Induction of colitis in cftr<sup>−/−</sup> mice results in bile duct injury

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Blanco, Paola G., Munir M. Zaman, Omer Junaidi, Sunil Sheth, Rhonda K. Yantiss, Iiad A. Nasser, and Steven D. Freedman. Induction of colitis in cftr<sup>−/−</sup> mice results in bile duct injury. Am J Physiol Gastrointest Liver Physiol 287: G491–G496, 2004. First published April 2, 2004; 10.1152/ajpgi.00452.2003.—It is unknown why some patients with inflammatory bowel disease develop primary sclerosing cholangitis. We have recently shown that patients with primary sclerosing cholangitis have an increased prevalence of mutations in the gene responsible for cystic fibrosis (CFTR) compared with individuals with inflammatory bowel disease alone. Our aim was to examine whether induction of colitis by oral dextran leads to bile duct injury in mice heterozygous or homozygous for mutations in CFTR. The effect of oral administration of docosahexaenoic acid to correct a fatty acid imbalance associated with cystic fibrosis was also examined to determine whether this would prevent bile duct inflammation. Wild-type mice and mice heterozygous and homozygous for CFTR mutations were given dextran orally for 14 days to induce colitis. Bile duct injury was quantitated by blinded histological scoring and measurement of serum alkaline phosphatase activity. The effect of pretreatment with docosahexaenoic acid for 7 days was examined. Treatment of mice with 100 mg dextran/day for 9 days followed by 85 mg/day for 5 days resulted in a significant increase in bile duct injury as determined by histological scoring in homozygous cystic fibrosis mice compared with wild-type mice (P = 0.005). The bile duct injury seen in cystic fibrosis mice was reflected in a threefold increase in serum alkaline phosphatase (P = 0.0006). Pretreatment with oral docosahexaenoic acid decreased both histological evidence of bile duct injury and serum alkaline phosphatase levels. In the setting of colitis, loss of CFTR function leads to bile duct injury.

Primary sclerosing cholangitis (PSC) is a slowly progressive cholestatic liver disease characterized by inflammation of the biliary tract leading to cirrhosis and portal hypertension and is a major indication for liver transplantation (14). Although the etiology of PSC is unknown, 2.5–7.5% of patients with inflammatory bowel disease will develop this inflammatory/fibrosing disease of the biliary system (14). Why a subset of patients with inflammatory bowel disease develop PSC is unknown, and lack of an experimental model of PSC has hampered progress in the study of its pathogenesis (25).

Of interest, PSC and cystic fibrosis liver disease have several findings seen in cystic fibrosis patients are similar to those observed in patients with PSC (7, 17, 20). Although the pathogenesis of PSC is not known, the presence of associated inflammatory bowel disease in the majority of patients with PSC suggests that intermittent portal bacteremia due to colitis might lead to chronic biliary tract infection, inflammation, and periductal fibrosis (14). If this were the sole mechanism, then all patients with inflammatory bowel disease should develop PSC. Given that only 3–7% of inflammatory bowel disease patients develop PSC, other mechanisms, such as genetic or environmental factors, must play a role in the development of PSC among these patients.

Since the cystic fibrosis gene product (CFTR) is expressed in the biliary tract (6, 12) and abnormalities in CFTR function play a key role in the development of cholestatic liver disease in some cystic fibrosis patients, we hypothesized that CFTR gene mutations predispose the biliary epithelium to injury. We have recently reported that genetic analyses of the CFTR gene in PSC patients compared with disease controls (primary biliary cirrhosis and inflammatory bowel disease) demonstrated a significantly increased number of mutations/variants in the PSC group (37 vs. 8.6% of disease controls; P = 0.02) (23). None of the PSC patients carried two mutations/variants, and 89% of PSC patients carried the 1540G variant-containing genotypes (which results in decreased functional CFTR) compared with 57% of disease controls (P = 0.03). Only 1 of 19 PSC patients had neither a CFTR mutation nor the 1540G variant. Measurement of CFTR chloride channel function by nasal potential difference testing demonstrated a statistically significant reduction in the median isoproterenol response in these PSC subjects compared with disease controls and healthy controls. Together, these data indicate that a single allelic mutation in the CFTR gene is associated with the development of PSC.

Because ~75–80% of cases of PSC are associated with inflammatory bowel disease (3, 8), we postulated that colitis in the setting of CFTR dysfunction would result in inflammation of the bile ducts. In the current study, this hypothesis was tested by inducing colitis in exon 10 cftr<sup>−/−</sup> transgenic knockout mice, a mouse model that does not develop spontaneous liver disease, to determine whether this leads to bile duct injury in the setting of one or two CFTR gene mutations. In addition, CFTR dysfunction in this exon 10 cftr<sup>−/−</sup> transgenic knockout mouse is associated with alterations in fatty acid metabolism characterized by an increase in arachidonic acid (AA) and a decrease in docosahexaenoic acid (DHA) (9). Because correction of this fatty acid defect with oral DHA ameliorates the

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pathological changes in the pancreas and ileum and diminishes pulmonary inflammation in these transgenic mice, we also examined whether oral administration of DHA prevents bile duct injury in this model (9, 10).

MATERIALS AND METHODS

Breeding of mice. Experiments were carried out under protocols approved by the Beth Israel Deaconess Medical Center Animal Care Committee. A breeding colony was established with University of North Carolina heterozygous cftr\(^{+/−}\) exon 10 knockout mice (Jackson Laboratory, Bar Harbor, ME). The tails of 14-day-old mice were clipped and processed for analysis of genotype (27). Wild-type (WT), heterozygous (HT), and cftr\(^{−/−}\) (CF) mice were weaned at an average of 23 days and placed on a water and Peptamen (Nestle Clinical Nutrition, Deerfield, IL) diet ad libitum.

Oral administration of dextran sodium sulfate. On day 40, mice were fed Peptamen alone or Peptamen plus 100 mg dextran sodium sulfate (DSS)/day (ICN Biomedicals, Costa Mesa, CA) for 9 days followed by 5 days of 85 mg DSS/day. The mean weight between the WT and CF mice was not statistically different between the groups, with weights ranging from 11.55 to 20.4 g. To study the effects of DHA, another group of mice was treated similarly, with the exception that they were pretreated with 2, 10, or 40 mg DHA (Pure Encapsulations, Sudbury, MA) or 40 mg eicosapentaenoic acid (EPA; NuChek Prep, Elysian, MN) per day for 7 days, prepared daily as a stable emulsion in Peptamen, which was then continued during administration of the DSS as described above. The volume of Peptamen administered was measured on a daily basis by using specific feeders.

Sample preparation and analysis. After each specific treatment, the mice were euthanized with carbon dioxide. Blood was taken immediately after death by direct cardiac puncture and was centrifuged, and serum was stored at 4°C. Alkaline phosphatase activity was assayed by using a Cobas Bioanalyzer (Roche, Grenzacherstasse, Switzerland) using freshly reconstituted alkaline phosphatase reagent (Sigma, St. Louis, MO). Liver and colonic tissues were fixed in formalin, embedded in paraffin, cut in cross section, and stained with hematoxylin and eosin. These tissues were submitted for blinded histological examination by two hepatopathologists.

Bile duct scoring. Quantitation of bile duct injury in the liver specimens was based on examination of the following histological features: 1) epithelial injury, characterized by intraepithelial inflammatory mononuclear cell infiltration, 2) bile duct proliferation, 3) bile duct angulation, and 4) fibrosis. Each of these four features was scored as 0 (absent or mild) or 1 (moderate and severe) and then summed for a maximum score of 4.

Statistical analysis. Results are expressed as means ± SE. Significant differences were determined by using ANOVA.

RESULTS

Establishment of DSS model. Oral administration of DSS has been shown to reproducibly lead to an acute colitis in rodents (21). Rectal administration of 2,4,6-trinitrobenzenesulfonic acid-induced colitis was also examined, but this led to >80% mortality in CF mice. To determine the optimal conditions for induction of colitis, different doses of DSS ranging from 85 to 250 mg/day were administered to WT, HT, and CF mouse littersmates. Although DSS is typically given as a specific concentration in water, CF mice take in variable amounts of water, leading to a significant variation in the degree of colitis. Giving DSS as a specific dose in Peptamen eliminated this variability. The length of time DSS was administered (from 7 to 21 days) as well as up to three cycles consisting of 7 days of DSS followed by 5 days on Peptamen alone was also examined. Treatment of mice with 100 mg DSS/day for 9 days followed by 85 mg DSS/day for 5 days resulted in the lowest mortality rate of 10% in WT, HT, and CF mice. The degree of colitis was similar between these groups as determined by visualization of bloody diarrhea in all animals and histological evidence demonstrating features of chronic colitis with mononuclear cell infiltrates, loss of crypts, and mucosal ulcerations in the colonic resection specimens (Fig. 1). Higher doses of DSS, as well as different durations of DSS treatment, resulted in significantly higher mortality among the mice (data not shown). Hence, 100 mg DSS for 9 days followed by 85 mg DSS/day for 5 days was used for all subsequent experiments.

Bile duct injury. Treatment of mice with 100 mg DSS/day for 9 days followed by 85 mg DSS/day for 5 days resulted in a significant increase in bile duct injury in CF mice compared with WT littersmates (P = 0.005). Mice heterozygous for the null mutation in CFTR showed a higher mean value, although this was not statistically different from WT animals (Fig. 2). The histological features of the livers from these mice are shown in Fig. 3. WT and CF animals that did not receive DSS

Fig. 1. Effect of dextran sodium sulfate (DSS) on colonic histology. Representative hematoxylin and eosin-stained sections of colon from wild-type (WT; A), cftr\(^{−/−}\) (CF; B), and CF mice pretreated with docosahexaenoic acid (DHA; C) are shown. All mice were given 100 mg DSS/day for 9 days followed by 5 days of 85 mg DSS/day. Bars, 100 μm.
showed no evidence of bile duct injury (Fig. 3, A and B, respectively). WT mice that received DSS developed colitis but did not show any features of bile duct injury (Fig. 3, C and D). In contrast, CF mice that received DSS developed bile duct injury, characterized by mononuclear cell inflammatory infiltrates associated with bile duct proliferation and angulation involving most of the bile ductules (Fig. 3, E and F). No fibrosis was present.

**Alkaline phosphatase levels.** To assess biochemically the evidence of bile duct injury, alkaline phosphatase levels were measured in serum from mice treated in the absence or presence of 100 mg DSS/day for 9 days followed by 85 mg DSS/day for 5 days. The results are shown in Fig. 4. There was no significant difference in alkaline phosphatase values between WT and CF mice in the absence of DSS administration. Following DSS treatment, the mean values (IU/liter ± SE) were 212 ± 30.4 for WT, 270 ± 34.6 for HT, and 695 ± 110.1 for CF mice. The difference in mean alkaline phosphatase levels in CF mice compared with WT littermates was statistically significant (P = 0.0006).

**Effect of DHA pretreatment on bile duct injury.** Oral administration of 40 mg DHA/day for 21 days to WT mice did not produce any changes in the normal liver histology. Pretreatment of CF mice with DHA for 7 days, which was continued through the period of administration of DSS, resulted in a marked decrease in mononuclear cell infiltrate and bile duct proliferation/angulation, as shown in the representative micrographs in Fig. 5. There was no overt decrease in the degree of colitis (Fig. 1).

The effect of DHA on bile duct injury is shown quantitatively in Fig. 5. A statistically significant decrease in bile duct injury was observed only at 40 mg DHA/day (P = 0.03) and was not seen at lower doses. The mean bile duct injury score was also lower following oral administration of 40 mg EPA/day to CF mice given DSS, although these changes were not statistically significant (Fig. 6). There was greater variation in the bile duct injury scores for the EPA-treated mice as well as those treated with 2 and 10 mg DHA/day. This was reflective of a partial treatment response resulting in a more variable quantitative score.

In Fig. 4, the effects of DHA and EPA on serum alkaline phosphatase values under these same experimental conditions is shown. Similar to bile duct injury scores, DHA at 40 mg/day led to a significant decrease in the mean alkaline phosphatase levels (P = 0.04). There was no significant decrease following treatment with EPA. DHA administration itself had no effect on alkaline phosphatase measurements in WT mice in the setting of DSS administration.

**DISCUSSION**

We have recently published (23) that patients with PSC have an increased frequency of single allelic mutations/variants in the CFTR gene compared with healthy control subjects as well as those with inflammatory bowel disease and no liver disease. As proof of concept that loss of CFTR function in the setting of colitis can lead to bile duct injury, in the current study we have demonstrated that mice homozygous for CFTR mutations develop bile duct injury following the induction of colitis. In addition to demonstrating a role for CFTR in bile duct injury in the setting of colitis, this new model using CF mice allows further investigation into the pathogenesis as well as testing of novel therapies. Of note, HT mice did not develop a statistically significant increase in bile duct injury score or serum alkaline phosphatase under these experimental conditions. It should be emphasized that, although this animal model using CF mice demonstrates bile duct injury similar to that seen in PSC, the histological changes do not meet the criteria for PSC since no fibrosis was present. This is not unexpected given the relatively short duration of colitis. Examination of CF mice treated for longer time periods with DSS may be required to produce fibrosis.

The degree of bile duct injury was confirmed by using two different methods. The first was quantitation of the four hallmark features of bile duct injury by two hepatopathologists blinded to the groups analyzed. Since the injury seen in PSC patients is a combination of bile duct proliferation and mononuclear cell infiltration, the scoring system used allowed quantitative assessment of all aspects of bile duct injury. Therefore, the mean value of 2.0 in CF mice represents significant bile duct proliferation/angulation combined with infiltration of inflammatory cells as shown in Figs. 2, E and F. Although less sensitive, serum alkaline phosphatase activity was measured that correlated with the histological results.

In the liver, CFTR is expressed at the apical membrane of the bile duct epithelial cells (6, 12). As a result of CFTR dysfunction, chloride secretion into the bile canaliculi is decreased, resulting in hyperconcentration and acidification. The formation of inspissated secretions leads to obstruction of intrahepatic bile ductules and subsequent cholestasis (11). In some cystic fibrosis patients, this is followed by focal inflammation and scarring of the bile ducts and eventually focal or multilobular biliary cirrhosis. CFTR dysfunction, in addition to its effect on the viscosity of ductular fluid, also leads to an excessive host inflammatory response due to increased levels of proinflammatory cytokines and neutrophils (1, 18). This explains the presence of chronic inflammation in the lung even in the absence of infection in patients with cystic fibrosis (13).

It is thought that an excessive host inflammatory response in the setting of viscous lung secretions with trapped bacteria leads to progressive pulmonary fibrosis and loss of tissue. In an analogous manner, portal vein bacteremia as a result of colitis...
(19), in combination with an excessive biliary tract inflammatory response secondary to CFTR dysfunction, could predispose patients with inflammatory bowel disease to develop PSC. This may be perpetuated by the presence of viscous biliary secretions. However, whether portal vein bacteremia is an important pathogenic mechanism leading to PSC is controversial. PSC can occur in the absence or precede the onset of colitis or can occur following colectomy (4), suggesting that colitis itself may not be the critical factor. Our data would suggest that loss of CFTR function might be sufficient to initiate and sustain bile duct injury. This may not be the only mechanism involved in the development of PSC and does not

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**Fig. 3. Effect of DSS on liver histology.** Representative hematoxylin and eosin-stained sections of liver are shown. **A:** liver from a WT mouse in the absence of DSS treatment. The portal triad shows a normal-appearing bile duct with no inflammatory cells. A representative bile duct that lacks intraepithelial inflammation or ductular proliferation is shown in the inset. **B:** liver from a CF mouse in the absence of DSS treatment. The portal tract shows no inflammation, and there is no ductular proliferation. The bile duct epithelium is cuboidal without lymphocytic infiltration (inset). **C and D:** liver from a WT mouse treated with DSS. The bile ducts are uninflamed, and no ductular proliferation is identified. **E and F:** liver from a CF mouse treated with DSS. The portal triads are infiltrated with inflammatory cells (arrows), and proliferating bile ductules are evident (arrowheads). The biliary epithelium is infiltrated with mononuclear cells. Bars in **A and B**, 100 μm; bars in **C and E**, 250 μm; bars in **D and F**, 50 μm.
exclude other independent or coexistent disorders of immune regulation (14).

Although the current study as well as our prior data in humans demonstrates an association of CFTR dysfunction with bile duct injury/PSC, other genetic factors have been linked with this biliary tract disorder. This includes an increased frequency of human leukocyte antigens (HLA) DR3, DR6, DR2, and DR52a (see Ref. 2 for review). Inflammatory bowel disease itself is associated with DR3, DQ2, and DRB1*0103 (22). In addition, there is aberrant expression of HLA class II antigens on both bile duct cells (5) and hepatocytes (16) in patients with PSC. Increased numbers of suppressor/cytotoxic lymphocytes have been demonstrated in peripheral blood of PSC patients (15), although another study using immunohistochemical analysis of liver tissue found a preponderance of CD4 helper/inducer lymphocytes (26). Lastly, alterations in humoral immunity characterized by increased immunoglobulins, elevations in anti-nuclear and anti-smooth muscle antibodies, and cytoplasmic anti-neutrophil antibody have been reported (2). These abnormalities need not be mutually exclusive of the role that CFTR dysfunction may play in the development of PSC.

In this CF mouse model, phospholipid-bound AA is increased twofold with a reciprocal decrease in phospholipid-bound DHA in CF-affected tissues (9). Correction of this fatty acid defect with oral administration of DHA above 20 mg/day prevented the development of certain manifestations of cystic fibrosis (9). In the current study, we examined whether DHA, by correcting this biochemical defect associated with cystic fibrosis, would prevent bile duct injury. Treatment with DHA resulted in a significant decrease in bile duct inflammation as well as markers of injury including bile duct proliferation and angulation. The effects were seen only at doses that have been previously shown to ameliorate the pathology in the pancreas. Also, the effects were relatively specific for DHA in that EPA did not lead to a statistically significant decrease in bile duct injury.

Fig. 4. Effect of DSS on serum alkaline phosphatase levels. Mean alkaline phosphatase levels (IU/liter) ± SE are shown for WT, HT, and CF mice. The effect of no DSS (−) or 100 mg DSS/day for 9 days followed by 5 days of 85 mg DSS/day (++) is shown. In addition, the effect of different concentrations of DHA and eicosapentaenoic acid (EPA) is depicted. A minimum of 3 animals were used for each group. *P < 0.005 and **P = 0.04.

Fig. 5. Liver specimens from mice pretreated with DHA. Bile duct proliferation is not present. The biliary epithelium is relatively preserved. Mild portal inflammation is present but is less than that seen in animals that did not receive DHA. Bar, 250 μm in A and 50 μm in B.

Fig. 6. Quantitation of the effect of n-3 fatty acids on bile duct injury. Bile duct injury scores (mean ± SE) are shown as a function of DHA and EPA concentrations. *P = 0.04.
injury score or levels of serum alkaline phosphatase. Because both EPA and DHA decrease AA levels by competing for incorporation into the sn-2 position of phospholipids, these findings would suggest that simply decreasing levels of AA is not sufficient to reverse the inflammation. Whether DHA’s action is due to correction of the fatty acid imbalance or, alternatively, DHA is affecting other aspects of inflammation such as inhibiting prostaglandins as previously demonstrated in CF mice given aerosolized Pseudomonas (10) remains to be determined. Further studies are needed to determine whether DHA-based therapies may be of benefit in the treatment of PSC.

GRANTS

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