

Prevention of N-Methylnitrosourea-induced Colon Tumorigenesis by Ursodeoxycholic Acid in F344 Rats

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Bile acids are known to promote colon carcinogenesis. However, there is one study showing that ursodeoxycholic acid (UDCA) supplemented in the diet at the concentration of 0.4% prevented azoxymethane-induced rat colon tumorigenesis. The aim of our study was to explore the inhibitory effect of a much smaller dose of UDCA on colon carcinogenesis in rats. One hundred 7-week-old F344 rats were given 2 mg of N-methylnitrosourea 3 times a week for 3 weeks by intrarectal instillation, and were fed a 0% (control), 0.4% or 0.08% UDCA-supplemented diet for the next 27 weeks. All the rats were killed and examined for tumor development at week 30. The tumor incidence and number were significantly lower and smaller, respectively, in the UDCA-fed rats than in the control rats: 40% and 36% vs. 68%; 0.5 ± 0.1 (mean \pm SEM) and 0.4 ± 0.1 vs. 1.0 ± 0.2 . All the tumors were located in the distal half of the colon and were plaque-shaped or polypoid, being well-differentiated adenocarcinomas restricted to the mucosa or submucosa. Bile acids in the feces and the blood obtained at weeks 20 and 30, respectively, were analyzed by HPLC. A significant increase of UDCA was confirmed in both the feces and the blood of the UDCA-fed rats compared with the control rats. The results suggest that the continuous feeding of a small dose of UDCA may prevent colon carcinogenesis.

Key words: Colon cancer — Cancer prevention — Ursodeoxycholic acid

Epidemiological studies have indicated that a diet rich in fat is associated with an increased risk of colon cancer.¹⁾ According to various lines of evidence, bile acids, which are required for dietary fat metabolism, may play an important role in colon carcinogenesis.²⁾ In humans and rodents, a diet with a high content of fat causes a physiological increase of bile acids in the bile and of their excretion into feces. Studies with rats have shown that bile acids, particularly secondary bile acids (deoxycholic acid and lithocholic acid), contribute to the promotion of colon carcinogenesis.³⁻⁶⁾ In those studies, bile acids were applied directly on the colonic mucosa via the rectum or indirectly by adding them to feed. However, Czygan *et al.* reported that among rats administered 1,2-dimethylhydrazine to induce colon cancer, those fed a diet supplemented with 1% ursodeoxycholic acid (UDCA) showed no increased development of colon tumors, compared to control rats fed a standard diet, while rats fed a diet supplemented with 1% chenodeoxycholic acid, an isomer of UDCA, showed a significantly enhanced tumor development.⁷⁾ Recently, Earnest *et al.* reported a significant reduction of azoxymethane (AOM)-induced colon tumors in rats fed a diet supplemented with 0.4% UDCA, but not in those fed a diet containing 0.2% UDCA.⁸⁾ Moreover, it

was reported that a diet containing 0.4% UDCA suppressed the formation of AOM-induced aberrant crypt foci (putative preneoplastic lesions) in the rat colon, while feeding of other bile acids increased the lesions.⁹⁾ Our preliminary short-term (5 weeks) experiment showed that the number of N-methylnitrosourea (MNU)-induced aberrant crypt foci in the colon of rats fed diets supplemented with 0.4%, 0.08% or 0.016% UDCA was 76%, 77% and 92% of that in the control rats without UDCA feeding, respectively (unpublished data).

The present study was conducted to examine the inhibitory effect of UDCA administered as a supplement in the diet at the levels of 0.4% and 0.08% on MNU-induced colon tumorigenesis in rats. UDCA inhibited colon tumor development at both dose levels. The results are different from those reported by Earnest *et al.*⁸⁾ Serum and fecal bile acids were also measured to analyze the mechanism of the inhibitory activity of UDCA.

MATERIALS AND METHODS

Female F344/NSlc rats (Shizuoka Laboratory Animal Center, Hamamatsu), 7 weeks of age at the start of the experiment, were used. They were housed, 5 rats per cage, in plastic cages with sterilized wood-chip bedding in a specific-pathogen-free room under constant environmental conditions with a 12 h light and dark cycle, a tempera-

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ture of 22°C and a relative humidity of 50%. They had free access to a standard pelleted chow CE-2 (CLEA Co., Tokyo) and drinking water. The body weight and food intake were measured once a week. Two test diets containing UDCA (purity 99.5%; Tokyo Tanabe Co., Tokyo) in CE-2 chow at the level of 0.4% or 0.08% (w/w) were prepared at CLEA Co. The concentrations of UDCA in these diets were confirmed by HPLC.

One hundred rats were given 0.5 ml of 0.4% aqueous solution of MNU (Nacalai Tesque, Kyoto) 3 times a week for 3 weeks by intrarectal instillation. The solution was prepared immediately before use. A metal feeding tube 8 cm long was inserted two-thirds into the colon lumen through the anal orifice, and the solution was injected.³⁾ The solution filled the distal half of the colon where the tumors develop. After completion of treatment with the carcinogen, the rats were divided into 3 groups fed diet supplemented with 0%, 0.4% or 0.08% UDCA (the control, UDCA (h) and UDCA (l) groups, respectively) for 27 weeks. The feces were collected for 3 days at week 20, stored at -80°C and analyzed for bile acid content.

At the end of week 30, all the rats were killed 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 of bile acids. After laparotomy, the colon was excised, cut open along its length, rinsed with 0.9% saline solution, and inspected macroscopically. 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.

Bile acids in the serum and the feces were determined by HPLC followed by enzymatic and fluorescence detection, as described previously.¹⁰⁾ Briefly, 250 mg of lyophilized feces was extracted with 5 ml of ethanol under reflux for 1 h, then centrifuged at 1,800g for 10 min, and

the supernatant was transferred into another test tube. The residue was further extracted twice with 5 ml of ethanol. The combined supernatants were evaporated to dryness. One milliliter of the serum was extracted with the same volume of ethanol, then evaporated to dryness. The extracts from the feces and the serum were redissolved in 2 ml of methanol and centrifuged at 1,800g for 10 min. The supernatant (100 µl) was mixed with 23-nordeoxycholic acid (100 µl of 60 µmol/liter methanolic solution) as an internal standard, and 20 µl of this solution was used for bile acid analysis by HPLC.

The statistical significance of differences 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

The body weight gain was similar in all groups. The mean weight was 115 g at week 1, and 214–216 g at week 30 in all groups. The values of mean food intake were 9.6, 9.5 and 9.9 g/day/rat in the control, UDCA (h) and UDCA (l) groups, respectively. Thus, the mean amount of ingested UDCA was computed to be 200 mg/kg body weight/day in the UDCA (h) group and 41 mg/kg body weight/day in the UDCA (l) group.

Data regarding the development of colon tumors are summarized in Table I. The incidence and number of tumors were significantly lower in the UDCA (h) and UDCA (l) groups than in the control group. However, 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, and were plaque-shaped or polypoid. Histologically, all the tumors were well-differentiated adenocarcinomas. Most of them were small, less than 10 mm in diameter, and restricted to the mucosa or submucosa. No metastases to lymph nodes or other organs were observed because the experiment was terminated after only 30 weeks. No distinct differences among the groups were observed in the location, shape, size, depth

Table I. Development of Colonic Tumors in F344 Rats Treated with N-Methylnitrosourea and Then Fed a Diet Containing Ursodeoxycholic Acid

Treatment groups ^{a)}	Number of rats examined	Number of rats with tumors	Number of tumors/rat	Number of tumors/tumor-bearing rat
Control	25	17 (68%)	1.0±0.2 ^{b)}	1.5±0.1
UDCA (h)	50	20 (40%) ^{c)}	0.5±0.1 ^{c)}	1.3±0.1
UDCA (l)	25	9 (36%) ^{c)}	0.4±0.1 ^{c)}	1.2±0.1

a) All rats were given an intrarectal dose of 2 mg of MNU 3 times a week for 3 weeks, and then fed a diet containing 0% (control group), 0.4% (UDCA (h) group) or 0.08% (UDCA (l) group) UDCA for 27 weeks. All rats were killed at the end of week 30.

b) Mean±SEM.

c) Significantly different from control group: *P*<0.05 or 0.01.

Table II. Analysis of Bile Acids Excreted into Feces from F344 Rats Fed a Diet Containing Ursodeoxycholic Acid

Treatment groups ^{a)}	α - & β -MCA	ω -MCA	HyoDCA	DCA	12-Keto-LCA	UDCA	LCA	Total bile acids
Control <i>n</i> =15	0.08±0.02 ^{b)}	0.28±0.03	0.55±0.08	0.26±0.06	0.45±0.07	0.06±0.004	0.21±0.01	1.86±0.26
UDCA (h) <i>n</i> =15	0.64±0.07*	1.71±0.12*	1.27±0.17*	0.56±0.02*	0.80±0.01*	0.48±0.05*	6.98±0.18*	12.43±0.44*
UDCA (l) <i>n</i> =15	0.30±0.06*	0.64±0.16	1.13±0.18*	0.51±0.03*	0.83±0.04*	0.15±0.01*	1.57±0.06*	5.13±0.05*

a) See text or Table I.

b) Mean±SEM, μ mol/g dry feces.

* Significantly different from control group: $P < 0.05$ or more.

MCA: muricholic acid, DCA: deoxycholic acid, LCA: lithocholic acid, UDCA: ursodeoxycholic acid.

Table III. Analysis of Bile Acids in Serum of F344 Rats Fed a Diet Containing Ursodeoxycholic Acid

Treatment groups ^{a)}	α - & β -MCA	CA	CDCA	HyoDCA	DCA	UDCA	LCA	Total
Control <i>n</i> =8	1.7±0.3 ^{b)}	3.3±0.5	1.8±0.5	1.1±0.2	0.1±0.05	ND ^{c)}	ND	8.0±1.1
UDCA (h) <i>n</i> =8	3.5±0.8	0.6±0.1*	3.7±0.7	0.7±0.2	0.2±0.1	6.3±0.8*	0.7±0.1*	15.3±2.3*
UDCA (l) <i>n</i> =8	2.9±0.6	3.4±0.7	2.4±0.6	1.4±0.3	0.1±0.04	2.4±0.5*	0.2±0.1*	12.8±2.7

a) See text or Table I.

b) Mean±SEM, nmol/ml.

c) Not detected.

* Significantly different from the control group: $P < 0.05$ or more.

MCA: muricholic acid, CA: cholic acid, CDCA: chenodeoxycholic acid, DCA: deoxycholic acid, UDCA: ursodeoxycholic acid, LCA: lithocholic acid.

of invasion or histological type of tumors. There were no other pathologic findings in the gastrointestinal tract or other organs in any of the groups. Thus, the data clearly demonstrate that both high and low doses of UDCA inhibited the development of MNU-induced colon tumors.

The mean weight of feces excreted at week 20 was 2.8 g/day/rat (1.7 or 1.8 g dry weight/day/rat) in all the groups. The results of fecal bile acids analysis are summarized in Table II. The amounts of individual and total bile acids were significantly larger in the UDCA groups than in the control group. It was noted that excessive amounts of ω -muricholic acid, hyodeoxycholic acid and lithocholic acid, which are derived from UDCA in the colon, were excreted in the feces of rats in the UDCA groups. The serum bile acids showed significant increases of UDCA and lithocholic acid in the UDCA groups and of total bile acids in the UDCA (h) group, compared with the control group (Table III). In particular, an excess amount of UDCA was noted. The level of cholic acid, a primary bile acid, was significantly lower in the UDCA (h) group than in the control group.

DISCUSSION

The present study demonstrated that UDCA administered as a supplement in the diet at concentrations of 0.4% and 0.08% inhibited the development of MNU-induced colon tumors in rats. The inhibitory effect was similar at both dose levels. These results were in agreement with those of our preliminary study, in which formation of MNU-induced colonic aberrant crypt foci was suppressed in rats fed the same diets for 5 weeks. The effect of UDCA on AOM-induced colonic aberrant crypt foci in rats was also confirmed in a recent study.⁹⁾ Earnest *et al.* reported that the development of AOM-induced colon tumors was inhibited in rats fed a diet containing 0.4% UDCA, but not in those fed a diet containing 0.2% UDCA.⁸⁾ The discrepancy between their results and ours seems likely to be due to the difference in the carcinogens used, i.e., subcutaneous AOM and intrarectal MNU. Our results constitute evidence of the inhibitory effect of UDCA at the low dose level of 41 mg/kg body weight/day on colon carcinogenesis in an animal model.

The present study confirmed that orally administered UDCA results in a marked increase of UDCA and lithocholic acid, which was derived from UDCA in the colon, as well as total bile acids in the feces and in the blood. UDCA was shown to enhance apoptosis of human colon cancer cells in an *in vitro* study, while lithocholic acid did not show such effect.¹¹⁾ On the other hand, deoxycholic acid had no effect on apoptosis of colon cancer cells at physiological low doses, but induced apoptosis as well as cytotoxicity at very high doses.^{12–15)} Failure of damaged cells to undergo apoptosis is now believed to be responsible for the growth of neoplasms, including colon tumors. UDCA suppressed AOM-induced activation of protein kinase C, an intermediate biomarker of tumor promotion, in the colon mucosa of rats.¹⁶⁾ We observed in rats that UDCA did not enhance colon mucosal ornithine decarboxylase activity as a biomarker of tumor promotion, while deoxycholic and chenodeoxycholic acids significantly enhanced it.¹⁷⁾ Furthermore, UDCA was shown to inhibit angiogenesis in chick embryo chorioallantoic membrane.¹⁸⁾ It is established that secondary bile acids, deoxycholic acid and lithocholic acid, enhance colonic cell proliferation and colon carcinogenesis.^{19,20)} Those

studies suggest that UDCA has a specific biological activity, different from other bile acids, in suppressing the promotion and/or progression phases of colon carcinogenesis. However, we have no data on colonic cell proliferation and apoptosis in the present study with an animal model. Further studies are necessary to clarify the possible mechanism of the chemopreventive action of UDCA on colon carcinogenesis.

In conclusion, the continuous feeding of a diet containing a low dose of UDCA (0.08%) inhibited MNU-induced colon cancer development in rats to the same extent as did that of a diet containing a high dose of UDCA (0.4%). It is expected that much smaller doses of UDCA might prevent colon carcinogenesis via an alteration in bile acid composition in the blood and feces, influencing the interaction with colonic cells.

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