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Assessment of CFTR localisation in native airway epithelial cells obtained by nasal brushing

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Abstract

Reliable methods for determining the localisation of mutant CFTR protein in native cells from CF individuals are necessary to allow the degree of mislocalisation of any genotype to be defined and to assess the effect of therapeutic agents on CFTR trafficking. Here, we present procedures for obtaining ciliated epithelial cells from CF patients by nasal brushing and a description of protocols for immunolocalisation of CFTR. The protocols are a consensus, following comparison of some aspects of methods currently used in the authors' laboratories.

Keywords: CFTR; CFTR antibody; Immunolocalisation; Nasal brushing; Airways

1. Introduction

Wild-type (wt) CFTR functions at the apical membrane of epithelial cells, where it acts as a cyclic AMP-dependent chloride (Cl^-) channel [1] and regulates other ion channels [2] and protein secretion [3]. By far the most common disease-causing mutation in CFTR is F508del, present on 70–80% of CF chromosomes worldwide. Cells transfected with F508del-CFTR show reduced Cl^- channel activity compared to wt since the protein is incorrectly processed, retained within the endoplasmic reticulum (ER) and degraded to a greater extent than wt protein [4]. Native F508del/F508del CF epithelial cells also have reduced Cl^- channel activity at the cell membrane [5]. However, the degree of mislocalisation of F508del-CFTR as demonstrated by immunostaining varies among different tissues and types of preparation [6–11]. Nevertheless, the majority of immunolocalisation studies have indicated that although some F508del-CFTR was detected at the apical region, a substantial amount was present within the cell.

It is very important to have reliable methods for determining the localisation of mutant CFTR protein in native cells from CF individuals. This allows: (a) the degree of mislocalisation of any genotype to be defined; (b) the effect of drugs, or other therapeutic strategies, on CFTR trafficking to be assessed following in vitro or in vivo administration. Although the degree of glycosylation of CFTR has been used to determine whether a particular mutation results in mislocalised CFTR [4], this technique is only suitable for use in cell lines in which enough protein is expressed for biochemical analysis (see Ref. [12]). Studies in native, non-cultured, non-transfected cells should be considered the "gold standard" for CF research and for assessing the efficacy of new therapies. The study of CFTR localisation and function in native airway epithelial cells has been considerably advanced by the ability to

Abbreviations: Ab, antibody; ER, endoplasmic reticulum; PBS, phosphate buffer saline; TCE (cells), tall columnar epithelial cells.

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obtain cells from the nasal epithelium by a brushing procedure [13,14]. This technique has the advantage that a range of subjects, in terms of age, sex, genotype and disease conditions can be studied. Additionally, nasal epithelium is considered to be representative of CF disease in the lower airways [15]. The nasal brushing technique, although far less invasive than those that sample bronchi or lungs, gives some discomfort to the subject and studies will usually require approval from local ethics committees. It is important that harvesting cells from the brushes should be rapid and cause minimal disturbance of the cells before fixation for immunolocalisation. This will often have to be balanced with the need to obtain enough cells for meaningful testing of, for example, the effects of drugs on CFTR location ex vivo. Our experience is that the technique, as described here, can yield highly polarised cells in which quantitation of the percentage of cells with a defined CFTR localisation can be determined. The technique thus allows assessment of the effects of CFTR trafficking or premature stop mutations, as well as those of drugs that promote the trafficking of such mutant proteins to the apical region [10,16].

2. Towards a consensus protocol

The present article is based on the protocols described in detail at the European Working Group on CFTR Expression website [17]. The following sections will consider the three steps of the technique, namely: (1) obtaining cells from patients, (2) harvesting cells prior to experimentation and (3) immunolocalisation of CFTR. For steps 2 and 3, we have compared the distribution of cell types obtained and the localisation of CFTR, in cells from CF patients and non-CF controls by two published methods [9,10].

2.1. Obtaining cells from patients

For many researchers, this is dependent on collaboration with clinical colleagues. Clinicians skilled in the art will have their own methods but the protocol described provides a detailed description.

2.1.1. Materials

Instruments that may be used alternatively to perform nasal brushing: (1) interdental brushes: 2.5 mm diameter for children, 3.0 mm diameter for adults (Paro-Isola, Thalwil, Switzerland); (2) cytology brushes (Surgipath, C-E brush #01970, Peterborough, UK)[1]; (3) rhino-probe® nasal curette (Arlington, IL, USA). Before use, brushes should be washed thoroughly with 70% ethanol, rinse in distilled water and sterilised under UV light overnight.

Collecting medium for living cells: Ham's F12 or DMEM/F12 culture media, containing (penicillin 100 U/ml and streptomycin 100 μg/ml) or phosphate-buffered saline, PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4).

2.1.2. Procedure

It is recommended that no anaesthesia should be used when obtaining cells by nasal brushing.

1. *Using an interdental brush*: (a) Ask the subject to blow the nose and thoroughly clean it and sit them in a chair with the head against the wall in order to prevent head withdrawal and tilt the subject's head backward. (b) Introduce a rhinoscope (closed) into a nostril, open it slowly and inspect the inferior nasal meatus, using direct light. Continue if the mucosa looks clean and healthy, otherwise try with the other nostril. (c) Introduce the brush and rub it against the posterior part of the inferior nasal meatus (the medial and superior side of it), using rotatory and linear movements. The subject will get tears in the eye, but the discomfort will diminish rapidly.[2] (d) Take out the brush and put it in a tube containing 1 ml of collecting medium or smear directly onto the surface of a microscope slide (see below).[3]
2. *Using a nasal curette*: (a) The inferior nasal turbinate is directly visualized by a lightened nasal speculum and the curette is placed on the inferior medial surface (''scoop side up''). (b) The probe is then pulled forward and replaced three to four times to collect cells. (c) The curette is smeared directly onto the surface of a microscope slide.

2.2. Harvesting cells prior to experimentation

Cells can be harvested either by smearing directly onto coated microscope slides (Snowcoat X-tra microslides, Surgipath, Peterborough, UK) or by releasing cells from the interdental brush into collecting medium using a disposable pipette tip (described below). This has the advantage of giving the best yield of cells and thereby a greater

[1] The experience in Cardiff is that these softer brushes are easier for subjects to tolerate. This may be important if the technique is performed on children or requires recruitment of volunteers, as opposed to taking brushings as part of a clinical procedure to obtain cells for examination, e.g. for ciliary dyskinesis.

[2] The experience in Lisboa is that the subject is asked preferably to not blow the nose or to blow very gently, to avoid any nasal tissue disturbance. The brush is introduced into the nose without the help of a rhinoscope and the tip of the inferior turbinate and the adjacent lateral nasal wall are scraped using only linear movements.

[3] If transport between site of collection and laboratory is required, cells collected in medium can be kept on ice for up to 8 h. It should however be stressed that cells should be kept at 4 °C for as short a time as possible in order to avoid any artefactual maturation of mutant CFTR.

Table 1
Comparison of CFTR distribution in nasal TCE cells fixed by two protocols

Genotype	Method of fixation	Percentage of cells with distribution described			Number of cells counted
		Within the cell	Throughout the cell including the apical region	Focused at the apical region	
wt	acetic acid/ethanol	22	33	45	100
	formaldehyde/sucrose	24	31	45	61
F508del/F508del	acetic acid/ethanol	66	26	8	38
	formaldehyde/sucrose	66	27	8	52

Cells were obtained by nasal brushing from individuals with genotypes shown, as described [10]. Cells were smeared onto coated slides and fixed either with acetic acid/ethanol as described or with formaldehyde/sucrose as in the consensus protocol. All cells were then processed for immunocytochemistry as described [10] and cells categorised for CFTR distribution.

number of conditions can be tested for effects of drugs on CFTR localisation and function.[4]

2.2.1. Procedure

(a) Take a 200-μl disposable pipette tip and cut its narrowest tip with a hot blade in order to obtain a hole with a diameter almost equal to the brush's diameter and place it in a tube containing collecting medium. (b) Pass the brush up and down three to four times through the opening of the pipette tip, until the brush looks dry. (c) Cells can be washed and resuspended in different media according to needs by centrifugation (at $300 \times g$ for 5 min).

2.3. Immunocytochemical detection of CFTR

Cells spread onto microscope slides directly from the brushing instrument or through harvesting by the procedure described above (Section 2) can be subjected to different immunocytochemistry protocols. Thus, before describing the recommended procedure, the following sections show comparisons of the localisation of CFTR in wt and CF cells by two published methods [9,10]. The CFTR localisation data in Tables 1 and 2 show direct comparisons, using the MPCT-1 antibody (Ab) [10], carried out in the Cardiff laboratory on a limited scale. An important advantage of this technique is that the number of cells with a defined CFTR localisation can be quantified. Only well-defined tall columnar epithelial cells (TCE) are quantified and at least 100 cells per sample should be counted. This should be routinely possible using the consensus procedure described in Section 2; however, using the smear technique, fewer cells are obtained and less cells may have to be counted, as in Tables 1 and 2.

2.3.1. Comparison of fixation methods

The distribution of CFTR in ciliated epithelial cells from a non-CF and a F508del/F508del CF individual was compared following smearing onto coated slides and fixation in either acetic acid/ethanol [10] or formaldehyde/sucrose [9]. Cells were categorised for CFTR distribution as previously described [10]. As shown in Table 1, the method of fixation had no effect on CFTR distribution in cells from either individual. In non-CF cells, wt-CFTR was localised in the apical region in 70–80% of the TCE cells, with differing degrees of scatter throughout the cell. In F508del/F508del CF cells, F508del-CFTR was predominantly restricted within the cell[5] (65–70% of cells) with approximately 10% distinctly focused in the apical region. Recently, the Lisboa group compared four different fixing solutions in nasal epithelial cells from non-CF and F508del/F508del CF individuals, namely: (a) acetone at −20 °C, formaldehyde at 4 °C; (b) with and (c) without post-fixation with methanol at −20 °C; and (d) acetic acid/ethanol [11]. Overall, it can be concluded that none of the different methods of fixation tested affected CFTR distribution.

2.3.2. Comparison of harvesting methods

The distribution of CFTR in ciliated epithelial cells obtained at the same time from the same individual was compared following either smearing onto coated slides and fixation in acetic acid/ethanol [10] or using the harvesting procedure described above (Section 2) and fixation in formaldehyde/sucrose [9]. As shown in Table 2, essentially the same pattern of CFTR localisation was observed in the two populations. As described previously [10], the majority

[4] Cells smeared or centrifuged (see below) onto coated slides can be stained either by May-Grünwald's method as described [9] or with Toluidine Blue [10]. Data obtained in Cardiff laboratory using Toluidine Blue showed that there is no alteration on the distribution of cell type between the different harvesting procedures used (smearing onto slides or removing cells by passage through a pipette tip): >90% of the cells observed were epithelial being approximately 70% tall columnar cells (ciliated and non-ciliated) and 20% basal. Penque et al [9] described detailed analysis of the cell types obtained with no difference among populations from wt, F508del-heterozygotes or homozygous subjects. 80–95% of cells obtained were epithelial; approximately 65–70% were tall columnar cells, of which 60% were ciliated; approximately 20–25% were basal cells. The data was similar to that of Bridges et al. [13] and Danel et al. [14].

[5] It should be noted that the Lisboa group do not routinely observe intracellular staining that could be associated with the F508del trafficking defect in TCE cells from F508del homozygous CF patients as is described in Tables 1 and 2 and by other groups (see Refs [10,17]).

Table 2
Comparison of CFTR distribution in nasal TCE cells obtained by two protocols

Genotype	Protocol	Percentage of cells with distribution described			Number of cells counted
		Within the cell	Throughout the cell including the apical region	Focused at the apical membrane	
wt	Smear	22	33	45	100
	PT	27	28	45	100
F508del/F508del	Smear	66	26	8	38
	PT	69	19	12	100
F508del/4016ins	Smear	71	17	12	58
	PT	91	6	3	100

Cells were obtained by nasal brushing from three individuals with the different genotypes shown, as described [10]. Cells from one brush were smeared onto coated slides and fixed ('smear method'); cells from a second brush were harvested as described in Section 2 above (pipette tip (PT) method). All cells were then processed for immunocytochemistry as described [10] and cells categorised for CFTR distribution.

of CFTR in non-CF cells was located in the apical region whereas the majority of F508del/F508del cells showed F508del-CFTR restricted within the cell. The same pattern was seen in cells from a CF individual F508del/4016ins expressing only one copy of F508del-CFTR as in F508del/F508del cells (see also Ref. [10]). In addition, the action of known F508del-CFTR trafficking drugs such as MPB-91 [10] was also observed in cells isolated by either procedure (data not shown).

2.3.3. Comparison of antibodies

The distribution of CFTR in cells obtained from non-CF individuals or F508del-homozygous CF patients, either as described by the consensus protocol (Section 2 and Table 3)

Table 3
Outline of immunocytochemical detection protocols

Fixation and storage	
Suspend cells in 1 ml of freshly prepared, ice-cold fixative buffer (4% (v/v) formaldehyde + 3.7% (w/v) sucrose in PBS).	
Incubate for 30 min on ice for fixation.	
Centrifuge (300 × *g* for 1 min), remove fixative buffer and wash once with 1 ml of ice-cold PBS to remove fixative completely.	
Re-suspend cells gently with P1000 micropipette (three times up and down) between washes.	
Finally, re-suspend cells again in cold PBS (0.3 – 1.0 ml).	
Store at 4 °C until immunocytochemical analysis (maximum: 1 week)	Allow cells to adhere to slides coated with 'Cell-tak' (Beckton Dickinson Biosciences, Oxford, UK). Wash slides with PBS and stored in a moist chamber at 4 °C (maximum: 1 week)
Immunolocalisation	
Centrifuge approx. 100 µl of cell suspension in a Cytospin for 5 min at 2000 rpm for adherence to silane-coated slides.	Incubate slides in PBS/1% Tween-20 for 5 min.
Wash twice in PBS.	
Permeabilize in 0.2% (v/v) Triton X-100 for 20 min.	
Wash three times for 5 min each in PBS.	
Block non-specific staining with 1% BSA (w/v) for 45 min.	Block non-specific staining with goat serum (1:20 in PBS/1% Tween-20) for 20 min.
Incubate with primary Ab (diluted in 1% BSA (w/v)) overnight at 4 °C.	Incubate with primary Ab (diluted in PBS + 0.1% Tween-20/0.1% BSA) overnight at 4 °C.
Wash three times for 10 min each in PBS.	Wash three times for 10 min each in PBS/1% Tween-20,
Incubate with secondary Ab (diluted in 0.5% BSA (w/v)) for 45 min.	Incubate with secondary antibody (diluted in PBS + 0.1% Tween-20/0.1% BSA) for 30 min.
Wash three times for 10min each in PBS.	Wash three times for 10 min each in PBS/1% Tween-20 Add the red nuclear stain propidium iodide (Sigma, St. Louis, MO, USA) to cells for 5 min. Wash three times for 5 min each in PBS/1% Tween-20.
Mount slides with coverslips in Vectashield containing the nuclear stain, DAPI	Mount slides with coverslips using Fluorosave (Calbiochem, Nottingham, UK) to prevent excessive bleaching of fluorescence.

or as described by Dormer et al. [10] was compared using the MPCT-1 Ab. There was no difference in the percentage of wt or F508del/F508del cells showing CFTR in the apical region, irrespective of method of isolation used. A detailed comparison of the application of a wider range of CFTR Abs in immunolocalisation has recently been published [11,18] and is also summarised in an accompanying article in this Supplement [19].

2.3.4. Procedure

In view of the comparisons described above, the procedure shown in Table 3 offers two alternative protocols that give similar results on cells harvested from brushes as described in Section 2. Unless otherwise stated, all procedures are carried out at room temperature and solutions made up in PBS.

3. Discussion

The technique described above for immunolocalisation of CFTR in freshly isolated native airway epithelial cells from CF patients allows quantitation of the percentage of cells with a defined CFTR location. It is recommended that at least 100 columnar ciliated cells should be examined to give meaningful results. The consensus protocol gives similar results to other published methods that have used different methods of isolating and fixing nasal epithelial cells for immunolocalisation.

It is important that the effects of mislocalisation of mutant CFTR and its correction by drug or other therapies on CFTR function, should also be assessed in parallel. For this purpose, the Cl^- transport function of CFTR can be measured by imaging of single cells containing fluorescent Cl^- indicators. A protocol for this technique is presented in an accompanying article in this Supplement [20]. CFTR function can also be measured in vivo by nasal PD, a technique described in detail elsewhere [21]. Although CFTR function is normally measured in fewer cells from a given population than its location, assessment of the heterogeneity of cellular responses in cells with the same CF genotype is an important factor in determining the relationship between genotype and phenotype in this disease.

In summary, it can be concluded that: (1) a minority (10–20%) of native F508del/F508del CF airways cells show CFTR in the apical membrane. (2) Most F508del/F508del CF cells show mislocalised CFTR, which is retained within the cell.

The demonstration that the majority of F508del-CFTR is mislocalised emphasises that moving F508del-CFTR to the apical membrane is necessary for developing a drug treatment targeted at CFTR rescue. The consensus protocol described can be used to test for relocation of F508del-CFTR to the apical region caused by drugs aimed at pharmacological correction of the basic CFTR trafficking defect.

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