Administration of ursodeoxycholate failed to prevent sludge and/or gallstone formation in cholecystokinin-1(A) receptor-deficient mice

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ABSTRACT

Gallstone disease is one of the most prevalent digestive diseases. The frequency of gallstone disease is about 10% in middle-age persons and 20% in aged persons. Gallbladder dysmotility and stasis of bile flow promote sludge and/or gallstone formation. Gallbladder contraction depends on cholecystokinin (CCK) via CCK-1 receptors (R)s. Previously, we raised CCK-1R deficient (–/–) mice and observed sludge and/or gallstone formation in more than 30% at 12–24 months of age. As ursodeoxycholate (UDCA) is commonly used for patients with gallstone disease, we expected that continuous administration of UDCA could prevent sludge and/or gallstone formation in CCK-1R(–/–) mice. A diet containing 0.1% UDCA was administered in age-matched CCK-1R(–/–) and wild-type male and female mice for 8 months. Administration of UDCA decreased the frequency of sludge and/or gallstone formation compared with the control (CRF-1) diet (39%→26% in male, 35%→25% in female mice); however, these effects did not attain a level of statistical significance. Although the body weight was significantly higher in UDCA-fed than CRF-1-fed male mice regardless of genotypes, the plasma lipid concentrations did not differ between the two diets. In conclusion, administration of UDCA was less effective than expected at preventing sludge and/or gallstone formation in CCK-1R(–/–) mice.

Gallstone disease is one of the most prevalent digestive diseases. Although the frequency of gallstone disease (with or without symptoms) is about 10% in middle-age persons, the frequency is further increased in aged persons (20%) (7, 15). One of the reasons for the higher frequency of gallstone disease in the aged may be the decrease in gallbladder contraction, possibly due to the decrease in numbers of neurons in the gastrointestinal smooth muscles in aged persons (6). This is a special concern in Japan,

where people over 65 years old accounted for more than 22% of the population in 2008. Meanwhile, a recent report from the U.S. (14) shows that persons with gallstone disease have increased overall mortality compared to the general population, especially from cardiovascular disease and cancer. These reports suggest the importance of primary prevention of gallstone disease.

Three abnormalities have been proposed in the pathogenesis of gallstones: 1) supersaturation in the solutes that precipitate from bile, such as cholesterol, calcium salts *etc.*, 2) in the case of cholesterol cholelithiasis, accelerated nucleation of cholesterol crystals in the gallbladder, and 3) gallbladder dysmotility and stasis (5). Gallbladder dysmotility and stasis have been shown to accelerate the nucleation

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of cholesterol crystals in the gallbladder (11, 17–19). Gallbladder contraction mainly depends on cholecystokinin (CCK) and CCK-1 (formerly called CCK-A) receptors (R)s (10). We generated CCK-1R knockout ($-/-$) mice and found that gallbladder contraction was not induced by CCK in CCK-1R($-/-$) mice, while CCK strongly induced gallbladder contraction in wild-type mice (20). Previously, more than 30% of CCK-1R($-/-$) mice, but not all, showed sludge and gallstone formation at 12 and 24 months of age while no sludge or gallstone formation was observed at 6 months of age in either wild-type or CCK-1R($-/-$) mice (8, 16). CCK-1R($-/-$) mice did not show obesity, plasma lipid abnormalities, or disturbance of glucose tolerance (21).

Long-term administration of one of the hydrophilic bile acids, ursodeoxycholate (UDCA), has been shown to promote the dissolution of cholesterol gallstones in humans (12, 23). UDCA is usually used as a therapy for patients with gallstone disease in Japan because of its lower toxicity and fewer side effects compared to other therapies (22). Therefore, in the present study, we expected that continuous administration of UDCA could prevent gallstone formation in CCK-1R($-/-$) mice. Moreover, in Western countries, cholesterol gallstones are more common in women and are associated with multiparity and the use of contraceptive pills containing high doses of estrogen (3–5, 13). The genetic factors of gallstone disease vary by race, and the sex difference of gallstone disease is not as clear-cut in Japan (male : female = 1 : 1.2) (7, 15) as in Western countries. Therefore, we also examined whether a sex difference existed for the effect of UDCA on the prevention of gallstone formation, using both male and female CCK-1R($-/-$) mice.

MATERIALS AND METHODS

The present experimental protocol was reviewed and approved by the appropriate committee of the Tokyo Kasei University. We also followed the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Physiological Society.

Animals and materials. The progenitor strain for CCK-1R($-/-$) was C57BL/6J (16, 20). More than seven generations of backcrossing were completed. The male CCK-1R($+/-$) mice were bred with female CCK-1R($+/-$) mice to yield progeny with the genotype CCK-1R($+/+$)(wild-type), ($+/-$), and ($-/-$). After weaning, determination of genotype was conducted

using blood obtained from the tail vein of progeny, and CCK-1R($+/-$) mice were removed from the cage; therefore, some cages contained both wild-type and CCK-1R($-/-$) mice. Two to 3 mice were maintained in one cage. All animals were maintained in a temperature-controlled room at 23°C with $55 \pm 5\%$ humidity, and with a 12-h light/12-h dark photocycle (08 : 00–20 : 00) at the animal room of the Tokyo Kasei University. The cage size was $18 \times 30 \times 11$ cm.

The UDCA was a generous gift from Mitsubishi Tanabe Pharma Co., Tokyo, Japan. A diet containing 0.1% UDCA in the standard diet CRF-1 (Oriental Co., Tokyo) was prepared.

Experimental protocols. After weaning, CRF-1 was provided until 4–6 months of age in all animals; then, CRF-1 was changed to the UDCA diet. The frequencies of sludge and gallstone formations were determined after 8 months' administration of UDCA. Body weight and food intake were recorded once a week. We estimated daily food intake over 7 days by providing a single weighed (220–240 g) serving of food to mice in respective cages; after the 7-day period, the remaining food including crumbs was weighed, and the daily food intake was estimated.

Administration of CRF-1 was continued in 8 additional male CCK-1R($-/-$) mice, 9 female CCK-1R($-/-$) mice, 1 male wild-type mouse, and 3 female wild-type mice. They were sacrificed at 12–14 months of age. The present results of CRF-1 were combined with previous data for 12 and 24 months of age provided in references 8 and 16.

Mice were sacrificed by guillotine and the blood was collected into tubes containing EDTA to obtain plasma. Thereafter, the gallbladder was removed and classified as normal (clear gallbladder), clouded (sludge formation), and/or containing gallstone formations. The plasma levels of triglyceride and cholesterol were measured by enzymatic determination (2).

Statistical analysis. Values are expressed as means $\pm$ SE. The significance of the differences was assessed by one-way analysis of variance (ANOVA), followed by Fischer's protected least significant difference (PLSD) test. The frequencies of gallstones were analyzed using the chi-squared test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Comparison in male mice with or without UDCA

The body weight did not differ significantly between UDCA-fed wild-type and CCK-1R(-/-) male mice, or between CRF-1-fed wild-type and CCK-1R(-/-) male mice (Table 1). However, the mean body weight was significantly higher in UDCA-fed mice compared with CRF-1-fed mice regardless of genotype [$F(3,94) = 8.58$, $P < 0.0001$]. The mean values of daily food intake of UDCA diet in respective cages when mice were 8 months old were 2.9–3.3 g/mouse (0.85–0.97 g/10 g body weight). Since each cage contained both wild-type and CCK-1R(-/-) mice as mentioned above, we could not determine the real food intake/day in wild-type and CCK-1R(-/-) mice separately. The mean values of food intake of CRF-1 shown in a previous report (16) were similar to each other: about 0.96 and 1.03 g/10 g body weight for CCK-1R(-/-) and wild-type mice at 8 months of age, respectively.

The frequency of sludge and gallstone formation in CRF-1-fed CCK-1R(-/-) mice was 39% while no

sludge and/or gallstone formation was observed in CRF-1-fed wild-type mice. When a UDCA diet was provided, 5 of 19 (26%) CCK-1R(-/-) male mice showed cloudy gallbladder (= sludge formation) (Table 1 and Fig. 1). Solid-type gallstones were not observed. However, the difference between UDCA-fed and CRF-1-fed CCK-1R(-/-) mice was not significant ($P = 0.25$). Unexpectedly, clouded gallbladders were observed in 3 of 11 UDCA-fed wild-type mice.

The plasma concentrations of triglycerides and cholesterol did not differ significantly among the 4 groups of male mice (Table 1).

Comparison in female mice with or without UDCA

In contrast with the results of male mice, the body weight in female mice did not differ significantly among the 4 groups [$F(3,84) = 2.60$, $P = 0.058$] (Table 2). The mean values of food intake of CRF-1 shown in a previous report (8) were 0.89–1.37 g/10 g body weight for CCK-1R(-/-) and 1.16–1.68 g/10 g body weight for wild-type mice. The mean values of daily food intake of UDCA diet were 2.4–3.5 g/

Table 1 Body weight, sludge and/or gallstone formation, and plasma concentrations of triglycerides and cholesterol in male mice

Diets	UDCA		CRF-1*	
Genotype	Wild-type	CCK-1R(-/-)	Wild-type	CCK-1R(-/-)
Number of animals	11	19	40	33
Body weight before UDCA (g)	29.3 ± 1.0	28.9 ± 1.0	not recorded	not recorded
Body weight after UDCA (g)	44.4 ± 1.3**	40.2 ± 1.4**	34.5 ± 0.84	33.0 ± 1.1
Sludge and/or gallstone formation***	3/11 (27%)	5/19 (26%)	0/40 (0%)	13/33 (39%)
Cholesterol (mM)	2.61 ± 0.22	2.65 ± 0.17	2.51 ± 0.17	2.58 ± 0.16
Triglycerides (mM)	0.64 ± 0.08	0.64 ± 0.06	0.85 ± 0.14	0.59 ± 0.85

Values are the mean ± SE. *The data of CRF-1 were the sums of present results and previous results as described in the text. **Significantly different from values of CRF-1. ***The frequency of sludge and gallstone formation corresponds to the number of sludge and gallstone-formation cases per number of animals examined.

Table 2 Body weight, sludge and/or gallstone formation, and plasma concentrations of triglycerides and cholesterol in female mice

Diets	UDCA		CRF-1*	
Genotype	Wild-type	CCK-1R(-/-)	Wild-type	CCK-1R(-/-)
Number of animals	13	20	29	26
Body weight before UDCA (g)	22.5 ± 0.4	24.0 ± 0.2	not recorded	not recorded
Body weight after UDCA (g)	32.5 ± 1.5	29.8 ± 0.7	27.9 ± 0.83	29.5 ± 1.00
Sludge and/or gallstone formation**	0/13 (0%)	5/20 (25%)	1/29 (3%)	9/26 (35%)
Cholesterol (mM)	1.98 ± 0.10	2.03 ± 0.06	2.02 ± 0.15	2.33 ± 0.12
Triglycerides (mM)	0.41 ± 0.05	0.39 ± 0.0	0.46 ± 0.03	0.46 ± 0.07

Values are the mean ± SE. *The data of CRF-1 were the sums of present results and previous results as described in the text. **The frequency of sludge and gallstone formation corresponds to the number of sludge and gallstone-formation cases per number of animals examined.

Fig. 1 **A.** A normal gallbladder of UDCA-fed male wild-type mouse. **B.** A clouded gallbladder of UDCA-fed male CCK-1R(-/-) mouse. The body weight was 47.6 g, and the plasma concentrations of triglyceride and cholesterol were 0.37 mM and 1.14 mM, respectively.

Fig. 2 A black pigmented gallstone in UDCA female CCK-1R(-/-) mouse. The body weight was 32.5 g, and the plasma concentrations of triglyceride and cholesterol were 0.23 mM and 2.2 mM, respectively.

mouse (0.77–1.12 g/10 g body weight).

One CRF-1-fed wild-type female mouse showed a clouded gallbladder while sludge and/or gallstone formation was observed in 9 of 26 (35%) CRF-1-fed CCK-1R(-/-) female mice (Table 2). No sludge and/or gallstone formation was observed in UDCA-fed wild-type female mice, but UDCA had no statistical effect in wild-type mice. The frequency of sludge and/or gallstone formation in UDCA-fed CCK-1R(-/-) female mice was 25% (5 of 20); however, the difference in frequency between the two diets for female CCK-1R(-/-) mice was not signifi-

cant ($P = 0.40$). One UDCA-fed female CCK-1R(-/-) had a black stone rather than a cholesterol stone (Table 2 and Fig. 2).

The plasma concentrations of triglycerides and cholesterol did not differ among the 4 groups of female mice (Table 2).

DISCUSSION

The present study showed that 8-month administration of UDCA slightly decreased the frequency of sludge and/or gallstone formation compared with administration of CRF-1: from 39% to 26% in male and from 35% to 25% in female CCK-1R(-/-) mice, respectively. However these effects did not attain a level of statistical significance.

Unexpectedly, sludge formation was observed in 3 of 11 UDCA-fed wild-type male mice. Interestingly, the mean body weights of male mice obtained from the present study were significantly higher than those in a previous study (8), while the body weight of female mice was not significantly different. As we observed that male mice are more susceptible to environmental factors (9), the present experimental conditions might have promoted higher weight, although the precise mechanism of the weight gain is unknown. Since obesity and hyperlipidemia as well as insulin resistance and hyperglycemia are risk factors for gallstones (3, 4, 14), the higher body weight (obesity) might be responsible for the sludge formation in male wild-type mice. On the other hand, the frequency of sludge and/or gallstone formation in UDCA-fed CCK-1R(-/-) male

mice was not further increased, but rather decreased (39% vs. 26%), despite their higher body weight than CRF-1-fed CCK-1R(−/−) mice. Therefore, UDCA might have been slightly, but not significantly, effective at preventing sludge formation induced by obesity in CCK-1R(−/−) mice.

Wang *et al.* (25) reported increased intestinal cholesterol absorption by the retardation of small intestinal transit time in CCK-1R(−/−) mice. We did not determine the cholesterol concentrations in the bile; however, one female CCK-1R(−/−) did not show cholesterol gallstones but black stones. Administration of chow containing hydrophilic bile acids including UDCA [0.5% (by weight) for 7 days] inhibited cholesterol absorption in C57L/J male mice without change in intestinal transit times (26). Although we used a diet containing 0.1% UDCA, a larger dose might be required to obtain a beneficial effect. However, when the dose of UDCA used in the present study was estimated, it was about 1 g UDCA/10 kg body weight and would be equivalent to 6000 mg UDCA/60 kg/day. Thus, administration of a higher dose of UDCA may not make sense.

Recently, successful preventive effects of UDCA on gallstone formation were observed in humans. Oral administration of UDCA could prevent gallstone formation after bariatric surgery, because rapid weight loss increases the risk for gallstone formation (24). Ai *et al.* (1) reported that oral administration of UDCA (600 mg/day) prevented the development of gallstones after cardiac surgery and that the prophylactic administration of UDCA could result in a significant decrease in the incidence of gallstones after open cardiac surgery. Although administration of UDCA failed to prevent sludge and/or gallstone formation in mice without surgical manipulation, administration of UDCA might be effective after surgery even in mice. Therefore, we do not deny the possibility of the preventive effect of UDCA on sludge and/or gallstone formation in mice.

In conclusion, administration of UDCA slightly decreased the frequency of sludge and gallstone formation in CCK-1R(−/−) mice, but the effect was not statistically significant. Sex difference appeared not to be a factor in the frequency of sludge and/or gallstone formation.

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