACACAGC-3′ (antisense)), based on the cDNA sequences.[14, 34] PCR products were observed by electrophoresis in 2–3% ultra PURE agarose gel (Gibco BRL, Gaithersburg, MD) in Tris-borate/EDTA buffer containing 0.5 μg/ml ethidium bromide. Each band was visualized by UV light and photographed with a Kodak digital camera, then analyzed by image analysis software (BioMax 1D, Kodak, Rochester, NY). The density of each band was normalized with respect to that of the corresponding β-actin band. The sizes of PCR products (COX-1 359 bp, COX-2 411 bp, β-actin 263 bp) were confirmed by using a 1 kbp DNA ladder (Gibco BRL). In all experiments, controls having no cDNA in the PCR mixture were run under conditions identical to those used for the experimental samples, and no PCR products were observed in these controls.

Statistical analysis Data are presented as mean±SD and Student's *t* test or Welch's method was used to determine the significance of differences between groups. Differences were considered to be significant at the $P<0.05$ level.

RESULTS

There were no differences of body weight, liver weight or the relative ratio of liver weight to body weight among groups at the termination of the experiment (Table I). Histopathologically, no differences were seen between groups treated with and without NS-398. In this study, stomachs showed no erosive or ulcerative changes, and there were no toxic changes in the liver of any animal.

Table I. Body Weight, Liver Weight and Relative Ratio of Liver Weight to Body Weight at Experimental Termination

Experiment	Group/Treatment	No. of rats	Body weight (g)	Liver weight (g)	Relative ratio (L/B ×100)
I	1/AOM alone	10	227±15	8.7±1.1	3.8±0.3
	2/AOM+1 mg/kg NS-398	10	230±11	8.9±0.5	3.9±0.2
	3/AOM+10 mg/kg NS-398	10	227±14	8.9±0.9	3.9±0.3
	4/10 mg/kg NS-398 alone	5	224±17	9.0±1.1	4.0±0.3
	5/non treatment	5	222±3	8.9±0.7	4.0±0.3
II	6/AOM alone	10	244±17	8.8±0.7	3.6±0.2
	7/AOM with 10 mg/kg NS-398	10	259±16	9.3±0.7	3.6±0.4
	8/AOM→10 mg/kg NS-398	10	247±12	8.7±0.7	3.5±0.2

No significant difference between any groups.

Table II. AOM-induced ACF Formation in Experiment I

Group/Treatment	No. of rats	Examined colon length (cm)	Total number of ACF	ACF number per unit length (/cm)
1/AOM alone	10	6.24±0.53	51.7±19.0	8.37±3.50
2/AOM+1 mg/kg NS-398	10	6.24±0.59	34.1±10.0[a)]	5.50±1.60[a)]
3/AOM+10 mg/kg NS-398	10	6.05±0.57	27.0±10.2[b)]	4.42±1.50[b)]
4/10 mg/kg NS-398 alone	5	6.22±0.40	0	0
5/non treatment	5	5.82±0.28	0	0

a, b) Significant difference from group 1 (AOM alone) by Welch's method ($P<0.05$ and $P<0.01$, respectively).

Table III. Multiplicity of Aberrant Crypts per Focus in Experiment I

Group/Treatment	No. of foci containing			
	1 crypt	2 crypts	3 crypts	4 or more crypts
1/AOM alone	13±4.6	23±10	10±4.6	5.3±2.8
2/AOM+1 mg/kg NS-398	8.8±3.1[a)]	16±5	6.8±2.9	2.7±2.7[d)]
3/AOM+10 mg/kg NS-398	7.2±3.3[b)]	11±5.7[c)]	6.8±3.3	2.2±1.5[b)]

a, b) Significant difference from group 1 (AOM alone) by Welch's method ($P<0.05$ and $P<0.01$, respectively).
c, d) Significant difference from group 1 (AOM alone) by Student's *t* test ($P<0.01$ and $P<0.05$, respectively).

Table IV. AOM-induced Whole ACF Formation in Experiment II

Group/Treatment	No. of rats	Total number of ACF	ACF number per unit length (/cm)
6/AOM alone	10	123.3±11.7	5.62±0.63
7/AOM with 10 mg/kg NS-398	10	102.0±9.6[a)]	4.58±0.44[a)]
8/AOM→10 mg/kg NS-398	10	85.0±19.8[a, b)]	3.90±1.06[a)]

a) Significant difference from group 6 (AOM alone) by Student's *t* test ($P<0.01$).
b) Significant difference between groups 7 and 8 by Welch's method ($P<0.05$).

The number of ACF per measured colon, the mean number of ACF per unit length (cm) and the multiplicity of aberrant crypts per focus in experiment I are shown in Tables II and III. The number of ACF per unit length (cm) in group 1 was 8.37±3.50. Those in groups 2 and 3 were much fewer than that in group 1 (5.50±1.60 and 4.42±1.50, $P<0.05$ and $P<0.01$ by Welch's method, respectively). Namely, the mean occurrence of ACF in groups 2 and 3 was reduced to 65.7% and 52.8% of that in group 1, respectively (Table II). No ACF were detectable in rats not treated with AOM, regardless of NS-398 treatment. In Table III, the multiplicity of aberrant crypts per observed ACF is shown. The numbers of ACF consisting of 4 or more crypts as well as of one crypt in groups treated with NS-398 were also significantly decreased (Table III).

In experiment II, both the total number of ACF and the number of ACF per unit length in groups 7 (102.0± 9.6 and 4.58±0.44) and 8 (85.0±19.8 and 3.90±1.06) were reduced, compared with those in group 6 (123.3± 11.7 and 5.62±0.63) (each $P<0.01$ by Student's *t* test) (Table IV). In addition, the total number of ACF in group 8 treated with NS-398 in the post-initiation phase was fewer than that of group 7 treated in the initiation phase ($P<0.05$ by Welch's method) (Table IV). ACF with 4 or more crypts in groups 7 and 8 were also decreased (Table V). The number of ACF with one crypt in group 8 was significantly smaller than that in groups 6 and 7 (Table V).

In immunohistochemistry for PCNA, the appearance of PCNA-stained cells in groups 1, 2 and 3 was increased by AOM exposure (Table VI). However, PCNA-stained

Table V. Multiplicity of Aberrant Crypts per Focus in Experiment II

Group/Treatment	No. of foci containing			
	1 crypt	2 crypts	3 crypts	4 or more crypts
6/AOM alone	34.3±7.2	43.0±4.5	30.2±9.5	16.4±3.5
7/AOM with 10 mg/kg NS-398	35.3±7.1	35.2±6.3[a)]	20.7±4.6[b)]	11.3±4.9[c)]
8/AOM→10 mg/kg NS-398	21.0±1.4[d)]	36.1±4.2[a)]	20.6±7.1[a)]	7.1±4.8[a)]

a, c) Significant difference from group 6 (AOM alone) by Student's *t* test ($P<0.01$ and $P<0.02$, respectively).
b, d) Significant difference from group 6 (AOM alone) by Welch's method ($P<0.02$ and $P<0.01$, respectively).

Table VI. PCNA-stained Cell Index

Group/Treatment	No. of rats	PCNA-stained cell index (%)
1/AOM alone	10	8.54±2.43[a)]
2/AOM+1 mg/kg NS-398	10	6.34±1.86[b, c)]
3/AOM+10 mg/kg NS-398	10	6.17±1.49[b, d)]
4/10 mg/kg NS-398 alone	5	4.32±1.64[e)]
5/no treatment	5	3.98±1.32

a, b) Significant difference from group 5 as control by Student's *t* test ($P<0.01$ and $P<0.05$, respectively).
c, d) Significant difference from group 1 (AOM alone) by Student's *t* test ($P<0.05$ and $P<0.02$, respectively).
e) Not significantly different from group 5.

Table VII. Relative Ratio of COX-1 or COX-2 Expression to β-Actin Expression Determined by RT-PCR Analysis

Group/Treatment	No. of rats	COX-1/β-actin (%)	COX-2/β-actin (%)
1/AOM alone	10	60.3±11.7	51.5±18.9
3/AOM+10 mg/kg NS-398	10	52.5±8.0	44.8±4.9
4/10 mg/kg NS-398 alone	5	53.6±8.1	33.9±5.9[a)]
5/no treatment	5	57.4±14.8	37.6±13.9

a) Significant difference from group 1 (AOM alone) by Welch's method ($P<0.05$) or group 3 (AOM+NS-398) by Student's *t* test ($P<0.01$).
Others: no significant difference between any groups.

cell indices (6.34±1.86 and 6.17±1.49, respectively) in groups 2 and 3 treated with AOM and NS-398 were significantly fewer than that (8.54±2.43) of group 1 ($P<0.05$ and $P<0.02$, by Student's *t* test, respectively), whereas there was no difference between groups without AOM, regardless of NS-398 treatment (Table VI).

In RT-PCR analysis, the PCR (30 cycles) detecting COX-2 expression required 2 more reaction cycles than that (28 cycles) for constitutive COX-1 to observe a similar density of PCR product in electrophoresis. The inducible COX-2 was observed even in normal colonic mucosa by RT-PCR, in agreement with the previous finding.[22)] For COX-1, there was no difference between any of the groups, although the expression in groups treated with NS-398 showed a decreasing trend (Table VII). For COX-2, the expressions in groups 1 and 3 treated with AOM were slightly increased compared with those in groups 4 and 5, presumably due to the AOM exposure. In addition, NS-398 treatment tended to inhibit the expression of COX-2 (group 1 vs. 3 and group 4 vs. 5), but the effect was not statistically significant (Table VII). The results are consistent with the idea that NS-398 is selective for COX-2 rather than COX-1.

DISCUSSION

NSAIDs have been shown to have chemopreventive activity in animal models and human trials, especially against colon carcinogenesis.[2, 4, 9)] However, the prolonged administration of NSAIDs is well known to have side effects such as gastrointestinal ulceration and renal toxicity.[35, 36)] Since most NSAIDs inhibit COX-1 rather than COX-2,[37)] the mechanism of these side effects of NSAIDs is considered to be associated with an imbalance of PGs, which are produced by constitutive COX-1 in the above tissues.[38, 39)] It was reported that COX-2 expression was increased in colon cancers, rather than COX-1 expression.[19–21)] Intestinal epithelial cells overexpressing COX-2 gene showed altered adhesion properties and resistance to apoptosis induced by butyrate.[40)] Therefore, selective COX-2 inhibitors may have chemopreventive potential with significantly reduced unwanted effects on the stomach and kidney.

NS-398 is a new NSAID with activity similar to that of indomethacin in analgesic and anti-inflammatory tests in rats, but no significant gastric lesions were seen even when NS-398 was given at 1000 mg/kg b.w. as a single oral dose.[23)] Moreover, this compound inhibited the

COX-2 of sheep placenta with a potency equal to that of indomethacin, but had no effect on COX-1 in ram seminal vesicles.[25] This COX-2 selectivity is approximately 80-fold higher than that of indomethacin when assessed with platelet COX-1 and monocyte COX-2.[41] Although NS-398 has not been fully assessed from the viewpoint of safety for human use, we utilized its characteristics as a selective COX-2 inhibitor to investigate the influence of COX in colon carcinogenesis. In this study, NS-398 inhibited the development and growth of AOM-induced ACF in rat colon.

The inhibitory mechanism of NS-398 may be related to the inhibition of the production of PGs in tissues, as in the case of other NSAIDs.[2-10] In this study, the PCNA-stained cell index in rats treated with AOM was decreased by NS-398 treatment, while no effect of NS-398 on cell proliferation in normal crypts was observed in terms of PCNA-immunohistochemistry. It is suggested that NS-398 inhibits cell proliferation by inhibiting the production of PGs induced by AOM. In experiment II, the inhibitory effect of NS-398 in the post-initiation phase was higher than that in the initiation phase. Therefore, NS-398 might be effective against the growth of ACF rather than their development. However, we could not observe significant changes of COX-2 mRNA expression levels in any group, regardless of NS-398, in this study, although colon neoplasms and the surrounding colonic mucosa in carcinogen-treated rats overexpressed COX-2 mRNA compared with the normal mucosa in the previous study.[22] The reasons may be as follows; (1) false-negative results due to the admixture of many normal crypts and few ACF, (2) NS-398 inhibits the activity of COX-2 for PG synthesis but not the expression of COX-2 itself, and (3) others. The second possibility seems likely. However, another mechanism may be related to the effect of apoptosis induced by NSAIDs, since recent studies have suggested that cell death caused by apoptosis may be responsible for the chemopreventive effects of NSAIDs such as sulindac, aspirin metabolites, naproxen, indomethacin and piroxicam on colorectal cancer cell lines.[42-44] We found that NS-398 induced apoptosis in colorectal cell lines as potently as indomethacin.[45] The fact that patients with familial adenomatous polyposis, who take NSAIDs, undergo a regression of intestinal adenomas[4,5] might be associated with the apoptotic effects of NSAIDs. DuBois *et al.*[46] showed that intestinal epithelial cells overexpressing COX-2 had a 3-fold increase in the duration of the G1 phase in the cell cycle. They suggested that the delay in G1 transit might be related to the resistance of the cells to programmed cell death, and that the resistance could affect the tumorigenic potential. If the slight increase of COX-2 expression caused by AOM exposure in this study is associated with the development and/or the growth of ACF, COX-2 inhibitors might be expected to inhibit the tumorigenicity. We are planning to examine the chemopreventive effect of NS-398 in colon carcinogenicity.

Takahashi *et al.*[26] and Reddy *et al.*[27] have reported inhibitory effects of other COX-2 inhibitors (nimesulide and SC-58635, respectively) on the formation of AOM-induced ACF, but these drugs were given in the diet. In this study, NS-398 inhibited the development of AOM-induced ACF in male F344 rats after oral administration. We think that NS-398, as a selective COX-2 inhibitor, might have potential as a chemopreventive agent against colon cancer.

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