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Effect of gallbladder hypomotility on cholesterol crystallization and growth in CCK-deficient mice

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ABSTRACT

We investigated the effect of gallbladder hypomotility on cholesterol crystallization and growth during the early stage of gallstone formation in CCK knockout mice. Contrary to wild-type mice, fasting gallbladder volumes were enlarged and the response of gallbladder emptying to a high-fat meal was impaired in knockout mice on chow or the lithogenic diet. In the lithogenic state, large amounts of mucin gel and liquid crystals as well as arc-like and tubular crystals formed first, followed by rapid formation of classic parallelogram-shaped cholesterol monohydrate crystals in knockout mice. Furthermore, three patterns of crystal growth habits were observed: proportional enlargement, spiral dislocation growth, and twin crystal growth, all enlarging solid cholesterol crystals. At day 15 on the lithogenic diet, 75% of knockout mice formed gallstones. However, wild-type mice formed very little mucin gel, liquid, and solid crystals, and gallstones were not observed. We conclude that lack of CCK induces gallbladder hypomotility that prolongs the residence time of excess cholesterol in the gallbladder, leading to rapid crystallization and precipitation of solid cholesterol crystals. Moreover, during the early stage of gallstone formation, there are two pathways of liquid and polymorph anhydrous crystals evolving to monohydrate crystals and three modes for cholesterol crystal growth.

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1. Introduction

Physical–chemical studies of biliary lipids have clearly shown that in bile, cholesterol is solubilized as a mixture of simple and mixed micelles as well as vesicles, all in a dynamic equilibrium [1]. Although this equilibration process starts after hepatic secretion of bile and continues in the biliary tree, the nucleation and crystallization of cholesterol molecules occurs predominantly in the gallbladder when cholesterol concentrations reach supersaturation. It has been found that gallbladder biles in some normal subjects and in patients with cholesterol gallstones are often supersaturated with cholesterol [2,3]. Furthermore, the precipitation of solid plate-like cholesterol monohydrate crystals from supersaturated bile is the first irreversible physical–chemical step in the formation of cholesterol gallstones [4,5]. Clinical studies have observed that cholesterol monohydrate crystals could be detected in fresh duodenal bile samples [6–8]. Also, aggregated cholesterol monohydrate crystals embedded in mucin gel could often be observed by ultrasonography as biliary sludge, as confirmed by polarizing light microscopy in human gallbladder biles that are obtained after cholecystectomy [9–11]. It has been established that gallbladder stasis is an important prerequisite for gallstone formation because cholesterol is often nucleated into solid monohydrate crystals when cholesterol supersaturation in gallbladder bile reaches beyond a limit, as well as cholesterol crystal growth and aggregation are accelerated by pronucleating proteins such as mucins [12]. However, little was known about how abnormal gallbladder motility influences crystallization and growth of excess cholesterol in gallbladder bile.

Cholecystokinin (CCK) is a gastrointestinal hormone that is produced and secreted by intestinal I cells. It has been found that a significant increase in plasma concentrations of CCK occurs after ingestion of a meal or fat, which can induce a significant contraction of the gallbladder [13,14]. A defective gallbladder motility has been strongly linked to the formation of gallstones [15], and abnormal gallbladder motility has been observed both in vivo and in vitro in subgroups of cholesterol gallstone patients [16], as well as in gallstone-free subjects under several conditions such as pregnancy, obesity, and diabetes. This, in turn, might represent a significant pathophysiologically relevant stimulus predisposing to gallstone

Abbreviations: CCK, cholecystokinin; CSI, cholesterol saturation index; KO, knockout; WT, wild-type

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formation [12]. As a result, a growing interest is focused on the lithogenic effect of gallbladder hypomotility as a key trigger condition.

A CCK-deficient mouse model has been established by a gene targeting strategy in mouse embryonic stem (ES) cells [17]. Because CCK can enhance gallbladder contraction by acting as an agonist at CCK-1 receptor, deletion of the *Cck* gene could result in a defective gallbladder motility. By using this unique mouse model, we systematically investigated the effect of dysfunctional gallbladder motility on cholesterol crystallization and growth during the early stage of cholesterol gallstone formation. In the present studies, we found that the lack of CCK impaired gallbladder motility function, enlarged gallbladder size, and enhanced cholesterol crystallization mostly by prolonging the residence time of excess cholesterol in the gallbladder lumen. All of these changes promoted cholesterol crystal growth and gallstone formation. We further observed that during the early stage of gallstone formation, there are two pathways of the liquid and the polymorph anhydrous crystals evolving to solid plate-like cholesterol monohydrate crystals, as well as three modes for cholesterol crystal growth.

2. Materials and methods

2.1. Animals and diets

The CCK knockout (KO) mice were generated by using a gene targeting strategy in which of lacZ reporter gene was inserted into the mouse *Cck* gene, resulting in a null mutation [17]. As a result, the CCK KO mice produced no functional CCK peptide fragments. All of the mice studied in these experiments were genotyped by PCR analysis of tail DNA to determine their genotype. Male CCK KO and wild-type (WT) mice on a pure C57BL/6J genetic background were studied. All mice were provided free access to water and the normal rodent chow diet containing trace (<0.02%) amounts of cholesterol (Harlan Teklad F6 Rodent Diet 8664, Madison, WI). During the cholesterol crystallization and gallstone studies, mice at 8–10 weeks old were fed a lithogenic diet containing 1% cholesterol, 0.5% cholic acid, and 15% butter fat for 15 days. All procedures were in accordance with current National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of Harvard University (Boston, MA).

2.2. Gallbladder contraction study

To explore whether gallbladder motility function was impaired due to loss of *Cck* gene expression, fasting and postprandial gallbladder volumes were measured in mice ($n=8$ per group) fed the lithogenic diet for 15 days. To study fasting gallbladder size, mice were fasted overnight but had free access to water. After weighing, mice were anesthetized with an intraperitoneal injection of 35 mg/kg pentobarbital (Abbott Laboratories, North Chicago, IL). Laparotomy commenced at 9:00 AM and was performed under sterile conditions through an upper midline incision. During laparotomy, the gallbladder was clearly exposed and its size was measured with a microcaliper. Gallbladder volumes were calculated using the following formula, assuming an ellipsoid shape of the organ [18]:

$$\text{Gallbladder volume } (\mu L) = \text{length (mm)} \times \text{width} \times \text{depth(mm)} \times \pi / 6$$

To determine gallbladder emptying function in mice ($n=4$ per group) in response to a high-fat meal, a PE-10 polyethylene catheter was inserted into the duodenum during laparotomy. The duodenal catheter was externalized through the left abdominal wall and connected to an infusion pump (Kent Scientific, Litchfield, CT). Following completion of all surgical procedures, gallbladder size was immediately measured with a microcaliper and gallbladder volumes were calculated using the above-mentioned formula. Then, mice were intraduodenally infused with corn oil (i.e., a high-fat meal) or 0.9% NaCl (as a control) at 40 μl/minute for 5 minutes. At 30 minutes after the duodenal infusion, postprandial gallbladder volume was measured with a microcaliper again. Gallbladder emptying function was determined by a difference in gallbladder size before and after the duodenal infusion of corn oil.

2.3. Collection of gallbladder bile and microscopic studies

Before (day 0) and at 6, 9, 12, and 15 days on the lithogenic diet, cholecystectomy was performed in overnight fasted mice ($n=4$ per group) after anesthetization. Fresh gallbladder bile was immediately examined according to the methods described previously [19]. After microscopic analysis, fresh gallbladder bile was harvested and stored at −20 °C for lipid studies. In brief, the entire gallbladder bile was placed on a glass slide at room temperature (~22 °C) and observed without a cover slip using a polarizing light microscope and phase-contrast optics. After a small hole was made in the fundus of gallbladder, bulk bile dribbled by gravity and mucin gel was pressed out digitally with the assistance of a 24-gauge needle. These fresh bile samples were examined by microscopic analysis for the presence of mucin strands, liquid and solid crystals, and sandy and true gallstones [19]. Mucin was observed as non-birefringent amorphous strands. Arc-like and tubular crystals (assumed to be metastable transitional forms of anhydrous cholesterol being hydrated to cholesterol monohydrate crystals), plate-like cholesterol monohydrate crystals, as well as small, aggregated, and fused liquid crystals were defined according to previously published criteria [19,20]. Sandy stones were irregularly shaped, and easily disintegrable agglomerates of cholesterol monohydrate crystals surrounded by mucin gel. As visualized under the microscope, individual cholesterol monohydrate crystals projected clearly from the edges of sandy stones, and grossly they displayed a yellow color. True gallstones were hard, ball-like objects, and light yellow in color with smooth curved surfaces. Because of scattered and absorbed light, they were opaque and black in color when observations were made with polarizing light microscopy. The images of cholesterol monohydrate crystals and gallstones were analyzed by a Carl Zeiss Imaging System with an AxioVision Rel 4.6 software (Carl Zeiss Microimaging GmbH, Göttingen, Germany).

2.4. Lipid analyses

Biliary phospholipid was measured as inorganic phosphorus by the method of Bartlett [21]. Cholesterol was determined using an enzymatic assay. Total bile salt concentration was measured enzymatically by the 3α-hydroxysteroid dehydrogenase method [22]. Gallstones were washed, air-dried at 22 °C, and the cholesterol content (wt./wt.) was determined by HPLC [19]. Cholesterol saturation index (CSI) of pooled gallbladder biles was calculated from critical tables [23] established for taurocholate, the predominant bile salts in mouse bile on the lithogenic diet. Relative lipid compositions of pooled gallbladder biles ($n=4$ per group at each time point) were plotted on condensed phased diagrams appropriate to their mean total lipid concentrations [20]. For graphic analysis, the phase limits of the micellar zones and the crystallization pathways were extrapolated from model systems developed for taurocholate at 37 °C [20].

2.5. Statistical method

All data are expressed as means ± SD. Statistically significant differences among groups of mice were assessed by Student's *t*-test, Mann–Whitney *U*-tests, or χ^2 tests. If the *F*-value was significant, comparisons among groups of mice were further analyzed by a multiple comparison test. Analyses were performed with a *SuperANOVA* software (Abacus Concepts, Berkeley, CA). Statistical significance was defined as a two-tailed probability of less than 0.05.

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