Published in final edited form as:

Am J Physiol Gastrointest Liver Physiol. 2008 June ; 294(6): G1411–G1420. doi:10.1152/ajpgi.00181.2007.

PATHOPHYSIOLOGIC BASIS OF LIVER DISEASE IN CYSTIC FIBROSIS EMPLOYING A $\Delta F508$ MOUSE MODEL

FOLKE FREUDENBERG^{1,2}, ANNEMARIE L. BRODERICK^{1,2,3}, BIAN B. YU², MONIKA R. LEONARD², JONATHAN N. GLICKMAN⁴, and MARTIN C. CAREY^{1,2}

¹Department of Medicine, Harvard Medical School and Harvard Digestive Diseases Center, Boston, MA, USA

²Department of Medicine, Gastroenterology Division, Brigham and Women's Hospital, Boston, MA, USA

³Combined Program of Gastroenterology and Nutrition, Children's Hospital, Boston, MA, USA

⁴Pathology Department, Harvard Medical School and Brigham and Women's Hospital, Boston, MA, USA

Abstract

The molecular pathogenesis of cystic fibrosis (CF) liver disease is unknown. This study investigates its earliest pathophysiologic manifestations employing a mouse model carrying $\Delta F508$, the commonest human CF mutation. We hypothesized that, if increased bile salt spillage into the colon occurs as in the human disease, this should lead to a hydrophobic bile salt profile and to “hyperbilirubinemia” because of induced enterohepatic cycling of unconjugated bilirubin. Hyperbilirubinemia may then lead to an increased bile salt to phospholipid ratio and, following hydrolysis, precipitation of divalent metal salts of unconjugated bilirubin. We document in CF mice elevated fecal bile acid excretion and biliary secretion of more hydrophobic bile salts compared with control wild type mice. Biliary secretion rates of bilirubin monoglucuronides, bile salts, phospholipids and cholesterol are increased significantly with an augmented bile salt to phospholipid ratio. Quantitative histopathology of CF livers displays mild early cholangiopathy in $\approx 53\%$ of mice and multifocal divalent metal salt deposition in cholangiocytes. We conclude that increased fecal bile acid loss leads to more hydrophobic bile salts in hepatic bile and to hyperbilirubinemia, a major contributor in augmenting the bile salt to phospholipid ratio and endogenous β -glucuronidase hydrolysis. The confluence of these perturbations damages intrahepatic bile ducts and facilitates entrance of unconjugated bilirubin into cholangiocytes. This study of the earliest stages of CF liver disease provides a framework for investigating the molecular pathophysiology of more advanced disease in murine models and in humans with CF.

Keywords

CFTR; gallstones; enterohepatic cycling; bilirubin; metal salts

Address correspondence to: Dr. Martin C. Carey, Brigham and Women's Hospital, Gastroenterology Division, 75 Francis Street, Boston, MA 02115, USA. Phone: (617) 732-5822; Fax: (617) 730-5807; E-mail: E-mail: mccarey@rics.bwh.harvard.edu.
Present addresses: Dr. F. Freudenberg, Division of Pediatric Gastroenterology and Hepatology, Dr. von Haunersches Kinderspital, Ludwig Maximilians University, Lindwurmstr. 4, D- 80337 Munich, Germany; Dr. A. Broderick, Our Lady's Hospital for Sick Children, Crumlin, Dublin 12, Ireland.

Introduction

Cystic fibrosis (CF), the most common monogenic inherited disease in humans with northern European ancestry, is caused by more than 1000 different mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene (53,57). *CFTR* is a chloride channel regulated by cAMP and is located on the apical plasma membranes of most absorptive and secretory cells including large cholangiocytes and cholecystocytes (19,20,34). Its dysfunction impairs Cl^- secretion and therefore $\text{Cl}^-/\text{HCO}_3^-$ exchange. The most common mutation in the *CFTR* gene, the ΔF508 mutation, involves about 70% of CF patients (57). Hepatobiliary abnormalities occur in approximately 13-27% of pediatric CF patients (29,50) and 30-72% of adults with CF (40,43,69) and include cholangitis, cholestasis, hepatic steatosis, focal biliary cirrhosis (40), and gallstones (2,43). The pathogenesis of any of these complications of CF is not understood. The aim of this study was to investigate the pathophysiological and histopathological changes in the hepatobiliary tree of a mouse model carrying the ΔF508 mutation in which liver disease is very mild or absent (76).

First, we assessed the histopathology of the liver, with particular reference to bile duct lesions. We also determined whether these CF mice demonstrate increased fecal bile acid loss and an altered bile salt profile, a notable feature of the human disease (49,58,74). We also characterized bilirubin molecular species and secretion rates, which can provide evidence for putative enterohepatic cycling of unconjugated bilirubin (UCB) since “hyperbilirubinemia” (increased secretion of conjugated bilirubins into bile) has been demonstrated previously in ileectomized rats (9) and patients with ileal Crohn's disease (10). Any degree of enterohepatic cycling of bilirubin mimics chronic mild hemolytic states (9) and places an animal at risk for intraductal hydrolysis of conjugated bilirubins and insoluble metal salt formation as well as precipitation of “black” pigment stones in the gallbladder (72).

We studied histopathological changes in both genders of mice as functions of age and demonstrate mild liver disease in approximately 53% of CF mice older than 100 days. Our work suggests that liver injury begins most likely at the level of the large cholangiocytes where the dysfunctional *CFTR* is located (1). We suggest that cholangiocytic injury is caused by a more hydrophobic bile salt pattern and an increased detergency from augmented bile salt to phospholipid ratio caused by hyperbilirubinemia. This leads, in turn, to increased intrahepatic hydrolysis by endogenous β -glucuronidase and precipitation of unconjugated bilirubin in cholangiocytes that, in contrast to hepatocytes, lack a “disposal” mechanism for the bile pigment. Our findings in this murine model of CF suggest that the liver disease is exceedingly mild, allowing for observation of an unperturbed “window” on its causation by altered bile salt physiology arising in the distal gut. These subtle but overt alterations in bile chemistry may be translatable to more severe animal models of CF liver disease and to humans with CF.

Materials and Methods

Animals

Heterozygous breeding pairs of ΔF508 mice (76) and wild type (WT) mice (background: 75%-C57BL/6, 25%-129SvEv) were kindly provided by Dr. Marie Egan, Yale University School of Medicine, New Haven, Connecticut. They were housed on corn cob bedding (The Andersons, Maumee, Ohio) in the animal facility of the Thorn Research Building at Brigham and Women's Hospital, Boston, MA. Mice were fed a diet containing 11% fat and replete with calories, vitamins and minerals (Mouse Diet 5015*, Labdiet®, Richmond, Indiana) and were maintained on a regular 12-hour light/dark cycle. Mice were given an oral isosmotic solution containing polyethylene glycol-3350 and electrolytes (GoLYTELY®, Braintree Laboratories, Braintree, Massachusetts) *ad libitum*. Histopathological studies and fecal bile acid analyses were performed on age- and gender-matched homozygous CF and control (wild type, WT) mice.

When we found little difference between genders and because CF mice were scarce, we utilized age-matched mice of either gender for the remaining experiments. All experiments were performed at the same time of day on mice in the non-fasted state. At three weeks of age, mice were genotyped after tail or ear clipping. DNA was isolated using DNeasy® kits (Qiagen, Valencia, California) and the PCR product was amplified using Amplitaq Gold Master Mix® (Applied Biosystems, Foster City, California) with the primer sequences GAG TGT TTT CTT GAT GAT GTG and ACC TCA ACC AGA AAA ACC AG. The amplified DNA was restricted utilizing the enzyme RsaI (Applied Biosystems) and separated by agarose gel electrophoresis. Mice were weighed prior to surgery. All experiments were performed following protocols approved by the Harvard University Medical Area Standing Committee on Animals.

Liver Histopathology

An age-matched study by a pathologist (J.N.G.) blinded as to genotype and mouse age was performed on 30 livers of CF mice and 48 livers of WT littermates aged from one day to approximately 400 days. After laparotomy and hepatectomy, livers were fixed at room temperature ($\approx 22^{\circ}\text{C}$) for a minimum of 12 hours in 10% formaldehyde, processed routinely, and embedded in paraffin. Five-micron thick sections were stained with hematoxylin and eosin (H&E) and special stains (see below) and examined. We employed a semi-quantitative scoring system to assess inflammation as follows: 0, absent; 1, either mild (scattered portal or lobular inflammatory cells in single or small clusters) or a few foci in a minority of lobules; 2, moderate (numerous inflammatory cells in clusters, involving the majority of lobules); or 3, severe (sheets of inflammatory cells, invariably involving the majority of lobules). Fibrosis was quantified using Masson's trichrome staining protocol employing the following scoring system: 0, no increase; 1, portal, pericentral or sinusoidal fibrosis; 2, septal fibrosis without bridging; 3, bridging fibrosis; 4, cirrhosis (regenerative nodule formation). Hepatocyte regeneration was evaluated by H&E and reticulin stains and graded as follows: 0, absent; 1, one or two foci only; 2, multifocal, involving a minority of lobules; 3, diffuse, involving the majority of lobules. Bile ductular proliferation was scored as absent, minimal (exemplified by prominent canals of Hering, increased cholangiocytes, but no extra ductules), 1+ (one to two foci and one extra bile ductule only), or 2+ (multifocal with multiple ductular profiles per focus). Scores were averaged for each group and reported as Mean Lesion Scores. Deposition of Fe and Ca bilirubinates in the liver was assessed by Prussian blue and von Kossa staining, respectively, as well as Hall's bilirubin stain.

Fecal bile acid excretion

Mice were housed individually in metabolic cages and given free access to GoLYTELY® and the high fat (11% by weight) diet. At 72 hours, three-day stools were collected and mice were weighed. Stools were dried under reduced pressure for 48 hours and then ground to a powder using a mortar and pestle. Alkaline hydrolysis was performed on 100 mg aliquots of homogenized, dried stool. After acidification with HCl, fecal bile acids were extracted with diethyl ether and measured by an enzymatic assay (52).

Biliary bilirubin outputs and molecular species

Under general anesthesia (ketamine:xylazine:atropine, 90:10:0.13 mg/kg BW) a midline abdominal incision was made, the cystic duct was identified and ligated, followed by ligation of the common bile duct near the Vaterian papilla. A 0.28 mm I.D. polyethylene catheter (Intramedic®, Becton Dickinson, Franklin Lakes, New Jersey) was inserted into the proximal common bile duct, and, after discarding the initial 5-minute drainage, bile was collected for ≈ 15 minutes into tared collection tubes. This procedure for bile collection minimizes interruption of the enterohepatic circulation. A 10- μl bile sample from each collection was

used to quantify bilirubin concentrations and molecular species by HPLC (63). Total bilirubin secretion rates were normalized to one hour of bile flow and 100 g body weight (BW).

Common biliary lipids in hepatic bile

Bile salt molecular species were determined by HPLC (56). Total bile salts were assayed by the 3 α -hydroxysteroid dehydrogenase method (67). Biliary phospholipids were measured as inorganic phosphorus (4), and biliary cholesterol was hydrolyzed and extracted (38) prior to HPLC analysis (70). Bile salt hydrophobicity was quantified as a hydrophobicity index according to the method of Heuman (35). Secretion rates of the major biliary lipids were calculated by normalizing concentrations per hour of bile flow and to 100 g BW. CSI's were calculated using critical tables (15).

Bile flow, hepatic bile pH, and electrolytes

Following bile collection, biliary pH values were measured immediately by microelectrode (Thermo Electron Corp., Beverly, Massachusetts), and bile volume was determined gravimetrically by numerically equating weight (g) with volume (ml). To obtain the 90- μ l volumes needed for biliary Cl⁻ measurements, hepatic bile was collected for approximately 1 hr. Samples were frozen at -20°C until analyses could be performed at the clinical chemistry Core Laboratory, Children's Hospital, Boston, MA on a cobas c 501 system (Roche Diagnostics, Indianapolis, IN) utilizing an ion-specific electrode and calibrated specifically for non-plasma, non-urine samples. Biliary Na⁺, K⁺, and PO₄³⁻ concentrations were measured in hepatic bile samples ranging in volume from 20-90 μ l on a cobas Integra 400 system (Roche Diagnostics, Indianapolis, IN). Samples were automatically diluted until values fell into the detectable range for the analyte. Both Na⁺ and K⁺ were measured by ion-selective electrodes, whereas measurement of phosphate was determined by a colorimetric reaction.

Ileal luminal pH values

After ligating the ileum at both the ileocecal valve and 5 cm cephalad from the initial ligation, 1.0 ml of 0.15 M NaCl solution was injected into the ileal lumen, followed by gentle manipulation. After three minutes of equilibration, which was shown in preliminary studies to lead to a steady state value, pH was measured intralumenally by means of a micro-glass electrode.

Statistical Treatments

Group values for each measurement are expressed as means $\pm$ SEM. For comparisons between CF and WT mice, statistical significance was assessed using an unpaired 2-tailed Student's *t* test. *P* values less than 0.05 are considered significant.

Results

General

Between 10 and 25 days, WT and CF mice were approximately the same weight. Over the course of this longitudinal study, all mice gained weight progressively (Figure 1) with WT mice diverging at the earliest time points and mean weights leveling off at about 250 days. After the first 25-day period both WT and CF mice displayed steady increases in weight, and differences between the groups remained similar. Of note is that mouse breeding (heterozygous male x heterozygous female or CF male x heterozygous female) yielded 40% fewer CF offspring than expected (data not shown). The mice studied in this work all appeared healthy up to surgery and sacrifice.

Liver histopathology

Sections of liver from representative CF (Fig. 2A) and WT (Fig. 2B) livers display variable, mild, patchy cholangiopathy characterized by reactive changes in the biliary epithelium, bile ductular proliferation and mild portal fibrosis. These findings were present only rarely in WT mice (Fig. 2B). Intracellular fatty deposits were not observed in any of the CF livers. Curiously, some older WT mice developed steatohepatitis, most likely from efficient absorption of the 11% fat diet. So-called “inspissated” plugs were not detected in the hepatobiliary tree of any animal. None of the mice manifested any advanced degree of liver fibrosis or cirrhosis, and evidence for apoptosis was found in only a single sample out of 30 (data not shown). There were no gender differences in the liver histopathology of either WT or CF mice.

Table 1 quantifies histopathologic changes (i.e., lobular inflammation, fibrosis, and bile duct alterations) in WT and CF mice as functions of age. At sacrifice, signs of lobular inflammation were found in approximately half of CF mice older than 100 days compared to nearly a third of age-matched WT mice, with both the percentage of affected CF mice and their Mean Lesion Scores reaching a plateau at 100 days; however, none of the differences between age-matched WT and CF mice were statistically significant. The percentage of both WT and CF mice affected with fibrosis and their corresponding Mean Lesion Scores demonstrated no age-related trend; however, the differences were significant for mice aged 100-200 days. Whereas the incidence and Mean Lesion Scores for bile duct alterations remained generally constant in WT mice, the proportion of affected CF mice and their Mean Lesion Scores rose steadily with passage of time. Differences for percentages affected and Mean Lesion Scores of bile ductular histopathology were statistically significant for comparisons of older age-matched CF and WT mice. Mean Lesion Scores for all histopathologic changes as functions of age indicate that liver disease was very mild in this CF model and remained so for the duration of the study. Nonetheless, 47% of CF mice did not exhibit any bile duct lesions even though some animals were more than 12 months old.

Prussian blue and von Kossa staining for Fe (Fig. 2C) and Ca (Fig. 2D) salts revealed multifocal hepatic deposition of both metals in cholangiocytes and adjacent portal tract connective tissue. Multifocal Fe deposition was found in 13% of CF and 6% of WT mice, and multifocal Ca deposition, in 25% of CF and 3% of WT mice. CF mice displayed a trend toward greater Fe deposition with increasing age (data not displayed). Hall's bilirubin stain was positive but insufficiently sensitive to the unaided eye to verify an appreciable difference between CF and WT mice.

Fecal bile acid excretion

Fig. 3 displays 24-hr fecal bile acid outputs for male and female WT and CF mice normalized per 100 g BW. Fecal excretion levels are significantly higher in all CF compared to WT mice, with values of 22.8 ± 3.1 and 12.7 ± 1.8 $\mu\text{mol/d/100 g BW}$ in CF and WT males, respectively ($n = 6$ per group), and 30.3 ± 4.8 and 15.8 ± 2.7 $\mu\text{mol/d/100 g BW}$ in CF and WT females ($n = 8$ per group); ($P = 0.02$ for both comparisons). Fig. 3 also shows that mean fecal bile acid excretions in female mice were somewhat higher than those of age-matched males; however, gender differences for CF and WT mice were not statistically significant.

Bile salt molecular species in hepatic bile

Table 2 displays percentages of the common bile salt species present in hepatic bile of CF and WT mice. Concentrations of tauro α -muricholate and tauro β -muricholate, both primary bile salts in the mouse (26), were combined because the peaks of these hydrophilic bile salts could not be baseline-separated by the HPLC method used (56). In CF mice, we found the percentage of tauromuricholates significantly decreased in CF compared to WT mice, with a reciprocal increase that was also significant for taurocholate. The secondary bile salt taurodeoxycholate

was increased in CF compared to WT mice, whereas the percentage of the primary bile salt taurochenodeoxycholate is similar in both CF and WT mice. Bile salt hydrophobicity indexes were significantly higher in CF mice compared to WT mice.

Bilirubin outputs and molecular species

Secretion rates of total conjugated bilirubins (Fig. 4A) are increased significantly in CF mice (35.6 ± 4.0 nmol/h/100 g BW, $n = 8$; compared to 22.4 ± 2.8 nmol/h/100 g BW in WT mice, $n = 7$; $P = 0.02$). This was due principally to the secretion rate of BMG (Fig. 4B), the principal bilirubin conjugate in mice, which was increased significantly in CF compared to WT mice (24.4 ± 2.7 and 16.0 ± 1.8 nmol/h/100 g BW, respectively; $n = 9$ and 7 ; $P = 0.03$). Secretion rates of total bilirubin diconjugates, i.e., bilirubin diglucuronide (BDG) plus all other diconjugates (BDX) (Fig. 4C), were appreciably increased (10.4 ± 1.7 in CF versus 6.4 ± 1.1 nmol/h/100 g BW in WT; $n = 8$ and 7 ; $P = 0.08$) without reaching significance. Although the secretion rate of UCB was nearly doubled in CF compared to WT mice (Fig. 4D) (0.7 ± 0.2 and 0.4 ± 0.1 nmol/h/100 g BW, respectively; $n = 9$ and 7 ; $P = 0.3$), that difference also failed to reach statistical significance. To determine if a correlation between liver histopathology and bilirubin secretion rates existed, we separated CF mice with histologic evidence of liver disease (CF + LD) from those with normal histology (CF - LD). Fig. 4E displays BMG secretion rates for both CF groups compared to WT mice. This reveals that BMG secretion rates are significantly elevated in CF mice with histopathological liver lesions (29.0 ± 2.6 nmol/h/100 g BW, $n = 6$; $P = 0.02$), whereas BMG secretion rates for CF mice without histologic changes (13.5 ± 1.5 nmol/h/100 g BW, $n = 2$) are similar to those of WT mice (16.0 ± 1.8 nmol/h/100 g BW).

Biliary pH and electrolytes

Fig. 5A demonstrates that pH values of hepatic bile, although slightly lower in CF mice, are not significantly different from those of WT mice (8.6 ± 0.06 and 8.7 ± 0.06 , respectively; $n = 11$ per group; $P = 0.3$). Hepatic bile Cl^- concentrations (Fig. 5B) show opposite trends, being higher (non-significantly) in CF than in WT mice (88.4 ± 3.7 and 81.5 ± 2.5 mM, respectively; $n = 8$ and 6 ; $P = 0.18$). No appreciable differences between CF and WT mice were found for PO_4^{3-} concentrations or concentrations of the monovalent counterions (Na^+ and K^+) (data not displayed).

Hepatic bile flow and biliary lipid secretion rates

Fig. 6A plots normalized hepatic bile flow and demonstrates that the values are significantly higher in CF than in WT mice (593.3 ± 73.2 and 335.2 ± 36.4 $\mu\text{L}/\text{h}/100$ g BW, respectively; $n = 9$ and 15 ; $P = 0.002$). The assayed absolute concentrations of bile salts, phospholipids, and cholesterol in hepatic bile are similar in CF and WT mice with mean CSI's of 0.65 and 0.67, respectively. However, there was a 19% increase in bile salt to phospholipid ratio in bile of CF compared to WT mice ($n = 6$ and 11 , respectively). Fig. 6B plots normalized bile salt secretion rates and shows a significant threefold increase in CF compared to WT mice (44.1 ± 12.3 compared to 15.6 ± 3.4 $\mu\text{mol}/\text{h}/100$ g BW; $P = 0.02$). Similarly, phospholipid secretion rates (Fig. 6C) are doubled in CF compared to WT mice (4.1 ± 0.9 compared to 1.9 ± 0.3 $\mu\text{mol}/\text{h}/100$ g BW; $P < 0.01$), and cholesterol secretion rates (Fig. 6D) are nearly tripled (0.91 ± 0.28 compared to 0.34 ± 0.05 $\mu\text{mol}/\text{h}/100$ g BW; $P = 0.02$). To obtain an estimate of the contributions of bile salt-dependent and bile salt-independent bile flows to the increased bile flow in CF mice, we plotted (Fig. 6E) the volume of bile flow (Fig. 6A) for both WT and CF mice against their respective bile salt outputs (Fig. 6B). The slope of each regression line (volume of hepatic bile water in microliters/ μmol bile salts) is a quantitative measure of the bile salt-dependent bile flow. The extrapolated y-intercept of each line (i.e., when bile salt output is theoretically zero) gives the bile salt-independent bile flow. Although the difference between slopes is not

quite significant, Fig. 6E suggests that bile salt-dependent bile flow in CF mice is less than 50% that of WT mice (slopes = 7.8 and 18.3; $n = 5$ and 10, respectively; $P = 0.096$), whereas the bile salt-independent bile flow in CF mice is nearly three times that of WT mice (y-intercept = 351 for CF and 125 for WT mice). These findings are consistent with the higher Cl^- values in the hepatic biles of CF mice that we documented in Fig. 5B.

Ileal luminal pH

As determined from preliminary experiments prior to measuring the pH by glass electrode, we allowed the injected saline solution to equilibrate in the doubly ligated, closed ileal loops for 3 minutes. The final recorded pH values in the distal ileum are significantly lower ($P = 0.003$) in CF (6.7 ± 0.1) than in WT mice (7.2 ± 0.1); $n = 8$ per group.

Discussion

This study provides a systematic description of the earliest and, in some cases, subtle alterations in the biochemistry, biophysics and pathophysiology of the small and large intestines and hepatobiliary system in a CF mouse model carrying the most common human CF mutation, ΔF508 . Although others have demonstrated advanced liver histopathology in a mouse model with more severe systemic CF disease (25), we chose for our experiments the mouse model described by Zeiher et al (76) in which liver disease is mild - if it occurs at all. This model allowed us to obtain valid indices for pH, electrolytes, and biliary outputs of flow, common biliary lipids, and especially bilirubins in hepatic bile without the values being perturbed by severe dysfunctional liver disease. The ΔF508 dysfunction is constructed on a 75% C57BL/6 - 25% 129SvEv background and resembles the human disease with the same genetic defect but with the difference that residual CFTR activity is present. As in the human disease, approximately 53% of these animals develop some histopathological evidence of liver disease, but many mice never acquire microscopic alterations in their livers. Interestingly, there is a high incidence of meconium ileus (in the absence of corncob bedding; see methods) in this model, and the ΔF508 mice exhibit significant growth retardation compared to WT mice (Figure 1). On the other hand, this ΔF508 murine model displays minimal pulmonary phenotype, most likely because of ancillary Cl^- channels and, in this regard, is markedly different from humans with the same *CFTR* mutation.

We documented the development of histopathologic changes in approximately 53% of CF animals, with morphologic features of cholangiopathy, including bile ductular proliferation and mild portal fibrosis (Fig. 2A), characteristics of very early and mild liver disease. As anticipated, no evidence was found histologically for bile ductular obstruction from so-called “inspissated” bile. As inferred histopathologically, this work suggests that the earliest evidence of hepatic disease appears to be damage to cholangiocytes, not hepatocytes. We proposed that the damage resulted from intraluminal alterations in the biochemistry and physical chemistry of biliary lipids and lipopigments, i.e., bilirubins, and focused on the possibility that there might be hyperbilirubinemia and deposition of salts of unconjugated bilirubin, and that hydrophobic bile acids and an altered bile salt/phospholipid ratio might be involved. This led us to focus on distal (i.e., ileal and colonic) causes for these alterations as the possible *fons et origo* of the liver insult.

We first examined steady state fecal bile acid outputs and biliary lipid compositions in CF and WT mice. As in the human disease where $\approx 30\%$ of CF patients exhibit bile salt malabsorption (49,74), we found that fecal bile acid excretion is increased approximately twofold in our CF mouse model (Fig. 3). Two principal hypotheses have been proposed for this observation in humans. i) Zentler-Munro et al (78) showed that increased acidity of the upper small intestine in CF leads to protonation and precipitation of glycine-conjugated bile salts as crystals (16, 60). However, acid-induced bile salt insolubility cannot explain our findings since the bile salt

pool consists mostly (>95%) of taurine-conjugated bile salts in mice. The sulfonate group of the side chain of taurine-conjugated bile salts cannot be protonated at gut luminal pH values found in CF mice since the pK'_a values are less than 2 (60). ii) Others have shown that increased fecal bile acid loss may be due to the binding of bile salts to undigested protein, starch, or lipids, thus preventing bile salt resorption in the ileum (49,73,77). This mechanism cannot apply either to our mouse model since the $\Delta F508$ mutation and several other CF mouse models exhibit no major pathologic changes in the pancreas, in contrast to humans (21,23,32,33,68,76). Although not a formal part of this study, we confirmed high levels of pancreatic amylase and lipase in the proximal small intestines of our CF mice (F. Freudenberg and M. C. Carey, unpublished observations). In addition, we found no evidence by Western blot for altered expression of the SLC10A2/ASBT receptor in the ileum (F. Freudenberg and M. C. Carey, unpublished observations). *Ex vivo* studies by Lack and Weiner (41) of the ileal mucosa's affinity for bile salts have shown that its function is highly sensitive to luminal pH, with small decreases resulting in a pronounced lower uptake. We interpolate from their published work (41) that the significant difference of approximately 0.5 pH units in ileal luminal pH (see Results) would decrease the transport of taurocholate by approximately 9% per enterohepatic cycle. This could explain most of the fecal bile acid loss in the CF mouse since the bile salt pool circulates frequently with nocturnal eating (36). Another plausible explanation for fecal bile acid loss could be the increased thickness of viscous intestinal mucin gel in the CF mouse model, but whether mucin gel binds bile salts appreciably is controversial (61,75). However, mucin gel may act as a diffusion barrier (6,42) to retard bile acid resorption by ileal SLC10A2/ASBT in both mice and humans. Nonetheless, we propose that the electrochemical reason noted above is the most likely cause for the approximate doubling of fecal bile acid loss (41) that we observed in our $\Delta F508$ mice (Fig. 3). The increased fecal bile acid excretion also reflects more secondary bile acid formation by bacteria in the colon and is consistent with the altered bile acid molecular species observed in hepatic bile (Table 2) since the murine liver is not 100% efficient in 7 α -rehydroxylation of the cholanoic acid nucleus. We propose that the excess bile acids in the colon are also responsible for induced enterohepatic cycling of bilirubin (9) found in these mice.

With further respect to the higher proportion of more hydrophobic bile salts (Table 2) in bile of CF mice, we note that the molecular bile salt profile found in a mouse model with severe fecal bile acid loss due to disruption of SLC10A2 is similar (22), and that more hydrophobic bile salts are better conserved by passive resorption in the colon (17). In addition, the higher bile salt to phospholipid ratio (30) may be another source of damage to cholangiocytes and may further aggravate cholangiocyte injury by facilitating the entry of locally formed UCB into cells. Most likely the increased bile salt to phospholipid ratio in hepatic bile is secondary to uncoupling of phospholipid from bile salt secretion at the canalicular level as a result of hypersecretion of conjugated bilirubins (3,71). These explanations validate, in part, the "classic" theoretical postulate that so-called "toxic" components of bile (mainly bile acids) might impair cholangiocyte integrity and cause bile duct damage leading to CF liver disease (29,62).

In addition to the secretion rates of the lipopigment molecules being significantly increased in $\Delta F508$ mice (Fig. 4A-C), we also found (Fig. 4E) a strong correlation between mice with elevated BMG secretion rates and those animals developing histopathological changes in the liver. Furthermore, in mice with mild CF liver disease we observed histological staining consistent with multifocal hepatic deposition, presumably as bilirubinates of Fe and Ca metal salts, in cholangiocytes and in portal tracts (Fig. 2C and D, respectively). It is unlikely that Ca or Fe precipitated with carbonate or phosphate since the pH of hepatic bile is not consistent with supersaturation with Ca salts of these anions (47, 48). Moreover, conjugated bile salts, even glycine-conjugated ones, are resistant to Ca precipitation in the typical concentrations found in hepatic bile (24, 39). In contrast, the exquisite sensitivity of UCB to forming insoluble

salts with Ca^{2+} is demonstrated by the ion product of $\text{Ca}(\text{HUCB})_2$ in model bile which is of the order of 10^{-15} M^2 (12). Hyperbilirubinemia of any cause is an accepted risk factor for increased intrahepatic hydrolysis by endogenous β -glucuronidase and deposition of UCB and possibly BMG (66) as metal salts, not only in the gallbladder as “black” pigment stones but also, as shown by us here, in cholangiocytes. Cholangiocytes lack a bilirubin conjugation/transport system and export pump (65) and therefore cannot dispose of UCB as effectively as do hepatocytes. Moreover, UCB is cytotoxic as well as being disruptive to plasma membranes (11, 13, 18, 51). We speculate that cytotoxicity of elevated UCB levels in cholangiocytes, either as the free dianion or as metal salts, might be a major contributor to CF hepatic liver disease (14), augmenting injury by the more hydrophobic hepatic bile (7).

The calculated relative lipid compositions expressed as CSI's of hepatic bile are similar in CF and WT mice (see results). Published studies in humans with severe CF disease indicate that significantly higher relative concentrations of bile cholesterol, secondary to bile salt malabsorption, are not always found (2,5,58,64). Nonetheless, because of increased bile flow, possibly secondary to alternate Cl^- channel compensation, the normalized (but not absolute) secretion rates of all three major biliary lipids (Fig. 6B-D) as well as bilirubins (Fig. 4) were significantly higher in CF compared to WT mice. Therefore, taking all data together, it is likely that the cholangiocyte damage in CF mice is related to increased detergent exposure over time, primarily to more hydrophobic bile salts (Table 2), and to hyperbilirubinemia, which, although innocuous *per se*, increases bile salt to phospholipid ratios and augments UCB formation.

It is evident (Fig. 6E) that bile salt-dependent bile flow (i.e., slope of the regression line plotting bile flow and bile salt output) is lower in CF than in WT mice. At the phospholipid/bile salt ratios in both CF and WT mice (values <0.15 ; data not shown), bile salt-dependent bile flow is predicated by the number of simple micelles (i.e., those not containing phospholipids) coexisting with mixed micelles (16,44). Because the number of these osmotically active particles increases with hydrophilicity (46), the decreased bile salt-dependent bile flow observed in the CF mice is consistent with a more hydrophobic bile salt profile (Table 2). The increased bile salt-independent bile flow in the CF mice is consistent with Cl^- levels being higher in CF than in WT mice. This suggests either that CFTR is stimulated by cholehepatic shunting of bile salts (37) or that alternate Cl^- channels are upregulated. It is interesting to note in this regard that, in two different ΔF508 mouse models (21,68), Bijvelds et al (8) showed that ileal bile salt absorption activates CFTR-mediated salt and water secretion. These authors suggested that an analogous ASBT/CFTR interaction might take place at the level of the intrahepatic bile ductules in CF.

We found only a slightly less alkaline hepatic bile pH in our mouse model (Fig. 5A), possibly due, as evidenced by our Cl^- data, from a compensation by other ductular Cl^- channels (27, 28,31,45,54,55,59). In addition to increasing bile salt-independent bile flow, this most likely normalized hepatic bile pH by providing ample Cl^- for $\text{Cl}^-/\text{HCO}_3^-$ exchange. However, the dysfunctional CFTR is also expressed on cholecystocytes, and we found that the pH of gallbladder bile in these CF mice was significantly more acidic than that of WT mice (F. Freudenberg and M. C. Carey, unpublished observations). If CFTR were the only Cl^- channel on cholangiocytes in humans, this would explain why CF liver disease is much more severe than in our mouse model. Contrariwise, if, in humans, there are individualized genetic mechanisms that maintain hepatic bile pH within the normal range, this could explain why some patients acquire liver disease and others, despite possessing the same mutation in the *CFTR* gene, do not.

In summary, this longitudinal study on a large cohort of ΔF508 CF mice of both genders suggests that several pathophysiological alterations in biliary lipid and lipopigment metabolism are likely responsible for early and mild histopathologic changes observed in 53% of CF mice.

Increased fecal bile acid loss in the CF mouse apparently results from decreased ileal pH that, in turn, leads to a more hydrophobic bile acid profile in hepatic bile from anaerobic bacterial catabolism of primary bile salts in the colon. Moreover, excess bile salt spillage into the colon also induces enterohepatic cycling of bilirubin, and the resulting “hyperbilirubinemia” is most likely responsible for the decreased biliary secretion rate of phospholipids compared to bile salts in $\Delta F508$ mice. Following β -glucuronidase hydrolysis, hyperbilirubinemia is also ultimately responsible for depositing insoluble metal salts of UCB, and possibly BMG, in cholangiocytes. This not only augments bile detergency but also impairs cholangiocyte integrity by a number of interrelated mechanisms. To further unravel the complexities of more severe liver disease in CF, systematic studies in mouse models with more advanced disease as well as studies in humans with CF are required.

Acknowledgments

We thank Dr. Marie Egan and Ms. Elisa Ferreira, Yale University Medical Center, New Haven, Connecticut, for providing heterozygous breeding pairs of CF mice together with helpful advice regarding their breeding and care. We are grateful to Dr. Paul A. Dawson, Wake Forest University School of Medicine, Winston-Salem, North Carolina, for providing antibodies to SLC10A2/ASBT; to Christopher MacDonald (Clinical Chemistry Core Laboratory, Children's Hospital, Boston, MA) for skilled assistance in measuring Cl⁻ concentrations; and to Dr. Vincent Ricchiuti (Director, General Clinical Research Center Core Laboratory, Brigham and Women's Hospital) and his laboratory for their expert measurement of Na⁺, K⁺ and PO₄³⁻ concentrations in hepatic bile samples.

Grants: This work was funded by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK036588, DK073687, and DK052911 (M. C. Carey), by Deutsche Forschungsgemeinschaft Grant FR 1733/1-1 (F. Freudenberg), and by Training Grant 5T32 DK07477 as well as a Foundation for Digestive Health and Nutrition/AstraZeneca Fellowship/Faculty transition award (A. Broderick).

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Fig. 1.

Age-weight comparisons of individual CF (○) and WT (□) mice obtained prior to surgery. At the earliest time points (circled), weights of CF and WT mice were similar, but by 50 days mouse weights had diverged. Weight gain for all mice followed a steady increase, thereafter leveling off at approximately 250 days. Second order, best-fit curves for CF (solid line) and WT (dotted line) mice demonstrate that weight differences between the genotypes remained similar for most of the study. It is clear that, on average, CF mice were substantially smaller than their age-matched WT controls.

Fig. 2.

Histopathology of the liver. Livers of CF (A) and WT (B) mice were fixed in formalin and embedded in paraffin. Five-micron thick sections were stained with hematoxylin and eosin. Original magnification is 200X. A representative CF liver (A) displays reactive changes in the biliary epithelium, bile ductular proliferation, and mild portal fibrosis (arrow), findings not present in WT (B) mice. To visualize Fe and Ca deposits, liver sections were prepared as described above and stained for Fe with Prussian blue stain or for Ca using the von Kossa protocol. Multifocal Fe deposition (C, arrow) was observed in 13% of CF and 6% of WT mice; multifocal Ca deposition (D, arrow) was found in 25% of CF and 3% of WT mice.

Fig. 3.

Fecal bile acid excretion. Fecal bile acid excretion expressed in μmol per day and per 100 g BW was measured by enzymatic assay of three-day stool collections. Bile acid excretion is increased approximately twofold in both female and male DF508 CF compared to WT mice. $P = 0.02$ for both; $n = 6$ per group (males) and 8 per group (females).

Fig. 4.

Bilirubin molecular species and secretion rates. Hepatic bile from 15-minute collections was immediately injected onto an HPLC column to separate and quantify bilirubin species. Bilirubin secretion rates were calculated for the various bilirubin molecular species and are displayed in nmoles per hour and per 100 g BW for WT and CF mice. (A) When secretion rates for all conjugated bilirubin species were combined, secretion rates of conjugated bilirubins were increased by $\approx 60\%$ in CF compared to WT mice ($n = 8$ and 7 , respectively; $P = 0.02$). (B) Secretion rates of BMG, the bilirubin species most prevalent in mice, were elevated by $\approx 50\%$ in CF ($n = 9$) compared to WT ($n = 7$) mice ($P = 0.03$). (C) Because of non-baseline separation of BDG and other bilirubin diconjugate peaks, we combined the concentrations of all bilirubin diconjugates (BDG+BDX) before calculating secretion rates. Although BDG +BDX secretion rates were also increased by $\approx 60\%$, the means of CF and WT groups ($n = 8$ and 7 per group, respectively) were not significantly different ($P = 0.08$). (D) Mean secretion rates of UCB were substantially increased in CF compared to WT mice, but this difference was also not statistically significant ($n = 9$ and 7 , respectively; $P = 0.3$). (E) Correlations between histopathological changes (“liver disease,” LD) and bilirubin secretion rates. By dividing CF mice into two groups based on the presence ($n = 6$) or absence ($n = 2$) of “liver disease,” we show that BMG secretion rates into bile are elevated only for CF mice with early “liver disease” (CF + LD), as inferred from histopathologic changes ($P = 0.02$), whereas in CF mice

without liver disease, i.e., normal histology (CF - LD), BMG secretion rates are statistically the same as in WT mice.

Fig. 5.

Biliary pH and Cl^- secretion rates in CF and WT mice. Hepatic bile pH was measured by microelectrode immediately following each 15-minute bile collection. (A) Although slightly lower in CF mice, hepatic bile pH is not significantly different in the CF and WT models ($n = 11$ per group; $P = 0.3$). (B) Cl^- concentrations of hepatic bile samples are non-significantly increased in CF mice ($n = 8$) compared to WT controls ($n = 6$); $P = 0.18$.

Table 1
Chronology of liver histopathology in WT and CF mice

Time intervals Mouse strain	Percentage of Mice Affected						Mean Lesion Score ^A					
	0-100 days		100-200 days		>200 days		0-100 days		100-200 days		>200 days	
	WT ^B	CF ^C	WT ^D	CF ^E	WT ^F	CF ^G	WT ^B	CF ^C	WT ^D	CF ^E	WT ^F	CF ^G
<i>n</i>	7	10	8	11	33	9	7	10	8	11	33	9
<i>Lobular inflammation</i>	43	20	25	55	33	56	0.6	0.4	0.3	0.6	0.3	0.6
<i>Fibrosis</i>	29	30	13	55 ^H	21	44	0.3	0.3	0.1	0.6 ^H	0.2	0.5
<i>Bile ductular proliferation and/or periductular fibrosis</i>	29	40	25	55	24	67 ^H	0.4	0.6	0.1	0.7 ^H	0.2	0.9 ^I

^A Scoring: 0, absent; 1, mild; 2, moderate; 3, severe. See Materials and Methods for more detailed explanation of scoring. Mean Lesion Scores were determined by averaging the Lesion Scores of all mice per group for each of the histopathologic properties under consideration.

^B *n* = 7
^C *n* = 10
^D *n* = 8
^E *n* = 11
^F *n* = 33
^G *n* = 9
^H *P* = 0.05
^I *P* < 0.001

Table 2Distribution of bile salt species and bile salt hydrophobicity indexes in hepatic bile of WT and $\Delta F508$ CF mice

<i>BILE SALT SPECIES</i>	Mean Molar Percent Bile Salt	
	<i>Wild Type</i>	<i>CF</i>
<i>Tauromuricholates (TaM + TβM)</i>	59.6 $\pm$ 2.1	38.8 $\pm$ 1.9*
<i>Tauroursodeoxycholate (TUDC)</i>	5.4 $\pm$ 1.1	3.4 $\pm$ 0.9
<i>Taurocholate (TC)</i>	31.8 $\pm$ 1.4	51.2 $\pm$ 1.9*
<i>Taurochenodeoxycholate (TCDC)</i>	2.4 $\pm$ 0.3	2.6 $\pm$ 0.4
<i>Taurodeoxycholate (TDC)</i>	0.7 $\pm$ 0.3	3.9 $\pm$ 0.9[†]
<i>HYDROPHOBICITY INDEX</i>	-0.48 $\pm$ 0.01	-0.29 $\pm$ 0.02*

Molar percentages of each bile salt are expressed as mean $\pm$ SE of total bile salt concentration. BS, Bile salt; WT, $n = 11$; CF, $n = 8$ * $P < 0.0001$ compared to WT[†] $P = 0.01$ compared to WT