Bovine gallbladder mucin promotes cholesterol crystal nucleation from cholesterol-transporting vesicles in supersaturated model bile

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Abstract This study examined the ability of purified gallbladder mucin to accelerate the nucleation of cholesterol monohydrate crystals from the cholesterol-transporting particles in supersaturated model bile. Mixed lipid micelles and cholesterol-phosphatidylcholine vesicles in supersaturated model bile were separated by Sephadex G-200 column chromatography. Mixed lipid micelles prepared by column chromatography had a low cholesterol-phosphatidylcholine ratio (0.30) and did not spontaneously nucleate cholesterol monohydrate crystals. In contrast, vesicles prepared by column chromatography had a cholesterol-phosphatidylcholine ratio of 1.00 and nucleated cholesterol crystals rapidly \( (P < 0.001) \). Nucleation of cholesterol crystals was significantly accelerated in a concentration- and time-dependent manner by purified bovine gallbladder mucin in cholesterol containing vesicles, but not in mixed lipid micelles \( (P < 0.001) \). A rapid filtration binding assay demonstrated significant binding of cholesterol and phosphatidylcholine in vesicles to gallbladder mucin but only minimal binding of cholesterol and phosphatidylcholine in mixed micelles. These data indicate that gallbladder mucin binds cholesterol and phosphatidylcholine in vesicles to gallbladder mucin but only minimal binding of cholesterol and phosphatidylcholine in mixed micelles. Nucleation of ChMC from supersaturated bile in vivo occurs primarily in the gallbladder rather than within the hepatic ducts. This occurs despite the presence of a higher cholesterol saturation index in hepatic bile \( (11) \), and the presence of Ch/PC vesicles in hepatic bile \( (2, 5-7) \). Gallbladder mucin, a high molecular weight glycoprotein secreted by the gallbladder epithelium, may be one of the factors responsible for the formation of cholesterol gallstones in the gallbladder rather than within the hepatic ducts \( (12-18) \). Gallbladder mucin hypersecretion and accumulation in the gallbladder as a viscoelastic gel precedes the formation of gallstones in both experimental animals \( (14, 17, 18) \) and in humans \( (19) \). Moreover, empirical observations have reported that the initial nucleation of ChMC occurs in the gallbladder mucus gel \( (13, 14, 17) \). Inhibition of gallbladder mucin secretion by aspirin prevents gallstone formation in the cholesterol-fed prairie dog \( (18) \) indicating the critical importance of gallbladder mucin for cholesterol crystal nucleation and gallstone formation in that model. The mechanism by which mucin accelerates ChMC nucleation, however, is not known.

Previous studies have demonstrated that gallbladder mucin accelerates the nucleation of ChMC in supersaturated native \( (20) \) and model biles \( (21, 22) \). This laboratory has also demonstrated binding of Ch and PC to gallbladder mucin in supersaturated model bile \( (22) \). Protease digestion of mucin decreases Ch and PC binding to mucin and eliminates the ability of mucin to accelerate ChMC nucleation \( (22) \). These findings suggest that the structural integrity of the hydrophobic domains on the mucin peptide core is essential for the pro-nucleating ef-

Supplementary key words phosphatidylcholine • cholesterol saturation index

Recent studies of the physical chemistry of native and model bile have demonstrated that cholesterol (Ch) is transported in mixed lipid micelles and also in a vesicular phase \( (1-10) \). The origin, structure, and function of lipid vesicles in hepatic and gallbladder bile are currently subjects of active investigation and controversy. Nevertheless, it is apparent that the nucleation of cholesterol monohydrate crystals (ChMC) occurs from the vesicular phase of model and native biles that are supersaturated with cholesterol \( (3, 8-10) \).

Abbreviations: Ch, cholesterol; PC, phosphatidylcholine; NaTC, sodium taurocholate; ChMC, cholesterol monohydrate crystals; BGM, bovine gallbladder mucin; CSI, cholesterol saturation index; HEPES, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid.
fect of mucin in supersaturated model bile. The goal of this study was to identify which of the cholesterol-transporting particles in model bile (mixed lipid micelles or vesicles) are sensitive to the nucleation-promoting effects of gallbladder mucin.

MATERIALS AND METHODS

Preparation of model bile

Cholesterol USP (Ch), phosphatidylcholine, egg yolk, type V (PC), and sodium taurocholate (NaTC) were obtained from Sigma Chemical Co., St. Louis, MO. Ch was purified by double recrystallization from hot 95% ethanol. NaTC was recrystallized twice by the method of Pope (23). PC was used as supplied in chloroform–methanol 9:1 (vol/vol). Lipids were stored at 4°C in the dark under N2 to avoid oxidation prior to use. The molar ratio of PC/(NaE plus PC) in model bile was 0.2. The total lipid concentration was 10 g/dl to reflect the average concentration of lipids in lithogenic human gallbladder bile (11). Cholesterol saturation indices were calculated from the critical tables of Carey (24). Trace amounts of [14C]cholesterol (57.7 mCi/mmol) and [3H]phosphatidylcholine (57.0 Ci/mmol) (New England Nuclear Research Products, Boston, MA) were added in the preparation of model biles prior to coprecipitation of the lipids from organic solvents. Following coprecipitation of the lipids, model biles were prepared by resuspension in a HEPES-saline buffer (10 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid–140 mM NaCl, pH 7.5). Model biles were then heated to 80°C for 1 hr to obtain an isotropic phase, filtered through a sterile 0.22-μm filter (Gelman Acrodisc, Gelman Sciences Inc., Ann Arbor, MI), and equilibrated at 37°C for 4 hr before use.

Separation of mixed micelles and vesicles

During thermal equilibration at 37°C for 4 hr, supersaturated model bile which was originally isotropic became opalescent indicating the formation of large lipid aggregates which have been identified by Mazer and Carey as vesicular microprecipitates (3). We used gel permeation chromatography to separate lipid vesicles from mixed lipid micelles based on their size difference (2, 7, 25, 26). Ultracentrifugation was also used to separate vesicles and mixed lipid micelles based on the difference in their buoyant density according to the method of Kibe et al (9). In our hands only incomplete separation of micelles and vesicles could be achieved (data not shown) and this technique was not used further.

Chromatography was performed using a Sephadex G-200 (Pharmacia, Uppsala, Sweden) column (85 cm × 1.0 cm) equilibrated and eluted in 10 mM HEPES, 140 mM NaCl, pH 7.5 buffer containing 5 mM NaTC. Supersaturated model bile (5 ml) with a cholesterol saturation index of 1.4 and a total lipid content of 10 g/dl was loaded on the column and eluted at a flow rate of 5 ml/hr. The distribution of radiolabeled lipids in chromatographic fractions was determined by liquid scintillation counting. After chromatographic separation, the vesicular and micellar fractions were concentrated to the initial volume (5 ml) of the original model bile using the Amicon concentrator (Diaflo ULtrafilters, Danvers, MA) with a PM10 membrane (molecular weight exclusion = 10,000) prior to nucleation or binding experiments.

Lipid analysis

The Ch, PC, and NaTC composition of the unfractinated model bile and the final mixed micellar and vesicular preparations isolated by gel permeation chromatography were determined as previously described (27–29).

Gallbladder mucin purification

Bovine gallbladder mucin was purified as previously described (30). Purified mucins were free of low molecular weight protein contaminants as gauged by sodium dodecyl sulfate polyacrylamide gel electrophoresis and contained less than 0.2% by weight as lipid contaminants (20, 30).

Nucleation experiments

Unfractionated supersaturated model bile and micellar and vesicular fractions prepared by gel permeation chromatography were incubated with or without purified gallbladder mucin (4 mg/ml unless otherwise specified) at 37°C without agitation. Cholesterol monohydrate crystals (ChMC) were identified by polarizing light microscopy as birefringent, notched rhomboidal plates and were quantitated daily for up to 10 days by counting in a hemocytometer chamber. The effect of gallbladder mucin on the nucleation of cholesterol monohydrate crystals was analyzed by Fisher’s exact test.

Rapid filtration binding assay

The relative binding of Ch and PC in the vesicular and micellar fractions prepared by gel permeation chromatography was examined using a rapid filtration binding assay which has been previously described (22). Purified, lyophilized mucin was reconstituted at a concentration of 4 mg/ml in 10 mM HEPES, 140 mM NaCl, pH 7.5, containing 5 mM NaTC. After fractionation of dual isotopically labeled model bile ([3H]PC and [14C]Ch) by Sephadex G-200 chromatography, aliquots of the vesicular and mixed micellar fractions were mixed with mucin and incubated at 37°C for 2 hr. The final volume...
of the mucin-lipid mixture was then brought to 250 μl with the HEPES buffer containing NaTC. A 200-μl aliquot of the mucin-lipid mixture was then vacuum-filtered over a cellulose nitrate filter with a 0.1-μm pore size (Sartorius Filters Inc., Hayward, CA). The filter was then washed by vacuum-filtering 2 ml of the HEPES-NaTC buffer in an identical fashion. Radioactivity remaining on the filter was quantitated by liquid scintillation counting to determine the amount of lipid bound to mucin. Each data point represents the mean of triplicate samples. The amount of PC and Ch bound to mucin was calculated from the critical tables of Carey (24). CSI was calculated from the critical tables of Carey (24) using an assumed total lipid concentration of 10 g/dl.

RESULTS

Separation of vesicles and mixed lipid micelles in supersaturated model bile

Sephadex-G 200 chromatography separated supersaturated model bile into two major cholesterol-containing fractions. One fraction eluted in the void volume of the column and was visibly opalescent, while a second, broader fraction eluted in the included volume and was optically clear (Fig. 1). The vesicular fraction of supersaturated model bile was harvested from the void volume and the micellar fraction from the included volume as indicated in the legend to Fig. 1. Examination of the chromatogram in Fig. 1 demonstrates that while the two cholesterol-containing peaks are distinct, overlap of these fractions did occur since the baseline between peaks did not reach zero. Only peak vesicular and micellar fractions were pooled to avoid cross-contamination of vesicles with micelles as much as possible.

Lipid composition of vesicular and micellar fractions

The total lipid concentration in the pooled vesicular and micellar fractions was significantly less than the original model bile (Table 1), secondary to harvesting only the peak vesicular and micellar fractions. Approximately 43% of the Ch in supersaturated model bile eluted in the void volume (vesicular fraction) of the Sephadex G-200 column while 57% of the Ch was recovered in the included volume (micellar fraction) under the conditions used in this experiment. Lipid analysis indicates that the micellar fraction was supersaturated with Ch following chromatography (CSI = 1.46, calculated on assumed

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**TABLE 1. Lipid analysis of model bile, vesicles, and micelles**

<table>
<thead>
<tr>
<th>Biliary Lipids</th>
<th>Bile Salt</th>
<th>Cholesterol</th>
<th>Phosphatidylcholine</th>
<th>Total Lipid</th>
<th>Ch/PC Mole Ratio</th>
<th>CSI</th>
<th>Mol % Ch</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>x 10^3 M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Model bile</td>
<td>92.0</td>
<td>17.44</td>
<td>36.46</td>
<td>10.0</td>
<td>0.48</td>
<td>1.58</td>
<td>11.95</td>
</tr>
<tr>
<td>Column chromatography</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vesicles (initial)</td>
<td>4.20</td>
<td>1.90</td>
<td>1.84</td>
<td>0.46</td>
<td>1.03</td>
<td>2.95</td>
<td>23.93</td>
</tr>
<tr>
<td>Vesicles (concentrated)</td>
<td>5.40</td>
<td>4.80</td>
<td>4.08</td>
<td>0.78</td>
<td>1.00</td>
<td>3.71</td>
<td>30.08</td>
</tr>
<tr>
<td>Micelles (initial)</td>
<td>6.20</td>
<td>1.09</td>
<td>3.69</td>
<td>0.68</td>
<td>0.30</td>
<td>1.19</td>
<td>9.93</td>
</tr>
<tr>
<td>Micelles (concentrated)</td>
<td>7.80</td>
<td>1.86</td>
<td>6.16</td>
<td>1.00</td>
<td>0.30</td>
<td>1.46</td>
<td>11.76</td>
</tr>
</tbody>
</table>

Model bile was prepared with a calculated CSI = 1.4 and a total lipid content of 10 g/dl. Vesicles and micelles were prepared by Sephadex G-200 column chromatography as shown in Fig. 1. Peak vesicle and micelle fractions were obtained as noted in the legend to Fig. 1. Lipid analyses were performed as described in Methods. CSI was calculated from the critical tables of Carey (24) using an assumed total lipid concentration of 10 g/dl.
total lipid concentration of 10 g/dl), but to a lesser degree than the vesicular fraction (CSI = 3.71, assumed total lipid = 10 g/dl). Vesicles had a Ch/PC molar ratio of approximately 1.0 after concentration of the pooled column fractions to the original volume of model bile (5 ml) by ultrafiltration (Table 1). The Ch/PC ratio of the mixed lipid micelle fractions was 0.30, lower than the Ch/PC ratio found in the original model bile (0.48) or the vesicular fraction. The mole % Ch in the vesicular fraction was 30.01, compared to 11.95 in the original bile and 8.02 in the micellar fraction.

Nucleation experiments

The first series of nucleation experiments sought to determine which cholesterol-transporting particles nucleate ChMC as separated under our experimental conditions. As shown in Table 2, there was no ChMC nucleation from the mixed lipid micelles prepared by chromatography during 5 days incubation at 37°C. Additional observations after 10 days failed to detect ChMC nucleation in the micelle preparation. In contrast, vesicles prepared by chromatography rapidly nucleated a large number of ChMC. The number of ChMC nucleated from vesicles prepared by chromatography was significantly greater than in the original model bile (P < 0.001) after both 2 and 4 days of incubation. No sample contained ChMC visible by polarizing microscopy on the first day.

The next series of nucleation experiments sought to determine the effect of purified bovine gallbladder mucin on nucleation ChMC in micelles and vesicles. No ChMC nucleation occurred in micelles prepared by chromatography even after 10 days incubation with gallbladder mucin at a concentration of 4 mg/ml. In contrast, gallbladder mucin caused a highly significant, concentration-dependent increase in the number of ChMC nucleated from vesicles prepared by chromatography (P < 0.001, Fig. 2).

Rapid filtration binding assay

A previous study has demonstrated that gallbladder mucin binds Ch and PC in model bile (22). In supersaturated model bile, mucin appears to bind nearly equimolar quantities of Ch and PC. Since vesicles prepared by chromatography had a Ch/PC molar ratio of 1.0, we examined the binding of Ch and PC in cholesterol-transporting vesicles to mucin. The relative binding of vesicles and micelles, prepared by chromatography, to gallbladder mucin was examined using a rapid filtration binding assay. Both PC and Ch transported in vesicles prepared by chromatography bound to gallbladder mucin (Fig. 3). The binding of PC and Ch in vesicles to mucin appears to be analogous to the binding of PC and Ch in unfractonated supersaturated model bile (ref. 22 and Fig. 2). Negligible amounts of PC and Ch transported in mixed lipid micelles prepared by chromatography bound to mucin. Although the concentration of the individual lipids was different in vesicular and micellar preparations (Table 1), the difference was not large enough to account for the increased binding of Ch and PC in vesicles when compared to micelles.

DISCUSSION

The goal of this study was to examine the effect of purified gallbladder mucin on ChMC nucleation from the cholesterol-transporting particles in supersaturated model bile. Previous studies from this laboratory have demonstrated that purified gallbladder mucin accelerates the

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**TABLE 2. Nucleation of cholesterol monohydrate crystals from vesicles and micelles**

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model bile</td>
<td>0</td>
<td>25.0 ± 6.2</td>
<td>41.0 ± 3.0</td>
</tr>
<tr>
<td>Model bile plus mucin</td>
<td>0</td>
<td>30.7 ± 8.6</td>
<td>64.3 ± 9.5*</td>
</tr>
<tr>
<td>Column chromatography</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Micelles</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Micelles plus mucin</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vesicles</td>
<td>0</td>
<td>78.3 ± 13.9**</td>
<td>823.3 ± 96.1**</td>
</tr>
<tr>
<td>Vesicles plus mucin</td>
<td>0</td>
<td>2500.7 ± 264.6***</td>
<td>3506.7 ± 220.3***</td>
</tr>
</tbody>
</table>

Model bile was prepared with a CSI = 1.4 and total lipid = 10 g/dl. Vesicles and micelles were prepared by Sephadex G-200 column chromatography as described in Fig. 1. Lipid preparations were incubated either with or without purified bovine gallbladder mucin at a final concentration of 4 mg/ml. Preparations were incubated under sterile conditions at 37°C and cholesterol crystals were quantitated by counting in a hemocytometer using polarizing microscopy. Values represent mean ± SEM. Difference among groups was determined by Fisher's t-test. *, P < 0.02 versus samples without mucin; **, P < 0.01 versus model bile; ***, P < 0.001 versus samples without mucin.
When studying cholesterol crystal nucleation in model bile, researchers used different physical separation techniques to fractionate the cholesterol-transporting particles in bile. The conditions under which model and native bile are fractionated appear to have a major influence on both the lipid composition of the vesicular phase and the relative quantity of cholesterol recovered in either vesicles or micelles. In the current experiments, however, vesicles were isolated by column chromatography in the presence of 5 mM NaTC after they had formed in the complex system of supersaturated bile. Caution is warranted in the interpretation of these results since the similarity in the physical chemical properties of vesicles isolated in this study with those in native bile has not been confirmed.

Table 2 demonstrates that, with the experimental conditions and techniques used in this study, vesicles prepared by chromatography were labile and rapidly nucleated ChMC. Fig. 3 demonstrates that gallbladder mucin accelerates ChMC nucleation from vesicles in a concentration- and time-dependent manner that is highly statistically significant ($P < 0.0001$ by ANOVA). The acceleration of ChMC nucleation by mucin in vesicles is analogous to the effect of mucin on ChMC nucleation in supersaturated model bile (21, 22). Additional caution must be applied to the interpretation of these results given the use of a single technique (column chromatography) under specific experimental conditions (elution buffer containing 5 mM NaTC) to separate vesicles and micelles.
The final experiment in this study examined the relative binding of vesicles and micelles to mucin using a rapid filtration binding assay. Ch and PC in vesicles bound to mucin in a concentration-dependent manner, while Ch and PC in the micellar fraction demonstrated significantly less binding to mucin. As in a previous study, the ability of mucin to bind Ch and PC correlated with the ability of mucin to accelerate ChMC nucleation (22). The current study extends previous observations regarding the nucleation-promoting properties of mucin by identifying Ch/PC vesicles in supersaturated model bile as the cholesterol-transporting particle which mucin binds and from which mucin accelerates ChMC nucleation.

The findings presented in this study may have direct relevance to understanding the pathogenesis of cholesterol cholelithiasis in humans. Gallbladder mucin appears to bind and accelerate ChMC nucleation from the cholesterol-transporting vesicles in supersaturated model bile that spontaneously nucleate in human gallbladder bile (8) and in model bile (3, 9, 10). The mechanism by which mucin promotes ChMC nucleation from these particles remains unclear. Recent investigations by Halpern et al. (8, 10) and Kibe et al. (9) have demonstrated that vesicle aggregation and fusion precedes ChMC nucleation in model bile. We hypothesize that mucus gel within the gallbladder lumen contains a hydrophobic milieu that is responsible for the sequestration within the mucus gel of the more hydrophobic components in the bulk aqueous phase of gallbladder bile. Evidence supporting this supposition comes from the following observations: 1) gallbladder mucin contains hydrophobic domains on the nonglycosylated portion of the mucin peptide core (30); 2) mucin binds unconjugated bilirubin (31); 3) the hydrophobic species unconjugated bilirubin and bilirubin monoglucuronide, but not the hydrophilic bilirubin diglucuronide, are present in the mucus gel of biliary sludge (32, 33); and 4) a macromolecular complex of mucin and unconjugated bilirubin is present in the organic matrix of cholesterol gallstones (34). Moreover, this laboratory has demonstrated that gallbladder mucin binds Ch and PC in supersaturated model bile. We postulate that the hydrophobic association of mucin and cholesterol-transporting vesicles in bile results in vesicle aggregation and fusion by
ChMC nucleation. One possible mechanism by which mucin may accelerate ChMC nucleation is suggested by the data in Fig. 3. PC in vesicles appears to have a slightly greater binding affinity for mucin than Ch. Preferential binding of PC in vesicles to mucin could potentially result in an increase in the Ch/PC molar ration in vesicles and hence accelerate vesicle fusion and nucleation.

Further elucidation of the role of gallbladder mucin in the pathogenesis of cholesrerol gallstones will require investigation of the mechanisms by which mucin promotes aggregation and fusion of cholesterol-containing vesicles in model and native bile.

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