Pathology of pancreatic and intestinal disorders in cystic fibrosis

Michael Wilschanski MBBS¹ Peter R Durie MD FRCP²

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INTRODUCTION

Cystic fibrosis (CF) is the most common fatal autosomal recessive disease of Caucasians with an incidence of up to 1 : 2000 in some populations¹. CF is a disorder of a single gene that affects many organ systems but chronic, progressive respiratory tract disease is the primary cause of mortality. The disease phenotype is characterized by viscid secretions that obstruct ducts within a variety of secretory epithelia. In the past three decades, symptomatic therapy has greatly improved overall health and survival. As median survival improves, gastrointestinal and hepatobiliary complications are becoming more problematic. The more common age-related intestinal and hepatic manifestations of cystic fibrosis are summarized in Figure 1. Liver disease is now the second most common CF-related cause of mortality². Gastrointestinal manifestations of CF may be due to primary malfunction of an organ (e.g. meconium ileus) or due to a secondary feature of the disease (e.g. signs of vitamin E deficiency due to pancreatic failure).

At birth, CF patients may or may not exhibit multiple symptoms of the disease and the severity is extremely variable. Nevertheless, almost all patients have increased concentrations of chloride in sweat. At birth, intestinal obstruction due to meconium ileus occurs in approximately 18% of newly diagnosed patients. During the first year of life, approximately 50% of patients exhibit signs and symptoms of nutrient maldigestion due to pancreatic insufficiency, inadequate weight gain, persistent cough or multiple bouts of pneumonia. However, the nature of the symptoms, their severity and the rate of progression of disease vary considerably among CF patients. Eventually, most patients who have CF will exhibit progressive lung disease. A small number of patients have been diagnosed with atypical signs and symptoms of CF disease and it is often unclear whether or not these patients truly have CF disease or some other illness. This includes males with

congenital bilateral absence of the vas deferens, other forms of obstructive azoospermia, and males or females with subtle respiratory manifestations or recurrent pancreatitis. The diagnosis of CF in these patients must be confirmed by evidence of abnormal ion transport, such as an abnormal sweat chloride test, or abnormal nasal potential difference measurement, or following identification of two CF-causing gene mutations. This article provides some insights into the current views concerning pathogenesis and variability of disease manifestations within the gastrointestinal tract.

CELLULAR PATHOGENESIS OF DISEASE

Although the varied and complex phenotype of CF has been documented for decades, only in the last 15 years have physicians and scientists appreciated that the common sites of disease in the affected organs are secretory surfaces limited to epithelial cells³. In the 1950s, documentation of elevated electrolytes in sweat established this test as the hallmark for diagnosis. In the early 1980s, Quinton's pioneering electrophysiologic studies of the sweat duct strongly suggested that CF was a primary defect of chloride conductance in epithelial cells³. Subsequently, the CF defect was localized to the apical cell membrane as an inability of intracellular cyclic adenosine monophosphate (cAMP) to regulate movement of chloride across affected epithelial surfaces.

Abnormalities of electrolyte transport by CF epithelia provides a unifying hypothesis about the biological defect in various affected organs. Quinton and colleagues found that sweat ducts of CF patients had a higher transepithelial voltage than normal ducts. When luminal chloride was reduced in normal ducts, luminal voltage became more negative suggesting that chloride is transported across the duct membrane electrically. In CF duct epithelia, however, reduced concentrations of chloride lead to a more positive transepithelial voltage, strongly indicating that the CF duct was impermeable to chloride.

Figure 2 provides a simplified concept of the mechanisms of ion transport at the cellular level in airway epithelia. In normal secretory epithelia, chloride ions (Cl^-) enter the cell across the basolateral membrane and Cl^- is maintained intracellularly above its electrochemical equilibrium by basolateral membrane transport processes.

¹Pediatric Gastroenterology Unit, Department of Pediatrics, Shaare Zedek Medical Center and The Hebrew University, Jerusalem, Israel; ²Division of Gastroenterology & Nutrition and The Research Institute, The Hospital for Sick Children; and the Department of Pediatrics, University of Toronto, Toronto, Ontario, Canada

Correspondence to: Dr Peter R Durie, Department of Gastroenterology & Nutrition, Hospital for Sick Children, 555 University Avenue, Toronto, Ontario M5G 1X8, Canada

Figure 1 Age-related intestinal and hepatobiliary complications in cystic fibrosis (CF). DIOS (MIE) = distal intestinal obstruction syndrome (meconium ileus equivalent)

Chloride exits the apical surface down an electrochemical gradient via Cl^- channels. Apical chloride transport is activated by phosphorylation in response to an increase in intracellular cAMP concentration. Sodium (Na^+) ions are passively transported through the apical surface of the cell via amiloride-sensitive Na^+ channels. In CF secretory epithelia the primary defect lies in the apical membrane due to the lack of a chloride channel or its dysfunction. There is failure of cAMP-regulated Cl^- secretion and increased basal Na^+ absorption. The net effect of disordered ion transport is the diminution of fluid secretion into the lumen. Knowles *et al.*⁴ evaluated electrolyte transport across airway epithelia *in vivo*, using a technique capable of measuring the transepithelial electrical potential difference in the trachea and inferior turbinate of the nose. He demonstrated a negative potential difference of approximately -10 mV in normal subjects. In contrast, the negative potential difference across the apical surface of the airway epithelium in CF patients was approximately two fold higher (-20 mV). The higher potential difference in CF was inhibited by the sodium channel inhibitor amiloride, which suggests a marked increase in inwardly directed Na^+ current in CF airway epithelia.

The exact relationship between the absence of the cAMP activated chloride function and enhanced amiloride-sensitive sodium absorption has not been elucidated. Various hypotheses have been proposed, including an increase in the number of sodium channels, enhanced ionic conductance or, as proposed by Boucher and co-investigators,

an increase in the open probability of sodium channels⁵ whose permeability is regulated by the CF chloride channel⁶. Substances that increase intracellular cAMP via adenylyl cyclase release fail to stimulate Cl^- secretion in CF epithelia, even when a favourable electrochemical gradient is established by superfusion of the mucosal surface with a Cl^- -free solution.

The intestinal tract exhibits a large diversity of both absorptive and secretory processes which show considerable regional variation. In CF, there is a consistent loss of cAMP-induced Cl^- secretion. In general, the mechanisms of intestinal fluid and electrolyte secretion appear to be similar to that of the airways, since Cl^- enters at the basolateral membrane via co-transport processes and Cl^- exits apically via chloride channels. Abnormalities of electrolyte transport by CF intestinal epithelia almost certainly lead to alterations in the composition of luminal contents. The lack of cAMP-induced Cl^- secretion, which normally counter balances intestinal absorption, could reduce fluid content in the intestinal lumen, causing dehydration and impaction of intestinal contents.

Similarly, the pancreatic manifestations of CF show that impaired pancreatic ductal fluid secretion in CF is a primary phenomenon⁷. In the apical membrane of the small pancreatic ducts, Cl^- channels work in parallel with chloride-bicarbonate exchangers to secrete a bicarbonate rich fluid. Chloride enters the duct lumen via apical cAMP responsive Cl^- channels and is recycled intracellularly through the apical membrane in exchange with intracellular

Figure 2 Cellular mechanisms of ion transport in airway epithelia.

In normal epithelia (top) chloride enters the cell via basolateral cotransport mechanisms and chloride concentration is maintained intracellularly above its electrochemical equilibrium. Chloride exits apically down an electrochemical gradient via chloride channels. Sodium ions are passively transported through apical amiloride-sensitive sodium channels. Basal potential difference across normal epithelia approximates -10 mV. In cystic fibrosis (CF) epithelia (bottom) there is failure or dysfunction of cyclic adenosine monophosphate-regulated chloride secretion and increased basal sodium absorption. Basal transepithelial potential difference (approximately -20 mV) is higher in CF than in normal subjects. CFTR = cystic fibrosis transmembrane conductance regulator

bicarbonate. Chloride secretion therefore, is responsible for increasing both the volume and the bicarbonate content of the luminal fluid. In the normal pancreas, stimulated acinar cells secrete fluid rich in enzymes and other proteins. Luminal proteins are maintained in solution by increased ductal fluid flow. In the CF pancreas, however, protein is secreted in normal quantities initially, but the volume and alkalinity of ductal fluid to maintain protein in solution is restricted by impaired anion transport. Consequently, highly concentrated pancreatic proteins that are relatively insoluble will precipitate obstructing the small ducts⁸. With

time this process produces destruction and atrophy of the acini and ducts.

Thus, the multisystem manifestations of CF is consistent with a general lack of success in maintaining the luminal hydration of secretions in various organs that contain macromolecules. In CF, the sweat duct is not affected pathologically, because its function does not involve secretion of macromolecules. Organs that are affected earliest, such as the exocrine pancreas and vas deferens of the male, appear to be most vulnerable to cystic fibrosis transmembrane conductance regulator (CFTR) related secretory problems, due to the tortuosity and length of their ducts and the high concentration of macromolecules in their secretions.

MOLECULAR PATHOGENESIS OF DISEASE

In the 1980s, the use of restriction fragment length polymorphism analysis led to the localization of the CF gene to the long arm of chromosome 7. A breakthrough in our understanding of disease pathogenesis occurred in 1989 when Lap-Chee Tsui and colleagues cloned the CF gene⁹⁻¹¹. The gene encodes 27 exons, separated by large introns, spread over 230 kb. This discovery permitted the direct study of the structure and function of the protein product which has been designated the CFTR. CFTR which is composed of 1480 amino acids, bears a striking structural similarity to a variety of bacterial, yeast, and mammalian transport proteins known as ABC transporters. P-glycoprotein is a notable example of these membrane transporter proteins. Like P-glycoprotein, CFTR contains two duplicated regions (Figure 3), each containing membrane-spanning domains with six transmembrane segments, and a nucleotide-binding domain. CFTR contains a unique regulatory, or R-domain, with an abundance of potential phosphorylation sites. There is now overwhelming proof that CFTR is anchored in the apical surface of the epithelial cell membrane and functions as a cAMP regulated Cl⁻ channel. Although it may have several regulatory and intracellular functions, the electrophysiological function of CFTR as an anionic membrane channel was elucidated *in vitro* following its expression in cells which do not normally express the protein. Utilizing electrophysiological techniques including patch-clamping and lipid bilayer studies, the regulatory and biophysical properties of CFTR were found to be identical to cAMP regulated Cl⁻ channels¹².

Since the *cftr* gene was cloned, almost 700 different mutations have been identified by members of the Cystic Fibrosis Genetic Analysis Consortium under the leadership of Dr Lap-Chee Tsui (personal communication). The major mutation causing CF is a three-base pair deletion, in the first nucleotide binding domain, at position 508 of the *cftr* gene ($\Delta F508$), which results in the loss of a single amino acid

Figure 3 The cystic fibrosis gene, which is on the long arm of chromosome 7, comprises 27 exons spanning 230 kb of DNA. The gene product, named CFTR (cystic fibrosis transmembrane conductance regulator), is a protein of 1480 amino acids. The predominant mutation which accounts for approximately 70% of all *cfr* gene mutations worldwide, is a three base pair deletion in exon 10 of the *cfr* gene resulting in a loss of a single amino acid, phenylalanine, at codon 508 ($\Delta F508$). CFTR contains two duplicated regions, each containing membrane spanning domains and a nucleotide binding domain. A unique regulatory or R-domain contains an abundance of phosphorylation sites. There is overwhelming proof that CFTR acts as a cyclic adenosine monophosphate-regulated chloride channel in the apical surface of epithelial cells. ATP=Adenosine triphosphate

(phenylalanine) in the protein product. $\Delta F508$ accounts for approximately 70% of all CF chromosomes worldwide, but the frequency varies in specific ethnic populations from as low as 30% among Ashkenazi Jewish patients to almost 90% among individuals of Scandinavian origin. On a worldwide basis, all other mutations are uncommon or rare, although the frequency varies considerably according to ethnic background. The next most common mutations are G542X, G551D, N1303K and W1282X, each of which account for 1% to 2.5% of CF chromosomes throughout the world. Two are missense mutations (G551D, N1303K) resulting in an amino acid substitution of the protein product, while G542X and W1282X are nonsense mutations which give rise to a premature stop codon, leading to the expression of no CFTR or a non-functional truncated protein. The next most common 19 mutations occur at frequencies of 0.1–0.7%. A higher frequency of certain *cfr* gene mutations occur in specific ethnic populations. For example, the stop mutation W1282X is present on approximately 50% of Ashkenazi Jewish chromosomes, making it the most common mutation in this particular population. Most of the remaining mutations are extremely rare, many of which are considered to be 'private' mutations. Following extensive genotype analysis in CF populations of multi-ethnic origin, *cfr* gene mutations have been identified in approximately 90% of CF

chromosomes. The remaining gene mutations, some of which may be in the promoter region of the gene or affect splicing efficiency between exons, await elucidation.

GENOTYPE-PHENOTYPE RELATIONSHIPS

The symptoms and severity of CF disease vary greatly among patients and the diagnosis may be established with a wide range of clinical features at various ages from the neonatal period to adulthood. Furthermore, there is considerable variability in the disease severity of involved organs. Attempts to examine the relationship between CF genotype and phenotype is complicated by possible effects of the environment, by the existence of hundreds of different *cfr* gene mutations and by other genetic modifiers, such as second-site mutations. For this reason, some of the initial studies have focused on the most common CF gene mutation $\Delta F508$, and its relationship to the CF phenotype¹³. More recently, attempts have been made to group *cfr* gene mutations according to their predicted functional effects.

Molecular consequences of *cfr* gene mutations

Several classification systems have been developed in an attempt to define the large number of *cfr* gene mutations on

Figure 4 Molecular consequences of cystic fibrosis transmembrane conductance regulator mutations. Different mutations can be classified according to the functional properties of the gene product with respect to chloride regulation. A modified classification system, originally proposed by Tsui (Ref 14) is shown (see the text for description of this classification system)

the basis of their functional effects on chloride conductance. Figure 4 shows five general mechanisms, originally proposed by Tsui¹⁴, which elucidate various ways *cfr* gene mutations may influence CFTR mediated apical Cl⁻ secretion.

Under normal circumstances, the *cfr* gene is transcribed into messenger RNA (mRNA), which in turn is translated into protein. Before CFTR becomes anchored in the apical portion of the cell membrane to function as a Cl⁻ channel, the protein undergoes post translational modification in the Golgi network. Class I, represents *cfr* gene mutations that would be expected to prevent transcription of *cfr* into a full-length mRNA. Thus, no protein or a truncated protein is formed which is unlikely to reach the cell surface. Most mutations belonging to class I possess nonsense codons which cause premature termination signals or have similar effects on mRNA synthesis due to splice-site or frameshift abnormalities. Since no functional CFTR protein is produced, apical CFTR Cl⁻ channel function is absent. Class II mutations represent mutant proteins which fail to reach the apical membrane under physiological conditions, due to a defect in intracellular trafficking. Instead, the nascent protein is degraded intracellularly due to improper folding. The most common *cfr* gene mutation, ΔF508, is a class II mutation. *In vitro* studies of epithelial cells expressing ΔF508 have shown that reduction of the incubation temperature from 37°C to between 23°C and 30°C facilitates effective passage of the mutant protein from the endoplasmic reticulum, through the Golgi to the apical surface of the cell membrane¹⁵. Once the mutated protein is positioned in the apical membrane it acts electrophysiologically as a normal chloride channel in response to appropriate agonists¹⁶. *Cfr* gene mutations belonging to class III include those that produce a protein capable of reaching the apical membrane but are unable to respond to activation by cAMP under physiological conditions. Some of

the mutated protein products belonging to this class appear to interfere with adenosine triphosphate phosphorylation in the first nucleotide binding fold. G551D is an example of a class III mutation.

Thus, in terms of functional effects, *cfr* gene mutations belonging to classes I, II, III result in the loss of cAMP regulated Cl⁻ channel function in affected epithelial surfaces. Theoretically, class IV and class V mutations are less severe. Class IV mutations produce protein that reaches the apical membrane, are capable of generating a cAMP regulated apical membrane chloride current, but have altered ionic properties resulting in a reduction in the amount of current; an example is the R117H mutation. Most mutations of the class IV category are missense mutations, where amino acid substitutions appear to affect the ionic properties of the transmembrane segments of the protein product. Class V mutations result in reduced synthesis of normal functioning CFTR due to defective processing (A455E) or aberrant splicing at alternative sites (3849+10kbC→T). Mutations in the promoter region of the *cfr* gene might be expected to have the same effect.

Recently, we tested the hypothesis that different functional classes of *cfr* gene mutations would have variable effects on epithelial chloride conductance *in vivo*¹⁷. Since the sweat gland is not affected pathologically, we examined sweat chloride concentrations. The first abnormal (>50 mmol/L) sweat test was analysed in 455 patients who had two identified CF mutations, at least one of which was ΔF508 (Figure 5). There was no significant difference in the mean sweat chloride values between mutations belonging to classes I, II or III. The patients with class IV mutations, all of whom carried the pancreatic sufficient phenotype had a significantly lower mean sweat chloride level than those in classes I–III. Interestingly, the sweat chloride values of patients in class V, all of whom were pancreatic sufficient were not significantly different from the mean values for classes I–III. This result may be anomalous because of the small number of patients and different mutations may have variable effects on Cl⁻ channel function within the sweat duct. Alternatively, a decreased number of normal chloride channels may have variable effects on the different organs influenced by the CFTR protein.

Classification of mutations into functional classes provide useful insights into the relationship between genotype and clinical phenotype¹⁴. Patients with classes I, II and III mutations are similar in that there is no functional Cl⁻ channel. However, patients with class IV or V mutations in which some Cl⁻ channel function is retained, might be expected to have a less severe phenotype. As we will describe in greater detail below, patients who have class I, II, and III mutations in both alleles are likely to have severe pancreatic disease, whereas those with at least one

Figure 5 In cystic fibrosis the sweat duct is not affected pathologically. We tested the hypothesis that different classes of cystic fibrosis transmembrane conductance regulator (CFTR) mutations would have variable effects on sweat duct chloride concentrations. Sweat chloride concentration in mmol/L (mean $\pm$ 1 SD) was analysed in 455 patients who had two identified CFTR mutations at least one of which was Δ F508. There was no significant difference in mean sweat chloride values in patients with mutations belonging to classes I, II or III. The patients with class IV mutations had significantly lower sweat chloride levels than those in class I-III ($P < 0.01$). Of interest, mean sweat chloride values of patients in class V were not significantly different from the mean values for classes I-III

class IV or V mutation are predicted to have a milder pancreatic phenotype. Similarly, patients with monosymptomatic or atypical CF, such as those who present with recurrent pancreatitis or male infertility have class IV or V *cfr* mutations on one or both alleles.

Pancreatic disease

Cross-sectional evaluation of a large CF population revealed that approximately 85% of patients had evidence of maldigestion due to pancreatic insufficiency necessitating exogenous pancreatic enzyme replacement therapy with meals¹⁸. The remaining patients had more subtle evidence of pancreatic disease when evaluated by sophisticated tests

of exocrine pancreatic function, but possessed sufficient exocrine pancreatic reserve to permit normal digestion without the need for pancreatic enzyme supplementation. The latter group of patients were considered to be pancreatic sufficient. As a group, patients with the pancreatic sufficient phenotype have better pulmonary function, lower sweat electrolyte concentrations and better overall survival than patients who have pancreatic insufficiency¹⁸, although pulmonary function tends to be extremely variable from patient to patient. Data from the (US) CF patient registry (1995) indicate that median survival for patients with pancreatic insufficiency was 29 years of age compared with 56 years of age for those with pancreatic sufficiency.

Before the *cfr* gene was cloned, we observed a remarkable concordance of pancreatic function status among CF patients within a given family¹⁹. This strongly suggested that genetic factors, due to the CF gene, directly influenced the pancreatic phenotype. When the CF gene was cloned, it was possible to evaluate large patient populations to refine our understanding of the genetic factors that influence the level of pancreatic function in the CF patients. It was hypothesized that the pancreatic sufficient phenotype would be conferred by a mild allele which would be dominant over a 'severe' allele¹¹. Thus, only patients who have two copies of severely mutated alleles would be pancreatic insufficient. This hypothesis was confirmed by studies that demonstrated that patients homozygous for the Δ F508 mutation (class II) almost invariably have pancreatic insufficiency¹³. Table 1 lists several mutations classified as 'severe' or 'mild' with respect to pancreatic function²⁰. A small number of more

Table 1 Classification of cystic fibrosis gene mutation as severe, mild or indeterminate with respect to pancreatic function

Severe (classes I, II or III)	Mild (classes IV or V)	Variable (classes IV or V)
Δ F508	R117H	G85E
1148T	R334W	2789+5G $\rightarrow$ A
G480C	R347P	
G551D	A455E	
R560T	P574H	
N1303K	3849+10kb C $\rightarrow$ T	
G542X	G551S	
W1282X	P5748	
621+1G $\rightarrow$ T	R352Q	
1717-1G $\rightarrow$ T	T3381	
556delA		

Adapted from Ref 20 with permission

recently described mutations [G85E and 278+5G→A] are less clearly determinant with respect to the pancreatic sufficient and pancreatic insufficient phenotypes. Pancreatic sufficient patients with 'mild' mutations who have been followed for many years, show little or no deterioration of exocrine pancreatic reserve over an extended time period²¹. These patients, however, are susceptible to developing bouts of recurrent acute pancreatitis or chronic pancreatitis. Only a small subset of patients with two severe alleles have been noted to be pancreatic sufficient at diagnosis. Certainly, age of diagnosis influences this observation, since a much higher percentage of asymptomatic patients with two severe alleles detected by newborn screening have a pancreatic sufficient phenotype at diagnosis. These patients, most of whom have extremely low exocrine pancreatic function, are expected to show deterioration of function within a relative brief period of time leading to pancreatic insufficiency²². Thus, on the basis of the functional consequences of *cfr* gene mutations (see Figure 2), pancreatic insufficiency is strongly associated with homozygosity or compound heterozygosity for severe mutations in classes I, II or III. Since severe mutations, including $\Delta F508$ account for about 92% of CF chromosomes, approximately 85% of CF patients (92×92) would be predicted to have the PI phenotype. Conversely, 15% of patients with one or two mild mutations belonging to class IV or class V should be pancreatic sufficient.

Meconium ileus

Meconium ileus, which is the earliest clinical manifestation of CF, develops prenatally with obstruction of the distal ileum by thick adherent meconium. The presence of meconium ileus in the newborn period is strongly suggestive of CF, but meconium ileus may be seen in its absence. CF meconium is markedly abnormal; it is viscid and strongly adherent to the intestinal epithelium. Altered physical properties of CF meconium is attributable to failure of the intestinal epithelium to secrete Cl^- which in turn might result in reduced intestinal water content and high concentrations of intraluminal macromolecules which include undegraded serum proteins, intestinal brush border enzymes, lysosomal enzymes and intestinal mucins. Meconium ileus occurs predominantly in patients with the pancreatic insufficient phenotype, but it is not a mandatory feature^{13,23}. One-fifth of pancreatic insufficient patients present with this intestinal phenotype and there is only a 30% concordance of meconium ileus among families with more than one affected sibling²³.

Thus, patients carrying two 'severe' mutations on both alleles, either homozygous or compound heterozygous for classes I, II or III appear to be susceptible to this complication. An early study suggested that the nonsense

mutation G542X is associated with an increased risk of meconium ileus, but this association has not been confirmed in a study of larger patient cohort. The CF genotype-phenotype consortium has reported a lower incidence of meconium ileus among compound heterozygotes carrying G551D/ $\Delta F508$ (6.4%) in comparison with 19.5% in patients homozygous for $\Delta F508$ ²⁴. Nevertheless, as will be discussed below, elegant studies in a CF mouse model, strongly suggest that second site mutations or 'modifier' genes are required for the clinical expression of meconium ileus.

Clues to the pathogenesis of meconium ileus-modifier gene effects in the CF mouse

Advances in gene targeting technology have resulted in the development of several mouse models of CF. These include 'Knock-out' CF mice generated by targeted disruption of the *cfr* gene²⁵, several mouse models with known *cfr* gene mutations [$\Delta F508$ ²⁶, G551D²⁷] or models where the normal human *cfr* gene is inserted into the mouse genome following targeted disruption of murine *cfr*²⁸. In the majority of mouse models, the affected homozygotes die of intestinal disease immediately after birth or at weaning after introduction of solid food. The gastrointestinal lesion is obstructive in nature, closely resembling meconium ileus. The distal ileum or proximal colon contains obstructive inspissated faecal material (Figure 6) which causes death following perforation and peritonitis. In contrast, most existing CF mouse models have minimal pancreatic and lung disease. Clarke *et al.*²⁹ suggested that an alternative, Ca^{2+} -activated Cl^- conductance present in the airways and pancreas, compensated for the lack of CFTR mediated Cl^- channel function in the exon-10 knockout mouse model. The absence of an alternative Ca^{2+} -activated chloride transport mechanism in the intestinal epithelial cells was

Figure 6 Intestinal obstruction resembling meconium ileus in the distal small intestine of a cystic fibrosis mouse. (From Dr R Rozmahel, with permission)

believed to explain the severe intestinal pathology in these mice. Furthermore, the lack of severe pancreatic disease in the CF mouse argues against a direct cause-effect relationship between pancreatic insufficiency and meconium ileus. An exon-1 knockout CF mouse developed by Rozmahel *et al.*³⁰ offers an explanation of the variable expression of the intestinal phenotype. Severe intestinal obstruction occurred in the majority of the homozygous mice causing death either a few days after birth or following the time of weaning. Interestingly, one-third of the mice survived on normal mouse chow well past 6 weeks of age. Thus, survival appeared to be determined by the severity of the intestinal phenotype, because none of the CF mice developed significant pulmonary or pancreatic disease.

Since these mice were bred in a mixed genetic background it was hypothesized that the prolonged survival of a sub-group of mice was genetically determined by an unlinked modifier gene. Electrophysiological studies were undertaken to establish the molecular basis for the prolonged survival. Nasal and rectal potential difference measurements, performed in a blinded fashion, showed that the long-living mice had an increased basal potential difference and an increased amiloride-sensitive component, when compared to wild-type mice but less than that observed for the more severely affected exon-10 knockout mice³¹. This suggested that a compensatory mechanism could be partially ameliorating the ion transport abnormalities caused by the absence of CFTR in the long-living mice. Whole-cell patch clamp studies and iodide efflux studies performed on crypt cells isolated from the long-living mice confirmed the presence of a Ca^{2+} -dependent Cl^- transport which was not present in wild-type intestinal cells³⁰. Rectal potential difference measurements of the long-living CF mice showed that chloride transport was stimulated by uridine triphosphate which stimulates Ca^{2+} and was inhibited by a non-CFTR Cl^- channel inhibitor. This effect was not observed in the wild-type mice nor in the severe exon-10 knockout mice. This observation provided direct *in vivo* evidence of the existence of calcium-mediated alternative chloride channels in the animals with the mild intestinal phenotype, which may be responsible for ameliorating the intestinal disease³¹.

Back-crossing experiments of long-living male mice with carrier females produced a higher proportion of long-living animals, which points to the presence of genetic modifier(s) for disease severity in mice. Molecular linkage analysis has shown an association between CF disease severity in the gastrointestinal tract and markers on mouse chromosome 7. The region containing the candidate modifier gene corresponds to human chromosome 19q13. Several candidate genes from this region are being investigated as potential modifier genes. These studies provide the first

clear evidence that the severity of CF may be modified by another gene locus and form the basis of ongoing studies by sib-pair analysis of the contribution of an intestinal modifier locus at human 19q13. Preliminary studies strongly demonstrate that genetic background will influence the disease phenotype even in patients with the same CFTR mutation and that a number of modifier genes are likely to influence various phenotypic manifestations of CF.

The CF mouse model has provided additional advances in our understanding of the relationship between genetics and intestinal disease. Gabriel *et al.*³² showed that the small intestine of the exon-10 knock-out mouse fails to secrete fluid in response to cholera toxin, a potent stimulator of cAMP chloride secretion. Heterozygote mice expressing half the normal amount of CFTR secreted 50% of the normal fluid volume in response to cholera toxin³². This provocative study lends support to the hypothesis that human CF heterozygotes possessed a genetic advantage by surviving the potentially life-threatening effects of infectious diarrhoeal illnesses induced by cAMP activated toxins.

Atypical disease

Certain clinical conditions are now associated with increased frequency of CF gene mutations. These include male genital defects causing obstructive azoospermia, recurrent pancreatitis, nasal polyposis and a variety of chronic respiratory conditions. In the individual patient, the relationship between a particular clinical condition and CF disease may be difficult to resolve unless genotyping reveals

Figure 7 The severity of disease in various organs affected by cystic fibrosis (CF) may be modulated by intrinsic (genetic) or extrinsic (environmental, iatrogenic or therapeutic) factors. Genetic factors include the functional effects of mutations within the *cftr* gene as well as modulating effects by other genes, as suggested by a CF murine model. CFTR = Cystic fibrosis transmembrane conductance regulator

the presence of two known CF-causing gene mutations or if there is clear evidence of elevated sweat chloride concentrations or abnormal ion transport across the nasal epithelium. It should be recognized, however, that the absence of a known CF gene mutation does not eliminate the diagnosis, since many CF gene mutations remain unidentified.

CONCLUSION

From a conceptual point of view (Figure 7), three factors appear to contribute to the variability of the CF phenotype. First, the specific pair of *cftr* gene mutations that each patient inherits clearly influences disease manifestations. The best direct association between genotype and phenotype is in the exocrine pancreas. Even variations within a single CFTR allele can influence the phenotype. The phenotypic effects of R117H (class IV missense mutation) is modulated by the number of thymidine repeats in intron-8 which acts as an acceptor-splice site for exon 9^{33,34}. When R117H is associated with 5-thymidines (5T variant) only about 40% of the CFTR mRNA transcripts are full length. In contrast, with R117H with 7-thymidines (7T variant) the intron acceptor, 90% of transcripts are full length. R117H associated with the 5T variant is more likely to occur in a pancreatic sufficient CF patient or in a male with congenital bilateral absence of the vas deferens. Secondly, the patient's genome appears to affect the CF phenotype. The exciting studies of intestinal disease in CF mice strongly suggest that genes other than *cftr* may compensate for absent or dysfunctional CFTR Cl⁻ channels. Lastly, the CF phenotype is almost certainly influenced by environmental factors or the effects of therapeutic interventions. The CF airways may be more susceptible to environmental toxins or environmental pathogens than, for example, the liver or the pancreas. The wide range of pulmonary disease seen in patients with the same CFTR gene mutations may at least in part be explained by environmental factors.

In conclusion, tremendous progress has been made since the discovery of the CF gene, concerning the relationship between the CF gene and phenotypic variability. This is particularly true for the pancreas and the intestinal tract. We are learning from the mouse model that secondary genetic factors influence the phenotype. Clearly, a variety of therapeutic interventions can be seriously contemplated based upon knowledge of the functional defect. Integration of clinical research with fundamental discoveries will help to guide our attempts to achieve a cure or control of disease affecting patients with CF.

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