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Enrichment of the More Hydrophilic Bile Acid Ursodeoxycholic Acid in the Fecal Water-soluble Fraction after Feeding to Rats with Colon Polyps[1]

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ABSTRACT

We recently showed that feeding the cytoprotective bile acid ursodeoxycholic acid (UDCA) to rats resulted in significant reduction in polyps and especially cancers, both in number and size (D. L. Earnest *et al.*, Cancer Res., *54:* 5071–5074, 1994). Because fecal secondary bile acids [particularly deoxycholic acid (DCA)] are considered to promote formation of colon adenomas and cancer, we have now attempted to find a relationship between polyp reduction and fecal secondary bile acids after feeding UDCA to these rats. We examined the fecal bile acids in rats with polyps and compared them with fecal bile acids in control rats and also determined the bile acid composition in fecal aqueous phase, which is in direct contact with the colon epithelium and may be physiologically more active. Treatment with azoxymethane did not significantly alter fecal bile acid composition in the rats. Cholic acid feeding resulted in greatly increased proportions of DCA (82% of total bile acids *versus* 18% in control rats). On the other hand, UDCA feeding significantly reduced the proportion of fecal DCA (2% in control rats fed UDCA and 3% in rats also treated with azoxymethane). In control rats, 96% of the bile acids were present in the water-insoluble fraction and 4% in the water-soluble fraction. The major insoluble bile acids included DCA and hyodeoxycholic acid (73% of total bile acids). In contrast, the muricholic acids were concentrated in the soluble fraction (37%). When 0.4% UDCA was added to the diet, lithocholic acid increased in the insoluble fraction (40 *versus* 1%), but the hydrophilic UDCA and muricholic acids were enriched in the water-soluble fraction (37 and 43%, respectively). Thus, the hydrophobic bile acids were distributed predominantly in the water-insoluble fraction, whereas the hydrophilic bile acids were distributed preferentially in the water-soluble fraction. These data suggest that UDCA may prevent colon tumors and polyps by countering the toxic effect of DCA and enhancing the possible cytoprotective effects of UDCA and muricholic acids in the water-soluble fraction in the feces of rat.

INTRODUCTION

Secondary bile acids have long been implicated in colorectal cancer as cocarcinogens, and some people with colorectal cancer are shown to excrete higher levels of secondary bile acids in their stool (1, 2). Diets high in fat tend to increase biliary secretion of bile acids, thereby increasing fecal secondary bile acids, and may be a factor in colonic carcinogenesis (3, 4). High concentrations of secondary bile acids, in particular DCA, may damage colonic epithelium to accelerate carcinogenesis (5–7). In most cases, colonic adenoma or polyp formation precedes colorectal cancer formation. These tumors can be successfully induced in experimental animals, usually by dimethylhydrazine, azoxymethane, methylazoxymethane, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, or *N*-methyl-*N*-nitrosourea (8), and effects of chemopreventive drugs can then be studied in these animals. We studied the effect of feeding a cytoprotective bile acid, UDCA,[3] to rats and found significant reduction in polyps and especially cancers in these rats, both in number and size (9, 10). To find a relationship between polyp reduction and fecal secondary bile acids after feeding UDCA, we examined the fecal bile acids in these rats and compared them with bile acids found in feces of control rats. We also have separated the fecal water-soluble fraction from the fecal solid material and determined the bile acid composition in this fraction, which is in direct contact with the colon epithelium and may be physiologically more active.

MATERIALS AND METHODS

Chemicals. Cholic and UDCA were obtained from Ciba-Geigy (Summit, NJ). Reference standards of α-, β- and ω-muricholic acids were prepared according to Iida *et al.* (11). All other bile acids used as reference standards were purchased from Steraloids, Inc. (Wilton, NH).

Animals. Male albino Fischer 344 rats, initially weighing approximately 90–130 g, were used in the studies. The animals were kept in metabolic cages and were fed AIN-76 standard diet with or without added bile acids. All diets were obtained from Bio-Serv (Frenchtown, NJ). The study protocol and handling of the animals is described in an earlier publication (9). For fecal bile acid measurements, 24-h fecal collections were made a day before animals were sacrificed, and all samples were stored on dry ice until analyzed.

GLC. A Hewlett-Packard model 5890A gas chromatograph equipped with a flame ionization detector and an injector with a split/splitless device for capillary columns was used for all separations. The chromatographic column consisted of a chemically bonded fused silica CP-Sil-5 CB (stationary phase, 100% dimethylsiloxane) capillary column (25-m length × 0.22-mm inner diameter; Chrompack, Inc., Raritan, NJ), and helium was used as the carrier gas at a flow rate of 1 ml/min. The GLC operating conditions were as follows. Injector and detector temperatures were 260°C and 290°C, respectively. After injection, oven temperature was kept at 100°C for 2 min and then programmed at a rate of 35°C/min to a final temperature of 278°C (12). The bile acids (5–100 μg) were treated with 3% anhydrous methanolic hydrochloric acid to obtain the methyl esters, which were then reacted with 100 μl of Sil-Prep (hexamethyldisilazane:trimethylchlorosilane:pyridine, 3:1:9; Alltech Associates, Inc., Deerfield, IL) for 20 min at 55°C. Solvents were evaporated at 55°C under N_2, the trimethylsilyl ether derivative formed was taken in 100 μl of hexane, and 1–2 μl were injected into the GLC column simultaneously with nor-deoxycholic acid used as the internal standard.

Gas Chromatography-Mass Spectrometry. Mass spectrometry of the bile acids, when needed, was carried out on a Hewlett-Packard model 5988 gas chromatograph-mass spectrometer using a 25-m fused silica CP-Sil-5 CB capillary column.

Separation of Fecal Aqueous Fraction and Solid Material. The 24-h feces were pooled and diluted with an equal weight of water and homogenized by vortexing for 10 min. The homogenized feces were then centrifuged at 100,000 × *g* for 30 min. The supernatant water layer was removed (fecal aqueous phase), and the pellet containing the solid material was weighed (fecal wet solid), freeze-dried, and weighed again (fecal dry solid). By subtraction of the weight of the fecal dry solid from the fecal wet solid, weight of the fecal aqueous phase adhering to the solid material was obtained. Aliquots from the aqueous and the solid fractions were used for bile acid analysis.

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[3] The abbreviations used are: UDCA, ursodeoxycholic acid; GLC, gas-liquid chromatography; PKC, protein kinase C.

Table 1 *Effect of UDCA on bile acids in fecal solid and aqueous phases in the rat*[a]

Treatment	LCA[b]	DCA	HyoDC	UDCA	β-MC	ω-MC	Unknown	Total[c]
Bile acids in fecal aqueous fraction								
STD[d]	1 ± 1	8 ± 3	38 ± 6	6 ± 2	10 ± 3	27 ± 7	10 ± 5	0.25 ± 0.07
UDCA[d]	1 ± 1	1 ± 1	10 ± 3[e]	37 ± 5[e]	17 ± 4	26 ± 4	9 ± 5	0.76 ± 0.16
AOM[f]	1 ± 1	9 ± 3	39 ± 8	5 ± 2	9 ± 3	27 ± 5	10 ± 3	0.36 ± 0.07
AOM + UDCA[f]	1 ± 1	2 ± 1	8 ± 3[e]	32 ± 4[e]	12 ± 4	37 ± 8	9 ± 4	0.56 ± 0.14
Bile acids in fecal solid fraction								
STD[d]	3 ± 1	19 ± 5	54 ± 12	7 ± 2	3 ± 1	8 ± 3	7 ± 5	5.70 ± 1.00
UDCA[d]	40 ± 12	3 ± 1	14 ± 2[g]	31 ± 8[g]	3 ± 1	5 ± 1	5 ± 4	24.87 ± 4.30
AOM[f]	3 ± 1	19 ± 6	52 ± 12	6 ± 2	2 ± 1	7 ± 4	11 ± 5	5.34 ± 0.91
AOM + UDCA[f]	51 ± 12	3 ± 1	8 ± 2[g]	21 ± 5[g]	2 ± 1	7 ± 3	9 ± 4	26.12 ± 5.20

[a] Seven rats were included in each treatment regimen. Values are given as percentage mean ± SD.
[b] LCA, lithocholic acid; HyoDC, hyodeoxycholic acid; UDCA, ursodeoxycholic acid; β-MC, β-muricholic acid; ω-MC, ω-muricholic acid.
[c] mg/ml fecal aqueous phase and mg/g dry solid feces.
[d] Rats were fed standard diet (STD) or diet supplemented with UDCA (0.4%). Feces were collected after 28 weeks of feeding and frozen immediately.
[e] Differs from group 1 and group 3; $P < 0.001$.
[f] Rats were fed standard diet (STD) or diet supplemented with UDCA (0.4%) and were given weekly s.c. injections of azoxymethane (AOM; 0.15 mg/kg body weight) during the first 2 weeks. Feces were collected after 28 weeks of feeding and frozen immediately.
[g] Differs from group 5 and group 7; $P < 0.001$.

Bile Acid Analysis. To an aliquot (50 μl) of the fecal aqueous phase or the fecal solid material (25 mg) obtained above was added nor-deoxycholic acid (8.5 μg in 100 μl methanol), and then 0.5 ml of 3 N sodium hydroxide was added, and the contents were heated in an autoclave at 110°C for 3 h. After cooling, the products were diluted with 1 ml of water and extracted four times with *n*-hexane (2 ml each). The aqueous phase was cooled in ice, acidified with 50% hydrochloric acid to pH 1, and extracted four times with ethyl acetate (3 ml each). The organic layer was washed with water to neutrality and evaporated at 50°C under nitrogen. The product was treated with 3% methanolic hydrochloric acid for 4 h at room temperature, solvent evaporated at 55°C under nitrogen, silylated, and subjected to GLC. For calculation of bile acids present in the fecal solid and aqueous phases, bile acids present in the water adhering to the wet fecal solid were also calculated and added to the bile acids in the fecal aqueous layer and were subtracted from the bile acids in the fecal solid material.

Statistics. Data are reported as means ± SD. Statistical significance was evaluated by ANOVA followed by post hoc Bonferroni test and was accepted at the level of $P < 0.01$.

RESULTS

We have shown in a previous study that 47% of the rats fed control diets supplemented with azoxymethane developed colonic tumors, which increased to 72% when 0.4% cholic acid was also added to the diet (9). On the other hand, supplementation with 0.4% UDCA reduced the number of tumor-bearing rats to 22% of control, showing a tumor-reducing effect of UDCA.

In this retrospective study, we have now compared the proportion of fecal bile acids in the water-soluble "active" fraction and the "inactive" water-insoluble fraction. The fecal water-soluble bile acids were found to constitute approximately 4 and 6%, respectively, of the total bile acids in the feces in the control and azoxymethane-treated rats, and 3 and 2%, respectively, after feeding 0.4% UDCA to rats in each group (Table 1). Although the total bile acids excreted in the feces increased approximately 5-fold after UDCA treatment, the water-soluble bile acids increased only 2–3-fold, the increase being mainly due to the addition of hydrophilic UDCA and β- and ω-muricholic acids (Table 1). The ratio of these hydrophilic bile acids: hydrophobic DCA and lithocholic acid in the water-soluble fraction increased from 5 to 40 after feeding UDCA and from 4 to 27 in rats also treated with azoxymethane (Table 1). On the other hand, the hydrophilic and the hydrophobic bile acids in the water-insoluble fraction were present in similar proportions before and during UDCA treatment (0.8 *versus* 0.9 in the control rats before and during UDCA treatment and 0.7 *versus* 0.55 in rats also treated with azoxymethane; Table 1).

As expected, lithocholic acid, the least soluble bile acid at fecal pH, was almost exclusively present in the inactive solid phase. In spite of a large increase in lithocholic acid in the solid phase bile acid pool after UDCA feeding (due to bacterial 7β-dehydroxylation of UDCA), its amount in the water-soluble phase did not change significantly. The proportion of DCA was greatly reduced in both water-soluble and -insoluble fractions after UDCA treatment. The absolute amount of DCA was reduced 62 and 65%, respectively, in the solid phase and 31 and 23%, respectively, in the aqueous phase after UDCA feeding to control rats and rats treated with azoxymethane (Table 1).

In Table 2 are listed the fecal total bile acids in the rats fed the various diets. There was no significant difference in fecal bile acid composition between control rats and rats injected with azoxymethane. Mainly the secondary bile acids, hyodeoxycholic acid, DCA, and ω-muricholic acid, were excreted in the feces in each case. Feeding cholic acid resulted in very efficient 7α-dehydroxylation, with formation of DCA as the major fecal bile acid. Only small amounts of hyodeoxycholic acid were present, and ω-muricholic acid was barely detectable ($P < 0.001$), suggesting the virtual saturation of the intestinal 7β-dehydroxylating bacteria by the ingested cholic acid. On the

Table 2 *Effect of various treatment regimens on fecal bile acids in the rat*[a]

Diet	LCA[b]	DCA	CA	HyoDC	UDCA	β-MC	ω-MC	Unknown
STD[c]	2 ± 1	18 ± 3		53 ± 6	7 ± 2	3 ± 1	9 ± 3	8 ± 3
STD + CA		82 ± 5[d]	6 ± 3	2 ± 1[d]	1 ± 1	3 ± 1	[d]	5 ± 3
STD + UDCA	39 ± 10[d]	2 ± 1[d]		14 ± 5[d]	31 ± 12[d]	3 ± 2	6 ± 2	5 ± 3
AOM[e]	3 ± 1	19 ± 3		51 ± 12	6 ± 2	3 ± 1	8 ± 3	11 ± 5
AOM + CA		77 ± 12[d]	3 ± 3	2 ± 1[d]	4 ± 1	2 ± 1	2 ± 1[d]	10 ± 5
AOM + UDCA	50 ± 13[d]		8 ± 3[d]	21 ± 5[d]	2 ± 1	8 ± 4	8 ± 4	

[a] Seven rats were included in each treatment regimen. Values are given as percentage mean ± SD
[b] LCA, lithocholic acid; CA, cholic acid; HyoDC, hyodeoxycholic acid; UDCA, ursodeoxycholic acid; β-MC, β-muricholic acid; ω-MC, ω-muricholic acid.
[c] Rats were fed standard diet (STD) or diets supplemented with cholic acid (0.4%) or UDCA (0.4%). Feces were collected after 28 weeks of feeding and frozen immediately.
[d] Differs from group 1 and group 4; $P < 0.001$.
[e] Rats were fed standard diet (STD) or diets supplemented with cholic acid (0.4%) or UDCA (0.4%) and were also given weekly s.c. injections of azoxymethane (AOM; 0.15 mg/kg body weight) during the first 2 weeks. Feces were collected after 28 weeks of feeding and frozen immediately.

other hand, UDCA feeding resulted in only partial 7β-dehydroxylation, and increased amounts of both lithocholic acid and UDCA were excreted in the feces (Table 2). There was a slight reduction in the absolute amount of DCA; however, its proportion was greatly reduced when UDCA was given ($P < 0.001$). Substantial amounts of hyodeoxycholic acid and ω-muricholic acid were still formed. Bacterial 7β-dehydroxylation of UDCA increased somewhat in azoxymethane-treated rats as compared with control rats fed UDCA (increased fecal lithocholic acid and reduced UDCA), although the difference was not statistically significant (Table 2).

DISCUSSION

In humans, approximately 600 mg of the primary bile acids, chenodeoxycholic acid and cholic acid, escape daily reabsorption from the ileum and are exposed to colonic bacteria, by which they are extensively metabolized. The predominant secondary bile acids formed are the 7α-dehydroxylated products lithocholic acid and DCA. These secondary bile acids have been implicated with colon carcinogenesis (1, 2), and glycodeoxycholic acid is shown to cause hepatocyte injury by apoptosis (13). It is believed that due to their detergent nature, these hydrophobic bile acids damage cells by dissolving lipids in the cell membrane (14). Conditions that increase the formation of these secondary bile acids likely result in greater cell damage, whereas UDCA with its equatorial 7β-hydroxyl group is a poor detergent and has been shown to be cytoprotective to the cell (15, 16).

The purpose of this study was to determine whether a correlation existed between colonic adenoma reduction after UDCA feeding to rats and their fecal secondary bile acids. UDCA has been shown to suppress azoxymethane-induced polyp formation in rats (9). Wali *et al.* (10) have suggested that azoxymethane treatment results in an increase in specific isoforms of PKC, particularly PKC-α, PKC-β_{11}, and PKC-ζ, which may be involved in polyp formation. Treatment with UDCA has been shown to suppress their formation (10).

Azoxymethane treatment did not significantly change fecal bile acid concentrations in control rats. Cholic acid feeding resulted in similar increases in fecal DCA output in control rats and rats treated with azoxymethane (Table 2); however, the 7β-hydroxy UDCA, which is shown to be more resistant to bacterial 7β-dehydroxylation (17), was only partially dehydroxylated to lithocholic acid when fed to the azoxymethane-treated rats. More lithocholic acid was formed after feeding UDCA to the azoxymethane-treated animals as compared to control rats (Table 2); however, the difference did not reach statistical significance, and substantial amounts of UDCA were still present in the colon.

A bile acid must be both ionized and in solution to obtain contact with the colonic epithelium. Thus, the portion of fecal bile acids that is not in solution may have little physiological significance, and it appears more important to determine this "active" water-soluble fraction of fecal bile acids than the total bile acids present in the feces, most of which are present in an "inactive" insoluble fecal solid state. Obviously, the bulk of lithocholic acid and a major fraction of DCA in the feces are present only in the insoluble fraction. Only the proportion of these bile acids in solution can cause cellular damage through direct contact with the colonic epithelium.

Although hyodeoxycholic acid was the major fecal water-soluble bile acid, significant amounts of muricholic acids, particularly ω-muricholic acid, also were present. Feeding of UDCA resulted in significant changes in both the fecal aqueous phase and insoluble bile acids. Substantial amounts of this bile acid were present in the aqueous fraction, and although a significant portion of UDCA was 7β-dehydroxylated to lithocholic acid, there was no significant increase in the proportion or the absolute amount of lithocholic acid in the fecal aqueous phase from its pretreatment levels. On the other hand, the proportion of DCA in the water-soluble fraction was reduced from pretreatment levels (Table 1). After feeding, the proportion of UDCA in the insoluble fraction rose from 7 to 31%, whereas the proportion of lithocholic acid rose from 3 to 40%, suggesting a substantial but incomplete 7β-dehydroxylation of UDCA by the intestinal bacteria. The proportion of the "inactive" UDCA increased in the feces of azoxymethane-treated rats to 21%, whereas lithocholic acid rose to 51% of the total insoluble bile acids, suggesting that treatment with azoxymethane might enhance bacterial 7β-dehydroxylation of UDCA. Increased formation of ω-muricholic acid after UDCA feeding suggests 6α-hydroxylation of UDCA in the rat. 6α-Hydroxylation of UDCA has been observed in patients with primary biliary cirrhosis treated with UDCA wherein small amounts of ω-muricholic acid were found in the urine (18).

6-Hydroxylation of bile acids increases their water solubility and hydrophilicity, and both β- and ω-muricholic acids are more water soluble and more hydrophilic than UDCA. Their increased formation in the feces of rats given UDCA results in increased amounts of these bile acids in the water-soluble fraction and increased overall hydrophilicity of the aqueous fraction in the feces. It is possible that in addition to the cytoprotective effect of UDCA (15, 16), the more hydrophilic β- and ω-muricholic acids also may enhance this effect (19) on the rat colonic epithelium and thus contribute to polyp reduction in these animals. In conclusion, the beneficial effect of UDCA lies in the cytoprotective effect of its increased intraluminal concentrations, by the reduced proportion of cytotoxic DCA and presumed cytoprotective effect of the increased amounts of the muricholic acids in the active water-soluble bile acid fraction in the feces of rat.

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