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Animal models for cystic fibrosis liver disease (CFLD)

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Abstract

Liver disease is a severe complication in patients with Cystic Fibrosis (CF), a genetic disease caused by mutations in the gene encoding for cystic fibrosis transmembrane conductance regulator (CFTR) channel. The sequence of events leading to CFLD is still unclear and has limited the development of more specific treatments other than the bile acid UDCA. However, in the last twenty years, several gaps have been filled, which have mainly been possible due to the availability of different animal models that mimic CF. CF mice, although they lack a spontaneous liver manifestation, have been essential to better understand the multiple functions of CFTR expression on the apical membrane of cholangiocytes, from chloride channel to regulator of epithelial innate immunity. Additionally, we have learned that the gut microbiota might be a pathogenetic factor for the development of liver disease. The recent creation of novel CF animal models (i.e. pig and ferret) that better reproduce the human disease, will allow for comparative studies with species that spontaneously develop the liver disease and will hopefully lead to novel therapeutic treatments. In this review, we have compared and summarized the main features of the current available CF animal models and their applicability for the study of the liver phenotype.

Keywords

Cholangiocytes; microbiota; inflammation; CFTR; biliary secretion

1. Introduction

Cystic Fibrosis (CF) is a life-threatening autosomal recessive disease with high incidence in the Caucasian population[1]. CFTR, the gene mutated in CF encodes a PKA/cAMP-activated chloride ion channel belonging to the family of the ATP binding cassette (ABC) transporter superfamily. CFTR is commonly expressed by secretory epithelia and CF is considered a multi-organ disease[1–3].

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Liver disease is a severe complication in cystic fibrosis and represents the third most common cause of death for non-pulmonary causes[4]. Cystic Fibrosis Liver Disease (CFLD) is phenotypically characterized by distinct liver alterations that may vary from abnormal liver enzymes, cholestasis, sclerosing cholangitis type of lesions that progress to focal biliary cirrhosis and often in association with micro gallbladder and liver steatosis[5, 6].

CFTR is specifically expressed on the apical membrane of biliary epithelial cells (i.e cholangiocytes) where it maintains the chloride ion gradient that drives the secretion into the bile of bicarbonate by anion exchange-2 (AE2) and consequently the level of alkalization and hydration of the bile[7, 8].

The pathogenetic mechanisms leading to the liver disease are still under investigation. A defective biliary secretion caused by the CFTR dysfunction is the primary event but secondary insults are at a play to drive the progression and severity of the disease[9, 10].

Important advances in the field have been possible thanks to the availability of animal models. CF researchers have made available a wide range of animal models that include different species from small rodents to larger mammals. Each of these models reproduce different aspects of the disease, however, together they can be informative on the impact of CFTR function in pathophysiology of the disease and how it correlates with different phenotypes[11, 12]. The development of novel genome targeting technique will bring new advances to already existing models and possibly help the search for curative options[13].

This review will make a critical comparison of the different experimental animals available or in development to model CF with particular emphasis on their capability to reproduce the liver phenotype.

2. Murine models and hepatobiliary disease development

Mutations of CFTR result in different grades of dysfunction of the channel at the membrane of secretory epithelia[14]. To date, approximately 2,000 mutations have been identified, but unfortunately the functional importance is known for only about 200 of them. Based on their functional alterations, most common mutations have been grouped into six different classes that include mutations with a complete absence of CFTR protein (Class I), with an altered traffic of a misfolded protein that does not reach the apical membrane (Class II), where CFTR protein is produced and inserted at the membrane but lacks channel function (Class III), and with different degree of reduction of the channel function (Classes IV-VI)[15]. The most common mutation is the deletion of a phenylalanine at the position 508 (F508del) that accounts for about 75% of mutated alleles in the European and North American population. This class II mutation produces a misfolded protein (not trafficking to the plasma membrane), with defective gating and decreased CFTR half-life when rescued. Class I-III mutations are causing the most severe defects[14, 15].

An important landmark in CF research has been the cloning of the CFTR gene in 1989[16]. Soon later, several CF mutant mouse models were generated[17]. Initially researchers concentrated their efforts on the production of null mice by deleting specific portions of the murine homolog gene to CFTR (*cftr*). In these mice, the CFTR protein is not detectable and

therefore they are comparable to a stop codon (Class I) mutation[18]. CFTR null mice were generated in different genetic backgrounds[19]. Since these models were not completely representative of the complexity of the CF human disease with the different spectrum of mutations, other models were created that mimic the class II mutations F508del and G480C and the class III mutation G551D[20–22].

The use of the mouse to model CF has certain advantages including the short time to generate the animals and the relatively contained cost of maintenance. However, physiological differences between the human and the mouse species and the restricted environment to which mice are exposed to has been a major challenge in the attempt to reproduce a complex multi-organ disease as CF. Indeed, CF mice present a mild (i.e G480C, G551D) to severe (i.e null, F508del) intestinal phenotype, similar to human patients, with intestinal obstructions at the weaning, nutrient malabsorption, mucus accumulation and small intestinal bacterial overgrowth (SIBO)[17, 20–22]. The incidence of developing intestinal obstruction (i.e meconium ileum, MI) at the time of weaning is quite high and to decrease the rate of mortality in these mice, their diet is supplemented with laxatives (i.e Poly Ethylene Glycol; PEG) or substituted with a complete liquid diet (Peptamen, Nestle) [17]. To overcome this complication, Whitsett lab were able to introduce the expression of human CFTR using a fatty acid-binding protein promoter in intestinal villus cells in null mice and generated a gut-corrected strain where the intestinal phenotype is completely recovered[23]. Although not many studies have been performed using the gut corrected strain, this is the first proofing that transfer of the human CFTR gene in epithelia that lack the expression of the protein can correct the physiologic defect.

Despite the intestinal disease, CF mice lack the spontaneous development of lung, pancreas and liver phenotypes unless they are aged (12–24 months) or challenged with toxic agents[24]. However, the G551D mice represent an exception; in fact, a liver phenotype is described although in a small percentage of animals[21]. The failure of these models to reproduce the human CF disease has been mainly attributed to the physiological differences in species. Specifically, mice show a higher presence of CFTR-independent Calcium activated Chloride Conductance (CaCC) that can mediate chloride efflux when CFTR is not functional[11]. More recently, a discovery of TMEM16A as the possible chloride channel that mediate CaCC [25] and its expression has also been confirmed in biliary cells[26].

Pulmonary disease is the primary cause of mortality in CF patients and therefore the need to reproduce the lung phenotype in CF animal models is a high priority and has dictated most of the advances in the field.

Studies of the lung have shown that when CF mice are challenged with either bacterial pathogens or their components (i.e Pseudomonas aeruginosa, LPS), they develop an excessive inflammatory response with a decreased clearance of bacteria as compared to normal mice[27]. This observation suggests that the inflammatory response to pathogens in the lung of CF mice is altered compared to normal mice, and that external challenges are essential toward the development of a phenotype.

The liver complication has often been overshadowed, however, mice models have also been used to study the liver disease and despite the lack of a spontaneous phenotype, several physiological similarities are in common with the human disease.

Bile efflux and bicarbonate secretion are both decreased in CFTR null and ΔF508 mice confirming the presence of a secretory defect [28, 29] and providing an in vivo model to test molecules or drugs able to correct the defect. In this regard, our group has identified glibenclamide, a compound belonging to the family of sulfonylureas and known to potentiate insulin secretion in β-cells, as a choleretic agent that stimulate bile flow with a mechanism independent of CFTR function. Our findings suggest that glibenclamide acts by stimulating a Ca2+ and cAMP independent vesicular transport pathway that is preserved in the biliary epithelium of CFTR null mice[29].

CF mice were also useful to clarify the pharmacologic action of bile acids (i.e. UDCA and nor-UDCA). UDCA is the elective treatment for CF patients[30], however, a recent revision of the literature has shown a lack of long-term clinical studies that support the efficacy of this treatment[31]. UDCA has shown to stimulate bile secretion in vivo in mice, but CFTR null and ΔF508 mice lack its choleretic effect. Indeed, data from our group show that the mechanism of action of UDCA relies on a CFTR-dependent secretion of ATP that stimulates purinergic signaling on the apical membrane of cholangiocytes and that this effect is blunted in CF[32]. On the contrary, norUDCA, (a side chain-modified UDCA derivate, whose chemico-physical properties predispose to cholehepatic shunting), was able to stimulate cholangiocyte secretion in CFTR-KO mice, suggesting its potential beneficial effect in CF[28].

Despite the presence of a secretory defect, Bodewes et al. has also shown that the bile acid composition of CFTR null mice differs from normal mice[33]. The defect starts from the intestine, with an increased fecal bile acid loss similar to what is seen in clinic in CF patients. On the other hand, this decreased amount of intestinal bile salt is followed by a compensatory hepato-biliary production of primary bile acids enriched in hydrophilic bile salt such as ursocholic acid (UCA) and cholic acid (CA)[33]. A similar bile salt composition is also present in the feces of these mice. Since a more hydrophilic bile is conventionally less toxic, contrary to their expectations, the authors exclude bile salt cytotoxicity as a main pathogenetic mechanism in CFLD. Remarkably, this study points out that the different bile pool composition might be the result of a different biotransformation of bile acids by an intestinal microflora with distinctive metabolic properties in CF mice[33]. Similar alterations in the bile acid composition of CF mice were described also by Debray et al. and explained by an alternative mechanism. The authors show that the gallbladder of CFTR null mice is enlarged and defective in emptying. This defective function of the gallbladder results in a reduction of bile acid flux into the intestine that reduces their enterohepatic circulation and favor a colecystohepatic shunt that results in a decreased formation of secondary bile acids[34].

Among the different gastrointestinal manifestations, the presence of a dysbiotic gut accompanied with increased intestinal permeability has been described in CF patients and

further confirmed in CF mice, which represents an important factor in explaining the development of CFLD[35, 36].

In this regard, our group and others have shown that in the CFTR-KO model, induction of colitis with dextran-sodium-sulfate (DSS), a protocol known to cause increased intestinal permeability and portal release of bacterial products, triggers biliary damage and inflammation in CFTR-KO, but not in WT mice exposed to the same treatment[37, 38]. DSS induces a biliary damage, characterized by the expansion of the ductular reactive component and portal inflammation, with extensive infiltration of neutrophils, similar to the focal lesion described in patients. However, in this DSS model, administration of choleretic bile acids (i.e norUDCA) that improve bile secretion, have no effect on the liver damage, suggesting that the secretory defect itself is not sufficient to develop the liver disease. Instead, treatment with broad-spectrum antibiotics causes a significant improvement of both the liver damage and inflammation further reinforcing the link between the gut and the liver[38]. Preliminary unpublished observations from our group show that the gut microbiota component might not only have a local effect on the bile acid metabolism as shown in the study by Bodewes et al. [33], but possibly also play a causal role in the development of the liver disease. Indeed, our data not only confirm the presence of a dysbiotic gut in CFTR-KO mice but also point out the presence of a more pro-inflammatory microflora that in the setting of increased intestinal permeability would activate specific immune responses when translocated to the liver.

Remarkably, the original manuscript from 1996, describing the presence of focal biliary cirrhosis in a small percentage of G551D mice, reports that these mice were first generated in a pathogen-free facility and followed by a move to a normal facility, where they were fed a different diet[21]. Two decades later, our knowledge in the microbiota field would certainly confirm that these mice were exposed to microbiota changes further suggesting the involvement of a liver-gut axis.

CF mice are also being used as an important source for cell isolation. Our group has used both null and F508del mice for the isolation of primary cultures of cholangiocytes[38–40]. In vitro data using isolated cholangiocytes show that loss of CFTR at the membrane causes important structural and innate immune changes that predispose the biliary cells to overreact when exposed to gut-derived endotoxin. These results are a confirmation of the liver phenotype previously described in CF mice treated with DSS. In fact, cholangiocytes isolated from CFTR-KO mice have increased NF-kB activation and secrete more pro-inflammatory cytokines compared to normal ones that would create a local inflammatory milieu[38]. Interestingly, our molecular data show that CFTR is part of a complex at the membrane, with proteins that are important to maintain an endotoxin tolerance against gut-derived endotoxins. In CF cholangiocytes, this mechanism of tolerance is altered and when the cells get in contact with bacterial derived components they over-react[40]. These observations impose that CFLD might be the result of a double component that involves the biliary cell in the liver and the microbiota in the gut and explain how a second “hit” in addition to the genetic mutation is necessary to develop the liver disease. New studies in the mouse model are needed to address the potential causative role of the microbiota in the liver disease.

3. Large animal models

The use of mouse models in the last decade or so has undoubtedly contributed to the understanding of many aspects of CF pathology and has clarified that dysfunctions of different organs might be somehow linked. However, the fact that several important biologic divergences exist between mice and humans, together with a lack of spontaneous manifestation of the CF disease in the mouse, has prompted researchers to search for larger and more representative mammal models such as the pig and the ferret [41].

Both these models are more representative of the CF manifestations seen in patients but still present some challenges[11].

Pigs have several analogies with humans in terms of life span, anatomy, physiology, size and genetics. The first CFTR null piglets were born about ten years ago at the University of Iowa[42]. They presented a very severe intestinal phenotype at birth characterized by meconium ileus similar to what is observed in about 15% of CF patients. However, in the piglets the defect is even more severe with a penetrance of 100%, and they require almost immediate surgical procedure to alleviate the intestinal obstructions and increase their survival. The same group also generated the pig model carrying the most common ΔF508 CFTR mutation[43]. Similar to the null allele, these piglets also suffered from meconium ileus, which suggested that the residual activity of CFTR protein that escape the ER and reach the membrane is not sufficient to prevent the intestinal phenotype. Beside the disadvantage of the intestinal complication, as predicted, both pig models spontaneously develop lung disease and present significant alterations of the pancreas and liver similar to human patients[42–45].

The histological evaluation of the liver shows the presence of chronic cellular inflammation at the level of the portal space with a focal distribution in both null and ΔF508 mutated piglets[42–45]. More interesting is the finding that after a few months after birth there is a progression of the liver phenotype with the appearance of bridging fibrotic areas and steatosis, very consistent with what is seen in patients. Also similar to CF patients, the CF piglets present a micro gallbladder filled with a dense mix of mucus and bile[42–46].

Additional analysis of different epithelia, other than the intestine, in piglets carrying the ΔF508 mutation confirmed the presence of a residual CFTR function (about 6% of the wild-type function), as result of a small fraction of ΔF508-CFTR that escapes the ER and reaches the apical membrane[44]. This result confirms previous in vitro data in cell lines carrying the same mutation and suggests that such a CFTR residual activity is not sufficient to prevent the different phenotypes in the pig.

An interesting anatomical feature of the pig is that the common bile duct and the pancreatic duct openings to the intestine are anatomically distinct[45]. Therefore, researchers were able to perform a detailed analysis of the pancreatic and biliary fluid separately in CFTR-KO animals. The volume and pH of pancreatic fluid was found to be lower compared to WT, and protein content was increased. Bile volume in resting conditions was similar between CF and WT and the increase in response to the physiologic hormone secretin was observed only in WT piglets confirming the secretory defect in CF pigs[45].

Although the CF pig model is probably the most representative of the human disease, it is also the most difficult to work with. The husbandry cost and the specialized facilities needed for these large animals certainly limit the widespread use of these animals by researchers in the field.

A second CF animal model recently generated is the ferret[47]. The ferret was chosen because, contrary to the mouse, its lung anatomy and cell biology closely resemble those of humans. Their gestation time of 42 days is close to the mice. The first ferret was obtained by insertion of a stop codon and the neomycin cassette into exon 10 of CFTR gene that generates a null genotype.

Similar to the mice and the pig, the intestinal phenotype in the CFTR-KO ferret is severe and about 75% of the kits dye within 24 hours after birth. A disadvantage of the ferret, compared to the pig is that the small size of a ferret kit prevents the possibility of a surgical treatment. In addition to the development of meconium ileus, the nutritional status of newborn CF ferrets is severely compromised and reflects anatomical differences in their intestinal tract, mainly the lack of a cecum and a shorter intestinal transit. In those animals that were able to survive, the pancreas shows histological lesions as seen in CF patients but less severe compared to the pig model. While the liver histology of CFTR-KO ferrets appears normal, the serum analysis reveals increased levels of ALT and bilirubin with decreased levels of cholesterol[12, 47].

The CFTR-KO ferrets were treated with UDCA and proton-pump inhibitors that were able to normalize the liver enzymes and improve their nutritional status, but the cholesterol remained low[47]. Survival in these animals was also compromised by development of multifocal bronchopneumonia with severe lung damage, inflammation and bacterial colonization. Interestingly, a second study has shown that several inflammatory pathways and airway innate immune mechanisms are altered in the KO ferret before and immediately after birth.

Certainly the ferret has given encouraging results in reproducing CF human disease and represents a good compromise compared to the pig and its demanding costs. However, less tools are available to study the ferret (i.e specie-specific antibodies, growth factors, recombinant proteins), which limit the isolation of cells. In addition, more work has to be done to create ferret models carrying different CFTR human mutation.

4. New models and future directions

With the advantage of novel genetic engineering approaches, new CF animal models are currently under development (i.e rat, rabbit)[13]. These animals are still being characterized and there are no data related to the liver phenotype.

The rat CFTR-KO model has been generated using Zinc-finger endonuclease (ZFN) technology[48]. This model has all the advantages of the mouse including the short time for breeding, contained husbandry costs and the availability of specie-specific molecular tools that for example are still missing for the ferret (i.e antibodies, recombinant proteins). Some

anatomical similarities with the humans, not present in the mouse, such as the presence of submucosal glands were attracting for lung researchers.

Indeed, the first results report that the rat reproduces several features of CF human airway disease (i.e mucus plugging, nasal and tracheal electrophysiological defects). However, similar to the mouse there was not evidence of spontaneous infections or inflammation in rat airways[49].

A second model recently developed using CRISPR/Cas9 is the CF rabbit, both CFTR null or carrying the ΔF508 mutation[50]. This model was also generated because of the need of an easy animal model that reproduces the lung phenotype. The CF rabbit is still at the initial characterization stage, but preliminary evidence shows development of a lung phenotype including the presence of bacteria infection in the airways.

More recently CF researchers have been trying to generate a humanized mouse model that express the human CFTR gene[51]. This has been possible by using bacteria artificial chromosomes (BAC) or yeast artificial chromosome (YAC) that allow the transfer of a large genomic sequence, including regulatory elements such as promoters, introns and flanking regions[52, 53]. In the future, it will be likely possible to use the CRISP/Cas9 gene editing technology to introduce specific CFTR mutations and use this system for testing new treatments.

5. Conclusions

CF is a multi-organ disease and the use of in-vivo animal models is necessary to have a more comprehensive understanding of the disease to apply for the search of new therapies. Both small animal models (i.e. mouse, rat) and larger mammals (i.e. rabbit, ferret, pig) have been developed. Although all of these models have limitations and do not fully recapitulate the human disease, the comparative analysis of CF biology among species has been the most useful tool to understand the pathophysiologic processes of CFTR in different organs (see table 1).

In regard to the liver, most of these models have been shown to recapitulate the secretory defect of the biliary epithelium. In the mouse, the biliary secretory defect is not sufficient to spontaneously develop the liver disease but it can be induced by treatment with DSS. In both pigs and ferrets, that have a more severe intestinal phenotype, the liver disease, similar to what is seen in the CF patients, appears earlier in life and in the pig can progress to fibrosis. Several observations have suggested that changes in the liver-gut axis play a role in the pathogenesis of liver disease in CF and future studies will better clarify this aspect.

In summary, all the CF animal models developed so far will continue to be an important tool to assist the CF researchers and in the era of genome editing we expect they will generate new systems to test potential treatments.

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Abbreviations

CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CFLD	Cystic Fibrosis Liver Disease
UDCA	ursodeoxycholic acid
AE2	anion exchanger 2
DSS	dextran sodium sulfate
CaCC	calcium activated chloride conductance
BAC	bacterial artificial chromosome
YAC	yeast artificial chromosome
DIOS	distal intestinal obstruction syndrome
SIBO	small intestine bacterial overgrowth

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Highlights

- Liver disease in CF is a severe complication that can progress to biliary cirrhosis.
- Several animal models are available to study the pathophysiology of CFLD.
- Altered biliary innate immunity and pathogenic gut microbiota play a role in CFLD.
- Genome editing technology will generate new useful models for drug testing.

Table 1.

CF liver and intestinal phenotype in human and animal models

	Liver	**Intestine**
Human	Cholestasis, focal biliary cirrhosis, microgallbladder, steatosis	Meconium ileus in 15% of patients, DIOS, SIBO
Mouse	Low grade steatosis, Biliary abnormalities in a small percentage of G551D mice, macrogallbladder	DIOS at weaning depending on the background, SIBO
Pig	Focal biliary cirrhosis, microgallbladder	Meconium ileus with 100% incidence
Ferret	Elevated liver enzymes (i.e ALT, bilirubin)	Meconium ileus with 75% incidence
Rat	Not described	DIOS at weaning
Rabbit	Not characterized	Not characterized