Development of primary human nasal epithelial cell cultures for the study of cystic fibrosis pathophysiology

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Cystic fibrosis (CF) is characterized by chronic pulmonary infections and deteriorating lung function, which usually leads to the death of the patients. The relationship between the abnormal CF transmembrane conductance regulator (CFTR) gene product, the development of inflammation, and the progression of lung disease in CF is not fully understood (25, 38). Investigation of human airway epithelial cells in vitro provides useful information about the interactions between CFTR and other ion channels as well as the inflammatory response to infection. Primary cells were first obtained from cultured nasal polyps (4). Studies using cells derived from nasal polyps usually employ outgrowth culture techniques or enzymatic cell isolation. Carrabino et al. (7) demonstrated that there was greater IL-8 release spontaneously from cultures derived from CF patients compared with controls. However, there are two limitations with this approach: firstly, although nasal polyps are reputed to be common in patients with CF, in Northern Ireland we only have approximately one polypectomy per year.

Secondly, the cells obtained from nasal polyps may not be entirely normal as they are associated with additional ongoing inflammatory processes (40). Most CF studies with primary epithelial cells have used cells obtained from lungs taken at transplantation (2). Although a lot of cells can be harvested using this technique; the cells have come from patients with end-stage lung disease and it is uncertain whether they are representative of cells from patients with milder disease. In addition, many laboratories do not have access to transplant lungs. We therefore adopted an approach that we have successfully used in asthmatic patients by obtaining cells using cytological brushings (12). However, instead of using bronchial brushings we studied nasal brushings from patients with and without CF. We had previously attempted to perform bronchial brushings but found that there was a low acceptance rate for bronchoscopy in these patients (31). We hypothesized that primary cell cultures from epithelial cells obtained from nasal brushings would have the appropriate bioelectric phenotype of normal CFTR function in healthy volunteers and abnormal CFTR function in patients with CF. The use of nasal epithelial cells offers a number of advantages for example cultures can be obtained from patients with a variety of genotypes, which can prove difficult when using donor lungs. In addition, nasal potential difference measurements could be compared with the bioelectric responses of the cells from the same person. Patients with CF have an elevated baseline potential difference, a larger response to amiloride, and a reduced or absent response to low chloride solutions and isoprenaline (44). This study describes the methods for nasal epithelial cell culture and some preliminary experiments investigating the electrophysiological properties of these cells.

Materials and Methods

Subjects. Patients (F508del homozygous) attending the adult CF center at Belfast City Hospital were recruited. No specific exclusion criteria were applied. Healthy controls were age and gender matched and recruited from staff within the research group, families, and friends. Both CF and non-CF subjects gave written informed consent to participate in the study in accordance with research ethics granted by Office for Research Ethical Committee of Northern Ireland.

Nasal brushings were obtained and cells grown successfully from 12 F508del homozygous patients [5 male; mean age of 25.2 ± 1.7 yr; forced expiratory volume in 1 s (%predicted) 62.7 ± 5.6%; 6 infected chronically with Pseudomonas aeruginosa] and 11 healthy controls (6 male; mean age of 28.4 ± 2.1 yr) using a standardized procedure. No subject refused to have nasal brushings performed.

Nasal brushing. Before the procedure, each participant blew their nose to eliminate any mucus lining the nasal cavity. A 3-mm diameter...
bronchial cytology brush (Diagmed Healthcare, Thirsk, UK) was used to obtain two brushings from the medial wall and the inferior turbinate of each nostril.

**Sample processing.** Epithelial cells were detached from the brush tip by gentle agitation using a sterile Pasteur pipette. Cell suspensions were centrifuged (114 g, 5 min, room temperature). The cell pellets were resuspended in a final total volume of 2 ml of airway epithelial growth medium (Promocell, Heidelberg, Germany) containing antibiotics [penicillin (100 IU/ml)/streptomycin (100 μg/ml) and Primocin (100 μg/ml)] and seeded into sterile plastic collagen (PureCol; Inamed Biomaterials, Fremont, CA)-coated flasks with a growth area of ~25 cm² (Orange Scientific). This flask was called passage (P) 01. After 24 h, culture media and unattached cells were removed from the P01 flask and replaced with fresh medium. The culture media removed from P01 was transferred into a second 25-cm² flask for further attachment and growth and refreshed with 1 ml of fresh media; this flask was called P02. The same procedure was carried out at 48 and 72 h to produce P03 and P04 flasks. After establishment of these cultures, cells were incubated (37°C, 5% CO₂) and the culture medium was changed every second day until the cells were ~80% confluent. Cells were then trypsinized from the T25 flasks and seeded into T75 flasks to form P1. Primocin was not needed in the cultures from this point. When confluent, the cells were either used immediately in experiments or stored in liquid nitrogen until needed.

Cells for use in patch-clamp experiments were detached using trypsin (0.05% of trypsin-EDTA solution) and plated on glass coverslips forming the bottom of 0.5-ml chambers that were formed using rubber seals. Once the cells were attached to the coverslip, the culture medium was replaced with standard external solution for patch-clamp recording of tight-seal recording techniques (19) at room temperature with heat-polished borosilicate pipettes (Harvard Apparatus, Edenbridge, UK) of 2–4 MΩ resistance using an Axopatch 200B (Molecular Devices, Sunnyvale, CA) voltage-clamp amplifier. The external solution contained the following (in mM): 126 NaCl, 2.5 CaCl₂, 1.2 MgCl₂, 12 glucose, and 10 HEPES (pH 7.4 with NaOH). Pipettes were filled with the following solution (in mM): 80 CsCl, 1 Mg-ATP, 5 creatine, 5 D-glucose, 10 HEPES, 10 BAPTA, and 4.6 CaCl₂ (intracellular Ca²⁺ concentration clamped at 100 nM), pH 7.4 with CsOH.

Voltage-clamp pulses or ramps were generated and data were captured using a Digidata 1322A interfaced to a computer running the Axon Clampfit software (Molecular Devices). For all data, current was filtered at 1 kHz and sampled at 5 kHz.

**For immunocytochemistry studies, cells were differentiated for 21 days, fixed and double immunostained against MUC5AC (green, goblet cells), acetylated-α-tubulin (yellow, ciliated cells), and actin (red) proteins. Image analysis was from a healthy control and B is derived from a patient with cystic fibrosis (CF).**
pClamp 9 program (Molecular Devices). Currents were filtered at 1 kHz and sampled at 5 kHz. Current kinetics and voltage-dependent properties were assessed by applying voltage steps of 1-s duration from a holding potential of −60 mV to test potentials ranging from −100 to 120 mV with 10-mV increments. To monitor the time course of current responses, we used voltage ramps from −100 to 120 mV of 0.5-s duration applied at 10-s intervals. Series resistance was compensated by 70−80%.

**Ussing chamber studies.** Snapwell inserts were mounted in Vertical Diffusion Chambers (Costar) and were bathed with continuously gassed Ringer solution (5% CO2 in O2; pH 7.4) maintained at 37°C containing the following (in mM): 120 NaCl, 25 NaHCO3, 3.3 KH2PO4, 0.8 K2HPO4, 1.2 CaCl2, 1.2 MgCl2, and 10 glucose. In some studies, cells that had been cultured at a reduced temperature (27°C) for 24 h before use were maintained at 27°C in the Ussing chambers throughout the experimental procedure. The solution osmolarity was always between 280 and 300 mosmol/kgH2O for all physiological salt solutions used. Cells were voltage clamped to 0 mV (model EVC4000, WPI), and the short-circuit current (Isc) was measured. Transepithelial resistance (Rt) was measured by applying a 1-mV pulse at 30-s intervals and calculating Rt by Ohm’s Law. Data were recorded using a PowerLab workstation (ADInstruments). Amiloride (10 μM) was added to the apical side of each insert once the baseline Isc had stabilized (typically 250–500 s following imposition of the voltage clamp). A supramaximal concentration of forskolin was added to both the apical and basolateral sides of wild-type (0.6 μM) and CF cells (20 μM) once the response to amiloride had stabilized. Genistein, an isoflavone compound that is used widely to indicate of any difference in the amiloride-sensitive short-circuit current (Isc) was measured. Transepithelial resistance (Rt) was measured by applying a 1-mV pulse at 30-s intervals and calculating Rt by Ohm’s Law. Data were recorded using a PowerLab workstation (ADInstruments). Amiloride (10 μM) was added to the apical side of each insert once the baseline Isc had stabilized (typically 250–500 s following imposition of the voltage clamp). A supramaximal concentration of forskolin was added to both the apical and basolateral sides of wild-type (0.6 μM) and CF cells (20 μM) once the response to amiloride had stabilized. Genistein, an isoflavone compound that is used widely to potentiate F508del CFTR (22), was added to both the apical and basolateral sides of the CF-derived NEC at 6, 18, and 54 M. CFTRinh172 (inh172) and UTP were added apically, both at 30 μM.

**Statistical analysis.** For ion transport and patch-clamp recordings, values are given as mean ± SE; n represents the number of measurements. Data from the patch-clamp studies were analyzed and plotted using the Origin 8.5 software (OriginLab, Northampton, MA). Student’s t-test was used for statistical comparisons, and differences were considered to be statistically significant at two-tail P value <0.05. The data from the IL-8 assays were analyzed by the Kruskal Wallis ANOVA test followed by Dunn’s multiple comparison test.

**RESULTS**

**Primary cell recovery and culture.** The nasal brushing procedure was well tolerated by both patients and control subjects. Indeed, participants declared themselves willing to undergo a further brushing and no marked nasal bleeding was observed. Successful cultures were obtained from 100% of control subjects. For the patients with CF, 16 patients were initially recruited but 4 samples failed to grow because of infections. Two of these four patients were chronically infected with *Burkholderia cenocepacia*. Cells from patients and controls grew similarly in culture reaching 80% confluence typically after 7−10 days in culture in both groups.

**Differentiated cells.** Culture of NECs at the air-liquid interface resulted in the formation of a confluent epithelium that included goblet and ciliated cells as shown by immunocytochemistry (Fig. 1). The ion transport characteristics of these NECs isolated from four healthy (wild type) and four F508del donors were studied (Table 1). Four to six inserts of NEC were used to calculate the mean for each individual donor. Donors were selected at random. Each of the donors formed a confluent epithelium with a classical “cobblestone” appearance that remained dry during air-liquid interface culture (i.e., there was no evidence of media on the apical surface). For this reason, inserts were not excluded from the final analysis based on a perceived low or high Rt value. The Rt values for these donors are shown in Table 1. The aims of the ion transport studies were to 1) assess any differences in the magnitude of the amiloride-sensitive and forskolin-stimulated currents between non-CF and F508del-derived NEC, and 2) to address whether a CFTR-mediated Isc response in the F508del cells could be enhanced by a short-term low temperature culture, as has been observed in both primary CF airway epithelial cell culture models and also numerous engineered cell lines.

All healthy controls developed an amiloride-sensitive short-circuit current (Isc) together with an anion secretory response following the addition of forskolin (Table 1). Figure 2A illustrates a typical raw Isc data trace from a healthy control. Nasal epithelial cells from F508del donors developed a similar transepithelial resistance to the wild-type cells (F508del: 633 ± 256 Ω cm²; wild type: 811 ± 246 Ω cm²; Table 1). There was no indication of any difference in the amiloride-sensitive Isc between the healthy (23.9 ± 8.5 μA/cm²) and F508del (25.0 ± 10.0 μA/cm²) donors in this small sample of the population (Table 1). In contrast, forskolin induced a mean increase in Isc of 0.7 ± 0.2 μA/cm² (n = 4 F508del donors) that compared with an increase of 16.1 ± 3.8 μA/cm² (n = 4) in the wild-type cultures (P = 0.007).

Reduced temperature culture of the F508del cells enhanced the short-circuit current response to both forskolin and genistein (Fig. 2B). Culturing F508del cells at 27°C for 24 h before assay increased the forskolin response from 0.7 ± 0.2 to 1.7 ± 0.3 μA/cm² (n = 4 matched donors; P = 0.027). In addition, the subsequent response to genistein was also elevated following low temperature culture (4.1 ± 0.5 vs. 2.0 ± 0.3 μA/cm²; P = 0.012). F508del cells also responded to stimulation with UTP with a transient increase in Isc. Following the post-UTP decline in Isc, there was a small residual inhibitory response to the CFTR inhibitor inh172.

**Patch-clamp recordings.** Nasal epithelial cells were highly suitable for patch-clamp examination of ion currents as tight electrical seals, in the range 5−10 GΩ with patch pipettes, could be routinely obtained on both control and CF cells. The two types of cells did not differ in size as was evident from similar membrane capacitance values: 29.6 ± 2.2 pF (n = 22).

### Table 1. Summary of ion transport data for wild-type and F508del CFTR NEC donors

<table>
<thead>
<tr>
<th>CF Status</th>
<th>Rt, Ω cm²</th>
<th>BL, μA/cm²</th>
<th>ΔAM, μA/cm²</th>
<th>ΔFSK, μA/cm²</th>
<th>ΔGen, μA/cm²</th>
<th>ΔFSK + Gen, μA/cm²</th>
<th>n</th>
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<tbody>
<tr>
<td>Non-CF</td>
<td>811 ± 246 (270−1,439)</td>
<td>27.7 ± 8.2</td>
<td>23.9 ± 8.5</td>
<td>16.1 ± 3.8</td>
<td>NT</td>
<td>NT</td>
<td>4</td>
</tr>
<tr>
<td>F508del</td>
<td>633 ± 256 (154−1,272)</td>
<td>31.2 ± 7.2</td>
<td>25.0 ± 10.0</td>
<td>0.7 ± 0.2</td>
<td>2.0 ± 0.3</td>
<td>2.7 ± 0.4</td>
<td>4</td>
</tr>
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</table>

Data represent the means ± SE values for the ion transport properties of cystic fibrosis (CF) and non-CF derived nasal epithelial cells (NECs) cultured at air-liquid interface. Data are derived from studies of 4 independent donors within each group. In addition, the upper and lower values for transepithelial resistance (Rt) are shown in parentheses. BL, baseline short-circuit current (Isc); Δ, change in response to stated treatment; n, number of independent donors; NT, not tested in this group; AM, amiloride; FSK, forskolin; Gen, genistein.
in control cells and 26.2 ± 3.7 pF (n = 11) in CF (F508del) cells (P = 0.408). To isolate the epithelial sodium channel (ENaC) and CFTR currents, we used a Cs+-based pipette solution with internal calcium “clamped” at 100 nM using 10 mM BAPTA/4.6 mM CaCl2 mixture. Thus any K+ currents were abolished while contribution of any Ca2+-dependent conductances was minimized. In nonstimulated cells, whole-cell currents had similar amplitude and kinetics properties (Fig. 3). These background currents tended to be somewhat smaller in CF cells (F508del) although this was not significant. Patch-clamp measurements revealed that control cells responded to forskolin (10 μM) with an increase in chloride current (Fig. 4, A and B) while CF cells were completely nonresponsive (n = 6), even to 30 μM forskolin (Fig. 4, C and D). In contrast, an amiloride-sensitive ENaC current was identified in both control and CF cells (Fig. 4, A and C). On average, 10 μM amiloride reduced current amplitude at 120 mV by 65 ± 6% (n = 4; range 31–73%) in control cells and by 55 ± 7% (n = 5; range 33–65%) in CF cells. The difference between the two cell types was not significant (P = 0.329); however, large cell-to-cell variability in the size of the amiloride-sensitive current component was present in each group making evaluation of any difference a very difficult task. Thus “Power and Sample size” calculations in Origin 8.5 indicate insufficient power (0.29) at the sample size n = 9 and calculated pooled standard deviation of 14.2, while increasing power to 0.95 would require sample size of 54, which is not feasible in patch-clamp measurements.

**IL-8 responses.** Cytomix induced significant IL-8 release from both control and CF epithelial cells compared with the spontaneous release (Fig. 5). However, although spontaneous and induced IL-8 release was higher from control epithelial cells than from CF cells, this did not reach statistical significance.

**DISCUSSION**

The method described in this study enables the culture of CF and control nasal epithelial cells. Since the harvesting of the cells is without marked trauma or stress to the patient or volunteer, it enables easy comparison of cells from patients with different CF mutations as well as the inclusion of age- and sex-matched control subjects. We believe that this method may help to investigate some of the challenging problems associated with the bioelectrical function of CF epithelium and the relationship to infection and inflammation.

This is not the first report to use nasal brushings as a source for epithelial cells. Noncultured brushings have been used to determine ciliary beat frequency (39) for the assessment of cell...
populations (4) and the determination of CFTR location and function (13, 20). Lopez-Souza et al. (28) described novel methods to establish three-dimensional cultures obtained by scraping the nose with a curette and subsequent electrophysiological investigations. However, these were healthy control subjects. Although both nasal and bronchial brushings have been used to establish cultures in other diseases (12, 30, 37), to our knowledge there are only two previously published reports of cultured nasal epithelial cells from young children with CF (3, 33). The success rate in the work of Mosler et al. (33) was lower than in this paper (confluence achieved in only 7 of 17 cultures from 2 CF patients and 5 non-CF patients) and they only reported on inflammatory mediator release from 3 cultures (2 non-CF and 1 CF). In our hands, the CF cultures which failed were due to infection, whereby two of the subjects were already colonized with *Burkholderia cenocepacia*, which may explain their failure to grow in vitro. None of the subjects in the paper by Mosler et al. (33) were infected with this organism. Black et al. (3) performed nasal brushings on children who were undergoing clinically indicated bronchoscopy. They did not report any failures from the 13 subjects studied (7 with CF).

Patch-clamp examination of the primary epithelial cell cultures from both CF and control subjects was performed without difficulty. At least 75% of cells developed tight seals in excess of 5 &OHM; resistance. In three separate experiments, we were able to patch &gt;30 cells obtained from 4 control and 3 CF donors. Thus this new human model system is of considerable promise for the use in Automated Patch-clamp Systems (APS), like the PatchXpress 7000A, which have recently revolutionized ion channel drug discovery in the pharmaceutical industry (45). In our experiments, ion current recordings were aimed at establishing appropriate conditions rather than at any detailed study of a particular current type. Nevertheless, both control and CF cells exhibited amiloride-sensitive currents of comparable magnitude, presumably carried by the ENaC channels, while CF cells lacked any measurable forskolin-induced current that could be attributed to CFTR activation. Interestingly, the ENaC current tended to be smaller in CF cells, but our current sample size had insufficient power to detect any statistically significant difference. Our calculations showed that sample size would have to be at least $n = 54$ to conclude about such difference.

ENaC and CFTR channel currents are often studied in heterologous expression systems or in transformed immortalized airway epithelial cell lines such as bronchial epithelial cell lines 16HBE14o-, CFBE41o-, 9HTEo-, and CF15 (6, 14).

Fig. 4. Effects of amiloride and forskolin on whole cell currents in normal and CF nasal epithelial cells. A and C: time course of a typical current response to amiloride and forskolin applications in a normal and CF (F508del) cell, respectively. Current responses were monitored by applying voltage ramps at 10 s interval and shown at −100 mV (∙) and 120 mV (∙). Forskolin activated CFTR current in normal cells (A), but, as expected, it was without effect in CF cells (C; $n = 6$ in both cell types). B and D: examples of current-voltage relationships measured at the corresponding moments (denoted a-c) in A and C, respectively.

Fig. 5. IL-8 release from wild-type and control nasal epithelial cells (NECs). Comparison of IL-8 release from primary nasal epithelial cell cultures derived from patients with CF ($n = 12$) and control subjects ($n = 8$), both spontaneous (∙) and stimulated with cytomix (see text for details; ▲, ●). Data were analyzed by the Kruskal Wallis ANOVA test followed by Dunn’s multiple comparison test. $^*P < 0.01$.  

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Primary cultured human NECs have been also used in patch-clamp (26) or in Ussing chamber measurements (32), but in both studies the cells were derived from surgically removed polyps or turbinates. We have already discussed several advantages and disadvantages of this approach. To our knowledge only one earlier patch-clamp study employed brushed human nasal epithelial cells (18), but this was limited to normal donors only.

The ion transport characteristics of the F508del homozygote epithelia were similar to previous reports using primary human airway epithelial cell cultures and native epithelium (10, 23, 35). The small or absent anion secretory responses to forskolin and genistein in the F508del cells (cultured at 37°C throughout) are consistent with either a lack of, or reduced expression of, CFTR at the apical membrane of the epithelia. Thus the combination of forskolin and genistein was able to induce an increase in $I_{sc}$ of $\sim 2.5$ μA/cm², suggesting that there is likely some expression of F508del CFTR at the plasma membrane, consistent with previous reports (5, 35). In contrast, all wild-type epithelia studied demonstrated a robust forskolin-stimulated response ($\sim 16$ μA/cm²) without the requirement for further “potentiator” by genistein. Together, these data would suggest that genistein may be able to potentiate F508del activity to $\sim 15\%$ of the wild-type control in this model system.

The amiloride-sensitive component of the nasal potential difference has been demonstrated to be elevated in CF (27). Some cultured CF airway epithelial cell models have also reported an enhanced ENaC-mediated Na⁺ transport. Two of the largest published data sets that have examined large numbers of donors have indicated some elevation of amiloride-sensitive $I_{sc}$ in F508del derived primary airway epithelia but in the absence of a change in Na⁺ conductance (10, 23). To assess any such difference in amiloride-sensitive current in CF NEC using the present assay format will require the characterization of additional donors to increase the study power. However, the data obtained to date suggest than any putative elevation of ENaC-mediated $I_{sc}$ in this CF NEC model is likely to be relatively modest.

We found no statistical difference in IL-8 released from control and CF epithelial cells either spontaneously or after stimulation. Similar results were reported by Black et al. (3) who found no difference in spontaneous or TNFα induced IL-8 release between patients with CF and from their control population. In contrast, Carrabino et al. (7) found raised basal release of IL-8 from primary CF epithelial cells obtained ex vivo from nasal polyps. This may reflect the active inflammatory state within the polyp. Overall, the data in the literature are somewhat variable with increased, decreased, and similar findings being reported for IL-8 release from CF cells compared with controls (2, 3, 7). A recent study using pediatric airway epithelial cells obtained by bronchial brushing also reported no difference between cells from controls and those from patients with CF with respect to basal or stimulated IL-8 release (42). Similar controversy surrounds the interaction between CFTR and ENaC. One previous study using large numbers of donors has indicated a significant elevation of amiloride-sensitive $I_{sc}$ in F508del-derived primary airway epithelia (10), while other studies reported inhibition, stimulation, and no specific influence of CFTR on ENaC function (34). Our present results from both $I_{sc}$ and patch-clamp measurements agree on no obvious difference in amiloride-sensitive current between healthy and CF cells. However, given large cell-to-cell variations in the ENaC size, we cannot exclude that such differences may be revealed with more measurements, which should involve additional donors to increase the study power.

Conclusion. Nasal brushing is a relatively simple method to obtain epithelial cells from patients with CF and control subjects. Patients and control subjects agreed to have the procedure repeated. These cells grow well in culture and investigation of ion transport using patch-clamp techniques or $I_{sc}$ measurements can be readily performed and the cells respond as predicted: amiloride-sensitive ENaC current of similar size was present in both cell types while forskolin-activated CFTR current was lacking in CF cells. The cells can be used to examine mediator secretion. We believe that this system offers an excellent opportunity to compare epithelial cells from patients with different mutations and as a novel system to test new drugs.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


