

Chemoprevention of N-Methylnitrosourea-induced Colon Carcinogenesis by Ursodeoxycholic Acid-5-aminosalicylic Acid Conjugate in F344 Rats

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Bile acids enhance colon carcinogenesis in animal models, whereas ursodeoxycholic acid (UDCA) suppresses it. Nonsteroid anti-inflammatory drugs prevent colon cancer development in animals and humans. The aim of the present study was to explore the inhibitory effect of UDCA conjugate with 5-aminosalicylic acid (5-ASA), UDCA-5-ASA conjugate (UDCA-5-ASA), against colon carcinogenesis in rats. One-hundred-and-twenty-nine 7-week-old F344 rats received an intrarectal instillation of 2 mg of N-methylnitrosourea 3 times a week for 3 weeks, and were fed a 0% (control), 0.11% or 0.02% UDCA-5-ASA-, 0.08% UDCA- or 0.03% 5-ASA-supplemented diet for the next 27 weeks. The test diets contained an equimolar amount of a test agent, 2.0 mmol/kg diet, except for the 0.02% UDCA-5-ASA diet. The tumor incidence and the mean number of tumors/rat at week 30 were significantly lower and smaller in the UDCA-5-ASA diet groups, 48% and 0.7 in both, and marginally lower in the UDCA and 5-ASA diet groups, 56% and 0.9, and 64% and 0.8, compared to the control group, 83% and 1.3. All the tumors were polypoid in shape, and most of them were differentiated adenocarcinomas restricted to the mucosa or submucosa. An analysis by HPLC for bile acids and 5-ASA in the feces and serum collected at week 30 showed that one-half of ingested UDCA-5-ASA was cleaved into UDCA and 5-ASA in the colon. Thus, the two moieties may have independently affected the promotion stage of carcinogenesis.

Key words: Colon cancer — Chemoprevention — Ursodeoxycholic acid — Aminosalicylic acid — Ursodeoxycholic acid-5-aminosalicylic acid conjugate

Epidemiological studies have indicated that a diet rich in fat is associated with an increased risk of colon cancer.^{1,2} The high fat diets cause a physiologic increase of bile acids in the bile and their excretion into the intestinal tract and feces. Various lines of evidence have suggested that bile acids, particularly the secondary bile acids deoxycholic acid and lithocholic acid, may play an important role in the promotion stage of colon carcinogenesis.¹ Animal studies showed that bile acids applied directly into the colonic lumen through the anus or indirectly by adding them to feed result in an increase of carcinogen-induced colon cancer development.^{3–6} However, Earnest *et al.*⁷ reported that ursodeoxycholic acid (UDCA) supplemented in the diet reduced the azoxymethane-induced colon tumor yield in rats, while cholic acid increased it. Our previous study also demonstrated a significant decrease of N-methylnitrosourea (MNU)-induced colon tumors in rats fed diets supplemented with a high dose (0.4%) or a low dose (0.08%) of UDCA.⁸ These studies revealed that UDCA may prevent colon carcinogenesis by counteracting the tumor-promoting activity of other bile acids.

On the other hand, it has been established that non-steroid anti-inflammatory drugs (NSAIDs), including aspirin (acetylsalicylic acid), have an anticarcinogenic effect, particularly against colon cancer, in rodents.^{9,10} A number of epidemiologic studies have indicated that the regular use of NSAIDs lowered the incidence of colon cancer by 40–60%.^{9,10} We observed that an intrarectal administration of 5-aminosalicylic acid (5-ASA), a NSAID, significantly reduced MNU-induced colon cancer development in rats (in preparation). 5-ASA has been widely used for the treatment of active inflammatory bowel diseases, ulcerative colitis and Crohn's disease, and for the prevention of their relapse. However, because of the absorption of a significant amount of 5-ASA from the small intestine and rapid excretion into the urine, the effective colonic dose is reduced. In order to target this drug to the colon, 5-ASA is conjugated via an azo bond with other amines (e.g., sulfasalazine), or formulated as a dimer with two units of 5-ASA combined via a diazo bond, or modified in other ways (methylcellulose-coated time release formulation and rectal suppositories). Those drugs are poorly absorbed from the intestine before being cleaved by colonic bacteria to release 5-ASA in the colon. We are interested in a conjugate of 5-ASA with UDCA via an amide bond (Fig. 1),¹¹ because this unique conjugate may facilitate the transport of both UDCA and 5-ASA to the

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colon, and could exhibit a cooperative anticarcinogenic effect in the colon.

The purpose of the present study was to examine the protective effect of UDCA-5-ASA conjugate supplemented in the diet against colon carcinogenesis in rats, comparing it with the individual effects of UDCA and 5-ASA. Also, the levels of bile acids and 5-ASA in the feces and serum, and the number of colonic mucosal aberrant crypt foci (ACFs), an intermediate biomarker relevant to carcinogenesis,¹²⁾ were analyzed. The results support a protective action of UDCA-5-ASA against colon carcinogenesis, and indicate that UDCA-5-ASA is more effective than UDCA or 5-ASA alone. To our knowledge, this is the first study to demonstrate a protective effect of UDCA-5-ASA against colon carcinogenesis in animal models.

MATERIALS AND METHODS

Animals, chemicals and diets Female F344/NSlc rats (Shizuoka Laboratory Animal Center, Hamamatsu), 7 weeks of age at the start of experiment, were used. They were housed, 5 rats per plastic cage with sterilized wood-chip bedding, in a specific-pathogen-free room under constant environmental conditions with a 12 h light and dark cycle, a temperature of 22°C and a relative humidity of 50%. They had free access to a standard pelleted diet CE-2 (CLEA Co., Tokyo) and drinking water. The body weight was measured once a week. The rats were maintained according to the standards set forth in the Guidelines for the Care and Use of Laboratory Animals of the Experimental Animal Facility, Akita University School of Medicine.

UDCA was a gift from Tokyo-Tanabe Pharmaceutical Co., Tokyo (purity 99.5%). 5-ASA was purchased from Sigma Chemical Co., St. Louis, MO (purity 99.0%). UDCA-5-ASA was synthesized by us (purity 98.3%) following the procedure described by Batta *et al.*¹¹⁾ The carcinogen MNU was obtained from Nacalai Tesque, Kyoto.

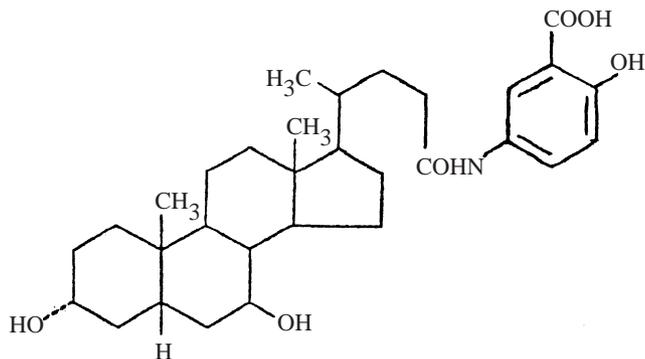


Fig. 1. Chemical structure of ursodeoxycholic acid-5-aminosalicylic acid conjugate.

Three test diets containing 0.11% UDCA-5-ASA (UDCA-5-ASA diet), 0.08% UDCA (UDCA diet) and 0.03% 5-ASA (5-ASA diet) in CE-2 pelleted diet were prepared at CLEA Co. The amounts of the agents supplemented in the UDCA-5-ASA, UDCA and 5-ASA diets were equivalent in molar level at 2.0 mmol/kg diet. A fourth diet contained 0.02% UDCA-5-ASA (UDCA-5-ASA(I) diet). The food intake was measured once a week.

Animal treatment and colon tumor examination A total number of 129 rats received an intrarectal instillation of 0.5 ml of freshly prepared 0.4% aqueous solution of MNU 3 times a week for weeks 1 to 3 to induce colon cancer, during which time they were fed CE-2 diet. A metal feeding tube 8 cm long was inserted two-thirds into the colon lumen through the anal orifice, and the solution was injected. The solution filled the distal half of the colon, where the tumors developed. After completion of the carcinogen treatment, the rats were divided into 5 groups, and given the CE-2, UDCA-5-ASA, UDCA-5-ASA(I), UDCA or 5-ASA diet during weeks 4 to 30. The feces were collected at weeks 15 and 30, and stored at -80°C until analysis of bile acids and 5-ASA.

At the end of week 30, all the rats were sacrificed by exsanguination from the abdominal aorta after laparotomy under intraperitoneal pentobarbital anesthesia (40 mg/kg body weight). The blood was centrifuged, and the serum was collected and stored at -30°C until analysis for bile acids and 5-ASA. The colon was excised, cut open along its length, and inspected grossly. The location, shape and size of colon tumors were recorded. Then, the colon was fixed in 10% formalin solution. All the tumors and macroscopically abnormal tissues or organs were examined microscopically after standard processing, sectioning and staining with hematoxylin and eosin.

Examination of colonic mucosal ACFs ACFs were examined in 5 or 6 rats without colon tumors at week 30 in each group. The distal half of the colon, 10 cm long, where the tumors developed, was flattened on filter paper and placed in 10% neutral formalin solution for one day. The fixed colon was stained with 0.2% methylene blue dye solution for 5 min, and ACFs were scored under light microscopy at $\times 40$ magnification by two of the authors. The criteria for aberrant crypts (AC) forming ACF were enlarged crypt opening, thicker surface epithelial lining, and extended pericryptal zone.¹²⁾ The average of two observers' results was recorded as the number of ACFs in each colon.

Assay of bile acids and 5-ASA in serum and feces The assay of bile acids including UDCA-5-ASA was carried out by HPLC.^{13, 14)} Briefly, 50 mg of lyophilized feces was extracted with 10 ml of methanol, and centrifuged at 1600g for 10 min. Two milliliters of the supernatant was supplemented with tauro-23-nordeoxycholic acid (0.1 ml of 85 μ M ethanolic solution) as an internal standard, then

evaporated to dryness. In the case of serum, 0.2 ml of sample was diluted with 2 ml of 0.1 M phosphate buffer (pH 7.2), then the same unit of internal standard as for the feces was added, and the mixture passed through a Bond Elute C18 cartridge. The cartridge was washed with water, and bile acids were eluted with 3 ml of methanol, then evaporated to dryness. The residues from the feces and the serum were dissolved in 0.2 ml of 20 mM diammonium hydrogen-phosphate-acetonitrile-methanol mixture (2:1:2, v/v/v), then the solution was centrifuged at 1600g for 10 min at 5°C, and 15 µl of the supernatant was injected into the HPLC column. The HPLC system was an LC-10A equipped with an RF-10AXL fluorescence detector (Shimadzu Co., Kyoto), STRODS-II separation column (Shimadzu Co.) and an E-3α-HSD enzyme column (Sekisui Chem. Co., Osaka). The flow rate was 1.0 ml/min at 20°C.

The assay of 5-ASA was carried out by HPLC. Briefly, 2 ml of the supernatant of fecal extract and 0.1 ml of the serum were supplemented with 2,4-dinitrofluorobenzene (0.1 ml of 5 mM ethanolic solution) as an inducer and phenylbenzoate (0.5 ml of 30 µM methanolic solution) as an internal standard, then evaporated to dryness. Each residue was dissolved in 0.5 ml of water-acetonitrile mixture (4:1, v/v), and centrifuged at 1600g for 10 min at 5°C. Twenty-five microliters of the supernatant was injected into the HPLC column. The HPLC system was an LC-10A equipped with an SPD-10A UV-VIS detector (Shimadzu Co.) and a Symmetry Shield RP18 separation column (Waters Assoc., Millford, MA). The flow rate was 0.9 ml/min at 40°C.

Levels of 5-ASA <5 nmol/ml serum and <50 nmol/g dry feces, and levels of bile acids <0.1 nmol/ml serum and <1.0 nmol/g dry feces were not detectable, and these values were recorded as 0.

Statistical analysis The statistical significance of differ-

ences was tested by use of the χ^2 test and Student's *t* test. The criterion of significance was a *P* value of <0.05.

RESULTS

General observations The body weight gains were similar in all the groups. The mean weight was 92–93 g at week 1, and 211–213 g at week 30. The mean food intake throughout the experiment was similar in all the groups, 10.3–10.6 g/day/rat. Thus, the mean amount of ingested test agents, UDCA-5-ASA, UDCA and 5-ASA, was computed to be 61 mg or 0.11 mmol/kg body weight/day in the UDCA-5-ASA group, 11 mg or 0.02 mmol/kg body weight/day in the UDCA-5-ASA(l) group, 45 mg or 0.11 mmol/kg body weight/day in the UDCA group and 15 mg or 0.10 mmol/kg body weight/day in the 5-ASA group.

Colon tumors The colon tumor incidence was significantly lower in the UDCA-5-ASA, UDCA-5-ASA(l) and UDCA groups than in the control group (Table I). It was also lower in the 5-ASA group, but the difference did not reach statistical significance. The tumor multiplicity (number of tumors per rat) was significantly smaller in the UDCA-5-ASA, UDCA-5-ASA(l) and 5-ASA groups than in the control group. It was also smaller, but not significantly so, in the UDCA group. The number of tumors per tumor-bearing rat was almost the same in all the groups. The tumors were located diffusely in the distal half of the colon, 0–10 cm proximal to the anus, and were plaque-shaped or polypoid. Histologically all the tumors were well- and moderately differentiated adenocarcinomas, except one signet-ring cell carcinoma in the UDCA-5-ASA group and one mucinous carcinoma in the 5-ASA group. Most of them were less than 10 mm in diameter, and restricted to the mucosa or submucosa (Table II). Metastases to lymph nodes or other organs were not observed, except that peritoneal dissemination was found

Table I. N-Methylnitrosourea-induced Colon Tumors at Week 30 in F344 Rats Treated with UDCA-5-ASA Conjugate, UDCA or 5-ASA

Treatment groups ^{a)}	No. of rats examined	No. of rats with tumors (%)	No. of tumors per rat	No. of tumors per tumor-bearing rat
Control	29	24 (83)	1.3±0.1 ^{b)}	1.5±0.1
UDCA-5-ASA	25	12 (48) ^{c)}	0.7±0.2 ^{c)}	1.4±0.2
UDCA-5-ASA(l)	25	12 (48) ^{c)}	0.7±0.2 ^{c)}	1.4±0.1
UDCA	25	14 (56) ^{c)}	0.9±0.2	1.6±0.2
5-ASA	25	16 (64)	0.8±0.1 ^{c)}	1.3±0.1

a) All rats received an intrarectal dose of 2 mg MNU 3 times a week for 3 weeks, and then fed the control CE-2 diet (control group), or the diet supplemented with 0.11% UDCA-5-ASA conjugate (UDCA-5-ASA group), 0.02% UDCA-5-ASA conjugate (UDCA-5-ASA(l) group), 0.08% UDCA (UDCA group) or 0.03% 5-ASA (5-ASA group) for 27 weeks. All the rats were killed at week 30.

b) Mean±SEM.

c) Significantly different from the control group: *P*<0.05.

in one rat of the 5-ASA group. No distinct differences among the groups were observed in the location, shape, size, invasion or histological type of tumors. There were no other pathologic findings in the gastrointestinal tract and other organs including the lung, liver and kidney, macroscopically or microscopically, in any of the groups. Thus, the test agents did not exhibit chronic toxicity.

These data clearly demonstrate that UDCA-5-ASA significantly inhibited colon cancer development induced by MNU, but UDCA and 5-ASA did so only marginally. **ACFs** The growth of ACFs was detected in the distal colon of all the rats examined. The total number of ACFs/colon was significantly greater in the UDCA-5-ASA(l), UDCA and 5-ASA groups, and slightly but insignificantly

Table II. Size and Depth of Invasion of N-Methylnitrosourea-induced Colon Tumors at Week 30 in F344 Rats Treated with UDCA-5-ASA Conjugate, UDCA or 5-ASA

Treatment groups ^{a)}	Size in diameter			Depth of invasion		
	1-3 mm (%)	4-6 mm (%)	7-20 mm (%)	Mucosa (%)	Submucosa (%)	Muscle/Serosa (%)
Control	17 ^{b)} (46)	14 (38)	6 (16)	23 (62)	11 (30)	3 (8)
UDCA-5-ASA	6 (35)	8 (47)	3 (18)	3 (76)	4 (24)	0
UDCA-5-ASA(l)	9 (53)	4 (23)	4 (23)	11 (65)	5 (29)	1 (6)
UDCA	9 (39)	8 (35)	6 (27)	15 (65)	6 (26)	2 (9)
5-ASA	8 (38)	9 (43)	4 (19)	10 (48)	8 (38)	3 (14)

a) See Table I or text.
b) Number of tumors.

Table III. N-Methylnitrosourea-induced Aberrant Crypt Foci at Week 30 in F344 Rats Treated with UDCA-5-ASA Conjugate, UDCA or 5-ASA

Treatment groups ^{a)}	No. of aberrant crypt foci consisting of			Total number
	1-3 aberrant crypts	4-9 aberrant crypts	10 or more aberrant crypts	
Control	3.4±0.7 ^{b)}	9.0±0.9	3.2±0.8	15.6±1.4
UDCA-5-ASA	9.2±1.7 ^{c)}	10.7±1.9	3.7±0.8	23.5±3.7
UDCA-5-ASA(l)	11.0±2.6 ^{c)}	11.0±1.5	2.8±1.1	24.8±3.1 ^{c)}
UDCA	7.5±1.4	13.5±2.0	3.3±0.6	24.3±3.0 ^{c)}
5-ASA	11.2±1.4 ^{c)}	12.2±2.0	2.8±1.1	26.2±3.5 ^{c)}

a) See Table I or text. Analysis was done in 5 or 6 rats in each group.
b) Mean±SEM.
c) Significantly different from the control group: $P < 0.05$.

Table IV. Fecal Bile Acids at Week 30 in F344 Rats Treated with N-Methylnitrosourea, and Then with UDCA-5-ASA Conjugate, UDCA or 5-ASA

Treatment groups ^{a)}	UDCA ^{b)}	HDCA	DCA/12kLCA	LCA	Others	UDCA-5-ASA	Total
Control	0.22±0.02 ^{c), A}	44.9±3.1 ^A	28.2±1.9 ^A	24.3±1.6 ^A	4.3±0.4 ^A	0 ^A	102.0±7.0 ^A
UDCA-5-ASA	2.84±0.37 ^B	73.2±4.7 ^B	50.8±2.4 ^B	157.5±7.3 ^B	31.3±2.0 ^B	5.56±0.40 ^B	321.2±15.9 ^B
UDCA-5-ASA(l)	0.45±0.07 ^C	52.2±4.6 ^A	39.1±2.6 ^C	46.6±3.2 ^C	5.5±0.2 ^C	0.98±0.07 ^C	144.9±10.3 ^C
UDCA	2.31±0.39 ^B	79.9±2.7 ^B	54.1±2.8 ^B	151.1±4.8 ^B	3.1±0.3 ^D	0 ^A	290.7±9.5 ^B
5-ASA	0 ^D	52.7±4.9 ^A	34.8±2.0 ^{A, C}	29.4±2.5 ^A	11.3±0.5 ^E	0 ^A	128.2±8.9 ^C

a) See Table I or text. Analysis was done in 6 or 5 rats in each group.
b) UDCA, ursodeoxycholic acid; HDCA, hyodeoxycholic acid; DCA, deoxycholic acid; 12kLCA, 12-keto-lithocholic acid; LCA, lithocholic acid; Others, including muricholic acid, hyocholic acid, cholic acid and chenodeoxycholic acid; UDCA-5-ASA, ursodeoxycholic acid-5-aminosalicylic acid conjugate.
c) Mean±SEM, μmol/g dry feces. Means not sharing a common superscript (A-D) between groups in a vertical column are significantly different: $P < 0.05$.

Table V. Serum Bile Acids at Week 30 in F344 Rats Treated with N-Methylnitrosourea, and Then with UDCA-5-ASA Conjugate, UDCA or 5-ASA

Treatment groups ^{a)}	UDCA ^{b)}	HDCA	DCA	LCA	Others	UDCA-5-ASA	Total
Control	0.55±0.26 ^{c), A, B, C}	1.85±0.33 ^A	0.27±0.01 ^{A, B}	0 ^A	18.0±4.7 ^A	0 ^A	20.7±5.0 ^A
UDCA-5-ASA	1.75±0.57 ^{A, C}	3.38±0.74 ^A	0.36±0.07 ^A	0.11±0.04 ^B	22.5±5.2 ^A	0.33±0.12 ^B	28.4±6.6 ^A
UDCA-5-ASA(I)	0.63±0.13 ^C	2.37±0.51 ^A	0.28±0.07 ^{A, B}	0 ^A	17.2±3.6 ^A	0 ^A	20.5±4.2 ^A
UDCA	1.15±0.13 ^A	0.60±0.14 ^B	0.13±0.03 ^B	0.05±0.02 ^{A, B}	5.4±1.0 ^B	0 ^A	7.3±1.3 ^B
5-ASA	0.31±0.02 ^B	2.23±0.33 ^A	0.32±0.04 ^A	0 ^A	17.5±1.8 ^A	0 ^A	20.8±2.4 ^A

a) See Table I or text. Analysis was done in 8 rats in each group.

b) UDCA, ursodeoxycholic acid; HDCA, hyodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; Others, including muricholic acid, hyocholic acid, cholic acid and chenodeoxycholic acid; UDCA-5-ASA, ursodeoxycholic acid-5-aminosalicylic acid conjugate.

c) Mean±SEM, nmol/ml. Means not sharing a common superscript (A,B,C) between groups in a vertical column are significantly different: $P < 0.05$.

greater in the UDCA-5-ASA group, than in the control group (Table III). The number of small-sized ACFs with one to three ACs increased significantly in the UDCA-5-ASA, UDCA-5-ASA(I) and 5-ASA groups, and slightly but insignificantly in the UDCA group, compared with the control group, while the number of large-sized ACFs with 4 or more ACs was almost the same in all the groups. The data seem to suggest that the test agents, UDCA-5-ASA, UDCA and 5-ASA suppressed the growth of small-sized ACFs to large-sized ACFs and thence to tumors.

Bile acids and 5-ASA The results of bile acid analysis for the serum and feces collected at week 30 are summarized in Tables IV and V. In the feces, a 3-fold increase of total bile acid in the UDCA-5-ASA and UDCA groups compared to the control group was noted. There was a considerable increase of lithocholic acid, and a moderate increase of other secondary bile acids, hyodeoxycholic acid, and deoxycholic acid and its metabolite 12-ketolithocholic acid. A specific feature was an elevated level of the primary bile acid chenodeoxycholic acid in the UDCA-5-ASA group compared with the control group: 27.4±1.6 vs. 3.5±0.4, $\mu\text{mol/g}$ dry feces. Each bile acid in the UDCA-5-ASA(I) group was significantly lower than in the UDCA-5-ASA and UDCA groups. In the serum, there was a 30% decrease of total bile acid in the UDCA group, compared to the control group. This was mainly due to the decrease of cholic acid: 2.6±0.7 vs. 12.3±3.7 nmol/ml. A very small amount of lithocholic acid, even in the UDCA-5-ASA and UDCA groups, was specifically noted. It was confirmed that the levels of UDCA in the feces and serum were significantly and insignificantly higher in the UDCA-5-ASA and UDCA groups, respectively, than in the control and UDCA-5-ASA(I) groups, though the absolute amounts were small. A small amount of UDCA-5-ASA was found in the feces of the UDCA-5-ASA and UDCA-5-ASA(I) groups in a dose-dependent manner, while in the serum, it was detected only in the UDCA-5-ASA group.

A very small amount of 5-ASA was found only in the feces of the UDCA-5-ASA group, 0.05±0.01 $\mu\text{mol/g}$ dry feces, while none was detected in the serum of any group. The analyses of bile acids and 5-ASA in the feces collected at week 15 (data not shown) showed basically the same results as those of the feces at week 30.

The overall results seem to indicate that a very small amount of UDCA-5-ASA was absorbed in a concentration-dependent manner, and some was excreted into the feces without metabolic change. The other was deconjugated into free UDCA and 5-ASA in the colon. A portion of free UDCA was absorbed from the colon to enter the enterohepatic cycle, and the rest was 7 β -dehydroxylated into lithocholic acid, which was poorly absorbed and was mostly excreted in the feces.

The mean daily amounts of ingested UDCA-5-ASA and its excretion into the feces were computed (Table VI). The amount of excreted feces was similar in all groups, 2.9–3.1 g/day/rat in wet weight and 1.7 g/day/rat in dry weight. The data showed that 45.2% and 42.1% of ingested UDCA-5-ASA recovered in non-hydrolyzed form in the feces of the UDCA-5-ASA and UDCA-5-ASA(I)

Table VI. Mean Amounts of Dietary Intake and Fecal Excretion of UDCA-5-ASA Conjugate, UDCA and 5-ASA at Week 30 in F344 Rats

Treatment groups ^{a)}	Dietary intake ($\mu\text{mol/day/rat}$)	Fecal excretion ($\mu\text{mol/day/rat}$)		
		UDCA-5-ASA	UDCA	5-ASA
Control	—	0	0.3	0
UDCA-5-ASA	20.8	9.4	4.7	0.08
UDCA-5-ASA(I)	3.8	1.6	0.7	0
UDCA	20.4	0	3.8	0
5-ASA	19.6	0	0	0

a) See Table I or text. Analysis was done in 6 or 5 rats in each group.

groups, respectively. Again, small amounts of 5-ASA were detected only in the feces of the UDCA-5-ASA group, but none in the serum of any group, perhaps because of its good absorption and acetylation in the colonic epithelial cells, followed by rapid excretion into the urine.¹⁵⁾

DISCUSSION

The present study clearly demonstrated that UDCA-5-ASA conjugate significantly inhibited MNU-induced colon cancer development in rats, but UDCA and 5-ASA did so only marginally, when the same molar dose of these agents was supplemented in the UDCA-5-ASA, UDCA and 5-ASA diets, respectively. The low dose of UDCA-5-ASA provided in the UDCA-5-ASA(I) diet exhibited much the same effect as the high dose in the UDCA-5-ASA diet. Taking these results into consideration, it appears likely that UDCA-5-ASA is a far more efficient agent than UDCA and 5-ASA to prevent colon cancer development by affecting the promotion stage of carcinogenesis.

It was noted that the numbers of small-sized ACFs, as well as the total numbers of ACFs, scored in the colon without tumors at week 30 were larger in the UDCA-5-ASA, UDCA and 5-ASA fed rats compared to the control rats, whereas the numbers of large-sized ACFs, which would progress further into malignant growth, were not different. In short-term (6–12 weeks) assay studies, UDCA decreased ACFs formation induced by azoxymethane or MNU in the rat colon,^{16–19)} while other bile acids increased them. For NSAIDs as well, there have been a number of short-term assay studies in which the formation of carcinogen-induced ACFs in the rat colon was reduced.²⁰⁾ Thus, our results seem to indicate that UDCA-5-ASA suppressed the growth of small-sized ACFs to large-sized ACFs, as well as further neoplastic progression, by the same mechanisms as UDCA and 5-ASA.

UDCA-5-ASA is poorly absorbed from the intestine, and is transported into the colon where it is partially hydrolyzed by the intestinal bacteria to release UDCA and 5-ASA.^{11,21)} In our present results, 45% of ingested UDCA-5-ASA was recovered as the conjugate form in the feces. This is consistent with the finding of Batta *et al.*,¹¹⁾ i.e., 63% in the feces. Thus, the data indicate that almost half of UDCA-5-ASA was deconjugated, and contributed to the production of high concentrations of UDCA and 5-ASA in the colon. However, the feeding of UDCA-5-ASA and UDCA resulted in a great increase of lithocholic acid, a potent tumor promoter, in the feces, though a low concentration in the serum was noted in the present study. A likely explanation for this discrepancy is that a large proportion of UDCA is 7 β -dehydroxylated to lithocholic acid by intestinal bacteria, and this hydrophobic bile acid is hardly reabsorbed under conditions where an appreciable amount of the hydrophilic and much more absorbable bile

acid UDCA is present.²²⁾ UDCA in the aqueous phase of the feces is in direct contact with the colon epithelium, and may be physiologically more active. Thus, a beneficial effect of UDCA and 5-ASA released from UDCA-5-ASA in the colon through their direct action *in situ* on the colonic mucosa could be expected in this study.

In the current study, we reconfirmed that a relatively small dose of 0.08% UDCA supplemented in the diet inhibited colon cancer development to the same extent as in our previous study.⁸⁾ Various lines of evidence have indicated that UDCA has specific biological activities different from those of other bile acids, that is, growth suppression and apoptosis induction in human colon cancer cells *in vitro*^{23,24)} and in rat colon mucosa *in vivo*.^{25–28)} On the other hand, it is established that NSAIDs may prevent carcinogenesis in various organs, especially the colon, in humans and animals.^{9,10)} Treatment with an appropriate dose of 5-ASA or sulfasalazine was shown to suppress the growth of colon tumors induced with 1,2-dimethylhydrazine in rats.^{29,30)} 5-ASA blocked bile acid-induced reactive oxygen formation, DNA-base hydroxylation and subsequent proliferative response in biopsied human colonic mucosa and scraped rat colonic mucosa.^{31,32)} The precise mechanisms of the prevention of colon carcinogenesis by 5-ASA have not yet been clarified, but perhaps they are common to those of other NSAIDs. Thus, the anticarcinogenic effect of UDCA-5-ASA could be attributable to anti-promoting action of UDCA plus 5-ASA directed on the colon mucosa *in situ*.

It is noteworthy in the present study that no dose-dependency of UDCA-5-ASA activity against colon cancer development and ACFs formation was observed. The high and low doses of this agent showed the same efficacy. Further trials are needed to find a lower or minimum effective dose before considering clinical application to humans.

In conclusion, UDCA-5-ASA conjugate may be an ideal agent in that both UDCA and 5-ASA moieties may be independently beneficial for prevention of colon carcinogenesis and small doses may be sufficient. In particular, this agent may be a promising candidate for the treatment of longstanding ulcerative colitis, since a case-control study stressed the lower incidence of colon cancer in patients receiving pharmacological treatment with sulfasalazine (relative risk: 0.34), comparing to patients without such treatment.³³⁾

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