Targeted disruption of the murine mucin gene 1 decreases susceptibility to cholesterol gallstone formation[1]

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Abstract **Gallbladder mucins play a critical role in the pathogenesis of cholesterol gallstones because of their ability to bind biliary lipids and accelerate cholesterol crystallization. Mucin secretion and accumulation in the gallbladder is determined by multiple mucin genes. To study whether mucin gene 1 (*Muc1*) influences susceptibility to cholesterol cholelithiasis, we investigated male *Muc1*-deficient (*Muc1*$^{-/-}$) and wild-type mice fed a lithogenic diet containing 1% cholesterol and 0.5% cholic acid for 56 days. Gene expression of the gallbladder *Muc1* and *Muc5ac* was significantly reduced in *Muc1*$^{-/-}$ mice in response to the lithogenic diet. *Muc3* and *Muc4* levels were upregulated and were similar between *Muc1*$^{-/-}$ and wild-type mice. Little or no *Muc2* and *Muc5b* mRNAs were detected. *Muc1*$^{-/-}$ mice displayed significant decreases in total mucin secretion and accumulation in the gallbladder as well as retardation of crystallization, growth, and agglomeration of cholesterol monohydrate crystals. At 56 days of feeding, gallstone prevalence was decreased by 40% in *Muc1*$^{-/-}$ mice. However, cholesterol saturation indices of gallbladder biles, hepatic secretion of biliary lipids, and gallbladder size were comparable in *Muc1*$^{-/-}$ and wild-type mice. We conclude that decreased gallstone formation in mice with disrupted *Muc1* gene results from reduced mucin secretion and accumulation in the gallbladder.**—Wang, H. H., N. H. Afdhal, S. J. Gendler, and D. Q-H. Wang. **Targeted disruption of the murine mucin gene 1 decreases susceptibility to cholesterol gallstone formation. *J. Lipid Res.* 2004.** 45: **438–447.**

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Accumulated evidence suggests that gallbladder mucins play an important role in the early stages of cholesterol gallstone formation (1–3) and are a potent pronucleating/crystallizing agent for accelerating cholesterol crystallization in native and model biles (4–6). Indeed, hypersecretion of gallbladder mucins is a prerequisite for gallstone formation, and increased amounts of gallbladder mucins are consistently observed in gallbladder bile of several animal models of gallstones (7–9). Also, mucins are found within cholesterol gallstones, where they act as a matrix for stone growth (10).

Gallbladder mucins, a heterogeneous family of *O*-linked glycoproteins, are divided into two classes: epithelial and gel-forming mucins (11). It has been proposed that the epithelial mucins produced by mucin gene 1 (*Muc1*), *Muc3*, and *Muc4* do not form aggregates and are integral membrane glycoproteins located on the apical surface of epithelial cells. The gel-forming mucins, *Muc2*, *Muc5ac*, and *Muc5b*, secreted by specialized gallbladder mucin-producing cells provide a protective coating to the underlying mucosa. They form disulfide-stabilized oligomers or polymers, a phenomenon that accounts for their viscoelastic properties.

Mucin secretion and accumulation in the gallbladder are determined by multiple mucin genes (12–15). Although the regulation of gallbladder mucin secretion and accumulation and its role in gallstone pathogenesis have been intensively studied in vivo and in vitro, no information is available on how individual mucin genes contribute to cholesterol gallstone formation and whether the epithelial mucins influence susceptibility to cholesterol gallstone formation. The *Muc1* gene regulates a membrane-associated mucin that is abundant in the secretory epithelia of the gallbladder (12, 16). In an early study, Spicer and coworkers (17) found that mice deficient in Muc1 mucin appear to develop normally and are healthy and fertile,

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Abbreviations: CCK, cholecystokinin; *Muc1*, mucin gene 1.

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and these mice show a significantly slower growth rate of primary breast tumors. Although in cystic fibrosis, mucin accumulation is abnormally high, resulting in severe intestinal obstruction, disruption of the *Muc1* gene significantly decreases total amounts of mucins in the gastrointestinal tract of cystic fibrosis mice (18). Because gallbladder mucins are of crucial importance in the pathogenesis of gallstones, we hypothesized that reduced Muc1 mucin in the gallbladders of mice with disrupted *Muc1* gene could decrease susceptibility to cholesterol gallstone formation. In the present study, we investigated male *Muc1* knockout ($Muc1^{-/-}$) and wild-type mice fed a lithogenic diet for 56 days. Our results show that targeted disruption of the *Muc1* gene significantly decreases mucin secretion and accumulation in the gallbladder of $Muc1^{-/-}$ mice compared with wild-type mice. Also, gene expression of the gallbladder *Muc5ac*, a gel-forming mucin gene, was significantly reduced in $Muc1^{-/-}$ mice in response to the lithogenic diet. As a result, cholesterol crystallization and the development of gallstone formation are significantly retarded.

MATERIALS AND METHODS

Animals and diets

$Muc1^{-/-}$ mice in a FVB/NJ background were generated by targeting mutation of the *Muc1* gene (17). The wild-type mice displayed normal *Muc1* expression in the same FVB/NJ background (Jackson Laboratory, Bar Harbor, ME). Inbred FVB/NJ strain is a gallstone-susceptible mouse (19). Male $Muc1^{-/-}$ and wild-type mice at 8–10 weeks of age were fed normal rodent chow (Harlan Teklad Laboratory Animal Diets, Madison, WI) containing trace (<0.02%) amounts of cholesterol or a semisynthetic lithogenic diet containing 1% cholesterol, 0.5% cholic acid, and 15% butter fat (8). 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.

Collection of gallbladder biles and gallstones and microscopic studies

Cholecystectomy was performed in fasted animals (n = 20 per group), and gallbladder volume was measured by weighing the whole gallbladder and its contents and equating gallbladder weight with gallbladder volume. Fresh gallbladder biles were examined by polarizing light microscopy (Nikon Instruments, Melville, NY). Mucins, solid and liquid crystals, and gallstones were defined according to previously established criteria (8). Gallstone size was determined with microcalipers. Gallbladder mucin gels were observed as nonbirefringent amorphous strands, and mucin gel accumulation scores were defined semiquantitatively by microscopy as follows: 0, no mucin gel; 1, 25% of the gallbladder filled with mucin gel; 2, 50% of the gallbladder filled with mucin gel; 3, 75% of the gallbladder filled with mucin gel; and 4, gallbladder completely (100%) filled with mucin gel. The score corresponded well with the mucin content in gallbladder biles, as verified by periodic acid/Schiff staining (8). Pooled gallbladder biles were frozen and stored at −20°C for further lipid analyses (see Lipid and mucin analyses below).

Collection of hepatic biles

Additional groups of $Muc1^{-/-}$ and wild-type mice (n = 5 per group) were used for biliary lipid secretion studies according to published methods (20). The first hour collection of hepatic bile was used to measure biliary lipid outputs. To determine the circulating bile salt pool size, 8 h biliary "washout" studies were performed (20). During hepatic bile collection, mouse body temperature was maintained at 37 ± 0.5°C with a heating lamp.

Gallbladder contraction study

To explore whether gallbladder emptying was altered by disruption of the *Muc1* gene, we measured gallbladder contraction function in response to exogenously administered cholecystokinin (CCK) in $Muc1^{-/-}$ and wild-type mice (n = 5 per group) before (on chow) and at day 56 of feeding the lithogenic diet. In brief, after fasted mice were anesthetized with pentobarbital, the right or left jugular vein was cannulated. We measured gallbladder volume gravimetrically at time 0 (control) and after intravenous injection of 17 nmol/kg body weight of sulfated CCK octapeptide (CCK-8) dissolved in 100 μl of PBS solution or 100 μl of PBS solution only. Gallbladder contractile function was determined by comparing gallbladder volumes of the CCK-8-injected mice with those of the control and the PBS-injected mice according to published methods (21).

Measurement of glycoprotein secretion by gallbladder

The rate of glycoprotein secretion by the mouse gallbladder was determined in organ culture according to published methods (7). The entire gallbladder (n = 5 per group) was incubated with 10 μCi/ml [^{3}H]glucosamine (NEN Life Science Products, Boston, MA), and unlabeled glucosamine was added to give a final glucosamine concentration of 1 mM. Incubations were carried out in an atmosphere of O_2/CO_2 (95:5, v/v) at 37°C for culture periods of up to 24 h. At the end of the culture period, histological examination of the tissue revealed excellent morphologic preservation of the epithelial cells of the gallbladder mucosa. Incorporation of radioisotope into tissue and secreted (medium) glycoproteins was measured after trichloroacetic acid precipitation (22).

Quantitative real-time PCR assays of gallbladder mucin genes

Total RNA was extracted from pooled gallbladder samples (n = 8 per group) using RNeasy Midi (Qiagen, Valencia, CA). Reverse-transcription reaction was performed using the SuperScript II First-strand Synthesis System (Invitrogen, Carlsbad, CA) with 5 μg of total RNA and random hexamers to generate cDNA. Primer Express Software (Applied Biosystems, Foster City, CA) was used to design the primers (**Table 1**). Real-time PCR assays (23) for all samples were performed in triplicate on the GeneAmp 5700 Sequence Detection System (Applied Biosystems). Relative mRNA levels were calculated using the threshold cycle of an unknown sample against a standard curve with known copy numbers. To obtain a normalized target value, the target amount was divided by the endogenous reference amount of rodent glyceraldehyde-3-phosphate dehydrogenase as the invariant control.

Preparation of mucin gel

Fresh mucin gel was obtained from lithogenic diet-fed mice (n = 20 per group). To dissolve crystalline and bound lipids, mucin gel was suspended in 10 volumes of 100 mM Tris buffer (pH 7.4) containing 10 mM taurocholate and was washed three times with 100 mM Tris buffer containing taurocholate according to published methods (24). Then, mucin gel was washed three times with 100 mM Tris buffer (pH 7.4) without taurocholate by centrifugation at 10,000 *g* for 1 h followed by dialysis and freeze-drying. Mucin gel was verified to be free of phase-separated lipids by polarizing light microscopy (×800 magnification) and was stored at 4°C until crystallization experiments were performed.

TABLE 1. Primer and probe sequences used in mRNA quantification by real-time PCR

Gene	Accession Number	Forward	Reverse	Probe
Muc1	NM_013605	5′-GGTTGCTTTGGCTATCGTCTA-TTT-3′	5′-AAAGATGTCCAGCTGCCCATA-3′	5′-AGTGTGCCAGTGCCGCCGA-AAG-3′
Muc2	NM_023566	5′-GTCTGCCACCTCATCATGGA-3′	5′-CAGGCAAGCTTCATAGTAGTG-CTT-3′	5′-CCTATTCTCCCAGTGCCACGC-CTTC-3′
Muc3	AF027131	5′-GTGGGACGGGCTCAAATG-3′	5′-CTCTACGCTCTCCACCAGTT-CCT-3′	5′-AGTGCACCAGCCTCTTCTATGG-GCC-3′
Muc4	NM_080457	5′-TCTTTCTGTCTCAACTGTTGAAT-CAGA-3′	5′-CGTGGCCAGGATGTCAAAC-3′	5′-TGAAGACTTGGCCTCTGGGTG-CAAAG-3′
Muc5ac	L42292	5′-GTCTGGCAGAAACAGTGGAG-ATT-3′	5′-TCGTGGCTTCTCACAGAAC-TTG-3′	5′-TGACACCTTTGTGAACCTGAGA-TCCAAAGG-3′
Muc5b	NM_028801	5′-CAGATCCATCCATCCCATT-TCT-3′	5′-TATCTGACTACCACTTGTTGAT-GTGACT-3′	5′-ATTTCCAAAAGCAACTCTATGT-TGCCTAGTAGGCC-3′

Muc1, mucin gene 1.

Cholesterol crystal appearance time assays

Model micellar bile was prepared by coprecipitation of cholesterol, lecithin, and taurocholate from $CHCl_3$-methanol (2:1, v/v) to yield a relative composition of 8.5, 22.9, and 68.6% (all mol%) according to published methods (25). The total lipid concentration was 29.3 g/dl. To induce supersaturation, stock solutions were diluted 4-fold with 0.15 M NaCl (pH 7.0) to 7.3 g/dl, and this dilution should increase the cholesterol saturation index from 0.97 ± 0.03 to 1.21 ± 0.05. Also, model bile samples were supplemented with mucin gel and soluble mucins (final concentration = 1.5 mg/ml; see Fig. 2A for rationale) purified from *Muc1*$^{-/-}$ or wild-type mice and were incubated individually at 37°C. This time point was taken as the initiation of all crystallization studies (n = 3 per group). Detection of plate-like cholesterol monohydrate crystals was performed by polarizing light microscopy at ×400 magnification every 2 h (24, 25). At 48 h of the crystallization studies, crystal numbers were counted in 5 μl bile samples using a microscopic field of 1 cm^2 (×400 magnification).

Lipid and mucin analyses

Total and individual bile salt concentrations were measured by reverse-phase HPLC (26). Biliary phospholipids were determined as inorganic phosphorus by the method of Bartlett (27). Bile cholesterol as well as cholesterol content in gallstones and the lithogenic diet were determined by HPLC (8). Cholesterol saturation indices in pooled gallbladder biles were calculated from the critical tables (28). After delipidation, soluble mucin concentrations were determined by a fluorometric method (29), and using an anti-Muc1 monoclonal antibody (CT2), Muc1 mucin levels were determined by Western blot analysis (30).

Statistical methods

All data are expressed as means ± SD. Statistically significant differences among groups of mice were assessed by Student's *t*-test or Chi-square tests. Analyses were performed with SuperANOVA software (Abacus Concepts, Berkeley, CA). Statistical significance was defined as two-tailed $P < 0.05$.

RESULTS

Mucin gene expression in the gallbladder

Figure 1 shows expression levels of *Muc1*, *Muc2*, *Muc3*, *Muc4*, *Muc5ac*, and *Muc5b* mRNAs, as measured by quantitative real-time PCR, in the gallbladders of wild-type (Fig. 1A) and *Muc1*$^{-/-}$ (Fig. 1B) mice as a function of days on the lithogenic diet for 56 days. The data are expressed relative to the level of the *Muc5b* transcript in the wild-type mice on the chow diet, and its mRNA expression is set at 1. At day 0 (on chow), the wild-type mice displayed high levels of *Muc1* and *Muc3* and low levels of *Muc4*, *Muc5ac*, and *Muc5b*. Expression levels of *Muc5ac* mRNA were increased significantly ($P < 0.0001$) in *Muc1*$^{-/-}$ mice. As expected, feeding the lithogenic diet induced significant ($P < 0.0001$) increases in expression levels of gallbladder *Muc1*, *Muc3*, *Muc4*, and *Muc5ac* mRNAs in the wild-type mice. Furthermore, in *Muc1*$^{-/-}$ mice, feeding the lithogenic diet significantly ($P < 0.0001$) increased expression levels of *Muc3* and *Muc4* and significantly ($P < 0.0001$) decreased mRNA levels of *Muc5ac*. We also measured gene expression of *Muc5b* and found that it was very low at base-

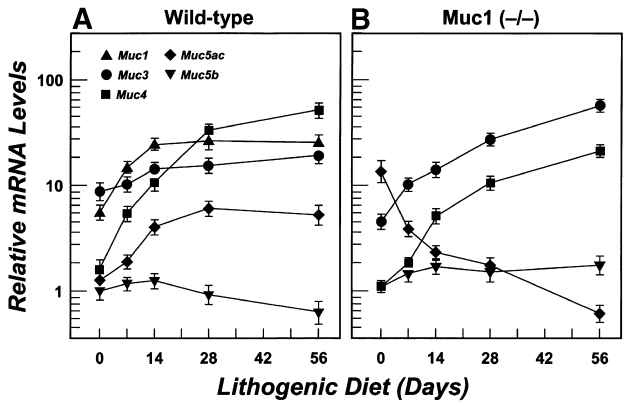

Fig. 1. The mRNA expression of mucin gene 1 (*Muc1*), *Muc3*, *Muc4*, *Muc5ac*, and *Muc5b* in the gallbladders of wild-type (A) and *Muc1* knockout (*Muc1*$^{-/-}$) (B) mice as a function of days on the lithogenic diet for 56 days. The data are expressed relative to the level of the *Muc5b* transcript in the wild-type mice on the chow diet. Each value represents the mean ± SD of data that were measured in triplicate by quantitative real-time PCR assays from the pooled gallbladder tissues (n = 8 per group). Of note is that the absence of *Muc1* mRNA in the gallbladders of *Muc1*$^{-/-}$ mice confirmed the complete knockout of the *Muc1* gene. The mRNA expression of the *Muc2* gene (data not shown) was absent from the gallbladders of both *Muc1*$^{-/-}$ and wild-type mice on chow or fed the lithogenic diet.

line and remained unchanged in response to the lithogenic diet. The mRNA expression of the *Muc2* gene (data not shown) was absent from the gallbladders of both *Muc1*$^{-/-}$ and wild-type mice on chow or fed the lithogenic diet.

Biliary soluble and Muc1 mucin concentrations

Figure 2 exhibits soluble and Muc1 mucin concentrations in pooled gallbladder biles (n = 20 per group) as functions of mouse strain and time on the lithogenic diet. On chow (day 0), soluble mucin levels were slightly higher in wild-type mice than in *Muc1*$^{-/-}$ mice (Fig. 2A). During the lithogenic diet feeding, soluble mucin concentrations gradually increased in both strains of mice and were comparable in *Muc1*$^{-/-}$ and wild-type mice. In contrast, the absence of Muc1 mucin in the gallbladder biles of *Muc1*$^{-/-}$ mice assayed by Western blot hybridization using an anti-Muc1 monoclonal antibody (30) verified complete knockout of the *Muc1* gene (Fig. 2B). However, in response to the lithogenic diet, wild-type mice increased Muc1 mucin levels by 2-fold at day 7 and by 3-fold at day 14 compared with the chow diet.

Gallbladder glycoprotein secretion

Figure 3 shows that the secretion of glycoproteins by individual whole gallbladders was investigated in mice on chow or fed the lithogenic diet. For each comparison, glycoprotein secretion in wild-type mice at day 0 (on chow) is set at 100. At day 0, glycoprotein secretion in *Muc1*$^{-/-}$ mice was slightly (P = NS) lower than that in wild-type mice. Compared with control values, a significant ($P <$ 0.001) increase by ~200% was observed in wild-type mice at 28 and 56 days of the feeding. Also, the lithogenic diet resulted in significant ($P < 0.01$) increases in glycoprotein secretion in *Muc1*$^{-/-}$ mice compared with the chow diet. Of note, however, is that in *Muc1*$^{-/-}$ mice, gallbladder glycoprotein secretion was ~75% of wild-type levels ($P <$ 0.05).

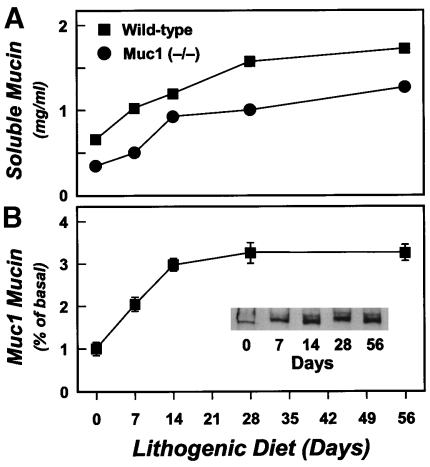

Fig. 2. Soluble and Muc1 mucin concentrations in pooled gallbladder biles (n = 20 per group) of *Muc1*$^{-/-}$ and wild-type mice before and during the lithogenic diet feeding. A: Compared with the chow diet (day 0), feeding the lithogenic diet gradually increases soluble mucin concentrations in both strains of mice. Soluble mucin levels are slightly higher in wild-type mice than in *Muc1*$^{-/-}$ mice. B: The absence of Muc1 mucin in the gallbladders of *Muc1*$^{-/-}$ mice assayed by Western blot hybridization using an anti-Muc1 monoclonal antibody (30) confirms the complete knockout of the *Muc1* gene. In contrast, in the wild-type mice, feeding the lithogenic diet markedly increases Muc1 mucin levels by day 14 compared with the chow diet. Each value represents the mean ± SD of data.

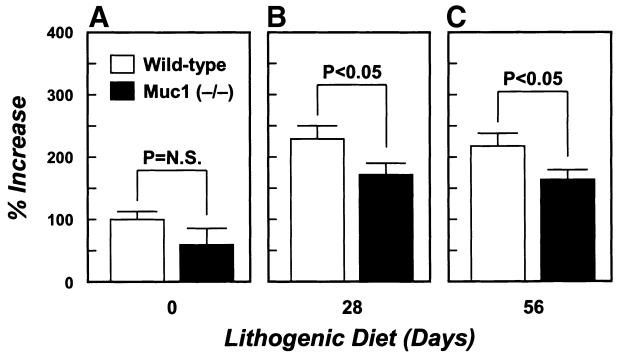

Fig. 3. Comparison of glycoprotein secretion by the gallbladders (n = 5 per group) of *Muc1*$^{-/-}$ and wild-type mice before (day 0) and at 28 and 56 days of feeding the lithogenic diet. A: For each comparison, glycoprotein secretion in wild-type mice on chow (day 0) is set at 100. At day 0, glycoprotein secretion in *Muc1*$^{-/-}$ mice was slightly (P = NS) lower compared with that in wild-type mice. B and C: Compared with the chow diet, the lithogenic diet resulted in significant ($P < 0.01$) increases in glycoprotein secretion in both strains. In particular, gallbladder glycoprotein secretion in *Muc1*$^{-/-}$ mice was ~75% ($P < 0.05$) of wild-type levels. Each value represents the mean ± SD of data.

Gallbladder accumulation of mucin gel

At day 0 (on chow), the gallbladder wall was thin and transparent. Macroscopic and light microscopic examination of gallbladder biles showed no evidence of mucin gel, solid and liquid crystals, or gallstones. **Figure 4** illustrates the distribution of the mucin gel accumulation scores in wild-type (Fig. 4A) and *Muc1*$^{-/-}$ (Fig. 4B) mice during feeding of the lithogenic diet for 56 days. At day 7, mucin gel accumulation scores were between 1 and 3 in both strains of mice, and 20% of wild-type mice and 40% of *Muc1*$^{-/-}$ mice were mucin gel-free. At day 14, the gallbladders of *Muc1*$^{-/-}$ mice displayed much lower mucin gel accumulation scores compared with those of wild-type mice. Most notably, 45% of *Muc1*$^{-/-}$ mice had a mucin gel accumulation score of 1, and 10% were mucin gel-free. In contrast, 40% and 30% of wild-type mice had mucin gel accumulation scores of 2 and 3, respectively. All gallbladders contained mucin gel after day 28 in both strains of mice. At day 28, gallbladder was completely filled with mucin gel in 30% of wild-type mice and in 20% of *Muc1*$^{-/-}$ mice. At day 56, the wild-type mice exhibited much greater mucin gel accumulation, and 20, 35, and 40% of mice had mucin gel accumulation scores of 2, 3, and 4, respectively. Of note is that *Muc1*$^{-/-}$ mice displayed much lower mucin gel accumulation scores, with 40, 25, and 30% of mice having mucin gel accumulation scores of 2, 3, and 4, respectively.

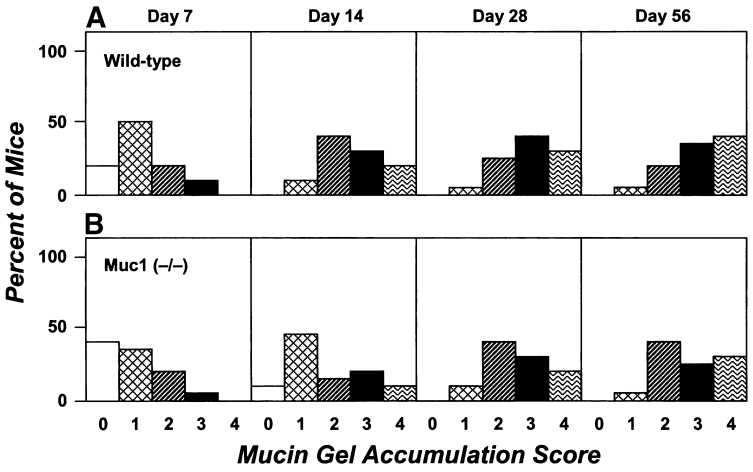

Fig. 4. Distributions of the mucin gel accumulation scores (see Materials and Methods for definitions) in wild-type (A) and *Muc1*$^{-/-}$ (B) mice (n = 20 per group) fed the lithogenic diet for 56 days. At day 7 of the feeding, 50% of wild-type mice and 35% of *Muc1*$^{-/-}$ mice displayed mucin gel accumulation scores of 1. Of note is that 20% of wild-type mice and 40% of *Muc1*$^{-/-}$ mice were mucin gel-free. During 56 days of lithogenic diet feeding, mucin gel accumulation scores were greatly increased. By the end of the feeding, all gallbladders of wild-type and *Muc1*$^{-/-}$ mice contained mucin gel. Most notably, mucin gel accumulation scores in wild-type mice were between 3 and 4, whereas in *Muc1*$^{-/-}$ mice, the corresponding values were 2 and 3, suggesting that gallbladders of *Muc1*$^{-/-}$ mice contained less mucin gel compared with those of wild-type mice.

Cholesterol crystallization sequences and gallstone formation

By day 7, 35% of wild-type mice and 25% of *Muc1*$^{-/-}$ mice formed cholesterol monohydrate crystals in gallbladder biles. With the passage of time, numbers of solid crystals as well as aggregated and fused liquid crystals increased, usually within mucin gel. At day 28, gallstones formed in 25% of wild-type mice and 15% of *Muc1*$^{-/-}$ mice (P = NS). After 56 days on the lithogenic diet, 60% of wild-type mice and 25% of *Muc1*$^{-/-}$ mice formed gallstones ($P < 0.05$). As shown in **Fig. 5**, the crystallization, growth, and agglomeration of cholesterol monohydrate crystals, as well as the development of gallstones, were significantly retarded in *Muc1*$^{-/-}$ mice (Fig. 5B) compared with wild-type mice (Fig. 5A).

Furthermore, gallstone sizes (0.21 ± 0.12 mm at 28 days and 0.31 ± 0.18 mm at 56 days) in *Muc1*$^{-/-}$ mice were significantly ($P < 0.05$) smaller compared with those in wild-type mice (0.37 ± 0.17 mm at 28 days and 0.43 ± 0.26 mm at 56 days). Notably, the frequency distribution of gallstone number at 56 days were between 2 and 4 in *Muc1*$^{-/-}$ mice, whereas the corresponding values were 5 and 9 in wild-type mice. The cholesterol extracted from these stones constituted more than 99% of stone weight.

Influence of mucin gel on cholesterol crystallization and solid cholesterol crystal number

For mucin gel addition experiments, the lipid-free mucin gel obtained from wild-type and *Muc1*$^{-/-}$ mice was added into model bile samples. After mucin gel was recon-

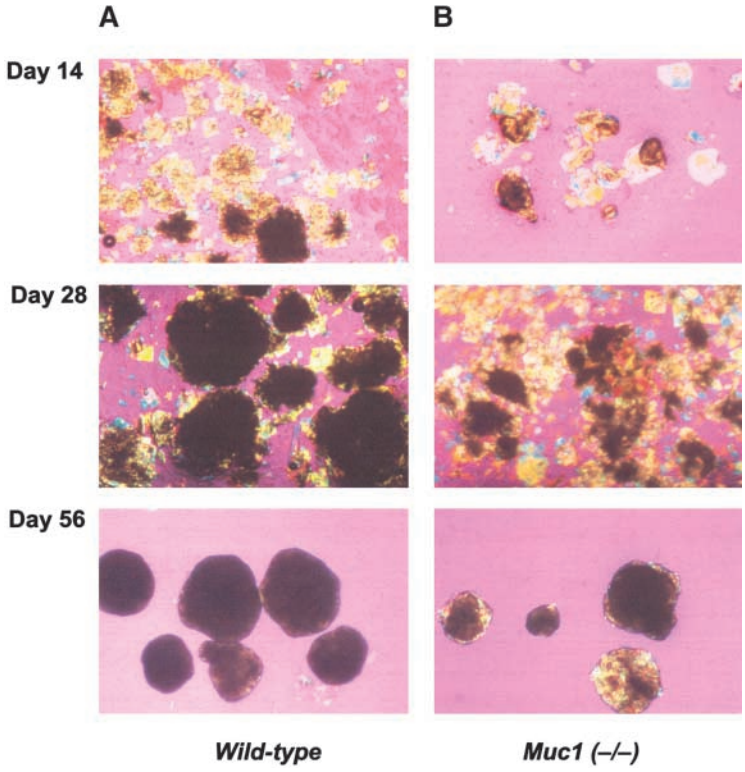

Fig. 5. Photomicrographs of cholesterol monohydrate crystal and gallstone formation observed in wild-type (A) and *Muc1*$^{-/-}$ (B) mice during the lithogenic diet feeding. At day 14, agglomerated cholesterol monohydrate crystals were surrounded by nonbirefringent amorphous mucin gel. At day 28, disintegratable amorphous sandy stones were surrounded by mucin gel, with individual cholesterol crystals projecting from the edges. At day 56, gallstones exhibited rounded contours and black centers from light scattering/absorption. Of note is that the crystallization, growth, and agglomeration of cholesterol monohydrate crystals, as well as the development of gallstones, were significantly retarded in *Muc1*$^{-/-}$ mice compared with the wild-type mice. All magnifications are ×400 except for the day 14 panels, which are ×800, by polarizing light microscopy.

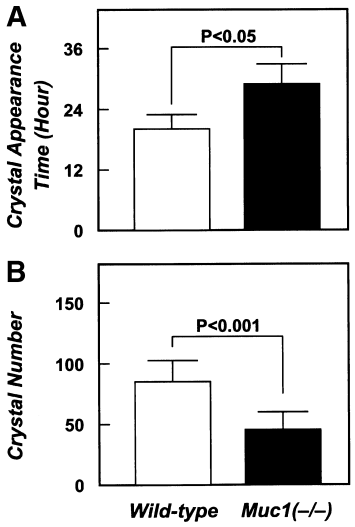

Fig. 6. Influence of mucin gel purified from wild-type (open bars) and *Muc1*$^{-/-}$ (closed bars) mice on cholesterol crystal appearance time, and effects of mucin gel on the number of cholesterol monohydrate plate-like crystals at 48 h of the crystallization studies (n = 3 per group). Note that appearance times of cholesterol monohydrate crystals were significantly ($P < 0.05$) decelerated (A) and the numbers of solid cholesterol crystals were significantly ($P < 0.001$) smaller (B) in *Muc1*$^{-/-}$ mice compared with wild-type mice. Each value represents the mean ± SD of data.

stituted in the bile samples, a viscous translucent gel formed immediately. After 8 h of incubation at 37°C, model biles became markedly sticky and several liquid crystals with Maltese crosses formed within the mucin gel. As displayed in **Fig. 6A**, the appearance times of cholesterol monohydrate crystals (29 ± 4 h) were significantly ($P < 0.05$) slower in *Muc1*$^{-/-}$ mice compared with those in wild-type mice (20 ± 3 h). Mucin gel purified from the wild-type mice increased solid cholesterol crystal number appreciably over the next 24 h, and individual cholesterol monohydrate crystals enlarged in size and became consolidated by mucin gel as agglomerates of 1–3 μm in diameter. In contrast, these effects were abated in model bile samples with the addition of mucin gel purified from *Muc1*$^{-/-}$ mice. Figure 6B shows that at 48 h of the crystallization studies, total numbers (46 ± 14 in 5 μl of bile) of solid cholesterol crystals were significantly ($P < 0.001$) lower in *Muc1*$^{-/-}$ mice compared with wild-type mice (85 ± 17 in 5 μl of bile).

Gallbladder volumes and emptying

On chow (day 0), gallbladder volumes were similar between *Muc1*$^{-/-}$ mice (16 ± 5 μl) and wild-type mice (15 ± 4 μl). The administration of CCK-8 significantly ($P < 0.05$) increased gallbladder emptying, and gallbladder volumes were reduced to 4–5 μl in both strains of mice. After the 56 day lithogenic diet feeding, gallbladder sizes in *Muc1*$^{-/-}$ mice were increased to 21 ± 4 μl, being the same as those in wild-type mice (23 ± 7 μl). However, CCK-8 treatment did not significantly induce gallbladder emptying (14–20 μl) in *Muc1*$^{-/-}$ or wild-type mice. As expected, PBS administration did not influence gallbladder sizes in either group.

Lipid compositions of gallbladder biles

Table 2 shows biliary lipid compositions of pooled gallbladder biles (n = 20 per group) before and during feeding of the lithogenic diet for 56 days. At day 0, *Muc1*$^{-/-}$ mice displayed similar cholesterol level (1.93 mol%) and cholesterol saturation index (0.46) compared with wild-type mice (2.24 mol% and 0.51). From day 7 on the lithogenic diet, both strains of mice developed cholesterol-supersaturated gallbladder biles. It is interesting that at day 56, cholesterol level (8.22–8.70 mol%) and cholesterol saturation indices (1.59–1.62) displayed identical values between *Muc1*$^{-/-}$ and wild-type mice. Furthermore, the mole percent of phospholipid and bile salts in pooled gallbladder biles of *Muc1*$^{-/-}$ mice were similar to those in wild-type mice.

Bile flow, biliary lipid secretion rates, and bile salt pool sizes

As shown in **Table 3**, mean bile flow rates on chow were identical (64.7–65.4 μl/min/kg) between *Muc1*$^{-/-}$ mice

TABLE 2. Biliary lipid composition of pooled gallbladder biles during gallstone formation

Day	Cholesterol	Phospholipid	Bile Salt	PL/(PL+BS)[a]	Total Lipid Concentration	Cholesterol Saturation Index[b]
		mol%			*g/dl*	
Wild-type						
0	2.24	10.63	87.12	0.109	10.36	0.51
7	5.12	11.96	82.92	0.126	10.83	1.07
14	6.26	12.63	81.11	0.135	11.11	1.24
28	7.01	13.07	79.92	0.141	11.39	1.35
56	8.70	13.21	78.09	0.145	11.76	1.62
Muc1$^{-/-}$						
0	1.93	10.81	87.26	0.110	8.65	0.46
7	4.82	11.59	83.60	0.122	9.47	1.07
14	5.71	11.52	82.77	0.122	10.48	1.24
28	6.70	12.42	80.88	0.133	10.60	1.34
56	8.22	12.84	78.94	0.140	11.02	1.59

Values were determined from the pooled gallbladder biles (n = 20 per group). *Muc1*$^{-/-}$, *Muc1* deficient.
[a] PL/(PL+BS), phospholipid divided by (phospholipid plus bile salt).
[b] Mean index values of the pooled gallbladder biles calculated from the critical tables (28).

and wild-type mice and remained unchanged (68.1–73.3 μl/min/kg) on the lithogenic diet for 56 days. Also, Table 3 compares outputs of the three biliary lipids at day 0 (baseline, on chow) and at 28 and 56 days on the lithogenic diet. On chow, *Muc1*$^{-/-}$ mice displayed similar secretion rates of biliary cholesterol, phospholipid, and bile salts compared with wild-type mice. Although lithogenic diet feeding induced significant increases in biliary secretion rates of cholesterol ($P < 0.01$) and phospholipid ($P < 0.01$) compared with the chow diet, no differences in these biliary secretion rates were observed between *Muc1*$^{-/-}$ and wild-type mice. Furthermore, on day 0, circulating bile salt pool sizes (Table 3) in *Muc1*$^{-/-}$ mice (2.4 ± 0.3 μmol) were similar to those in wild-type mice (2.6 ± 0.3 μmol), increasing slightly (P = NS) in both strains (2.7–2.8 μmol) at 56 days of the feeding. The total bile salt pool sizes, i.e., the circulating bile salt pool size plus the bile salt pool in the gallbladder, were 3.7–4.2 μmol in *Muc1*$^{-/-}$ mice and 3.9–4.3 μmol in wild-type mice.

Bile salt species

The HPLC analysis revealed that all bile salt species (Table 3) in hepatic biles were distributed similarly between *Muc1*$^{-/-}$ and wild-type mice. On chow, the predominant molecular species were taurocholate (49.7–51.1%) and tauro-β-muricholate (41.6–42.2%) in both strains of mice. As expected, on the lithogenic diet, taurocholate (57.5–69.6%) became the major bile salt, with significant ($P < 0.05$) increases in taurodeoxycholate (8.7–11.3%) and taurochenodeoxycholate (13.4–24.0%) and a significant ($P < 0.001$) decrease in tauro-β-muricholate (3.0–6.1%). Moreover, no differences in bile salt composition were found between *Muc1*$^{-/-}$ and wild-type mice at 56 days on the lithogenic diet.

DISCUSSION

In the present study, the most important findings are that *i*) targeted disruption of the murine *Muc1* gene significantly decreases susceptibility to cholesterol gallstone formation by reducing gallbladder mucin secretion and accumulation, leading to the retardation of crystallization, growth, and agglomeration of cholesterol monohydrate crystals in biles as well as the development of gallstones; *ii*) appearance times of cholesterol monohydrate crystals are significantly decelerated and numbers of solid cholesterol crystals are significantly smaller in model biles with the addition of mucin gel purified from gallbladder biles of *Muc1*$^{-/-}$ mice; and *iii*) knockout of the *Muc1* gene influences neither cholesterol saturation indices of gallblad-

TABLE 3. Bile flow rates, biliary lipid outputs, and bile salt pool sizes and species

Parameter	Day 0	Day 28	Day 56
Wild type			
Bile flow (μl/min/kg B.W.)	65.4 ± 6.1	68.1 ± 7.9	73.3 ± 4.7
Biliary output			
Cholesterol (μmol/h/kg B.W.)	7.9 ± 1.5	14.2 ± 2.1[a]	16.6 ± 3.0[a]
Phospholipid (μmol/h/kg B.W.)	15.7 ± 2.9	35.5 ± 3.4[a]	45.5 ± 6.0[a]
Bile salt (μmol/h/kg B.W.)	112.7 ± 16.9	120.0 ± 22.1	123.5 ± 15.2
Circulating bile salt pool size (μmol)	2.6 ± 0.3	2.4 ± 0.3	2.8 ± 0.4
Total bile salt pool size (μmol)	3.9	4.1	4.3
Bile salt species			
Taurocholate	51.1 ± 6.5	68.8 ± 4.9	57.5 ± 3.5
Tauro-β-muricholate	41.6 ± 5.7	3.9 ± 0.5[b]	5.6 ± 1.4[b]
Taurochenodeoxycholate	0.4 ± 0.3	14.7 ± 1.3[b]	24.0 ± 2.1[b]
Tauro-ω-muricholate	2.1 ± 0.5	1.7 ± 0.4	0.4 ± 0.2
Tauroursodeoxycholate	2.3 ± 1.1	2.2 ± 0.7	2.3 ± 0.3
Taurodeoxycholate	2.5 ± 0.9	8.7 ± 2.2[c]	10.2 ± 1.2[a]
Muc1$^{-/-}$			
Bile flow (μl/min/kg B.W.)	64.7 ± 5.4	69.1 ± 5.7	73.0 ± 6.8
Biliary output			
Cholesterol (μmol/h/kg B.W.)	6.6 ± 1.3	13.9 ± 1.6[a]	15.5 ± 1.9[a]
Phospholipid (μmol/h/kg B.W.)	15.7 ± 3.1	34.2 ± 3.9[a]	43.4 ± 5.6[a]
Bile salt (μmol/h/kg B.W.)	104.9 ± 14.5	113.4 ± 9.8	123.6 ± 11.5
Circulating bile salt pool size (μmol)	2.4 ± 0.3	2.6 ± 0.4	2.7 ± 0.4
Total bile salt pool size (μmol)	3.7	4.1	4.2
Bile salt species			
Taurocholate	49.7 ± 5.9	69.6 ± 5.5	57.2 ± 1.5
Tauro-β-muricholate	42.2 ± 5.5	3.0 ± 0.9[b]	6.1 ± 1.2[b]
Taurochenodeoxycholate	0.7 ± 0.2	13.4 ± 1.7[b]	22.6 ± 1.8[b]
Tauro-ω-muricholate	1.4 ± 0.4	2.6 ± 0.3	1.0 ± 0.2
Tauroursodeoxycholate	2.6 ± 0.7	2.4 ± 0.5	1.8 ± 0.4
Taurodeoxycholate	3.4 ± 1.2	9.0 ± 1.0[c]	11.3 ± 1.1[a]

Values represent means ± SD of five animals per group. All mice were fed rodent chow containing trace (<0.02%) cholesterol (day 0) and the lithogenic diet containing 1% cholesterol, 0.5% cholic acid, and 15% butter fat for 56 days. B.W., body weight.

[a] Statistically different from day 0, $P < 0.01$.

[b] Statistically different from day 0, $P < 0.001$.

[c] Statistically different from day 0, $P < 0.05$.

der biles, biliary lipid secretion rates, bile salt species, and pool sizes, nor gallbladder sizes and emptying function.

Relatively high levels of steady state *Muc1* mRNA were observed in the gallbladders of the wild-type mice. In comparison, the absence of Muc1 mucin and *Muc1* mRNA in the gallbladders of *Muc1*$^{-/-}$ mice confirmed the complete knockout of the *Muc1* gene. Furthermore, our results demonstrate that the mouse gallbladder epithelium was characterized by the expression of a unique pattern of mucin genes, consisting of high levels of *Muc1* and *Muc3* mRNAs as well as low levels of *Muc4*, *Muc5ac*, and *Muc5b* mRNAs. No *Muc2* mRNA was present in the mouse gallbladder. Our results suggest that mouse gallbladder mucins are highly heterogeneous. We note that the expression pattern of gallbladder mucin genes in the mouse is different from that in human (15, 31–34), in whom MUC1, MUC3, MUC5AC, and MUC5B are the predominant mucin proteins in gallbladder and in bile as detected by Northern and Western blot analyses as well as by immunohistochemical methods. There are little or no MUC2 and MUC4 mucins in the human gallbladder. The reasons for these differences in mucin gene expression patterns may reflect unique cytoprotective needs attributable to exposure to high concentrations of hydrophobic bile acids and cholesterol-supersaturated biles in humans (35, 36). In contrast, under chow feeding conditions, mouse gallbladder biles contain large amounts of hydrophilic bile salts and considerably lower cholesterol content (37, 38).

Feeding the lithogenic diet caused significant increases in expression levels of *Muc1*, *Muc3*, *Muc4*, and *Muc5ac* mRNAs in the wild-type mice, with *Muc2* and *Muc5b* mRNA levels remaining unchanged. Furthermore, we observed that increased soluble and Muc1 mucin concentrations in bile, as well as mucin secretion and accumulation in the gallbladder, were associated with increased cholesterol saturation indices of biles (Table 2), which is consistent with previous findings in animal gallbladder biles (7, 8, 39). Our study suggests that increases in gallbladder mucins may be secondary to cholesterol supersaturation per se (7, 8, 39). The second possible stimulators of gallbladder mucin hypersecretion may be hydrophobic bile salts, because there is a significant shift in bile salt composition from tauro-β-muricholate to taurocholate and taurodeoxycholate in both strains of mice fed the lithogenic diet for 56 days. In contrast, in response to the lithogenic diet, mice with knockout of the *Muc1* gene displayed significant decreases in expression levels of *Muc5ac* and *Muc5b* mRNAs, with *Muc3* and *Muc4* being significantly increased. When examining fresh gallbladder biles by microscopy, we found that cholesterol monohydrate crystals and liquid crystals were appreciably less frequent in the early stage of gallstone formation in *Muc1*$^{-/-}$ mice compared with wild-type mice. Furthermore, our current in vitro experiments (Fig. 6) show that mucin gel purified from the wild-type mice acts as a strong pronucleating/crystallizing factor for accelerating solid cholesterol crystal appearance times and as a nucleus for holding cholesterol monohydrate crystals for their growth and agglomeration, resulting in increased cholesterol crystal number. In contrast, these effects of mucin gel were significantly diminished in *Muc1*$^{-/-}$ mice.

Although the gallbladder biles of *Muc1*$^{-/-}$ mice contained no Muc1 mucin, as confirmed by Western blot analysis (Fig. 2B), we did not examine other individual mucin concentrations in *Muc1*$^{-/-}$ or wild-type mice by the same method because of the lack of antibodies to Muc2, Muc3, Muc4, Muc5ac, and Muc5b. Nevertheless, we studied expression levels of these gallbladder mucin genes (Fig. 1) in both strains of mice, suggesting a possible change in the concentrations of each mucin in gallbladder biles. Most notably, during the 56-day lithogenic diet feeding period, the crystallization, growth, and agglomeration of cholesterol monohydrate crystals, as well as the development of gallstones, were significantly retarded in *Muc1*$^{-/-}$ mice (Fig. 5B). Consequently, at day 56 of the feeding, gallstone prevalence rates, as well as stone sizes and numbers, were significantly decreased in *Muc1*$^{-/-}$ mice. Because mouse gallbladder mucins are derived from a uniquely heterogeneous group of mucin genes, the mucin layer of the gallbladder may be composed of multiple layers: the epithelial mucins produced by *Muc1*, *Muc3*, and *Muc4* are located on the apical surface of epithelial cells, and the gel-forming mucins secreted by *Muc5ac* and *Muc5b* form the next layer. Our results demonstrate that the lack of the epithelial Muc1 mucin greatly reduces susceptibility to cholesterol cholelithiasis. In addition, a significant decrease in gel-forming mucin produced by the *Muc5ac* gene could contribute to reduced gallstone prevalence rates in *Muc1*$^{-/-}$ mice. In contrast, expression levels of the gel-forming *Muc5ac* gene, but not *Muc2* or *Muc5b*, were increased significantly in the gallbladders of wild-type mice in response to the lithogenic diet, indicating that Muc5ac mucin may play a major role in murine cholesterol gallstones.

Furthermore, our results suggest that there may be gene-gene interactions between *Muc1* and *Muc5ac* that might affect mucin secretion and accumulation in the gallbladder, and it remains unclear whether the knockout of the gallbladder *Muc1* gene or the lack of the membrane-associated mucin determined by the *Muc1* gene on the apical surface of epithelial cells in the gallbladder influences the expression and function of the *Muc5ac* gene. A recent study (40) by quantitative trait locus mapping in a backcross between SWR/J and AKR/J strains showed that polymorphisms in the *Muc3* gene or its promoter may be linked to gallbladder accumulation of mucin gel and affect cholesterol gallstone formation. Our results and those of Wittenburg et al. (40) support the concept that the epithelial mucin genes may influence gallbladder mucin accumulation by regulating the expression and function of the gel-forming mucin genes. In addition, because mucin secretion by the gallbladder is determined by multiple mucin genes (12–15) and some amounts of mucin accumulation in the gallbladder were still observed, a small number of stones with small sizes were found in *Muc1*$^{-/-}$ mice. Taken together, our findings demonstrate that knockout of the *Muc1* gene significantly reduces cholesterol gallstone formation by diminishing mucin secre-

tion and accumulation in the gallbladder and retarding cholesterol nucleation and crystallization in bile.

In agreement with previous studies (8, 20), we found that upon feeding the lithogenic diet, hepatic hypersecretion of biliary cholesterol and a rapid onset of cholesterol-supersaturated gallbladder bile occur, which are essentially similar between *Muc1*$^{-/-}$ and wild-type mice. Although the biliary bile salt pool was enriched with taurocholate, mainly attributable to the addition of 0.5% cholic acid to the lithogenic diet, there were no differences in bile salt species and pool sizes in these two strains of mice. Because gallbladder hypomotility is a crucial risk factor for gallstone formation, we investigated whether disruption of the *Muc1* gene influences gallbladder emptying. We found that on chow, *Muc1*$^{-/-}$ mice displayed identical gallbladder sizes and emptying function compared with wild-type mice, and feeding the lithogenic diet reduced gallbladder emptying in both strains, possibly attributable to impaired CCK-1 receptor function as well as reduced CCK-1 receptor number and/or CCK binding capacity of gallbladder CCK-1 receptors (41–43). Thus, our study shows that knockout of the gallbladder *Muc1* gene does not influence biliary cholesterol secretion, cholesterol saturation indices of gallbladder biles, or gallbladder size and emptying function before and during the lithogenic diet feeding, suggesting that these biliary and gallbladder factors are most likely not a major contributor to the retardation of cholesterol crystallization and gallstone formation in *Muc1*$^{-/-}$ mice.

In conclusion, compared with the wild-type mice, *Muc1*$^{-/-}$ mice display significantly decreased cumulative susceptibility to cholesterol gallstone formation by significantly reducing mucin secretion and accumulation in the gallbladder, which retards cholesterol crystallization and the development of gallstones. Our findings support the notion that completely blocking the secretion and accumulation of gallbladder mucins may prevent the formation of cholesterol gallstones.

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