Review Article

Recent advances in cystic fibrosis

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Introduction

The successful identification of the gene that is altered in cystic fibrosis (CF) represents the first example in which a disease gene was cloned on the basis of genetic linkage data.^{1–3} The success of this approach was facilitated by the availability of three-generation families with multiple affected individuals and the availability of objective criteria for diagnosing CF. In the 4 years since the cloning of the CF gene, our understanding of the molecular pathology of CF has emerged with remarkable speed. These advances are reviewed below.

The cystic fibrosis gene

The CF gene, now known as the cystic fibrosis transmembrane conductance regulator (CFTR) gene, is localized on the long arm of chromosome 7, spans approximately 230 kb of DNA and contains 27 exons.^{1–4} Initial evidence that the CFTR gene was the gene mutated in CF came from DNA sequence analysis of cDNA clones from CF patients. This comparison identified a 3 base-pair deletion in exon 10 which would result in the loss of a phenylalanine residue at position 508 (ΔF_{508}) in CF but not in normal individuals.² Since then, over 300 CFTR gene mutations have been detected (Cystic Fibrosis Genetic Analysis Consortium, unpublished). These consist of missense, stop-codon, frameshift and splice site mutations that occur throughout the gene. Missense mutations represent 55% of mutations identified so far, while the remainder of mutations are accounted for by nonsense, frameshift and splicing mutations. A single large deletion, deleting exons 4–7 and 9–13 has been identified on a Spanish CF chromosome;⁵ otherwise, no other gross deletions have been reported, which is surprising for a gene of this size. The distribution of these mutations within the CFTR gene is relatively symmetrical, with the majority occurring in exons 4, 7, 11, 13, 17b and 19.

The frequency of the ΔF_{508} mutation differs significantly among various ethnic groups, being highest in Denmark and lowest in Turkey. In Northern Europe, the overall frequency is approximately 71%, while in Southern Europe it varies from 45%–55%.⁶ Why this mutation should be so frequent among Caucasians is unclear. Heterozygote advantage in the form of resistance against enterotoxin-elicited diarrhoeas, or genetic drift represent the most likely explanations. The overall frequency world-wide of mutations other than the ΔF_{508} mutation is extremely low. Some of these rare alleles segregate with specific ethnic groups. For example, the W1282X mutation accounts for 48% of CF chromosomes in the Ashkenazi Jewish population, and the 621 +1G→T mutation for 23% of French Canadian CF chromosomes.^{7,8} Knowledge of the ethnic distribution of CFTR mutations is therefore essential for prenatal diagnosis and heterozygote screening.

Structure and function of CFTR protein

The CFTR coding region encodes a 1,500 amino-acid protein that shares sequence homology with a superfamily of proteins involved in the transport of molecules across membranes.² CFTR contains two repeated motifs, each containing a membrane spanning domain and a hydrophilic nucleotide binding domain (NBD). These two motifs are separated by a highly charged cytoplasmic domain, named the regulatory, or R domain.² The transmembrane domains and the nucleotide binding folds are shared by all members of this superfamily of proteins, whilst the R domain is unique to CFTR.² Cross-species analysis of the DNA sequence encoding CFTR shows significant conservation in structure between the human CFTR cDNA and its bovine, mouse, rat and the dogshark homologues, suggesting that the function of CFTR is conserved throughout evolution.⁹

The most consistent biochemical defect in CF epithelial cells is a marked reduction in the permeability of chloride (Cl^-) ions across their

apical membrane.¹⁰ Is CFTR a chloride channel, or is it a transport protein involved in the delivery of chloride channels to the apical membrane of epithelial cells? There is now little doubt that CFTR is a chloride channel. Three lines of evidence support this assertion. First, transfection of non-epithelial cell lines, which do not normally express CFTR, with the normal CFTR cDNA resulted in a plasma membrane chloride conductance that was stimulated by cAMP.¹¹ Secondly, certain mutations within the transmembrane region of the CFTR protein alter the interanionic selectivity of the channel without affecting its regulation.¹² Finally, reconstitution of purified CFTR into a lipid bilayer induced chloride channels with the correct properties.¹³

CFTR may have functions other than those of a small conductance chloride channel in the apical membrane of epithelial cells. A defect in endocytosis and exocytosis was demonstrated in a CF pancreatic cell line (CFPAC), which was corrected by the expression of wild-type CFTR.¹⁴ Membrane recycling regulates the secretion and localization of proteins through and on the cell membrane. A defect in this process may have diverse effects and could explain abnormalities in sodium transport and in the sialylation and sulphation of glycoproteins that are also found in CF. CF cells may also display a defect in the acidification of the trans-Golgi network, of prelysosomes and of endosomes because of diminished chloride conductance in the membrane of these intracellular organelles.¹⁵ However, it is unclear if this chloride conductance is directly mediated by CFTR.

The relationship between CFTR and other epithelial chloride channels is unclear. Observations from the study of 'knockout' mice lacking CFTR suggest that CFTR regulates the function of another distinct protein, the outward rectifying chloride channel.¹⁶ Comparison of the cell-specific expression of CFTR and the multi-drug resistance P-glycoprotein, a putative volume-regulated chloride channel with significant structural homology to CFTR, found that both genes have a complementary pattern of expression in all tissues studied. This led to the hypothesis that CFTR and P-glycoprotein serve similar roles in epithelial cells.¹⁷

Molecular mechanisms of CFTR chloride channel dysfunction

CFTR gene mutations are thought to disrupt CFTR function by a number of distinct mechanisms. The majority of mutations, including ΔF_{508} , cause disease by disrupting the intracellular transport and processing of the CFTR protein.¹⁸ Wild-type CFTR is glycosylated first in the endoplasmic reticulum and then in the Golgi, from

where it is ultimately transported to the plasma membrane.¹⁸ Most mutant forms of CFTR do not become fully glycosylated and are trapped either in the endoplasmic reticulum or the Golgi, and subsequently degraded.¹⁸ This appears to be a temperature-sensitive event. At 27°C, the mutant CFTR forms functionally active channels but at 37°C it is subjected to biosynthetic arrest.¹⁹ Immunohistochemical studies, which showed no CFTR at the apical membrane of the absorptive part of the sweat duct of CF patients who are homozygous for the ΔF_{508} mutation, or compound heterozygotes for the ΔF_{508} , 621 + 1G→T and 171 + 1G→A mutations, support this hypothesis.²⁰ These findings have important implications. In patients in whom no CFTR is correctly localized in epithelial cells, therapeutic measures should be directed towards the replacement or bypassing of the protein, or towards the development of drugs that can promote intracellular transport of CFTR. These approaches will only succeed if mutant CFTR, such as ΔF_{508} -CFTR, retains normal functional activity. Reconstitution of ΔF_{508} -CFTR in a lipid bilayer was associated with chloride channel function indistinguishable from wild-type CFTR, supporting this assertion.²¹

CF mutations can disrupt CFTR by three other mechanisms. Stop-codon and frameshift mutations are thought to cause CF by resulting in truncated or absent CFTR protein.²² Some mutations within the transmembrane domain of CFTR are associated with a chloride channel that is appropriately processed and regulated but result in abnormally low chloride currents per cell.²³ These mutations in homozygous or heterozygous form are found in pancreas-sufficient CF patients, a clinically less severe group. This would suggest that mutations associated with CFTR protein that retain significant chloride transport function are also associated with less severe clinical forms. Finally, CFTR mutations altering amino acids in the nucleotide binding fold of the protein may alter chloride channel function by disrupting its correct regulation by ATP.

Animal models

Three murine models of cystic fibrosis have been generated from an embryonic stem cell line in which the mouse *cftr* gene was disrupted at exon 10.^{24–26} All murine models display absent or blunted cAMP-mediated chloride conductance in airway and intestinal epithelial cells, comparable to the ion transport defect of human CF epithelial cells. Two sets of mutant mice are characterized by failure to thrive, and bowel obstruction resembling meconium ileus, while another set are characterized by no overt clinical disease up to 30 days

post-partum. The mild intestinal phenotype of these *cf/cf* mice is probably accounted for by expression of very low levels of residual wild-type protein.²⁵ In contrast to the human disease, the majority of animals analysed so far do not display pulmonary or pancreatic disease. These observations have implications for the debate on the relative contribution of the airway surface epithelium and of the submucosal glands to the development of lung disease in CF patients, since the mouse lower respiratory tract is devoid of submucosal glands. In relation to pancreatic involvement, mouse *cftr* mRNA and protein are found at low levels in the murine pancreas, which may explain the absence of pancreatic disease in all *cf/cf* mice.

Clinical research

These basic research findings are already changing clinical practice. The two main areas of advance are screening for CF and the design of new treatments. These include gene therapy, which could provide a virtual cure for the disease, ion transport drugs aimed at reversing the defect in epithelial cells, anti-inflammatory agents and DNase. For pre-terminal disease, these new treatments are unlikely to help but the early results of lung transplantation are most encouraging.

Screening

About 85% of carriers of the CF gene can now be detected by testing for the four commonest mutations. This allows three-quarters of couples at risk of having a CF child to be identified. Pilot screening programmes have been successful and guidelines for screening have been published.²⁷ At present screening tests are being used by those with a family history of CF but wider application in antenatal clinics is likely to come.

Gene therapy

The goal of gene therapy is to insert the normal gene for CFTR into affected cells, and so correct the ion transport defect and return the function to normal. The gene is now available in sufficient quantities, vectors have been developed, both adenovirus and liposome systems have been shown to work *in vitro*, and liposome-based systems have corrected the CF defect in transgenic mice.^{28,29} This shows that gene therapy is feasible and human studies are now beginning. The adenovirus vector appears safe at low doses, but local inflammation and an immune response may limit the use of

higher doses. Liposomes do not appear to cause inflammation but the effects of long-term repeat dosing have not yet been evaluated. There has not, to date, been a comparison of the two approaches for efficiency of DNA transfection. There is still a long way to go before this encouraging start is translated into realistic treatment. It is not yet clear which cell type within the lungs should be targeted and it may be that the submucosal glands are at least as important as the epithelium. Furthermore the duration of expression of the inserted gene is not known and this is essential information for working out dosing schedules. Finally the safety of gene therapy needs to be established both for single and multiple applications before phase 3 clinical trials can begin.

Ion transport therapy

The two airway ion transport abnormalities in CF (increased sodium transport and chloride impermeability) can both be modified with drugs. Nebulized ATP or UTP can stimulate chloride secretion by opening a channel distinct from CFTR,³⁰ while nebulized amiloride can block the increased sodium absorption. If, therefore, these defects are central to pathogenesis, their correction might be expected to prevent, or conceivably to treat CF lung disease.

There is conflicting evidence in favour of this form of treatment: amiloride has been shown to improve mucociliary clearance in one study,³¹ but to have no effect in another.³² Similarly, the only two clinical trials give different answers; the first showed a benefit when all other respiratory treatment had been withdrawn,³³ while the second showed no benefit from giving amiloride as an additional treatment.³² Larger studies are under way.

Anti-inflammatory agents

The airways in CF are colonized by bacteria, chiefly *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Initially, these remain in the airway lumen with little in the way of tissue invasion and inflammation. Over the years the number of organisms increases as does the inflammatory host response to their presence. It is probably this inflammation which causes the lung damage and a number of anti-inflammatory strategies are being developed. These include anti-proteinases (alpha-1-anti-trypsin,³⁴ secretory leucocyte protease inhibitor, etc.), dietary measures to enhance leukotriene production,³⁵ as well as the use of both steroidal and non-steroidal anti-inflammatory drugs. Encouraging data are accumulating to support these

approaches and a number of phase 3 trials are in progress or being planned. For example, in one study prednisolone was reported to improve a number of clinical and laboratory parameters in children with CF without adverse effects³⁶ but a subsequent larger study has failed to confirm these findings.³⁷ Trials of inhaled steroids are in progress.

DNase

Infected sputum contains DNA, derived chiefly from neutrophils, which increases the viscosity. This DNA can be broken down by the enzyme DNase, which has been shown to liquify sputum and to reduce its viscosity³⁸ and is available in the recombinant human form (rhDNase).

Phase 2 clinical studies have shown improvement in lung function following nebulized rhDNase³⁹ with forced expired volume in one second and forced vital capacity increasing by 13.3% and 7.2%, respectively, after 10 days of treatment. These changes reverted to pretreatment levels within a few days of stopping. A large multi-centre phase 3 study involving 968 patients has also shown encouraging results. rhDNase treatment was associated with improved lung function, fewer chest infections and a reduction in hospital days, as well as with a reduction in symptoms and an improvement in quality of life. There were no serious adverse effects and the only frequent side effect was voice alteration. These studies are all highly encouraging and suggest that rhDNase will be a real addition to existing treatments.

Transplantation

More than 100 people with CF have now received lung transplants (usually heart and lung) and the results have been encouraging.⁴⁰ The one year survival is around 65%, which is comparable with that for other disease groups receiving lung transplants, and there are long-term survivors who are

well and leading normal lives 7 years later. The chief problems are infection and rejection in the first postoperative year and obliterative bronchiolitis thereafter. While there are no universally agreed contraindications to transplantation, the risks of high-dose steroids, previous pleural surgery and active mycobacterial or fungal infection are usually considered unacceptable. Most centres do not intubate and ventilate patients who are dying while on the waiting list.

The most difficult problem is the shortage of donor organs, and there will never be enough to satisfy the needs of all people dying from respiratory failure. Research is, therefore, continuing into the possible use of animal organs for human transplantation.

Clinical trials

Even without these new treatments the outlook for a child born with CF has never been better, with an estimated life expectancy of 40. Existing treatments are effective and with the increasing use of home care systems it is becoming more and more possible for someone with CF to lead a relatively normal life. Against this background of improved prognosis there are some special problems in evaluating all these promising new approaches in clinical trials. In the first place there may be too many phase 3 trials competing for a limited number of suitable patients. Second, the very slow rate of progress of CF lung disease makes the value of any new treatment very difficult to prove without a very long period of study. Third, some new treatments (for example, gene therapy) may be most appropriate for presymptomatic disease and so raise difficult ethical problems. Finally, one of the chief priorities of CF patients may be for a new treatment to replace their existing regimen rather than an addition. These factors will combine to provide new and special challenges for the design of CF clinical trials in the future.

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