

Non-bronchoscopic sampling and culture of bronchial epithelial cells in children

G. M. Doherty^{*†}, S. N. Christie^{*†}, G. Skibinski[‡], S. M. Puddicombe[§], T. J. Warke[¶], F. de Courcey[‡], A. L. Cross[¶], J. D. M. Lyons[‡], M. Ennis[‡], M. D. Shields^{*†} and L. G. Heaney[¶]

^{*}Department of Child Health, Queen's University, Belfast, Northern Ireland, [†]Royal Belfast Hospital for Sick Children, Belfast, Northern Ireland, [‡]Respiratory Research Group, Department of Clinical Biochemistry, Queen's University, Belfast, Northern Ireland, [§]Respiratory Cell and Molecular Biology Division, School of Medicine, Southampton General Hospital, Southampton, UK, and [¶]Department of Medicine, Queen's University, Belfast, Northern Ireland

Abstract

Background The bronchial epithelium is likely to play a vital role in airway diseases in children, such as asthma and viral-associated wheeze. In adults, studies with primary bronchial epithelial cells cultured from samples obtained by fibre-optic bronchoscopy have provided key insights into the role of the epithelial cell. However, it is difficult to justify bronchoscopy in children to obtain epithelial cells for research purposes.

Objective To examine the possibility of retrieving and culturing viable epithelial cells using a blind non-bronchoscopic method from children undergoing elective surgery.

Methods Subjects were children undergoing elective surgery under general anaesthesia. Following intubation, non-bronchoscopic bronchoalveolar lavage and non-bronchoscopic bronchial brushing were performed. A sheathed bronchial cytology brush was advanced through the endotracheal tube, wedged and then withdrawn 2–3 cm before gentle sampling was used to collect bronchial epithelial cells. Initial samples were used to characterize the number, type and viability of epithelial cells recovered compared to a control group of adults undergoing standard bronchoscopic sampling. Subsequent samples were used to establish primary bronchial epithelial cell cultures in children both with and without wheezing illness.

Results A total of 63 children underwent bronchial brushing [38 male; median age 7.1 years (1.0–14.2 years)]. Initial samples ($n = 30$) showed recovery of viable epithelial cells comparable to that from a single brush obtained via a bronchoscope in an adult control group ($n = 11$). In 27 (82%) of the subsequent 33 samples obtained non-bronchoscopically from children, primary bronchial epithelial cell cultures were successfully established. There were no adverse effects attributable to sampling.

Conclusion We have shown that non-bronchoscopic bronchial brushing is a safe and effective technique for recovering viable bronchial epithelial cells that consistently yield primary cultures. This method will facilitate examination of the role of the epithelium in paediatric disease.

Keywords airway, asthma, cell culture, child, epithelium, inflammation, sampling

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Introduction

The bronchial epithelium forms the interface between the environment and the lungs. Airborne pollutants, allergens and respiratory viruses that penetrate the defences of the upper airways impact on the bronchial epithelium. Recent findings in adults show that the epithelial cells actively respond to this airborne assault and this response plays a central role in regulating airway inflammation [1–4]. The epithelium is an important source of pro-inflammatory molecules (such as TNF- α , IL-1 β , IL-6, IL-8, MCP1, RANTES, and eotaxin) [4, 5]. It also produces growth factors such as transforming growth factors (TGF- β 1 and 2) and insulin-like growth factor-1 (IGF-1) [6, 7].

Wheezing illness in children is increasing in prevalence and is a significant cause of morbidity [8–10]. Environmental triggers are thought to interact with a number of susceptibility genes to determine the expression of the clinical condition [11, 12]. Phenotypic differences have been demonstrated in epithelial cells from adults with asthma compared to normal adults: they show histological evidence of increased stress and persistent damage [13–15]; they are more permeable to irritant and oxidant gases such as NO₂ and O₃ [16]; and oxidant injury *in vitro* promotes greater apoptosis [2].

While the key role of the epithelium is undisputed, the nature of any primary defect in the epithelial cell has yet to be defined. Injury to the epithelium may initiate or perpetuate inflammation. Aberrant or disproportionate repair may result ultimately in basement membrane thickening and other structural changes in the airway [17, 18]. It is unclear whether the aberrant behaviour observed is inherited or acquired [19].

Correspondence: Dr L. G. Heaney, MD, Department of Medicine, Queen's University, Grosvenor Road, Belfast BT12 6BJ, Northern Ireland.

E-mail: liam.heaney@beh.n-i.nhs.uk

The role of the epithelial cell in childhood wheeze has not been addressed partly because of the ethical and practical difficulties associated with obtaining these samples from children. It is difficult to justify elective bronchoscopic procedures in children for research purposes. It is not known if those abnormalities demonstrated in adults with asthma are present early in the natural history of the disease, nor if the response of the epithelial cell differs in the different wheezing phenotypes.

The value of research in childhood is increasingly recognized [20, 21]. We have previously developed a non-bronchoscopic lavage sampling method in children attending for elective surgery, and using this have demonstrated a Th2-type immune response in the airways of children with chronic persistent atopic asthma [22, 23]. This is not seen in children with wheeze precipitated only by viral infection where the inflammation is characterized by neutrophilia [22].

Given the likely key role of the epithelium in children with asthma and viral-associated wheeze, we wanted to develop a safe, reproducible non-bronchoscopic method to sample epithelial cells from children attending for elective surgery and determine if primary epithelial cell cultures could be established using this method.

Materials and methods

Subjects

We recruited children attending hospital for elective surgery with the expectation that approximately one third of these children would have current 'asthma symptoms' with a range of disease severity, which reflects the prevalence and severity in the community. A detailed asthma and allergy questionnaire was administered by a physician, and children were classified into four groups, as previously described: atopic asthmatics, viral-associated wheezers, non-asthmatic atopics and normal (non-atopic non-asthmatic) controls [22]. Symptoms were considered current if present in the past year, or outgrown otherwise.

Written informed consent was obtained from the parents of the children prior to sampling, and from the children where they demonstrated an understanding of the procedure. The study was approved by the Research Ethics Committee of Queen's University Belfast.

Initially, children ($n = 30$) were studied to examine the feasibility of epithelial sampling using this method. For comparison purposes, adults ($n = 11$) attending for routine diagnostic bronchoscopy, for investigation of abnormal shadowing on chest radiograph or investigation of haemoptysis with no visible endobronchial disease were recruited and gentle epithelial brush sampling was performed under direct vision using the same type of brush as in the non-bronchoscopic method and processed in an identical manner. The total number and viability of the epithelial cells recovered in the two groups was compared using the Mann-Whitney test with an exact distribution (SPSS for Windows Version 11.0, SPSS Inc., Chicago, IL, USA).

We subsequently examined the feasibility of establishing primary epithelial cultures using the non-bronchoscopic samples.

Sample collection and processing

Each child was anaesthetized using standard techniques, with endotracheal intubation. Following intubation and pre-oxygenation, each child had a non-bronchoscopic broncho-alveolar lavage (BAL) performed as previously described. In brief, a sterile 8 FG graduated neonatal suction catheter (Portex Ltd., Hythe, Kent, UK) was inserted through the endotracheal tube and wedged in a distal airway. A single 20 mL aliquot of normal saline was instilled and immediately aspirated [22].

This was followed by a non-bronchoscopic bronchial brush sampling. Bronchial brushing was performed by the insertion of a single sheath bronchial cytology brush (TeleMed Systems Inc., Hudson, MA, USA) through the endotracheal tube and gently wedging its tip in a peripheral bronchus. The external diameter of the sheath is 1.8 mm; the diameter of the brush once extended is 3 mm. Once wedged, brush and sheath were withdrawn 2–3 cm before the brush was advanced 2–3 cm through the protective sheath and gentle brush sampling was performed. Care was taken not to advance the tip of the brush beyond the point where it had wedged. Force was not applied if resistance was encountered. The brush was then withdrawn into its protective sheath before removal. Samples were placed in 5 mL of sterile RPMI 1640 (Invitrogen, Paisley, Scotland) with 20% fetal bovine serum at 4 °C, the brush tip cut off and the process repeated once more.

Cell viability was assessed by exclusion of trypan blue dye. Coverslip preparations were made using the method of Lavolette et al. [24]. Cytospin preparations were made and stained with DiffQuik (American Scientific Products, McGaw, IL, USA) and used for immunocytochemistry (see below).

Epithelial cell immunocytochemistry

Epithelial cell immunocytochemistry was performed using standard techniques. In brief, epithelial cell cytopins were air-dried overnight and fixed in dry acetone (30 min). After air-drying and washing, endogenous peroxidase was blocked by incubation in 0.3% hydrogen peroxide in 1% sodium azide for 30 min. After washing in phosphate-buffered saline, non-specific binding was blocked (3% bovine serum albumin (BSA) for 30 min) and primary antibody was added [anti-cytokeratin-13 or anti-cytokeratin-18 (Sigma-Aldrich, Poole, Dorset, UK) diluted 1:100 in 3% BSA and incubated for 2 h]. After washing, horseradish peroxidase-conjugated secondary antibody was added and incubated for 1 h. After further washing, the slides were process developed (Sigma DAB substrate/buffer tablets, 10 min) and the reaction stopped using phosphate-buffered saline. After rinsing and drying, the slides were counterstained with Mayer's haematoxylin (Sigma-Aldrich).

For immunocytochemical staining of cultured cells, cells were grown directly onto sterile glass coverslips. Fixation in 3% formaldehyde with detection of the primary antibody by a fluorescently labelled secondary antibody (AlexaFluor 488; Molecular Probes, Eugene, OR, USA) was found to give better results for cultured cells than the horseradish peroxidase method [25].

Establishment of primary bronchial epithelial cell (PBEC) cultures

We established PBEC cultures using minor modifications of methods previously described by Lordan and co-workers [2, 26]. Epithelial cells were detached from the brush tips by gentle agitation. The cell suspension was centrifuged at 112 *g* for 5 min. The resulting cell pellet was resuspended in 2 mL of bronchial epithelial growth medium (BEGM: BioWhittaker, Verviers, Belgium) and seeded into sterile plastic flasks with a growth area of approximately 10 cm². After 24 h, the supernatant and unattached cells were removed and replaced with a fresh medium. The removed supernatant was seeded into a second 10 cm² flask to encourage further cell attachment and growth; this process was repeated at 48 and 72 h. Cells were incubated at 37 °C in 5% CO₂/95% air and cultured until 80% confluent.

Cells were then passed from 10 cm² flasks to flasks with a growth area of 75 cm². Cells were liberated using 0.05% trypsin–EDTA solution (Sigma-Aldrich, Poole, Dorset, UK) for three min with activity and quenched using an equivalent volume of RPMI with 20% fetal bovine serum. Cells were centrifuged as above and resuspended in BEGM. Initial samples were passaged three to six times to determine the point at which they became senescent. Epithelial cell purity was determined by examining for the typical morphological features of primary epithelial cells in culture and staining the cells with anti-cytokeratin antibodies.

Results

In this study, a total of 63 children underwent non-bronchoscopic bronchial brush sampling (38 male, median age 7.3 years, range 1.0–14.2). Of these 63 children, 16 had current asthma symptoms and two had outgrown their asthmatic symptoms, 12 had clinical evidence of atopy without asthma and three had viral-associated wheeze; 30 children had no current or past asthmatic symptoms or clinical evidence of atopy.

In the first 30 children (18 male) recruited, we examined the total number of epithelial cells retrieved, their viability, morphology and immunocytochemical staining for cytokeratin. The children were aged from 1.0 to 12.6 years (median 6.7 years). The comparison group consisted of adults (*n* = 11; median age 56.5 years, range 46–84) attending for routine diagnostic bronchoscopy. The total cell retrieval and viability are shown in Fig. 1. No statistically significant differences could be demonstrated between single brushings obtained from adult or paediatric subjects, although there was a non-significant trend towards lower viability of the cells recovered from children.

Morphology demonstrated both ciliated differentiated epithelial cells and a subpopulation of non-ciliated cuboidal cells consistent with epithelial basal cells. Immunocytochemical staining revealed that all cells were positive for cytokeratin 18 and only the basal epithelial cells were positive for cytokeratin 13.

There were no complications at the time of sampling in children. Blood staining of the bronchial brush was usual but minor. Postoperative observations in the 30 children in the first group were similar to our previous reference group of

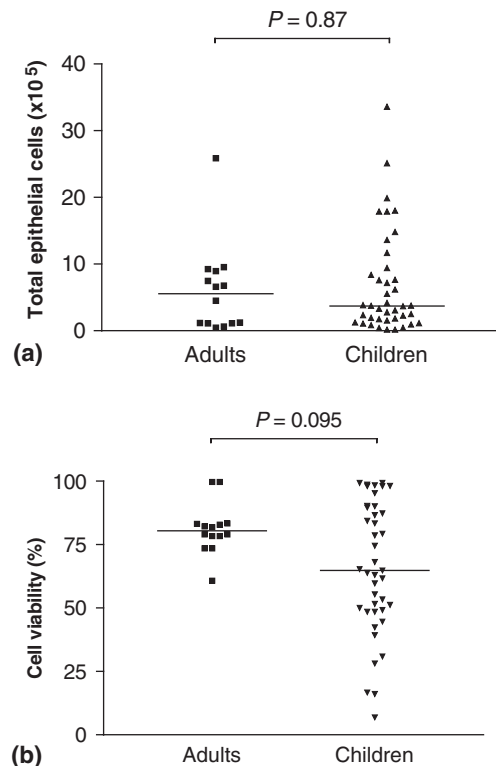


Fig. 1. Total number of epithelial cells recovered and viability in a group of 30 children undergoing non-bronchoscopic sampling and a control group of 11 adults undergoing bronchoscopic sampling. (A total of 48 brushings in the children's group, with seven excluded because very few cells were recovered for counting; and 20 brushings in the adult group with 6 excluded. Comparisons were made by the Mann–Whitney test using an exact distribution: SPSS for Windows Version 11.0.)

children undergoing general anaesthesia who did not have lavage or brush sampling performed [23].

We subsequently obtained bronchial brushings, which were seeded for culture, from 33 children (20 male, median age 8.5 years, range 3.1–14.2 years). These subjects included 15 children without symptoms, 10 with asthmatic symptoms, six with atopy but no history of wheeze and two with viral-associated wheeze. In most of these subjects two brush samples were obtained. In seven, however, only a single brush sample was obtained, usually because of time constraints during the elective surgical lists.

A total of 27 of 33 samples (82%) resulted in successful cultures. Successful cultures included 7 children with asthma, two with viral-associated wheeze and four with atopy but with no history of wheeze. Six cultures were unsuccessful (three from asthmatics, two from non-asthmatic atopics and 1 from a normal child). In two, very little material was recovered macroscopically or microscopically following brushing. Two samples succumbed to bacterial infection (one of these children had marked neutrophilia in BAL suggesting that there may have been a subclinical lower respiratory tract infection). In the other two, no obvious reason for the failure could be identified, although one of these samples was from an asthmatic teenager who smoked. (The only other sample from a child who reported smoking was successfully grown). In the seven children where only a single brush specimen could be obtained, successful cultures were established in six.

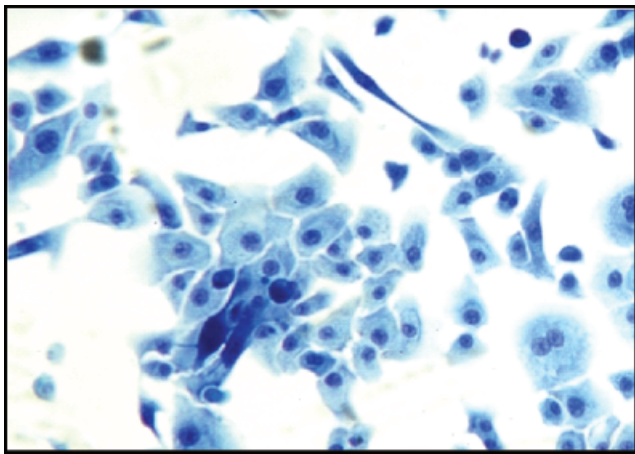


Fig. 2. Typical morphology of cultured epithelial cells (cultured on collagen-coated glass, DiffQuik staining, magnification $\times 200$).

Cells grew in monolayers and demonstrated typical morphology for epithelial cells in culture (see Fig. 2). Immunocytochemistry in selected cultures demonstrated positive staining for both cytokeratin 13 and cytokeratin 18. From seeding to at least 80% confluence over 10 cm^2 took a median of 8 days (range 6–23 days) ($n = 27$). The first seven samples were transferred at this stage from 10 to 25 cm^2 flasks. Growth was rapid, however, and the following 20 of 27 samples were transferred from 10 to 75 cm^2 flasks. Growth over this period was more consistent than at passage 0, with at least 80% confluence reached after a median of 7 days (range 4–11). Differences in the success rate of culture, or crude measures of growth (time to confluence at passage zero or passage one) between the different groups were not statistically significant. For example, the median time to confluence for subjects with wheeze vs. no wheeze was 8 vs. 8 days at passage 0 ($n = 27$; Mann–Whitney U -test: $P = 0.6$) and 7 vs. 6 days at passage 1 ($n = 20$; $P = 0.21$).

Cells continued to grow rapidly for several weeks, but thereafter underwent growth arrest and detachment after 9–13 weeks (corresponding to passage 3–6 depending on rate of growth). [$n = 5$].

Expansion of cultures provided homogenous samples of basal epithelial cells. Each successful culture produced between one and four flasks with a growth surface of 75 cm^2 at passage one. On average, approximately 2 million cells can be recovered from each of these flasks for experiments at passage two ($n = 16$; range 1.0–2.8 million).

Discussion

We have developed a safe non-bronchoscopic sampling method to obtain epithelial cells from children undergoing elective surgery with general anaesthesia and endotracheal intubation. Our comparative studies demonstrate that this method is almost as good in terms of yield of viable cells as a single brush specimen obtained from adults during bronchoscopy under direct vision. Our control group of adults undergoing brushings under direct vision was small and we may therefore be unable to demonstrate small differences between the non-bronchoscopic and bronchoscopic methods

in the viability and number of epithelial cells recovered. However, the rate of successful culture and the total yield of cells at the end of passage one is similar to that reported previously for culture of brushed bronchial epithelial cells using several brushings under direct vision [2]. The slightly lower viability of epithelial cells recovered from children probably reflects a difference in the non-bronchoscopic and bronchoscopic methods, rather than an underlying difference in adult and paediatric physiology. To confirm this would require a comparison group of adults undergoing non-bronchoscopic sampling.

In general, only procedures associated with minimal risk to the child are ethically justified for research purposes where any direct benefit to the child is unlikely [21]. Our previous experience and continued success with the use of a non-bronchoscopic bronchoalveolar lavage method convinced us that a non-bronchoscopic bronchial brushing could be performed safely in children [22, 23, 27–30]. The development of these methods will allow investigation of the epithelial cell in the airways of children.

It is also likely that this method will be applied to other patient groups. The expense and inconvenience of bronchoscopy is often compensated for by the detailed information and samples that can be obtained in each patient undergoing the procedure. However, experiments with sizeable cohorts may be required to detect small differences between groups. The size of the cohorts required may make bronchoscopic sampling practically difficult even in adult volunteers. A non-bronchoscopic method may simplify recruitment and collection using elective surgery as a window of opportunity to obtain cells for research purposes. Moreover, in our experience if there is macroscopically visible material recovered on a single brush specimen then this is usually sufficient for culture. Performing a single non-bronchoscopic brush sample on an intubated patient takes around 30 s. Using elective surgical lists overcomes the ethical difficulties in studying children with mild to moderate asthma during quiescent periods. The brevity of the procedure prevents disruption of the surgical lists.

The cells obtained are consistently epithelial cells. Cells recovered include basal undifferentiated epithelial cells and consistent with this, we have successfully expanded primary cultures from the samples obtained. Structurally and functionally these cells appear similar to samples retrieved by bronchoscopic methods.

Our samples were taken in conjunction with bronchoalveolar lavage samples from all children. This will allow the functional epithelial biology to be related to both cellular and soluble indices of inflammation and tissue repair present in BAL fluid at the time of sampling.

Given the low morbidity of the method, we believe this method will be generally acceptable and will remove any practical and ethical difficulties associated with obtaining these samples from children.

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