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EDITORIAL

Use of *in vivo* nasal transepithelial potential difference to evaluate efficacy in CF gene therapy Phase I trials

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The human nasal transepithelial potential difference: measurement and origin

The technique of *in vivo* measurement of nasal transepithelial electrical potential difference (PD) in humans was first described in 1981 by KNOWLES and coworkers [1, 2]. This technique consists in measuring the PD between a reference electrode in contact with the subcutaneous space of the forearm and an exploring electrode placed on the nasal mucosal surface. The initial studies showed that the magnitude of the PD depended upon the site of measurement in the nose, and was maximal on the innersurface of the inferior turbinate. There was a correlation between the magnitude of the PD and the percentage of ciliated cells in the epithelial region where the exploring electrode was placed. Reproducibility of the measurements was good.

This electrical PD implied the presence of electrogenic ion transport mechanisms, and their source was the epithelial layer, since abrasion of the epithelial abolished the PD. One of the most important results was that the nasal (and also tracheobronchial) PD was much higher in cystic fibrosis (CF) patients compared to non-CF subjects, including CF heterozygotes and patients with several bronchopulmonary diseases [2]. Furthermore, these two groups of subjects could be clearly distinguished on the basis of the nasal PD. This finding led to a series of studies on epithelial ion transport in human airways. The effect of various drugs and electrolyte solutions on the *in vivo* nasal PD were investigated, and *in vitro* studies were performed on excised tissues and on confluent cultures of airway epithelial cells [3].

The *in vitro* studies used increasingly sophisticated electrophysiological technologies, including Ussing chambers, nonselective and ion selective intracellular microelectrodes, and patch clamp techniques. In brief, it was shown that under most physiological conditions, Na^+ absorption from the airway lumen is the dominant active ion transport in human airway epithelia. Cl^- follows Na^+ passively, probably through the paracellular pathway. Under circumstances that provide an electrochemical gradient for Cl^- across the apical membrane (*i.e.* inhibitors of the apical membrane Na^+ conductance, superfusion of the mucosal surface with Cl^--free solutions, hyperpolarization of the basolateral membrane, activators of Cl^--conductive apical membrane channels, *etc.*), transcellular Cl^- secretion can occur. Thus, normal human airway epithelia are capable of salt and water absorption (driven by active Na^+ absorption), or secretion (driven by active Cl^- secretion) [3]. In CF airway epithelial cells, Na^+ absorption is increased and Cl^- secretion is defective [3–5]. Patch clamp studies of CF airway epithelial cells identified abnormalities in the Cl^- and Na^+ channels that are normally located in the apical membrane [3, 6, 7]. In addition, the product of the CF gene, cystic fibrosis transmembrane (conductance) regulator (CFTR), was recognized to be a cyclic adenosine monophosphate (cAMP)-activated Cl^- channel that may also regulate other ion channels and several cell functions (*e.g.* exocytosis/endocytosis, ...) [8, 9]. It is widely believed that abnormalities in ion transports in CF account for abnormal airway secretions, which in turn result in altered mucociliary clearance and susceptibility to airways infection.

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Use of *in vivo* measurement of the nasal PD in the CF gene therapy Phase I trials

The rapidly growing knowledge of mechanisms of CF airway disease has led to new therapeutic approaches. Among these, the identification of the CF gene has made it possible to propose gene therapy for treatment of the airway disease. Many *in vitro* studies have shown that gene transfer of the normal CFTR gene to CF cells can correct abnormalities in ion transport (both sodium hyperabsorption and defective chloride secretion) [10–12]. There are now several CF gene therapy trials underway in the US and Europe, using as delivery vehicles recombinant replication-deficient adenoviruses or liposomes [13]. The goal of these Phase I studies is to determine the feasibility of *in vivo* gene transfer for the correction of CF airway disease using these particular vectors, in terms of toxicity and efficacy. The lung has been the initial target for CF gene therapy, because the pulmonary manifestations of the disease are usually the more severe and are responsible for more than 95% of the mortality. However, most of these trials include a phase of gene delivery to the nose [14, 15]. The rationale for evaluating the consequences of gene transfer in the nose is that the epithelium of the nose shares many histological and functional similarities with the tracheobronchial epithelium [16]; in particular, these epithelia have similar ion transport capabilities and display similar ion transport abnormalities in CF patients [1, 15, 17]. In addition, the nasal epithelium is accessible for collecting cells and performing direct measurements of function. Finally,

the nasal mucosa in patients with CF is relatively free of infection, as compared with bronchi.

In the current trials, even those in which gene transfer is targeted to the bronchial epithelium, efficacy cannot be assessed on the basis of clinical data (however, clinical data are used to assess toxicity); clearly, in later trials, clinical data, such as pulmonary function and frequency of infections, will be major determinants. Evaluation of efficacy in the current CF gene therapy trials requires not only evidence of expression of the normal CFTR gene in a "large" number of epithelial cells, but also demonstration that at least one important abnormal cellular function has been partially or totally corrected, leading to correction of the resulting altered function of the epithelial layer. Since the ion transport abnormalities are the most widely studied abnormal cell function in CF airways, and since it is possible to estimate non-invasively the ion transport rates of the epithelial layer by measuring the nasal PD *in vivo*, investigators have opted to use this test as an end-point to assess the functional efficacy of *in vivo* gene transfer in the Phase I trials [14, 15]. Correction of the nasal PD would suggest that administration of the normal CFTR gene has corrected the CF electrolyte transport defect in the targeted nasal epithelium.

The basal nasal PD as a diagnostic test for CF

How reliable is the *in vivo* measurement of nasal PD for detecting correction of the CF phenotype? To address this question, we must first consider how well the *in vivo* measurement of the nasal PD discriminates between CF and non-CF subjects. The initial study by KNOWLES and co-workers [2] indicated that the raised nasal PD discriminated very accurately between CF patients and non-CF subjects, including patients with other lung diseases. This finding was confirmed in two studies [18, 19], but not in another study that found considerable overlap of values between CF and non-CF patients [20]. The latter group decided to modify their technique of measurement and, in two more recent large studies, they have found very little overlap in values of the nasal PD measured on the floor of the nasal cavity between the CF and non-CF subjects, including patients with various bronchial diseases [21, 22]. Based on these data, the nasal PD has been advocated as a diagnostic tool for CF. There has been no formal comparison of the diagnostic value of the sweat test and the *in vivo* nasal PD; there are a few reports of CF patients with a sweat test in the normal range and a high PD [23]. The nasal PD technique is also being used to explore genotype/phenotype relationships in CF.

Experimental protocols may improve the sensitivity and specificity of the nasal PD to detect correction of the CF phenotype

The basal nasal PD may not be the best indicator of functional efficacy in the gene therapy trials. Firstly, the nasal PD decreases during acute nasal infections [24], after mild trauma [1], and probably in a variety of nasal diseases, such as nasal polyps [25]. It is conceivable that nasal injury induced by the process of gene therapy (for instance vector-induced inflammation) may itself decrease the basal nasal PD. Secondly, in the case of partial correction of the ion transport abnormalities during the CF gene therapy trials, the basal nasal PD may not be sensitive enough.

Other options exist. The raised nasal PD in CF patients reflects the high Na^+ absorption and the low basal Cl^- permeability, with little or no response to cAMP-mediated agonists that stimulate Cl^- secretion across the normal epithelium. Na^+ absorption and Cl^- secretion can be explored *in vivo* using Na^+ transport inhibitors, Cl^- secretagogues and ion substitution protocols. The resulting changes in the nasal PD can be interpreted on the basis of a large *in vitro* database. In brief, Na^+ absorption can be assessed by the response to the Na^+ channel blocker amiloride; the absolute as well as relative response to amiloride is significantly greater in patients with CF [2, 25, 26]. The basal Cl^- permeability of the nasal epithelium can be assessed by the response to superfusion of a Cl^- free solution; in non-CF subjects, superfusion of a Cl^- free solution induces a hyperpolarization (lumen more negative), whereas CF subjects display little or no change in the nasal PD [26].

Assessment of Cl^- secretion is more difficult. *In vitro* studies on cultured preparations have found that Cl^- is approximately at electrochemical equilibrium across the apical membrane of the nasal epithelial cells [27], which means that there is no net driving force for active Cl^- movement. *In vitro* studies have also shown that a driving force can be created by luminal application of amiloride or a Cl^- free solution (or both). It is then possible to assess the effect on the rate of Cl^- secretion of cAMP-mediated agonists. Amiloride is useful not only to create a driving force for Cl^- secretion, but also to abolish Na^+ movement, which simplified the interpretation of the responses to drugs and solution changes.

Thus, apart for the basal PD, there are at least four types of protocol suitable for *in vivo* use that may help to differentiate CF and non-CF subjects: 1) response to amiloride; 2) response to a Cl^- free solution; 3) response to a cAMP elevating agent in the presence of amiloride; and 4) response to a cAMP elevating agent in the presence of amiloride and Cl^- free solution. Previous studies have shown that CF and non-CF nasal epithelia typically respond differently to each of these protocols, but their diagnostic values for discriminating between CF and non-CF subjects have not been clearly established and compared.

About a year ago, ZABNER *et al.* [28] reported the results of a clinical trial in which recombinant adenoviruses, containing the normal CFTR gene, were instilled onto the nasal epithelia of three CF patients. Their study included functional assessment of gene complementation using the *in vivo* measurement of the nasal PD. Prior to the use of nasal PD in the gene therapy trials, the investigators validated their technique by studying nine CF patients and seven non-CF subjects, and reported that basal PD was more electrically negative in CF patients, and that superfusion of terbutaline (10 μM) in the presence of amiloride (100 μM) induced a 1–5 mV

hyperpolarization in all non-CF subjects, whereas hyperpolarization was never observed in CF patients. Application of the adenovirus containing the normal CFTR gene resulted in a decrease in the basal PD in all three patients. In addition, terbutaline in the presence of amiloride induced a hyperpolarization in all three patients. These limited data support previous findings that both the basal PD and the response to terbutaline in the presence of amiloride discriminate CF from non-CF subjects, and suggest that nasal PD measurement with this protocol may be sufficiently sensitive to detect correction of CF nasal ion transport abnormalities by CFTR gene complementation.

MIDDLETON and co-workers, who are also involved in a CF gene therapy Phase I trial, formally determined the diagnostic value of protocols of superfusion of drugs and solutions, in particular in comparison with the basal PD. Their results are published in this issue of The Journal [29]. They measured the PD along the floor of the nasal cavity in 241 non-CF subjects and 146 CF patients. They found that the basal PD was clearly discriminative, but seven non-CF subjects fell into the lowest part (in absolute value) of the CF range, and 28 CF patients fell into the highest part of the non-CF range. The basal PD and the changes induced by superfusion of drugs and solutions were recorded in 16 non-CF volunteers and 27 CF patients homozygous for the ΔF508 mutation. They compared four protocols: amiloride (100 μM); amiloride + low Cl^- solution ([Cl^-] 6 mM); amiloride + isoprenaline (10 μM) or terbutaline (100 μM); and amiloride + low Cl^- solution + isoprenaline. Not all subjects participated in all protocols. The amiloride response (expressed as absolute decrement of PD) was significantly increased in the CF patients, but there were three overlap values out of 37 measurements in non-CF and CF subjects. Surprisingly, the β-adrenergic agents in the presence of amiloride had no significant effect in non-CF subjects but induced a small but significant depolarization in CF subjects. However, this unexpected result could not be used to differentiate non-CF and CF subjects on an individual basis, because there was a considerable overlap of values. Superfusion with the low Cl^- solution induced a hyperpolarization in non-CF subjects but no significant effect in CF patients. Addition of isoprenaline in the presence of low Cl^- solution (and amiloride) further hyperpolarized the nasal PD in non-CF subjects, without significant effect in CF patients. There was no overlap in the response to either solution between the non-CF and CF subjects.

Another unexpected result was a transient hyperpolarization induced by low Cl^- solution and by isoprenaline or terbutaline alone or in low Cl^- solution in CF subjects. This finding has not been reported and at present has no clear explanation. However, it implies that comparison between non-CF and CF subjects based on the response to low Cl^- solution and/or a β-adrenergic agent must be made after 5 min, *i.e.* when a steady-state is reached. The conclusions of this study are that: 1) basal PD and amiloride response do clearly distinguish non-CF and CF patients, but, as discussed above, in the context of gene therapy trials, a nonspecific decrease in the basal PD and in the amiloride response may be observed; and 2) the best protocol to discriminate non-CF and CF subjects explores the Cl^- secretion pathway using superfusion of low Cl^- solution containing 100 μM amiloride and 10 μM isoprenaline.

Remaining problems

Despite the importance of the results of this carefully conducted study, problems still persist, especially with regard to the gene therapy trials. Firstly, MIDDLETON *et al.* [29] observed unexpected responses, such as transient hyperpolarization induced by low Cl^- solution and β-adrenergic agents. They offer two possible explanations: liquid junction potential induced by the substitution of gluconate for Cl^- in the perfused solution, and activation of Ca^{2+}-mediated Cl^- secretion by isoprenaline. These effects need to be understood before these protocols are fully validated.

Secondly, there are notable differences between published studies. For instance, KNOWLES and co-workers [2] found that amiloride response expressed as percentage of the basal PD was significantly higher in patients with CF, whereas MIDDLETON *et al.* [29] found no significant difference. ZABNER *et al.* [28] found a small but significant effect of terbutaline (in the presence of amiloride) in non-CF subjects, whereas MIDDLETON *et al.* [29] found no significant effect. At present, these differences appear difficult to reconcile. They may, perhaps, be explained by differences in technique, site of measurement on the nasal mucosa, or composition of solutions.

Thirdly, to validate the technique, comparisons have been made between CF and non-CF subjects. However, in the CF gene therapy trials, the CF subjects will be used as their own control. Partial correction of the ion transport defects may be more difficult to detect. On the other hand, the use of the CF subject as his/her own control is likely to augment the power of the study, since the intrasubject variability is less than the intersubject variability [1, 22].

Furthermore, the nasal PD varies according to the site of measurement [1], which implies that, since serial measurements are necessary in the CF gene therapy trials, care must be taken to ensure that the PD is measured at the very same site.

Finally, the nose is not the real goal of CF gene therapy. Ultimately, the normal CFTR gene will be targeted to the bronchi. It is possible to measure the tracheobronchial PD through a fibreoptic bronchoscope [2, 17, 30, 31]. However, this technique is invasive and difficult to perform for obvious reasons (need for access to the subglottic airways, use of local anaesthetics which may affect the results [17], *etc.*) not to mention the need for repeated measurements. In addition, CF bronchi are often infected, which, as discussed above, may affect the value of the bronchial PD. Finally, this technique does not measure the transepithelial PD in small airways; even though that site may be crucial for the development of airways obstruction in CF, very little is known about ion transport activities in small airways.

In conclusion, the *in vivo* measurement of nasal

transepithelial PD is likely to be one of the best available tools to assess the efficacy of new therapeutic approaches in CF, including gene therapy, at the airway epithelial level. The optimal discriminative criterion is probably the assessment of Cl^- secretion by superfusion of a β-adrenergic agent in the presence of amiloride and, probably, in a low Cl^- solution; however, basal PD and response to amiloride provide additional and important information. More work needs to be carried out to explain the unexpected electrophysiological responses observed in this protocol and the differences between published studies, and to define rigorous criteria for efficacy testing using electrophysiological techniques in gene therapy studies. Finally, one should keep in mind that at the present time, the relationships between the cellular abnormalities in ion transport and the cascade of events that leads to lung destruction are ill-defined. Until we resolve this issue, it is difficult to evaluate the usefulness of correction of ion transport abnormalities as a surrogate for clinical efficacy.

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