Pre-clinical and clinical endpoint assays for cystic fibrosis gene therapy

Uta Griesenbach\textsuperscript{a,c,*}, A. Christopher Boyd\textsuperscript{b,c}

On behalf of the UK Cystic Fibrosis Gene Therapy Consortium

\textsuperscript{a}Department of Gene Therapy, Faculty of Medicine, Imperial College London, UK
\textsuperscript{b}Medical Sciences (Medical Genetics), University of Edinburgh, Molecular Medicine Centre, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, UK
\textsuperscript{c}UK Cystic Fibrosis Gene Therapy Consortium, UK

Received 7 April 2004; accepted 22 February 2005
Available online 23 May 2005

Abstract

The credibility and hence value of pre-clinical and clinical cystic fibrosis gene therapy studies depend on the assays used to evaluate gene transfer. Awareness of assay suitability, sensitivity and variability is therefore crucial to the design of experimental programmes. Here, we review the assays that are in use to assess the efficacy of gene transfer in pre-clinical and clinical CF gene therapy research, highlight their weaknesses and suggest possible new strategies that may help to overcome current limitations.

© 2005 European Cystic Fibrosis Society. Published by Elsevier B.V. All rights reserved.

Keywords: Cystic fibrosis; Gene therapy; Assays; Pre-clinical; Clinical

Contents

1. Introduction ............................................................ 90
2. Pre-clinical endpoints of airway gene transfer .......................................... 90
2.1. Gene transfer in wild-type animals ........................................................ 90
2.1.1. Reporter genes in lung or nasal turbinate homogenate ................................ 90
2.1.2. Historical analysis of reporter gene expression ...................................... 91
2.2. Assessment of CFTR expression and function in vitro or ex vivo .......... 92
2.3. CFTR gene transfer and endpoint assays in the CF mouse ...................... 93
2.3.1. Quantification of recombinant CFTR mRNA ........................................... 93
2.3.2. Quantification of recombinant CFTR protein ........................................... 93
2.3.3. Quantification of recombinant CFTR chloride channel function .......... 94
2.4. Airway xenograft model systems ............................................. 95
3. Clinical endpoints of CFTR gene transfer ............................................ 95
3.1. Direct assays of CFTR .................................................. 95
3.1.1. Quantification of CFTR mRNA .................................................. 95
3.1.2. Quantification of CFTR protein .................................................. 95
3.1.3. Quantification of CFTR chloride channel activity ................................ 96
3.2. Indirect assays of CFTR .................................................. 96

* Corresponding author. Department of Gene Therapy, Faculty of Medicine, Imperial College, 1B Manresa Road, London SW3 6LR, UK. Tel.: +44 207 351 8339; fax: +44 207 351 8340.
\textit{E-mail address:} u.griesenbach@imperial.ac.uk (U. Griesenbach).

1569-1993/$ - see front matter © 2005 European Cystic Fibrosis Society. Published by Elsevier B.V. All rights reserved.
Cystic fibrosis (CF) is the most common lethal mono- genic autosomal recessive disease in the Caucasian pop- ulation and affects approximately 70,000 individuals world- wide. Although several organs are affected, severe lung disease is the cause of most morbidity and mortality in CF individuals [1]. Overall the CFTR protein is not expressed in airway epithelial cells [2]. It is currently unclear which of these cell types should be the main targets for CF gene therapy, but considering that CF (at least in the early stages) presents as a small airway disease, we are working on the basis that airway epithelial cells (AECs) are highly relevant targets for CF gene therapy.

The gene underlying CF, the cystic fibrosis transmem- brane conductance regulator (CFTR), was cloned in 1989 and encodes a chloride channel, which is located in the apical membrane of epithelial cells [3]. Two competing theories attempt to explain the relationship between defective ion transport and CF lung disease. The “high salt” hypothesis postulates that airway surface liquid (ASL) in CF patients has a higher salt concentration than in non-CF individuals, due to the inability of airway epithelial cells to absorb chloride ions. This in turn may lead to impaired mucociliary clearance and reduced activity of anti-microbial peptides [4]. The “low volume” hypothesis (in our view, the one better supported by the available data) postulates that due to absent chloride transport and increased sodium absorption the height of the ASL is reduced, leading to impaired mucociliary clearance [5]. Reduced mucociliary clearance, which is explained by both theories, leads to formation of thickened dehydrated mucus, which provides an ideal environment for bacterial infection, leading to chronic inflammation and ultimately organ failure in the CF lung. Whether inflammatory pathways are dysregulated in CF airways independent of infection, or whether the inflammatory response following bacterial infection is exaggerated and disproportional, has been widely debated, but a conclusive consensus has not been reached.

Proof-of-principle for CFTR gene transfer was quickly established in vitro [6] and in an animal model [7]. The first clinical trials in CF patients were carried out in 1993 and to date about 30 trial protocols, most of which have been completed, are published (see www.wiley.co.uk). Although early hopes that CF gene therapy in the lung would be straightforward due to the topical accessibility of the lung were not confirmed, steady progress has been made over the years. Here, we review the assays that are in use to assess successful gene transfer in pre-clinical and clinical CF gene therapy research, but also highlight their weaknesses and suggest possible new strategies that may help to overcome limitations of the current assays.

The UK Cystic Fibrosis Gene Therapy Consortium (UKCGT) has been set up with the help of the UK Cystic Fibrosis Trust to combine the research efforts of three CF gene therapy groups in Edinburgh, London and Oxford, with the expressed aim of developing effective formulations for clinical use. This ambitious initiative has motivated us to channel substantial resources into developing and testing pre-clinical and clinical assays for CF gene therapy. Where relevant, the consortium’s developments in these areas will be described below.

## 2. Pre-clinical endpoints of airway gene transfer

### 2.1. Gene transfer in wild-type animals

#### 2.1.1. Reporter genes in lung or nasal turbinate homogenate

In the early years of gene therapy research, many viral and non-viral gene transfer agents (GTAs) were assessed in cell culture. However, it became quickly apparent that cell culture results do not predict the performance of GTAs in the lung. This is probably due to differences in proliferation status (most cells in the lung are terminally differentiated and do not divide), privileged access to the basolateral membrane in cell culture but not in vivo, and possibly a different distribution or number of receptors. As a consequence of this divergence, it has become essential to evaluate most GTAs for CF gene therapy in the airways of animal models.

The distribution of GTAs in animal models associated with specific administration techniques has to be carefully examined. The mouse is the most frequently used model and GTAs are often administered through bolus injections either into the nose [8], or directly into the trachea [9,10]. This type of administration may not be the most desirable for efficient airway gene transfer, because the GTA will have only a short contact time with the surface of airway epithelial cells and will mainly accumulate in the alveolar region (the so-called “pooling effect”). Although several groups have attempted to nebulise GTAs into the mouse lung [11,12], this has generally been inefficient.

The nasal epithelium of mice expresses CFTR [13], shows the characteristic bioelectric properties of humans and is therefore an appropriate surrogate organ. Slow
perfusion of GTA onto the mouse turbinate via a thin catheter ensures prolonged contact time with the epithelial surface [14] and allows characterisation of GTAs without the added complication of limited contact time. In addition, larger animal models such as primates [15,16], pigs [17] and most recently sheep [18] have been used for airway gene transfer and potentially allow testing of gene transfer using clinically relevant delivery methods such as nebulisation. An important advantage of the sheep and other large animal models are that they allow repeat delivery experiments on the same scales of material and time that are appropriate for clinical use [19], with clinically used delivery systems.

Reporter genes encoding chloramphenicol acetyl transferase (CAT), firefly luciferase (Lux) or β-galactosidase (β-gal) (Table 1) have been widely used for the identification and characterisation of effective viral and non-viral GTAs [10,14,20]. Typically, reporter gene activity is measured in tissue homogenates using commercially available non-radioactive chemiluminescent assays or ELISAs. In our experience, Lux is the fastest, cheapest and most sensitive assay (UKCFGT unpublished data). A major disadvantage of reporter gene assays carried out on tissue homogenates is that they do not reveal which cells have been transfected, nor how many or where they are. For example, a candidate GTA for CF gene therapy may be more efficacious in non-epithelial cells (which are not a target for CF gene therapy) and would give misleading results in any homogenate-based reporter gene assay.

Table 1

<table>
<thead>
<tr>
<th>Abbreviated name</th>
<th>Full name</th>
<th>Mainly used for</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
<td>Fluorescence microscopy</td>
</tr>
<tr>
<td>βgal</td>
<td>β-galactosidase</td>
<td>Histological staining</td>
</tr>
<tr>
<td>CAT</td>
<td>Chloramphenicol acetyl transferase</td>
<td>Chemiluminescence assay</td>
</tr>
<tr>
<td>Lux</td>
<td>Firefly luciferase</td>
<td>Chemiluminescence assay</td>
</tr>
</tbody>
</table>

More recently several groups, including our own, are attempting to answer the “which cells where?” question after non-viral gene transfer. This requires careful optimisation of the signal to noise ratio due to poorer transfection efficiencies. Cunningham et al detected β-gal expression in 46% of porcine airway epithelial cells with anti-β-gal antibodies after transfection with the non-viral vector LID [17]. However, it has proven difficult to reproduce these results with other non-viral vectors. Our group is currently evaluating more sensitive immunohistochemistry-based techniques such as tyramide signal amplification (TSA) [25] or rolling circle amplification (RCA) [26] for the detection of recombinant protein (reporter gene or CFTR) after gene transfer. However, all these techniques share the concern that protein expression required to see a positive signal may be much higher than the endogenous CFTR levels we are trying to reproduce in order to correct the defect. Ideally, screening procedures should avoid discarding GTAs that fail to give a positive reporter gene signal but are capable of delivering a therapeutic fraction (suggested to be 5% [27,28]) of the comparatively low CFTR expression levels seen in normal individuals. There is evidence that under certain circumstances overexpression of CFTR is deleterious [29,30]; also CFTR expression in vivo is markedly heterogeneous [31]. It is possible therefore that an efficient GTA would deliver too much CFTR, with unknown consequences. For this reason it is essential to further test any GTAs with CFTR as transgene, and ensure that normal CFTR function with no undesirable side-effects is observed (see below). If overexpression in vivo of CFTR proves to be a problem, we can address it in several ways. One is to use a weaker promoter to reduce transcription or to use the endogenous CFTR promoter, which would ensure appropriate expression in the right cell-type. However, although much interesting development has occurred [32–34], a functionally verified tissue-specific CFTR promoter suitable for gene therapy is currently not available. The

2.1.2. Histological analysis of reporter gene expression

Histological analysis of reporter gene expression using immunohistochemistry [17], green fluorescent protein (GFP) visualisation [21] or direct staining techniques [such as the use of 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal)], which stains cells that express β-gal (β-gal blue [22]) are more labour intensive, but also more informative than reporter gene assays on homogenates, as these may reveal which cells have been transfected. However, most of these techniques are not very sensitive and require fairly high expression of the reporter gene in each cell [23]. (Thus, for example, X-gal staining has been shown to underestimate transfection efficiency when compared to immunohistochemical lacZ detection [24].) They have therefore generally been limited in practical terms to the analysis of

**Fig. 1.** Efficient transfection of mouse nasal epithelium with recombinant Sendai virus expressing β-galactosidase. Close to 100% of airway epithelial cells express β-galactosidase, here visible as blue precipitate.
other is to change the codon usage of the transgene to reduce translation. Given these possible interventions, current efforts are aimed at maximising CFTR expression to compensate for the low efficiencies of (in particular) non-viral GTAs.

2.2. Assessment of CFTR expression and function in vitro or ex vivo

In addition to optimising gene transfer, the capacity of GTAs to make functional CFTR has to be determined. Non-viral GTAs usually contain eukaryotic expression plasmids carrying CFTR cDNA. If the same batch of plasmid is used in different formulations, it is generally only necessary to test each new batch of plasmid once. For this purpose the plasmid should be combined with a lipid or polymer formulation that is known to transfect the chosen in vitro cell type CFTR sequence disadvantages viral growth. This may be a particular problem if the presence of wild-type CFTR transgene during viral amplification by acquiring mutations. This is necessary because viruses may silence the CFTR transgene during viral amplification by acquiring mutations. This may be a particular problem if the presence of wild-type CFTR sequence disadvantages viral growth.

Several in vitro/ex vivo CFTR functional assays have been developed and their strengths and weaknesses will be briefly discussed.

(a) Agonist-mediated halide efflux: this assay, first introduced by Venglarik [35] is based on the cAMP-mediated efflux of halide through the CFTR channel from the cell interior into the extracellular medium. Medium is collected at frequent intervals from adherent cells preloaded with radioactive halide. Cyclic-AMP mediated chloride channels are more permeable to iodide than many other chloride transport processes [36], so $^{125}$Iodide (half life $= 59.6$ days) is conveniently used instead of the natural chloride radiotracer chlorine-$36$ (half life $= 300,000$ years). An alternative to radiotracer based halide efflux measurement of CFTR channel activity is the assessment of effluxed iodide/chloride using an ion-selective electrode (ISE). This is essentially an electrochemical sensor based on a thin film halide-selective membrane recognition element. Basically, the ISE produces a potential that is proportional to the concentration of the halide. The electrodes are relatively inexpensive and simple to use and have been shown to give equivalent results to the radiotracer ‘sample-replace’ assay described above [37].

(b) Cyclic-AMP-dependent halide CFTR channel activity assay using quinolinium salt-based halide-sensitive fluorescent probes (6-methoxy-N-(-sulphopropyl)quinolinium (SPQ) and N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE)). The assay, developed by Verkman and colleagues, follows the secretion of halide from epithelial cells by measuring dequenching of intracellular SPQ/MQAE fluorescence by the effluxing halide in response to cAMP-stimulation [38]. Many studies have used the fluorescence indicators to assess chloride channel efflux. In these studies, iodide is used as a convenient chloride analogue because SPQ/MQAE fluorescence is more effectively quenched by iodide than by chloride (halide ion selectivity: iodide $>$ bromide $>$ chloride) thus improving the signal to noise ratio in SPQ/MQAE spectrofluorimetry. Both SPQ and MQAE spectrofluorimetry has been used in numerous studies to assess halide efflux in adherent cultured cell lines and primary epithelial cells [9,39]. However, these fluorometric assays are more expensive and technically more difficult than the radioactive efflux assays. In addition, fluorophore loading is fairly invasive and the post loading recovery window needs to be optimised, to avoid passive leaking of fluorophores out of the cells. However, this time window is often not compatible with post-transfection protocols.

(c) Ussing chamber: this allows assessment of the ion transport properties of epithelial cell layers, either as native tissue samples or grown in vitro on permeable filters [40]. The change in short circuit current (ΔIsc) in response to IBMX (a phosphodiesterase inhibitor) and/or forskolin (a cAMP agonist) is the most used measurement (e.g. see [41]). ΔIsc is significantly lower in CFTR-deficient cells, so the efficacy of gene therapy intervention can be measured by an increase of ΔIsc towards normal levels. Importantly, it is comparatively easy to discriminate the response of CFTR from other CFTR-regulated channels, by using specific inhibitors such as DIDS (4,4-dioctanoylstilbene-2,2'-disulfonic acid) and glibenclamide, which inhibit the outwardly rectifying chloride channel (ORCC) and CFTR, respectively.

(d) Patch clamping: this is a demanding technique requiring the use of a microscope and a solute-containing glass pipette (which acts as an electrode) to isolate a cell or patch of membrane containing one or more ion channels. By this means, current across membranes of a single cell can be measured. Because single channels can be isolated, the properties of the channel can be examined in exquisite detail. Whole-cell patch clamp methods have been used to investigate CFTR gene transfer [42], but the time involved and difficulty of applying the method limit its usefulness in this domain. However it should certainly be considered as an endpoint in cases where indisputable evidence of functional CFTR at the apical membrane is required.

Overall, results obtained from radioactive and non-radioactive efflux assays and Ussing chambers described...
above represent average CFTR function in the entire sample population, whereas patch-clamping and SPQ/MQAE analysis assess CFTR function in individual cells.

For the purpose of vector testing we recommend the use of iodide efflux assays, as these are not technically difficult and can be done with relatively high throughput using transformed cell lines. However, one has to be certain that the cells to be transfected do not have any endogenous cAMP-dependent chloride channel activity. 293T cells (ATCC) are suitable for analysis of CFTR containing eukaryotic expression plasmids, as these cells do not have any endogenous cAMP-dependent chloride channel activity and transflect well (>90% of cells transfected) with Lipofectamine 2000 (Gibco Life technologies). When choosing a cell line for virus-mediated CFTR testing, one has to ensure that the virus transfects the cell line efficiently and this will likely vary depending on the virus to be tested. In our experience 293T cells (for example) are suitable for analysis of adenovirus and Sendai virus-mediated CFTR expression (Felix Munkonge, personal communication).

2.3. CFTR gene transfer and endpoint assays in the CF mouse

In our opinion, reporter gene expression in wild-type mice is of limited value, for the reasons highlighted above. Gene transfer experiments should use CFTR and relevant CFTR-based endpoint measurements wherever possible. Currently the CF knockout mouse is the only animal model for CF and, although these mice do not develop the characteristic CF lung disease, they have the same ion transport defect as CF patients in their nasal epithelium [43]. This, combined with the fact that the nasal epithelium can be exposed to GTAs in a time-controlled fashion (as described above), makes the CF mouse nose a useful model organ for assessing and optimising CFTR gene transfer.

Initially, it has been difficult and expensive to produce sufficient CF mice for large scale gene transfer screening programmes, because the mice were bred as heterozygotes, with only one in four pups being a homozygote CF knockout animal. In addition, most CF mice suffered from severe intestinal disease, required a special fibre-free diet and had a high post-weaning mortality rate. However, the gut disease in CF mice has been corrected through incorporation of a CFTR transgene under the control of the fatty acid binding protein promoter [44]. This promoter is thought to restrict CFTR expression to the gut and results in CF mice that are fertile, do not have gut disease and have normal post-weaning survival, but theoretically still have the ion transport defect in the nasal epithelium. These mice can be bred at similar cost to wild-type inbred animals and therefore make a large scale screening program in CF mice much more feasible. We are currently analysing the electrical properties of the nasal epithelium in these mice.

Two groups have recently shown that pre-treatment of CF mice with adenovirus-expressing CFTR ameliorates these exaggerated responses to *P. aeruginosa* and *B. cepacia* [45,46]. However, it is currently unclear how CFTR expression improves host response against bacteria, and beneficial pro-inflammatory responses to the adenoviral backbone cannot be completely ruled out.

2.3.1. Quantification of recombinant CFTR mRNA

Recombinant CFTR mRNA has traditionally been measured after gene transfer. This is either done as a semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) or more recently through quantitative real-time RT-PCR. Rose et al. have demonstrated that real-time TaqMan RT-PCR discriminates effectively between vector-specific and endogenous CFTR expression in the airways and quantifies both types of mRNA accurately [47]. However, RT-PCR assays (like the reporter gene assays described above) have so far been carried out on mRNA extracted from total tissue homogenates, and therefore suffer from the same limitations, i.e. that the identity of cells and the number of cells expressing the transgene cannot be determined. Laser capture microdissection (LCM) of murine bronchial epithelial cells coupled to real-time RT-PCR has previously been described as a way of measuring endogenous gene expression [48] and might be applicable to gene therapy as well. If successful, this would allow us to determine whether recombinant CFTR is made in airway epithelial cells and how the amount relates to endogenous CFTR expression.

The perceived difficulty of detecting CFTR protein has led to the use of in situ hybridisation as a means of measuring transgene expression. This technique has the theoretical advantage that probes can be made species or vector specific. The low abundance of CFTR message [49] means that in practice the method is difficult to perform; great care must be taken in order to separate any signal from background levels. Judicious design and preparation of a suitable probe is essential. Nevertheless, in situ hybridisation has been used successfully for CFTR analysis both in normal and transgenic animals, and in gene transfer experiments [49–51]. It is however an unusually demanding method and cannot be recommended as a routine endpoint assay.

2.3.2. Quantification of recombinant CFTR protein

Traditional methods for endogenous CFTR protein detection such as immunoprecipitation and Western blots have not been successful for the detection of recombinant CFTR protein after in vivo gene transfer, most likely related to the low sensitivity of these assays. In addition, they are generally carried out on tissue homogenates and will therefore suffer from the limitations already discussed. Western blotting has been used successfully to detect CFTR gene transfer in vitro [52,53], and once this efficiency is reached in vivo CFTR should be detectable by this means. An alternative method for protein detection is mass spectrometry. Most recently, a highly sensitive and high throughput SELDI-TOF (Surface Enhanced Laser Desorption/Ionization-Time Of Flight) system has been developed,
which is sensitive to the attomolar level [54]. In a popular embodiment of this technology, biological samples in solution are applied to a metal chip with surface characteristics that select out a desired subset of proteins. After further processing, the chip is subjected to laser-induced ionization for mass spectrometric analysis. Released proteins (which can range in size from peptides as small as 1.2 kDa up to large proteins of several hundred kDa) are fractionated by time-of-flight, which is directly related to molecular weight. The graphical output highlights each protein as a peak corresponding to a particular molecular size. Peak heights semi-quantitatively report on the relative amounts of protein in the sample. The main drawback is that identification of the proteins requires further analysis (e.g. microsequencing). However, in the context of CF gene therapy, this assay may provide useful information, especially if coupled to LCM. The UKCFGTC is actively exploring the use of SELDI-TOF technology for protein expression profiling.

Immunohistochemical detection of endogenous and recombinant CFTR expression has long been hindered by low expression levels, and poor antibodies. However, more recently several monoclonal and polyclonal anti-CFTR antibodies have been described [55] that show promise for immunohistochemical detection of CFTR. Endogenous CFTR can now be readily detected in human nasal brushings (Fig. 2). The levels of recombinant CFTR after non-viral gene transfer are likely to be lower than endogenous levels and more sophisticated amplification methods such as TSA and RCA will be helpful, and we are currently developing these. Immunohistochemistry may allow co-localisation of endogenous and recombinant CFTR if an epitope tagged CFTR cDNA [56] is used for gene transfer. This information would be very reassuring, as it would demonstrate that the protein defect can be corrected in “relevant” cells. Results from immunohistochemistry must be interpreted with great caution, however, for a number of reasons. One is that image interpretation is highly subjective. Because autofluorescence is usually present to some extent, it is important to conduct blinded analyses on randomised control and test samples. Another problem is that there is a non-linear relationship between signal and antigen concentration. This means, for example that a cell expressing CFTR at just below a certain threshold might be scored as negative, while a neighbouring cell expressing slightly more protein will be easily detected. Careful titration of antibody dilutions is one suggested way of approaching this problem [57].

It is currently unclear how much CFTR protein expression is required to correct the chloride and sodium transport defects. Importantly, the question of whether high-level expression in a few cells or low-level expression in many cells is required will remain unanswered until better animal models and assays become available.

2.3.3. Quantification of recombinant CFTR chloride channel function

As mentioned above, CF knockout mice show the characteristic CF ion transport defect (low chloride secretion, high sodium absorption) across the nasal epithelium [58]. Protocols for nasal potential difference (PD) measurements in mice have been developed by several groups and discriminate CF and non-CF mice reliably [59,60] There are several important differences between non-CF and CF animals: (1) CF mice have a higher basal PD than non-CF mice due to the increased sodium absorption; (2) after perfusion with a low chloride solution which generates a driving force for chloride secretion, non-CF mice respond with an increase in chloride secretion (visible by hyperpolarisation in PD), whereas CF mice do not show this change (Fig. 3). The absolute level of increase depends on

---

Fig. 2. Detection of endogenous CFTR in human airway epithelial cells using an anti-CFTR specific antibody. Apically localised CFTR protein is visible as a green layer and the DAPI-stained cell nucleus appears in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

---

Fig. 3. Nasal potential difference measurements in CF and non-CF murine nasal epithelium. Following measurement of basal PD (BL), a solution containing amiloride (Am) is perfused into the nasal cavity, which leads to a drop of PD in CF and non-CF mice. Subsequently the nasal epithelium is perfused with a low chloride solution (LC), which generates a driving force for chloride secretion and increased PD in non-CF, but not in CF mice.
where in the nose the PD is measured, and appears to be dependent on the relative distribution of respiratory and olfactory epithelium lining the nasal cavity. Parsons et al. suggested measuring nasal PD with catheters inserted about 2.5 mm into the mouse nose as this restricts readings to respiratory epithelium [61]. Interestingly, however, the greatest discrimination between CF and non-CF signals is at around 5 mm, where predominantly olfactory epithelium is being measured.

Recently, several reports have been published showing that the chloride transport defect in CF animals can be partially corrected after gene transfer of the CFTR gene [61–64] indicating that these measurements are suitable and relevant endpoint assays for CFTR function.

It is not clear how these ion transport defects relate to disease pathology. It would, therefore, be of great advantage to include more clinically relevant assays, based for example on bacterial adherence to epithelial cells and airway surface liquid height measurements, in the pre-clinical screening program. From a clinical point of view, bacteria-based assays are particularly important, because it is widely acknowledged that chronic bacterial colonisation leads to inflammation and ultimately severe lung damage in CF and it would therefore be encouraging to demonstrate that bacterial load or adherence to airway epithelial cells could be reduced through gene therapy. The UK Cystic Fibrosis Gene Therapy Consortium is investigating the use of these types of assays of CFTR molecular function.

Microarrays are powerful tools for profiling gene expression patterns, and it is natural to consider their applicability as gene therapy endpoints. It is first necessary to establish what effect CFTR mutations have on patterns of gene expression. Many groups (including colleagues in the Consortium) are assessing this and recently a comprehensive analysis of CFTR-related expression patterns in mice has been published [65]. It is possible to conceive of a custom-made chip, carrying robust secondary transcriptional markers of CFTR activity, that could be used to assess the efficacy of gene transfer. There are technological difficulties in this approach, however. Most importantly, the sample sizes obtainable from mouse nasal delivery experiments will be small, particularly if techniques such as LCM are used to isolate the epithelial cell fraction, and a microarray assay may not be sensitive enough to register the minute changes in RNA anticipated. It is likely that the use of increasingly sophisticated RNA amplification techniques will overcome this hurdle. Another consideration is that some changes at the level of transcription seen to be induced by CFTR transgene expression may occur in the absence of functional CFTR protein at the apical membrane. Finally, inter- and intra-animal variability in gene expression needs to be taken into account. Nevertheless, transcriptional profiling using microarrays could form the foundation of a powerful new kind of endpoint assay for gene therapy.

2.4. Airway xenograft model systems

A variety of other models have been developed for the study of CF. One of particular interest and potential for the development of CF gene therapy is the use of human airway xenografts on a nude mouse background. Such systems are excellent models of the in vivo human airway, and are already providing valuable insights into the processes of airway growth and regeneration [66].

3. Clinical endpoints of CFTR gene transfer

Once a GTA has satisfied designated criteria in pre-clinical studies and has passed toxicology testing, clinical trials in CF patients are warranted. The nasal epithelium has frequently been used as a surrogate for the lung, as it is likely to be a safer target for phase 1/2 trials and because certain endpoint assays can be carried out more easily, but a case can also be made for targeting the lung directly in such trials.

When considering clinical endpoints for CF lung gene therapy it is important to differentiate between single and repeat administration trials. In our view, single administration protocols are unlikely significantly to attenuate chronic lung inflammation or ameliorate lung function, so assays assessing inflammatory markers in lung tissue, bronchoalveolar lavage fluid (BALF) or exhaled breath condensate (EBC) may detect no change. However, in repeat administration phase 2/3 trials, bacterial colonisation, lung inflammation and lung function measurements will be more important.

3.1. Direct assays of CFTR

3.1.1. Quantification of CFTR mRNA

RT-PCR has been performed on human nasal and bronchial cells harvested by brushing after CFTR gene transfer [67–69]. However, the value of such data in the absence of knowing which cells have been transfected is limited. In our hands, a typical nasal sample contains up to 20% of non-epithelial cells (as judged by visual assessment of the proportion of columnar cells using for example antibody staining for epithelial cell markers such as cytokeratins (MAB 3412, Chemicon: Heather Davidson, personal communication)). It is therefore crucial to develop epithelial cell specific mRNA assays, and laser capture of epithelial cells from nasal and bronchial biopsies may significantly enrich samples for these relevant cell types.

3.1.2. Quantification of CFTR protein

Immunohistochemistry has been used with some success to detect CFTR expression after gene transfer in primary airway epithelia cells [68,70]. Hyde et al. reported recombinant CFTR protein detection in 6 out of 9 CF patients using a protocol optimised to preclude detection of endogenous
mutant (delta F508) CFTR. In this study the authors used Genzyme’s 3-1, a monoclonal antibody raised against the R domain and now available from Chemicon. However, the difficulty of CFTR immunodetection is well-known in the CF field, and to date there is no ideal antibody for all purposes. The low abundance and membrane localisation of CFTR contribute to the problems observed, though batch variation, non-specific staining and cross-reaction to other proteins are also troublesome. Because of these factors, it may be advisable to test a range of CFTR antibodies to identify those best suited to the application being considered. Doucet et al. [55] have provided an excellent guide on the range of CFTR antibodies currently available.

Except for functional assays of CFTR, immunodetection is the most convincing way of demonstrating the efficacy of gene transfer, since a necessary prerequisite for CFTR function is the correct localisation of protein at the apical membrane. Detection of CFTR mRNA alone is insufficient, since CFTR undergoes complex post-translational biogenesis [71]. We therefore believe that, despite the difficulties, investment of substantial effort into CFTR immunodetection as an endpoint is justified.

3.1.3. Quantification of CFTR chloride channel activity

Currently, PD measurements are the most important endpoint assay in phase 1/2 trials. Robust protocols have been developed in several laboratories that allow reliable discrimination between CF and non-CF nasal epithelium [72,73]. As in CF mice, sodium absorption is increased in CF patients, leading to increased basal PD, whereas chloride secretion is significantly reduced or absent. In the nose the electrical measurements do not require anaesthesia, are not invasive and do not cause great discomfort, and can therefore be repeatedly carried out in the same patients. This allows the assessment of CFTR function over time. However, damage to the nasal epithelium with the perfusion catheter may occur and could affect subsequent measurements, since assessment of ion transport relies on intact epithelium. Appropriate spacing of the sites of repeated measurements ought to allow healing of the epithelium, but this has not yet been systematically studied. Electrical PD measurements in the lung have been developed, and discriminate between CF and non-CF epithelium in all regions tested [74]. However, in contrast to measurement in the nose [75], PD measurements in the lung require anaesthesia and bronchoscopy and therefore cannot be carried out repeatedly after gene transfer. This, in addition to being technically more difficult, may explain why PD measurements in the lung have only been carried out in one trial [67].

In addition to PD measurements, chloride channel activity can be measured in primary airway epithelial cells using fluorescent chloride channel indicators. Cells are harvested through brushing of the nostrils or airways with small bronchoscopy brushes [39,74]. The cells are then loaded with a fluorescent halide indicator such as SPQ and analysis is carried out as described above. Although the assay is robust for cell lines, it is difficult to adapt for primary epithelial cells because of their poor adherence and ciliary motion. Blinded pre-clinical experiments that verify the protocol using CF and non-CF brushings should be undertaken and assessed before fluorescence efflux assays are adopted for trial use.

3.2. Indirect assays of CFTR

3.2.1. Invasive secondary endpoints

As described for the pre-clinical studies, there is clearly a need for secondary assays in clinical trials and we are actively developing a panel of potential assays based on bacterial adherence, mucociliary clearance and mucus composition. Davies et al. demonstrated that ex vivo CFTR gene transfer to CF nasal epithelial cell reduces the attachment of Pseudomonas aeruginosa to ciliated epithelial cells [39,76]. The assay was subsequently used as a secondary endpoint in a lung gene therapy trial and five out of six patients showed decreased bacterial adherence when compared with pre-treatment values [74].

Analysis of inflammatory markers in sputum and broncho-alveolar lavage fluid (BALF) may be an important secondary endpoint in repeat administration studies. Differences in BALF from CF and non-CF subjects have been reported for a large number of cytokines and inflammatory mediators including interleukin 8 (IL-8), tumor necrosis factor α (TNF-α) and interleukin 10 (IL-10) [77,78] and ELISA kits are available for easy quantification (Genzyme). Interestingly, a small change in cytokine expression has been reported in sputum after single administration of GTA in at least two trials. Alton et al. reported a decrease in sputum IL-8 after administration of liposome/CFTR gene transfer and Wagner et al. described an increase in the anti-inflammatory cytokine IL-10 after administration of aden-associated virus-CFTR (AAV-CFTR) to the maxillary sinus of CF patients [74,79]. These changes in cytokine expression may become more pronounced after repeat administration. More recently, the first repeat-administration lung trial (3 doses of nebulised AAV2, 1 month apart, in patients with mild lung disease) was carried out. The treatment was well tolerated and showed some evidence of a small improvement in lung function, and a reduction in IL-8 in induced sputum after the first, but not subsequent administrations. This reduction in efficacy on re-administration of the AAV vector may in part be caused by the development of an immune response after the first administration [80].

Custom-made chips for microarray analysis of gene expression after CFTR transfer may also play a role in clinical trials. However this would first require the purification of epithelial cells from contaminating cells; this is not easy and the yield is low. In brushing or biopsy analysis, sample size may again be a limiting factor, and there is the added complication that, unlike mice used for pre-clinical work, patients are not genetically homogeneous.
The chips would therefore need to detect relevant changes in gene expression against a much noisier background. Colleagues in the UKCFGTC are carrying out microarray experiments on CF and non-CF individuals in order to determine whether there are indeed markers robust enough to use in a clinical setting.

Similar considerations affect the possible clinical use of SELDI-TOF analysis. Again, colleagues are investigating the feasibility of using this technique to detect CFTR and other proteins in various types of human CF and non-CF sample. Sensitivity is likely to be the key limiting factor, though the success with which SELDI-TOF has been used to profile tumours in cancer research encourages us to pursue this approach [54].

A useful guiding principle of marker-based secondary assays for CF gene therapy is “more is better”, the notion being that simultaneous analysis of a series of markers after gene transfer will yield statistically more significant data on efficacy than observations of only one or two markers. However, care is required in selecting the panel of markers to assay. Because it is possible that the GTA itself may cause changes in inflammatory gene expression, the panel ought to include, in addition to markers of CF-associated inflammation, markers that relate to other aspects of CFTR function or maturation (e.g. trafficking [71]). Any change towards normal levels of such markers seen in a trial would be convincing evidence that effective expression of recombinant CFTR was responsible. We are only now beginning to identify appropriate markers.

3.2.2. Non-invasive secondary endpoints

The most relevant endpoint for successful CF gene therapy in later trial stages is an improvement or stabilisation of lung function (e.g. FEV1). However, this is a difficult endpoint to measure because (a) gene therapy will probably be most successful in patients (especially children) who already have good lung function, as gene transfer efficiency is likely to be higher in the absence of excessive sputum accumulation, and (b) lung function decline in most patients is not linear over time and patients may have stable lung function for many years before declining rapidly. CF gene therapy may have to be carried out in a large number of patients over a long period of time to see a significant effect on lung function. It is therefore of crucial importance to develop more short-term non-invasive endpoints to assess efficacy of gene transfer. The development of non-invasive assays for CF gene therapy is widely recognised to be a priority, and rapid advances are eagerly anticipated, particularly in the area of imaging [81]. Positron Emission Tomography (PET) and Computer Tomography (CT) scans, as well as white cell scans have recently been proposed.

Analysis of inflammatory markers in exhaled breath condensate (EBC) may fulfil the criteria outlined above. One attractive feature of EBC is that it is easy to collect and painless for the donor: it has been performed in children as young as 6 years and also offers the potential for repeated sampling. The composition of EBC is uncertain, but could conceivably include condensed gas and water exhaled from anywhere in the airway from the mouth to the alveoli. It may also contain microdroplets of airway surface lining fluid (ASL) shed from the airway during exhalation. The presence of microdroplets is supported by the detection of large non-volatile compounds in EBC (unpublished data). In addition, EBC analysis has revealed acidification in the airways of CF patients [82]. Another source of material for the assay of inflammatory markers is sputum. We are actively examining the potential of this source: preliminary results indicate that inflammatory markers are easier to detect in induced sputum than in EBC (Gordon MacGregor, unpublished data). Ordóñez et al have also recently demonstrated that induced sputum is a useful source of material for non-invasive outcome measures. Following a course of intravenous antibiotics, bacterial density, neutrophil counts and IL-8 decreased significantly, when compared to pre-treatment values [83].

SELDI-TOF mass spectrometry may permit the development of multiple assays of proteins as surrogate markers of CFTR function and inflammation. An attraction of the approach is that samples collected non-invasively (e.g. EBC or spontaneous sputum) could be suitable for analysis. Using tree-based statistical decision protocols, we aim to develop a protein profile scoring system to assess the efficacy of CF gene therapy. Bacterial colonisation, as assessed by the number of infective exacerbations or the need for IV antibiotics in a given time period, can easily be quantified. However, more direct quantification of bacterial load, in sputum for example, might also provide useful information although the poor sensitivity of such assays would necessitate the analysis of large numbers of individuals. Colonisation in CF is believed to be a function of poor mucociliary clearance: non-invasive techniques to measure clearance have previously been developed [84] and should also be considered as a potential endpoint.

Finally, we should consider the experiences of the subjects themselves. The feelings and opinions of patients before, during and after treatment may strike many as being too subjective a basis from which to draw scientific conclusions. However, we feel this is a neglected area and could provide valuable information about the progress of blinded, placebo-controlled, phase 2/3 gene therapy trials. We believe this aspect could best be approached through the use of a validated quality of life questionnaire building on previously published experience [85].

4. Conclusions

Here we have summarized the strengths and weaknesses of current pre-clinical and clinical assays for CF gene therapy...
and have highlighted areas that require further development. In addition to improving airway gene transfer, significant effort has to be invested into refining the endpoint assays before CF gene therapy can become a successful treatment. Importantly, power calculations based on data generated during assay development should routinely be carried out for all pre-clinical and clinical experiments to ensure that studies are performed with sufficiently high numbers and therefore lead to statistically meaningful results ensuring long term benefits for treated patients.

Acknowledgements

We thank Eric Alton, Jane Davies, Duncan Geddes, Deborah Gill, Steve Hyde and David Porteous for the critical evaluation of this manuscript and all members of the UK Cystic Fibrosis Gene Therapy Consortium (http://www.cfgenetherapy.org.uk) for their help and encouragement.

References


The image contains a page of a document with the following references:


