Long-term docosahexaenoic acid therapy in a congenic murine model of cystic fibrosis

Satti Beharry,1 Cameron Ackerley,5 Mary Corey,2,7 Geraldine Kent,3,11 Yew-Meng Heng,5 Hilary Christensen,5 Catherine Luk,3 Rhonda K. Yantiss,8 Imad A. Nasser,10 Munir Zaman,9 Steven D. Friedman,9 and Peter R. Durie1,4,6

Programs in 1Integrative Biology, 2Population Health Sciences, and 3Laboratory Animal Services, The Research Institute, and Departments of 4Pediatrics and 5Pathology, Hospital for Sick Children, and Departments of 6Pediatrics and 7Public Health Sciences, University of Toronto, Toronto, Ontario, Canada; 8Department of Pathology, Weill Medical College of Cornell University, New York, New York; Departments of 9Medicine and 10Pathology, Beth Israel Deaconess Medical Center/Harvard Medical School, Boston, Massachusetts; and 11Department of Animal Care and Veterinary Services, University of Western Ontario, London, Ontario, Canada

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Beharry S, Ackerley C, Corey M, Kent G, Heng Y-M, Christensen H, Luk C, Yantiss RK, Nasser IA, Zaman M, Freedman SD, Durie PR. Long-term docosahexaenoic acid therapy in a congenic murine model of cystic fibrosis. Am J Physiol Gastrointest Liver Physiol 292: G839–G848, 2007. First published November 9, 2006; doi:10.1152/ajpgi.00582.2005.—We used a congenic C57Bl/6J cystic fibrosis transmembrane conductance regulator (Cftr)−/− mouse model, which develops cystic fibrosis (CF)-like pathology in all organs, to evaluate the short- and long-term therapeutic effects of dietary docosahexaenoic acid (DHA). Thirty-day-old Cftr−/− mice and wild-type littermates were randomized to receive a liquid diet with or without DHA (40 mg/day). Animals were killed for histological and lipid analysis after 7, 30, and 60 days of therapy. DHA had no significant therapeutic or harmful effect on the lung, pancreas, or ileum of the Cftr−/− mice or their wild-type littermates. In contrast, dietary DHA resulted in highly significant amelioration of the severity of liver disease in the Cftr−/− mice, primarily a reduction in the degree of peri-portal inflammation. Additionally, these detailed measurements confirm our previous findings that Cftr−/− mice have significant alterations in the pancreas (except external acinar diameter), ileum, liver, lung, and salivary (except sublingual) glands at all ages compared with their age-matched wild-type littermates. In conclusion, inhibition of cytokines and/or eicosanoid metabolism and release of endogenous inhibitors of inflammation by DHA may account for the anti-inflammatory effects in the liver of this congenic murine model of CF. The potential therapeutic benefits of DHA in severe CF-associated liver disease remain to be explored.

cystic fibrosis transmembrane conductance regulator; postnatal organ development; anti-inflammatory effect; cystic fibrosis-associated liver disease

The first murine model of cystic fibrosis (CF) was established by gene targeting of embryonic stem cells to disrupt the murine cystic fibrosis transmembrane conductance regulator (Cftr) gene (31). Other knockout murine models and models mimicking human CFTR gene mutations have since been developed (6, 8, 10, 12, 13, 36, 37). Most CF animals develop severe CF-like intestinal obstruction at birth or when weaned to solid chow (19, 20). In contrast to the intestine, early reports found other CF-affected organs, including the lungs, pancreas, liver, and male genital tract, to be either mildly affected or normal (8, 18). It is noteworthy that most early publications of murine models of CF reported animals that were bred in a mixed genetic background. Rozmahel et al. (28) were the first to demonstrate that the genetic background of the murine CF model greatly influences disease severity. An exon-1 knockout CF mouse model (Cftrtm1HSC/Cftrtm1HSC) bred into different congenic backgrounds exhibited variable severity of intestinal disease, suggesting that modifier genes could, in part, compensate for the lack of functional intestinal CFTR. Subsequently, Kent et al. (23, 24) demonstrated that Cftr−/− (Cftrtm1UNC/tm1UNC) mice bred to congenicity into the C57Bl/6J background consistently developed spontaneous lung disease.

Our laboratory (14) has demonstrated that, with further aging, C57Bl/6J congenic Cftr−/− mice developed characteristic CF-like pathology in all organs affected by human CF disease (14). Features of lung disease included alterations in the appearance of the airway surface, defective mucociliary transport, alveolar distention, interstitial thickening with fibrosis, and inflammation. In the liver, there was acute and chronic portal tract inflammation with cholangitis, canicular, and small bile duct cholestasis, as well as bile duct proliferation, portal fibrosis, and, in some cases, biliary cirrhosis. Exocrine pancreatic disease began with intraluminal obstruction within the acinar lumina and small ducts, which led to intraluminal dilatation. In older animals, there was evidence of acinar atrophy, with replacement by fat and fibrous tissue. Similar alterations were observed in the small ducts of the parotid and submaxillary glands of the Cftr−/− mice, but the pathological effects were less severe than in the exocrine pancreas. After 3 mo of age, the vas deferens of the Cftr−/− males could not be identified.

It has been suggested that a defect in fatty acid metabolism plays a role in the pathogenesis of CF disease (15–17, 28, 32–34). Freedman et al. (17) evaluated an exon-10 knockout CF mouse model, bred into a mixed background (Cftrtm1UNC/tm1UNC), to provide evidence of a membrane lipid imbalance in cells from all organs affected by CF disease. These changes, characterized by increased arachidonic acid (AA) and decreased docosahexaenoic acid (DHA) concentrations, were observed in the Cftr−/− mice when compared with wild-type mice. Dietary supplements of
or DHA) and assessed after 7, 30, or 60 days of treatment. In a
mized to receive the two dietary regimens at 30 days of age (no DHA

cftr

 genetic drift, each new generation of mice was bred to wild-type
chow and continuously bred. To maintain congenic status and prevent
alternate breeding. Offspring were genotyped at 14 days of age by
PCR analysis of tail-clip DNA. To minimize bowel obstruction and
optimize long-term viability, 21- to 23-day-old CF mice (C57Bl/6J
tm1UNC/Cftr

) were maintained on regular mouse
chow and continuously bred. To maintain congenic status and prevent
genetic drift, each new generation of mice was bred to wild-type
C57Bl/6J mice, obtained from Jackson Laboratories (Bar Harbor,
ME). Male and female wild-type (cftr

/) animals were used in
alternating breeding. Offspring were genotyped at 14 days of age by
PCR analysis of tail-clip DNA. To minimize bowel obstruction and
optimize long-term viability, 21- to 23-day-old CF mice (C57Bl/6J
tm1UNC/Cftr

) and their cftr

/ littermates were weaned to
a liquid diet (Peptamen, unrestricted donation from Nestlé Nutrition)
provided ad libitum with feeder bottles (calibrated glass). Fresh diet
and feeders, sterilized by autoclave, were replaced daily. Mice and
their offspring were kept in a 12:12-h light-dark cycle, housed in
nonsterile microisolator cages in a conventional mouse housing room,
with corn cob bedding changed daily, and provided with sterile water
in addition to the liquid diet. The pathogen-free status of the colony
was confirmed by serological screening.

At 30 days of age, cftr

/ and cftr

/+ mice were randomly
assigned to receive Peptamen without or with 40 mg DHA/day
sonicated into the Peptamen. All mice were weighed weekly until
death at 37, 60, and 90 days of age. This corresponded to two
genotype groups (cftr

/+ and cftr

/), each of which were random-
ized to receive the two dietary regimens at 30 days of age (no DHA
or DHA) and assessed after 7, 30, or 60 days of treatment. In a
separate group of experiments, cftr

/ wild-type animals were
similarly treated with and without DHA and killed at 90 days of age.
Liver samples (~1 g) were harvested from three mice in each group,
placed in a sealed cryovial, and immediately frozen in liquid nitrogen
before determination of myeloperoxidase activity (3).

Tissue Collection and Preparation

The mice were anesthetized by intraperitoneal injection of pento-
barbital sodium (30 mg/kg), and the abdominal cavity was opened.
The inferior vena cava was cut, and 1% glutaraldehyde and 4%
paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) were perfused
through the left ventricle. The airways were perfused through the
larynx with the same fixative, and the tissues were maintained at a
constant pressure. The lungs, liver, pancreas, ileum, parotid gland,
and submaxillary and sublingual glands were harvested. Representative
samples of each organ were immersed in 10% formalin (in 0.1 M
phosphate buffer) for light microscopy, and separate pieces were
immersed in the perfusing fixative (1% glutaraldehyde, 4% parafo-
raldehyde) for potential ultrastructural morphology by electron
microscopy. Further processing included postfixation in 1% OsO4,
embedding in epon araldite, and the preparation of 0.5-μm sections
for toluidine blue staining for light microscopic evaluation. Separate
groups of similarly treated mice were sedated with carbon dioxide,
and blood was collected by cardiac puncture for membrane lipid
analysis. The pancreas, ileum, and portions of the lobes of the lungs
and liver of these animals were removed for lipid analysis as de-
scribed below.

Morphometric Assessment

All histological specimens were coded so that the observers were
blinded to the genotype, age, and dietary regimen of each mouse.
Specimens were prepared for light microscopic examination as de-
scribed by Durie et al. (14). Digital images of the pancreas and ileum
were acquired with a light microscope (Olympus BX 60; Carsen
Group) equipped with a charge-coupled device camera (Cool Snaps;
Roper Scientific) and used for morphometric analysis as described
below.

Pancreas. Random digital images of 0.5-μm-thick toluidine blue-
stained sections of pancreatic tissue were acquired with a ×40
objective lens. A minimum of 50 measurements of the luminal
diameters of the intralobular and interlobular ducts were made from
hematoxylin and eosin-stained sections of the pancreas. A minimum
of 100 measurements of the acinar lumen diameter and the external
acinar length were also recorded for each animal.

Submaxillary and sublingual glands. Images of the toluidine blue
sections of the submaxillary and sublingual glands were acquired with
a ×40 objective lens. Luminal diameters of the acini and ducts were
measured from these images as described for the exocrine pancreas.

Ileum. A ×40 objective lens was used to acquire digital images of
hematoxylin and eosin-prepared ileal sections. A computer program
(Scion) was used to measure the ileal mucosal height and intestinal
wall thickness (minimum of 25 villi per mouse), goblet cell-to-
epithelial cell ratio, and goblet cell number per villus from these
images.

Lung. Formalin-fixed, paraffin-embedded sections of the lung in-
terstitium were reticulin stained and analyzed by light microscopy
with a ×40 objective lens. A grid overlay was used to prevent
selection bias of a minimum of 10 measurements of the lung inter-
stitial thickness from different fields for each animal collected using
Scion Image for Windows.

Liver. Hematoxylin and eosin sections were assessed blindly by
two trained observers (C. Ackery and P. R. Durie) for the degree of
inflammation, biliary duct obstruction, fibrosis, and hepatosteatosis.
We applied a previously published numerical scoring system for each
parameter, using an arbitrary 0–4 scale, in which zero represents
normal histology and four represents the most severe pathology for
each parameter. A minimum of five portal tracts was assessed for each
mouse, and an average of the numerically scored parameters provided
a total overall score for each animal. The hepatic specimens were also
independently and blindly assessed by two experienced hepatop-
athologists (R. K. Yantiss and I. A. Nasser) who used a previously
reported scoring system (2, 16, 17).

Membrane Lipid Analysis

Red blood cells and plasma were frozen at −80°C and shipped in
batches to Boston, MA, for lipid analysis by Freedman et al. (17).
Lipids were extracted with 6 vol of a chloroform-methanol (2:1,
vol/vol) solution. The lower phase was collected and concentrated in
a heating block under a gentle stream of nitrogen. Extracted lipids
were methylated and analyzed for fatty acid methyl esters using a
Hewlett-Packard GC/MS (HP-5890 Series II/HP-5971) mounted with
a WCOT capillary column (Supelco-wax-10, 30 m × 0.53 mm
internal diameter). Results were expressed as a ratio of AA to DHA.

Biochemical Analysis

As a measure of inflammation, myeloperoxidase activity was as-
assessed at the Ontario Ministry of Agriculture, Food and Rural Affairs

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Laboratory (University of Guelph, Ontario, Canada), using a spectrophotometric technique with tetramethylbenzidine as the substrate (4). Thawed liver samples weighing ~100 mg were homogenized in buffer and centrifuged. Potassium phosphate buffer (1 mL) containing hexadecyltrimethylammonium bromide was added to the pellet and sonicated. The suspension was freeze-thawed once in liquid nitrogen, resolicited for 25 s on ice, and centrifuged. The supernatant was used for analysis. Each cuvette contained 20 mL of the undiluted sample, 20 mL of 16 mmol/L tetramethylbenzidine dissolved in dimethylformamide, and 175 mL of 220 mM potassium phosphate buffer containing 100 mM sodium chloride at pH 5.4. The reaction was initiated with 25 mL of 3 mM hydrogen peroxide. The protein content of liver tissue samples was determined by a BCA protein assay kit (Pierce Biotechnology, Rockford, IL) and a standard curve prepared with dilutions of BSA. Results are expressed as unit of enzyme activity/gram of tissue, where one unit of activity is defined as one unit change in absorbance per minute at 37°C.

Statistical Analysis

Morphometric measurements taken from each organ, expressed as means and 1 SD, were used in multiple regression models to analyze the effects of genotype, age, and diet regimen. The study design allowed this complex statistical analysis to first compute for each variable a full model containing the 3x3-way interaction (genotype x age x diet) and all two-way interactions. Where the three-way interaction was not significant, all combinations of the two-way design were evaluated. Interaction terms were used in the final ‘best fit’ model for each significant variable found in comparisons between mice killed at ages 37, 60, and 90 days (age effect), between untreated cftr−/− and untreated Cftr−/− mice (genotype effect), and between DHA-treated and age-matched untreated cftr+/+ and Cftr−/− mice (DHA treatment effect). P < 0.05 was considered significant. The analysis was also repeated with the cutoff for inclusion in the model raised to P = 0.1 to explore suggestive effects that may have had importance if the sample size were larger.

RESULTS

Animals

Untreated and DHA-treated cftr+/+ mice and their Cftr−/− littermates were killed for morphometric evaluation at 37 days (cftr+/+: n = 5, Cftr−/−: n = 7), 60 days (cftr+/+: n = 5, Cftr−/−: n = 5) and 90 days (cftr+/+: n = 6, Cftr−/−: n = 6) of age. Those used for lipid analysis were killed at 37 days (untreated cftr+/+: n = 5, untreated Cftr−/−: n = 5, DHA-treated cftr+/+: n = 2, DHA-treated Cftr−/−: n = 3) and 90 days (untreated cftr+/+: n = 5; untreated Cftr−/−: n = 4; DHA-treated cftr+/+: n = 5, DHA-treated Cftr−/−: n = 5) of age.

Morphometric Evaluation

The wild-type animals showed no major histological abnormalities in any of the organs evaluated, but some organs showed age-related alterations, which are described below. Also, comparative morphometric analyses of all organs were performed according to age, genotype, and therapy. In keeping with our previous observations (14), this blinded comparison of the untreated Cftr−/− mice shows characteristic and progressive CF-like age-related changes in all organs, which were significantly different from their age-matched cftr+/+ littermates. However, with the notable exception of the liver, in which DHA therapy showed a highly significant reduction in the degree of perportal inflammation, we were unable to demonstrate evidence of a short- or long-term morphological therapeutic effect in any other organ affected by CF disease.

Liver

Untreated wild-type and CF mice: age-related changes in organ morphology. In agreement with our previous observations (14), the cftr+/+ mice showed little or no histological abnormalities at any age. This is reflected by the numerical scores for total disease severity, as well as the separate scores for biliary obstruction and portal tract inflammation, which were zero or close to zero at all ages (Figs. 1 and 3A). In contrast, the Cftr−/− mice showed characteristic and progressive CF-like portal tract disease with features of biliary proliferation and inflammation. The overall numerical scores of disease severity increased between ages 37 and 60 days but remained the same between ages 60 and 90 days (Fig. 1A and Fig. 3, B and C). Total numerical scores in the Cftr−/− mice were significantly greater than in age-matched cftr+/+ littermates at all ages (P < 0.0001). Scores for biliary duct proliferation in the Cftr−/− animals showed a significant worsening with age, with higher values in the 90-day-old mice than in the 37- and 60-day-old Cftr−/− mice (P = 0.01 and P = 0.03, respectively). The degree of bile duct proliferation in the Cftr−/− mice was significantly greater at all ages than in their age-matched cftr+/+ littermates (P = 0.003). The severity of portal tract inflammation in the Cftr−/− mice did not vary with age but was significantly increased compared with their cftr+/+ littermates (P < 0.0001 at all ages). Hepatosteatosis was seen in the vast majority of the Cftr−/− mice but was rarely observed in their wild-type littermates.

Effect of DHA therapy. We report the results of the blinded morphological evaluation performed by P. R. Durie and C. Ackerely. Independent assessment by R. K. Yantiss and I. A. Nasser using a distinct but comparable numerical scoring system (2, 16, 17) yielded similar results (data not shown). There was a significant genotype treatment effect on hepatic morphology at all ages in the Cftr−/− mice (Figs. 2A and 3D). Total severity scores in the DHA-treated Cftr−/− mice were significantly decreased from the age-matched untreated Cftr−/− animals at 37, 60, and 90 days of age (P = 0.003). In contrast, there was no significant therapeutic or deleterious consequence of DHA therapy in the wild-type animals at any age (P = 0.1). Although the total severity score in the DHA-treated Cftr−/− mice was no different from the DHA-treated cftr+/+ mice (P = 0.5) at 37 days, the treated 60- and 90-day-old Cftr−/− mice showed persistent evidence of liver disease compared with their age-matched DHA-treated wild-type controls (Fig. 2A; P < 0.0001).

To determine which variable exhibited the greatest therapeutic effect, we assessed the age-related measurements of biliary duct obstruction (Fig. 2B) and portal inflammation (Fig. 2C) separately in the cftr−/− and Cftr−/− mice. Although the DHA treatment effect on bile duct proliferation did not reach significance, there was a suggestive effect at all ages when the DHA-treated Cftr−/− mice were compared with their age-matched untreated Cftr−/− littermates (P = 0.07). Similar trends were obtained for this measurement when the DHA-treated cftr+/+ mice were compared with their age-matched untreated cftr+/+ littermates. A larger sample size may have resolved whether DHA treatment had a significant effect on...
bile duct proliferation. Bile duct proliferation in DHA-treated Cfr<sup>−/−</sup> mice compared with their age-matched untreated Cfr<sup>−/−</sup> littermates remained significantly increased at all ages (<i>P</i> = 0.0002). There was no therapeutic or deleterious treatment effect at any age in the age-matched treated and untreated cftr<sup>+/+</sup> mice. However, the inflammatory scores of the DHA-treated Cfr<sup>−/−</sup> mice showed a highly significant reduction in inflammation after 7, 30, and 60 days of treatment compared with their age-matched untreated Cfr<sup>−/−</sup> littermates (<i>P</i> = 0.0002). There were no significant differences between treated and untreated cftr<sup>+/+</sup> mice (data not shown). DHA treatment significantly improved overall severity of liver disease and portal tract inflammation at all ages. *<i>P</i> ≤ 0.003, **<i>P</i> ≤ 0.0002, ***<i>P</i> ≤ 0.006. ***<i>P</i> ≤ 0.0001. Refer to text for detailed explanation regarding significant differences.

In contrast, we observed a clear DHA treatment genotype effect for portal tract inflammation. The DHA-treated Cfr<sup>−/−</sup> mice.
remained significantly higher than the scores of the age-matched treated cftr<sup>+/+</sup> littersmates at all ages (P = 0.006).

To biochemically quantify the effect of DHA on the degree of inflammation, we assessed myeloperoxidase activity in liver tissue extracted from a separate group of DHA-treated and untreated cftr<sup>+/+</sup> and Cftr<sup>−/−</sup> mice at 90 days of age. Myeloperoxidase activity was significantly increased in the untreated Cftr<sup>−/−</sup> mice when compared with their age-matched untreated cftr<sup>+/+</sup> littermates (24.4 ± 1.9 vs. 11.0 ± 1.9 units of activity/g of tissue; P < 0.02). DHA treatment of the Cftr<sup>−/−</sup> mice showed a highly significant reduction in inflammation after 60 days of treatment compared with their age-matched untreated Cftr<sup>−/−</sup> littersmates (15.4 ± 0.6 vs. 24.4 ± 1.9 units of activity/g of tissue; P < 0.007).

Pancreas

Untreated wild-type and CF mice: age-related changes in organ morphology. The intralobular duct and acinar luminal diameters of the cftr<sup>+/+</sup> mice revealed no significant age-related changes between 37, 60, and 90 days of age (Fig. 4, A and C). In contrast, the interlobular duct diameter measurements of the cftr<sup>+/+</sup> mice was significantly increased at 60 days of age compared with the values at ages 37 and 90 days (Fig. 4B). The changes in the interlobular duct diameter measurements in the cftr<sup>+/+</sup> mice between ages 37 and 60 days, 60 and 90 days, and 37 and 90 days were highly significant (P < 0.0001, P = 0.02, and P = 0.01, respectively). The same general pattern was observed for the external acinar diameter of the cftr<sup>+/+</sup> mice, but only the differences between 37 and 90 days of age and between 60 and 90 days of age were significant (P < 0.0001; Fig. 4D).

The Cftr<sup>−/−</sup> mice showed similar values for intralobular duct diameter measurements at ages 37 and 60 days (Fig. 4A). Although there was a tendency for the intralobular duct diameter to increase at 90 days, this value did not achieve significance compared with values at 37 or 60 days. The interlobular duct diameters of the Cftr<sup>−/−</sup> mice revealed age-related changes similar to their wild-type littermates, with a significant increase in diameter between 37 and 60 days (Fig. 4B; P < 0.0001) followed by a significant decrease at 90 days of age (Fig. 4B, 60 vs. 90 days, P = 0.02; and 37 vs. 90 days, P = 0.01). However, the intralobular and interlobular measurements in the Cftr<sup>−/−</sup> mice were significantly increased compared with their age-matched cftr<sup>+/+</sup> littersmates at all ages (P < 0.0001).

The acinar luminal diameter in the Cftr<sup>−/−</sup> mice revealed a significant increase in diameter between 37 and 60 days (Fig. 4C; P = 0.003) followed by a nonsignificant trend to decrease at age 90 days. Additionally, the acinar lumen diameter in the Cftr<sup>−/−</sup> mice was significantly increased compared with their age-matched cftr<sup>+/+</sup> littersmates (P < 0.0001) at all ages. The external acinar diameter of the 90-day-old Cftr<sup>−/−</sup> mice was significantly reduced compared with 37- and 60-day-old Cftr<sup>−/−</sup> mice (Fig. 4D, 37 vs. 90 days and 60 vs. 90 days; P < 0.0001). The external acinar diameter values in the Cftr<sup>−/−</sup> mice at 37 and 60 days were not different from each other (Fig. 4D); in addition, these values were no different from the measurements in the cftr<sup>+/+</sup> mice at any age.

Effect of DHA therapy. We were unable to demonstrate any significant morphological changes attributable to DHA therapy in the pancreas of the wild-type or Cftr<sup>−/−</sup> mice at 37, 60, or 90 days of age (Table 1). Although the intralobular duct diameter in the pancreas of the treated 90-day-old Cftr<sup>−/−</sup> mice showed a trend to be narrower than the age-matched untreated Cftr<sup>−/−</sup> mice (P = 0.07), suggesting a partial DHA treatment effect, the measurements showed considerable variability. In
contrast, the acinar luminal diameter tended to be greater in the 90-day-old DHA-treated cftr\(^{+/+}\) mice than in their 60-day-old DHA-treated cftr\(^{+/+}\) littermates. Although this difference was not significantly different between the DHA-treated 60- and 90-day-old cftr\(^{+/+}\) animals, this trend was inconsistent with the pattern (DHA-treated values unchanged or lower) in the DHA-treated Cftr\(^{+/+}\) and their age-matched untreated littermates at all ages as well as the 37- and 60-day-old DHA-treated cftr\(^{+/+}\) animals.

**Salivary Glands**

Untreated wild-type and CF mice: age-related changes in organ morphology. Because of technical difficulties, the number of salivary gland samples was insufficient for statistical analysis. Nevertheless, the diameters of the serous-type acinar cells and ducts of the submaxillary and parotid glands in the cftr\(^{+/+}\) mice showed no obvious variation with age. In contrast, the duct diameter of the sublingual gland in the cftr\(^{+/+}\) mice at 90 days of age appeared to be greater than the values at age 37 and 60 days, suggesting an age effect. In concert with our previous observations (14), there were no age-related changes in the duct diameters of the submaxillary and parotid glands in the Cfr\(^{-/-}\) mice. However, the ductal diameters of the submaxillary and parotid glands in the Cfr\(^{-/-}\) mice appeared greatly increased compared with their age-matched cftr\(^{+/+}\) littermates. The sublingual gland in the Cfr\(^{-/-}\) mice showed the same suggestive age-related change as their cftr\(^{+/+}\) littermates with a reduction in ductal diameter values between 37 and 60 days of age, followed by a marked increase at 90 days. However, the sublingual gland duct diameter measurements in the Cfr\(^{-/-}\) mice appeared to be the same as those of their cftr\(^{+/+}\) littermates at all ages.

Effect of DHA therapy. Observational assessment failed to identify any obvious therapeutic effect of any morphological measurement that might be attributed to DHA therapy.

**Ileum**

Untreated wild-type and CF mice: age-related changes in organ morphology. Ileal mucosal height of the cftr\(^{+/+}\) mice was similar at ages 37 and 60 days followed by a nonsignificant increasing trend at 90 days of age (Fig. 5A). The change in ileal mucosal height between 37 and 90 days and between 60 and 90 days of age showed a suggestive age effect (Fig. 5A; \(P = 0.06\)). However, measurements of intestinal wall thickness of the

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**Fig. 4.** Intralobular luminal duct (A), interlobular luminal duct (B), acinar luminal (C), and external acinar (D) diameters in the pancreas of untreated Cfr\(^{-/-}\) (CF) and cftr\(^{+/+}\) (WT) mice killed at 37, 60, and 90 days of age. The format is as described for Fig. 1. \(*P \leq 0.01.\) **\(P \leq 0.003.\) ***\(P \leq 0.0001.\) \#\(P \leq 0.0001.\) See text for detailed explanation regarding significant differences.
The lipid profiles in plasma and red blood cell membranes of the treated and untreated cftr<sup>+/+</sup> and Cftr<sup>-/-</sup> mice are shown in Table 3. The AA-to-DHA ratios were similar in the plasma and red blood cells of the untreated cftr<sup>+/+</sup> and Cftr<sup>-/-</sup> mice (cftr<sup>+/+</sup>: 2.9 ± 0.5 and 3.4 ± 0.3, Cftr<sup>-/-</sup>: 2.1 ± 0.2 and 3.1 ± 0.6, respectively). After 60 days of DHA therapy, the plasma and red blood cell AA-to-DHA ratios in the treated cftr<sup>+/+</sup> and Cftr<sup>-/-</sup> mice were similar (cftr<sup>+/+</sup> and Cftr<sup>-/-</sup>: 0.3 ± 0.1 and 0.5 ± 0.1), and both groups showed a highly significant treatment effect compared with their untreated littermates (P < 0.001). It should be noted that the plasma AA-to-DHA ratio in both the cftr<sup>+/+</sup> and Cftr<sup>-/-</sup> mice was similar at 7 and 60 days of DHA treatment, whereas the AA-to-DHA ratio in the red blood cells of both groups was significantly lower after 60 days of treatment than at 7 days (P < 0.02). The cell membrane AA-to-DHA ratios in tissue extracts from the pancreas, ileum, and lung of the cftr<sup>+/+</sup> and Cftr<sup>-/-</sup> mice were similar to the results found in the plasma and red blood cells (data not shown).
The mouse model of CF in this study exhibits the typical hallmarks of progressive CF-like disease in all organs that are affected in the human form of CF disease. Thus it offers an ideal model to evaluate the effects of DHA therapy on CF-like organ pathology. In this regard, after a brief 7-day period of DHA therapy with this particular murine model of CF, we were unable to confirm the previous observations of Freedman et al. (17). Similarly, when we extended therapy of DHA to periods of 30 and 60 days, the $Cfr^{-/-}$ mice showed no obvious improvements in morphology in the lungs, pancreas, or intestine. In striking contrast, there was a highly significant effect of DHA therapy on the progression of CF-like hepatobiliary disease. The reasons for the discrepancy between these findings and those of Freedman et al. (17) remain speculative. Both studies used the same diet and dose of DHA, which was randomized at 30 days of age. Because the animals used in both studies were CFTR knockouts, the only major difference between the studies was the genetic background of the mice. Freedman et al. (16, 17) used $Cfr^{-/-}$ mice ($Cfr^{-/-}$/tm1UNC/$Cfr^{-/-}$/tm1UNC) bred into a mixed genetic background, whereas all animals used in the present study were bred into a congenic C57Bl/6J background (C57Bl/6J $Cfr^{-/-}$/tm1UNC/$Cfr^{-/-}$/tm1UNC). The likelihood that the genetic background of the animals accounted for the discrepant findings observed in the ileum (reduced mucosal height), pancreas (decreased acinar lumen diameter), and lung (decreased inflammatory response) in Freedman et al.'s (17) 7-day DHA-treated $Cfr^{-/-}$ mice is supported by previous studies of murine models of CF (3, 9, 24, 33–36). $Cfr^{-/-}$ mice bred in a mixed genetic background show considerable variability in the severity and location of pathological changes in the multiple organs affected by CF disease (8).

We observed a good correlation between diet and lipid profiles in plasma and red blood cells. In this regard, we were able to see a therapeutic effect of DHA, with a marked decrease in AA concentration and a corresponding increase in DHA concentration in plasma and cell membranes derived from red blood cells and CF-affected tissues of the treated $Cfr^{-/-}$ and wild-type mice after 7, 30, and 60 days of therapy compared with the untreated age-matched $Cfr^{-/-}$ and wild-type littermates. Previous studies, including those of Freedman et al. (17), showed a good general relationship between lipid profiles in cell membranes of CF-affected tissue and those obtained in plasma and red blood cells (7, 26, 27). Furthermore, it has been demonstrated that Peptamen alone does not influence the cell membrane phospholipid profile (7). Similar to the study of Freedman et al. (17), we found no baseline

Table 2. **Ileal goblet cell-to-epithelial cell ratio**

<table>
<thead>
<tr>
<th>Age (days of treatment)</th>
<th>DHA</th>
<th>WT</th>
<th>CF</th>
</tr>
</thead>
<tbody>
<tr>
<td>37 days (7 days)</td>
<td>–</td>
<td>0.13±0.07</td>
<td>0.37±0.29</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.15±0.09</td>
<td>0.37±0.15</td>
</tr>
<tr>
<td>60 days (30 days)</td>
<td>–</td>
<td>0.19±0.03</td>
<td>0.32±0.11</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.30±0.07</td>
<td>0.23±0.06</td>
</tr>
<tr>
<td>90 days (60 days)</td>
<td>–</td>
<td>0.13±0.04</td>
<td>0.30±0.14</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.20±0.08</td>
<td>0.21±0.02</td>
</tr>
</tbody>
</table>

Values are means ± SD. Thirty-day-old $Cfr^{-/-}$ (CF) and $cfr^{-/-}$ (WT) mice were fed a liquid diet without DHA (−) and with DHA (+) for 7, 30, or 60 days (5–7 mice/group). The mean goblet cell-to-epithelial cell ratio of the DHA treated and untreated $Cfr^{-/-}$ and $cfr^{-/-}$ mice showed no age-related changes and no significant effect of DHA therapy. However, the goblet cell-to-epithelial cell ratio of the untreated $cfr^{-/-}$ mice was significantly increased compared with the untreated $cfr^{-/-}$ littermates ($P = 0.002$), demonstrating an absolute increase in the number of goblet cells in the $Cfr^{-/-}$ mice. See text for additional details.
DHA therapy induced significant short- and long-term amelioration of CFTR properties, which participate in the resolution phase after inflammation. These compounds act through the endogenous production of lipid mediators that have potent anti-inflammatory properties of DHA are due to downregulation of tyrosine kinase NF-kB, which in turn upregulates cyclooxygenase-2 (11). Cyclooxygenase-2 in turn drives the conversion of AA to many different angiogenic and proinflammatory eicosanoids and cytokines (11).

We took advantage of these experiments to quantify age-related changes in the CF-affected organs compared with the untreated wild-type littermates. These results provide valuable information, which should be taken into consideration for utilizing the CF mouse model for preclinical therapeutic trials or for developing strategies to identify modifier gene(s) studies of CF disease severity. First, to summarize, the normal age-related developmental changes were observed in the pancreas, ileum, and liver in the wild-type mice. For example, the pancreatic acinar lumen increased significantly between 37 and 60 days, whereas the external acinar diameter decreased between 60 and 90 days. Although there was no significant age-related change in the number of goblet cell per villus, ileal intestinal wall thickness increased significantly between 60 and 90 days. Furthermore, in the portal tracts of the liver, there was a moderate but significant increase in the degree of bile duct proliferation at 90 days.

There were striking differences in morphometric measurements between the untreated wild-type mice and their age-matched Cftr<sup>-/-</sup> littermates at all ages. These included highly significant differences at all ages for all pancreatic (except external acinar diameter), ileal, liver, and lung parameters in the untreated Cftr<sup>-/-</sup> mice compared with their untreated Cftr<sup>+/+</sup> littermates. Nevertheless, some of the morphometric measurements of Cftr<sup>-/-</sup> mice followed similar age-related patterns as the Cftr<sup>+/+</sup> littermates. For example, Cftr<sup>+/+</sup> and Cftr<sup>-/-</sup> mice exhibited no age-related changes in the degree of ileal goblet cell hyperplasia, portal tract inflammation in the liver, and lung interstitial diameter. Thus these data have provided additional quantitative confirmation of our previous observations (14) that loss of CFTR in this congenic model produced similar CF-like pathological changes that are observed in the human form of CF disease and confirms its value for elucidating the pathogenesis of CF disease and tailoring novel therapeutic drugs to specific organs.

In summary, we were unable to demonstrate a short- or long-term therapeutic benefit of dietary DHA in the lungs, pancreas, or intestine in this congenic C57Bl/6J CF mouse model. However, DHA therapy did induce a significant amelioration of the severity and progression of hepatobiliary disease, primarily by reducing the degree of inflammation. Finally, detailed quantitative age-related morphological measurements in specific organs of the untreated Cftr<sup>-/-</sup> and their untreated wild-type littermates would provide useful information to investigators who propose to utilize this congenic murine CF model to test novel therapies or examine disease pathogenesis.

Table 3. AA-to-DHA ratios in plasma and red blood cell membrane

<table>
<thead>
<tr>
<th>Therapy</th>
<th>AA-to-DHA Ratio (days of DHA treatment)</th>
<th>WT</th>
<th>CF</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHA</td>
<td>37 days old (7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.3±0.3</td>
<td>2.7±0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.6±0.3</td>
<td>0.4±0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.0±0.3</td>
<td>3.4±0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.7±0.9</td>
<td>0.9±0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Lipid analysis of plasma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid analysis of red blood cells</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD. Thirty-day-old Cftr<sup>-/-</sup> (CF) and cftr<sup>+/+</sup> (WT) mice were fed a liquid diet without DHA (−) or with DHA (+) for 7 or 60 days (2–5 mice/group). AA-to-DHA ratios of plasma and red blood cell membranes show significant differences between DHA-treated and untreated Cftr<sup>-/-</sup> and their cftr<sup>+/+</sup> littermates at all ages (P ≤ 0.05). See text for additional details.
GRANTS

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REFERENCES


