Effects of Oral Administration of Sulfolithocholic Acid Disodium Salt and Lithocholic Acid Sodium Salt on N-Methyl-N-nitrosourea-induced Colonic Tumorigenesis in Conventional Rats

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ABSTRACT

Effects of p.o. administration of sulfolithocholic acid disodium salt (SLCNa) and lithocholic acid sodium salt (LCNa) on N-methyl-N-nitrosourea (MNU)-induced colonic tumorigenesis were studied in conventional rats. Female F344 rats received either 0.5 ml of distilled water (DW) alone or DW containing 2.5 mg of MNU twice in 1 wk intrarectally. Then rats were fed freely on a basal diet (PCE-2) or PCE-2 containing LCNa or SLCNa (both at 0.5 mmol/100 g of PCE-2) for 40 wk. Thus, 6 groups were completed: MNU + PCE-2 (n = 30); MNU + LCNa (n = 29); MNU + SLCNa (n = 22); DW + PCE-2 (n = 17); DW + LCNa (n = 20), and DW + SLCNa (n = 19). Numbers of rats bearing colonic tumor were 3 (10%) in MNU + PCE-2, 2 (7%) in MNU + LCNa, and 8 (36%) in MNU + SLCNa group (uncorrected χ² = 9.35 among the 3 groups), but none in those groups without MNU. Total fecal bile acids in the rats given bile salts showed about 2-fold increase compared with those without bile salts. Fecal bile acid profiles between the LCNa and SLCNa groups were indistinguishable except for a slight increase of sulfolithocholic acid in the SLCNa groups. These results indicated that p.o. administration of SLCNa but not LCNa promoted MNU-induced colonic tumorigenesis in conventional rats. Fecal bile acid profiles did not support the higher tumor incidence in the MNU + SLCNa group compared with the MNU + LCNa group, which suggested an unrecognized mechanism probably relating to desulfation of SLCNa was involved in this phenomenon.

INTRODUCTION

Studies of etiological factors have indicated that high intake of dietary fat is associated with colonic carcinogenesis (1). Bile acids have been implicated in this event, since high fat diets increase bile acid outputs (2), and clinical and experimental evidence suggests that bile acids promote colonic carcinogenesis (3). Promoting capacity of bile acids was demonstrated in such compounds as LCA,2 deoxycholic acid, chenodeoxycholic acid, cholic acid, and 5/3-chol-3-en-24-oic acid (4–6). These compounds were dissolved in oil or water depending on their polarity and solubility in these solvents and given through an intrarectal route to attain an effective concentration of bile acids in the colon (4–6). Chenodeoxycholic acid and cholic acid were also examined by p.o. administration which was another route to attain a high concentration of bile acids in the colon (7, 8). In the p.o. experiments administered cholic acid acted efficiently, but chenodeoxycholic acid did insufficiently towards colonic tumor promotion (7, 8). These consequences were in contrast with those by the intrarectal route where chenodeoxycholic acid and cholic acid were equally effective in tumor promotion (5) or where chenodeoxycholic acid induced ornithine decarboxylase activity more strongly than did cholic acid (9). Such variant results between p.o. and intrarectal administration were ascribed to the different properties of the principal metabolites of these compounds by intestinal microflora; namely, deoxycholic acid from cholic acid was soluble in aqueous phases and, hence, active on the intestines (7), whereas LCA from chenodeoxycholic acid was mostly insoluble in aqueous phases and unavailable to act on the intestines (8). These results suggested that not only structure-activity of bile acids but also their interaction with intestinal microflora and microclimates was important in physiological or pathophysiologically action of bile acids. Particularly, action of LCA was largely dependent on these factors in such a way that it was inactive in physiological aqueous phases (8) despite the fact that it was active when dissolved in oil (4, 9). In view of these observations further consideration on the unique properties of LCA metabolism was warranted, since sulfation of LCA changes this less-polar compound to highly water-soluble SLCA, whereas amidation does not change its aqueous solubility (10). Sulfation of bile acids is thought to be one of the defense mechanisms by facilitating faster elimination of toxic LCA from the body into urine and feces (11–13). Conversely desulfation may imply retoxification of detoxified compounds. Indeed desulfation of SLCA by intestinal microflora liberates such tumor promoters as LCA or 5/3-chol-3-en-24-oic acid (14). Therefore, it is conceivable that administration of SLCA may have a promoting capacity in colonic tumorigenesis through interaction with intestinal microflora. This speculation prompted us to study the effects of SLCA on experimental colonic tumorogenesis in conventional rats. SLCA was administered p.o., since this route was appropriate to know the results of interaction with intestinal microflora. This speculation prompted us to study the effects of SLCA on experimental colonic tumorogenesis in conventional rats. SLCA was administered p.o., since this route was appropriate to know the results of interaction with intestinal microflora.

MATERIALS AND METHODS

Animals. One hundred thirty-seven 4-wk-old female F344/NS1c rats were obtained from Shizuoka Laboratory Animal Center (Hamamatsu, Japan) 4 wk before the experiment. Rats were housed in plastic cages (2/cage) with sterilized wooden chips as bedding in an air-conditioned room at 23 ± 2°C and 55 ± 5% humidity with 12-h alternating light and dark periods. Rats were first fed on a commercial pellet diet (CE-2; Clea Japan, Osaka, Japan) and tap water ad libitum. Two wk later the diet was changed from pellets to PCE-2 given in a glass container with a plastic cap. PCE-2 was renewed every other day, and intake of PCE-2 was monitored. Body weight and gross appearance were recorded periodically.

Bile Acids and Chemicals. LCA was obtained from Wako Pure Chemicals (Osaka, Japan), and LCNa was prepared with the equimolar sodium hydroxide. SLCNa was synthesized by the method described by Tserng and Klein (15). No impurity was seen in these products on thin-layer chromatography as the R<value of SLCNa relative to LCNa.
was 0.72 using a normal butyl alcohol:acetic acidwater (10:1:1) system (16). MNU was purchased from Nakarai Chemicals (Kyoto, Japan) and used by dissolving in DW immediately before intrarectal injection.

In preparation of diets, bile salts were mixed with PCE-2 sequentially to make up a final concentration of 0.5 mmol/100 g of PCE-2. This concentration was chosen from a previous study so as not to induce serious effects on rats (17). Bile salts were stable for at least 6 mo at room temperature as assessed on thin-layer chromatography (16).

Experimental. Eighty-one rats received intrarectal injections of 0.5 ml of DW containing 2.5 mg of MNU twice in 1 wk using a blunted gastric feeding metal tube by inserting 8 cm from the anus. Fifty-six rats received 0.5 ml of DW by the same manner. The rats were left without any treatment for a week after the second injection. Thereafter these rats were fed on PCE-2 alone, or PCE-2 containing LCNa or SLCA for 40 wk. Thus, 6 groups were completed in this study; Group I, MNU + PCE-2 (n = 30); Group II, MNU + LCNa (n = 29); Group III, MNU + SLCA (n = 22); Group IV, DW + PCE-2 (n = 17); Group V, DW + LCNa (n = 20); and Group VI, DW + SLCA (n = 19). At the end, rats were killed with diethyl ether, autopsied, and examined histologically with exactly the same method and criteria as described before (6).

Fecal Bile Acid Analysis. Feces were collected 1 wk before the intrarectal injection and 4 times during bile salts administration. Fresh feces were obtained by pushing fecal masses out of the rectum by hands. Feces before the intrarectal injection were all combined together, and those during were combined in each group. Feces were freeze-dried, pulverized, and stored at -20°C.

One hundred mg of dried feces were subjected to the analytical method as described in detail before (18). Briefly dried feces were refluxed in organic solvents and purified using Lipidex 1000 (United Technologies Packard, Downers Grove, IL) and Bond-Elut Carboxyl (Analytichem International, Harbor City, CA). Bile acids were then separated to their mode of conjugation on Lipidex-DEAP (Packard) into unconjugates, taurine conjugates, and sulfates. Conjugated fractions were subjected to solvolysis and/or hydrolysis, and liberated unconjugates were recovered. Unconjugates thus obtained were measured by capillary column gas chromatography (6, 18), and compounds were ascertained on mass spectrometry. Analyses were done in duplicate. Variation of duplicates was about 3%, and recovery was quantitative. Results were shown as the mean of duplicates per gram of dry feces without correction for recovery.

Statistical Analysis. The χ² test with Yates' correction was used as appropriate.

RESULTS

Record of Animal Care. During the experiment, there were no notable changes in gross appearance except for harsh hair in rats given bile salts. PCE-2 intakes were about 9.7 to 10.1 g/day/rat on average without any difference among the groups. Accordingly ingestion of bile salts showed only a slight difference as in the LCNa (50.7 μmol/day/rat) and SLCA (51.7 μmol/day/rat) groups. The body weight of rats increased by about 0.2 g/day/rat on average without any difference among the groups.

Autopsy and Histological Findings. Table 1 summarizes the number of rats bearing colonic tumors and characteristics of tumors. None of the rats without MNU had tumors, whereas those that received MNU had tumors in varied incidences as 10%, 7%, and 36% in Groups I, II, and III, respectively [uncorrected χ² = 9.35 among the 3 groups, and corrected χ² = 3.82 for Group I versus III (P < 0.1), and 5.14 for Group II versus III (P < 0.05)]. Histological examination showed that the tumors were all of epithelial origin. And one rat (3%) in Group I, 2 rats (7%) in Group II, and 3 rats (14%) in Group III had a tubular adenocarcinoma. The deepest invasion of the carcinomas was to the submucosa in one of Groups I and III. There was no metastasis to other organs including regional lymph nodes. The incidence of carcinoma in Group III was higher compared with the other groups; however, the difference was not significant (uncorrected χ² = 1.98 among the 3 groups). Nor was the difference significant with respect to the ratios between adenoma and carcinoma (uncorrected χ² = 3.11 among the 3 groups), although both tumors of Group II were carcinomas. The size of tumors in Group II was larger than the others, but the numbers of tumors were too small to compare with the other groups adequately. The tumors of Group II were pedunculated, while halves of tumors in the other groups were sessile (uncorrected χ² = 3.11). All of these tumors were distributed on the distal colon.

There were no noticeable macroscopic or microscopic changes in other organs except for a mild hydropic appearance and/or slight ducal cell reaction of the liver (17) microscopically in about 20% of rats given bile salts. There was no difference in these features with respect to LCNa, SLCA, or MNU. There was no extracolonic tumor in any rats.

Fecal Bile Acid Profiles. Excretions of total and principal bile acids in these rats are shown in Fig. 1. Total bile acids of rats given bile salts were almost double compared with those of rats without bile salts. Increased excretions of LCA and hyodeoxycholic acid were chiefly responsible for these results as can be assessed from the increased percentage of LCA and the unaltered percentage of hyodeoxycholic acid. Actual amounts of deoxycholic acid and other minor components were almost identical in all groups. With regard to conjugating forms, 94 to 98% were unconjugates, 1 to 5% were taurine conjugates, and 1 to 3% were sulfates. In the groups given SLCA, amounts of sulfated LCA were about 3- to 4-fold higher than those given LCNa or PCE-2 alone. However, as absolute amounts of sulfates were small, it did not make any difference in overall conjugating forms among the groups. 5β-Chol-3-en-24-oic acid was not detected in any of the feces in these groups. Although we examined fecal bile acids only in terms of per dry weight of combined feces in each group, there was no substantial difference in these results with respect to administration of LCNa and SLCA with or without MNU.

DISCUSSION

Effects of p.o. administration of bile acids on experimental colonic tumorogenesis have been studied in such compounds as cholic acid, chenodeoxycholic acid, and ursodeoxycholic acid, where the former 2 compounds showed an efficient or incomplete promoting effect (7, 8, 19, 20). Effects of p.o. administration of LCA and SLCA have not been examined, which is presumably because (a) LCA was already shown to be a promoter when it was given intrarectally in oil solution (4), (b) LCA feeding causes harmful effects to animals (17), (c) LCA is not expected to exert any significant effect on the large intestine for its low solubility in aqueous phases (8, 21), or (d) sulfation of bile acids is one of the defense mechanisms of the body (11, 12); hence, LCA is not expected to do any harm to animals. The present study showed that p.o. administration of SLCA but not LCNa promoted MNU-induced colonic tumorogenesis in conventional rats.

We used the same tumor initiating system as our previous study (6). The incidence of colonic tumor in the present control group with MNU alone (10%) was lower than in the previous one (17%) (6). A shorter experimental period and absence of an intrarectal maneuver could be responsible for this trend in the present study. The relatively smaller size of the tumors compared with the previous study (6) could also be due to the
compared with the others. The implication of these results is significant, and the numbers of tumors were not large enough to make further interpretation appropriate.

In addition, the differences were statistically insignificant, as this compound was detected so far only in the large intestine (23). Although there is no direct evidence yet, we speculate that, when SLCNa was desulfated just on the lipid bilayer, then liberated compounds like LCA would be able to act on the epithelial cells. Although LCA was unable to pass through the mucosa's resistance (21, 25) and might not be active on the intestines. Therefore, we assume that, when SLCNa was desulfated just on the lipid bilayer, then liberated compounds like LCA would be able to act on the epithelial cells. Although LCA was unable to pass through the mucosa's resistance (21, 25) and might not be active on the intestines.

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats</th>
<th>Colon tumor</th>
<th>Adenocarcinoma</th>
<th>Total no. of tumors</th>
<th>Size (mm)</th>
<th>Shape</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td>%α</td>
<td>No.</td>
<td>%α</td>
<td>Range</td>
</tr>
<tr>
<td>I (MNU + PCE-2)</td>
<td>30</td>
<td>3</td>
<td>10</td>
<td>1</td>
<td>3</td>
<td>3 (2/1)*</td>
</tr>
<tr>
<td>II (MNU + LCNa)</td>
<td>29</td>
<td>2</td>
<td>7</td>
<td>2</td>
<td>7</td>
<td>2 (0/2)</td>
</tr>
<tr>
<td>III (MNU + SLCNa)</td>
<td>22</td>
<td>8</td>
<td>36</td>
<td>3</td>
<td>14</td>
<td>9 (6/3)</td>
</tr>
</tbody>
</table>

* Uncorrected \( \chi^2 = 3.11 \) among the 3 groups (not significant).
α Uncorrected \( \chi^2 = 9.35 \) among the 3 groups, and corrected \( \chi^2 = 3.82 \) for Group I versus III (\( P < 0.1 \)), and 5.14 for Group II versus III (\( P < 0.05 \)).
β Uncorrected \( \chi^2 = 1.98 \) among the 3 groups (not significant).
γ Uncorrected \( \chi^2 = 3.11 \) among the 3 groups with respect to the adenoma/carcinoma ratios (not significant).

Fig. 1. Excretion of total bile acids (\( \mu \text{mol/g of dry feces} \)) and the percentage of principal bile acids in rats given intrarectal injection of either DW or MNU followed by p.o. administration of a basal diet (PCE-2), or PCE-2 containing LCNa or SLCNa.

same reasons. The histological characteristics of tumors in this study were not different in principle from our previous study (6) or from those in the literature (4, 5, 7), as the tumors were of epithelial origin including adenoma and carcinoma. The incidence of carcinoma in the rats with MNU + SLCNa was higher than the other groups. And the rats with MNU + LCNa had larger tumors and a different adenoma/carcinoma ratio compared with the others. The implication of these results is not certain. In addition, the differences were statistically insignificant, and the numbers of tumors were not large enough to make further interpretation appropriate.

Systemic effects of LCNa were similar to those reported before (17), but the extent was far less serious, presumably because of the lower dosage in our study. Our rats gained body weight normally. Systemic effects of SLCNa were the same as those of LCNa, which was suggestive of desulfation of SLCNa in the intestines as discussed below.

We found that fecal bile acid profiles were indistinguishable between the LCNa and SLCNa groups except for a slight increase of SLCA in the SLCNa groups. These results were compatible with the recent report (13) that the i.p. administration of radiolabeled taurine conjugates of LCA and SLCA resulted in the same fecal tracer pattern. Moreover, although the route of administration, dosage, and conjugating form were different, these two studies agreed that the predominant compounds in feces of these rats were LCA and hyodeoxycholic acid. These results indicated that efficient desulfation of free and amides of SLCA took place in the intestines liberating mainly LCA (11, 13). \( 5 \beta \)-Chol-3-en-24-oic acid was not detected in the present study. Whether this was due to extensive metabolism of this compound in rats (6) or to species difference is not certain, as this compound was detected so far only in the incubation with human intestinal microflora (14, 22) but not with rat flora (23).

The p.o. administration of LCNa and SLCNa increased the concentrations of LCA and hyodeoxycholic acid in feces. LCA was insoluble in aqueous phases (21), and hyodeoxycholic acid was inactive for ornithine decarboxylase induction (9). Therefore, these compounds were unlikely to be involved in tumor promotion. Deoxycholic acid and other bile acids in feces of rats given bile salts were similar to those of rats on the basal diet. Thus, our finding that p.o. administration of LCNa was associated with high LCA and hyodeoxycholic acid in feces but not with tumor promotion seemed to be in accordance with the previous observation (8).
in this case sulfate function of SLCA acts as a vehicle for LCA to carry it into the large intestine by avoiding absorption from the intestines (12) and to let it pass through the mucosal resistance against less-polar bile acids (21, 25) by modifying the aqueous solubility. Some of the less-polar bile acids are potent tumor promoters (4, 6) and induce colonic tumor promotion without appreciable alteration in bile acid metabolism (6). Therefore, increased concentrations of these bile acids acting on the mucosa, to which extent systemic bile acid metabolism is not altered, may cause significant effects on tumor promotion. This could possibly also account for some of the results of the fecal bile acid study in humans, where no substantial difference was observed quantitatively among patients with colonic tumors and healthy controls (27), despite the role of bile acids in colon carcinoma being apparent experimentally (3–7). Further study on mucosa-associated bacterial desulfation of SLCA and on gnotobiotic models is necessary to test this speculation.

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