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Stable ciliary activity in human nasal epithelial cells grown in a perfusion system

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Abstract

Purpose: Explore the usefulness of a perfusion system in order to establish human nasal epithelial cell cultures suitable for long-term in vitro ciliary beat frequency (CBF) and cilio-toxicity studies.
Methods: The cells were obtained by protease digestion of nasal biopsy material. The cells were plated at a density of $0.8–1 \times 10^6/cm^2$ on Vitrogen-coated polyethylene terephthalate membranes, and cultured under submerged conditions in a CO_2 incubator or in a perfusion system (initiated on days 8–9 after plating). The CBF was determined at $24.1 \pm 0.8\,^{\circ}C$ by a computerized microscope photometry system. The morphology of the cultured cells was characterized by transmission electron microscopy (TEM).
Results: Under CO_2 incubator culture conditions, stable ciliary activity was expressed and maintained from day 2 to day 24. Under perfusion system culture conditions, the CBF (mean ± S.D., $n = 4$) amounted to 8.4 ± 0.9 and 8.8 ± 0.4 Hz on days 7 and 14, respectively. These values were lower as compared to the corresponding CBF obtained in the CO_2 incubator cultures (9.5 ± 0.6 and 9.9 ± 1.0 Hz, respectively). Reference cilio-stimulatory (glycocholate) and cilio-inhibitory (chlorocresol) compounds were used to assess CBF reactivity. In the CO_2 incubator and 7- and 14-days perfusion system cultures, glycocholate (0.5%) showed a reversible cilio-stimulatory effect of 23, 26 and 21%, respectively, while chlorocresol (0.005%) exerted a reversible cilio-inhibitory effect of 36, 40 and 36%, respectively. TEM revealed polarized cuboidal to columnar epithelial morphology, with well-differentiated ciliated cells under CO_2 and perfusion system conditions (up to day 23).
Conclusion: Culturing human nasal epithelial cells on Vitrogen-coated polyethylene terephthalate membranes in submerged conditions in a CO_2 incubator and in a perfusion system offers the possibility for long-term preservation (up to 22–24 days) of stable and reactive CBF in vitro.

Keywords: Human nasal epithelial cells; Ciliary beat frequency; Perfusion system

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1. Introduction

Nasal mucociliary transport is one of the most important local defence mechanisms of the respiratory tract (Jorissen et al., 2000). The efficiency of the mucociliary clearance system depends on the physiological control of the ciliated cells and on the rheological properties of the mucus blanket (Jorissen, 1998). The coordinated ciliary activity is essential for efficient mucociliary clearance and the ciliary beat frequency (CBF) represents the basic and most widely studied functional ciliary parameter (Jorissen et al., 2000). The nasal mucociliary clearance system is susceptible to damage and could be affected by nasally applied drugs, air born particles, pollution, allergens, infection agents as bacteria and viruses. A number of cell culture systems (primary and cell lines) of nasal epithelial cells have been developed and extensively used, particularly for studies on cystic fibrosis, electrolyte transport, ciliogenesis and ciliary movement, mucus secretion, nasal mucosal infection, and the underlying cellular and molecular mechanisms.

A nasal epithelial cell culture suitable for CBF studies requires ciliated cells with stable, coordinated and reactive ciliary activity in the range of 7–12 Hz, as well as preservation of these parameters for a long in vitro period. Immersion feeding and air–liquid interface cultures, established on extracellular matrix, coated or uncoated microporous or non-permeable supports have been used for CBF measurements. The main disadvantages of these systems are the unstable ciliary activity, progressive ciliary loss, lowering of CBF, increased interindividual variability with time in culture, and their short lifespan (Rautiainen et al., 1993; Agu et al., 2001). However, the cells retain some functional characteristics and can be used for CBF measurement at well-defined time points after plating (Haxel et al., 2001). Contradictory results have been reported concerning the effect of time in culture on CBF of human nasal epithelial cell monolayer cultures on microporous cell-support materials. CBF of human nasal epithelial cells cultured on a floating collagen gel has been shown to be 18, 10 and 17 Hz on days 8, 10 and 14, respectively (Yoshitsugu et al., 1994). In cells cultured in an air–liquid interface system, a time-related increase in the ciliary activity has been observed by Rhee et al. (2001), while Agu et al. (2001) and Rautiainen et al. (1993) reported a time-dependent decrease. This functional cilia instability complicates long-term functional and cilio-toxicity investigations in monolayer cultures.

Normal and stable CBF after ciliogenesis in suspension culture has been demonstrated (Jorissen et al., 1989; Jorissen and Bessems, 1995a). The in vitro induced ciliogenesis results in normal (around 7.9 Hz) and intercellularly coordinated ciliary activity for up to 6 months (Jorissen et al., 2000). The suspension culture approach is a useful tool in studying ciliogenesis, ciliary beat frequency, ciliotoxicity and the mechanism involved in their regulation (Jorissen et al., 1989, 2000; Jorissen and Bessems, 1995a; Jorissen, 1998; Agu et al., 1999, 2000; Million et al., 1999; Laoukili et al., 2000). The main disadvantages of the subsequent monolayer-suspension culture are that it is not suitable for transport studies and that it is a time-consuming and costly procedure.

The main purpose of this study was to explore the usefulness of a perfusion system in order to establish a human nasal epithelial cell culture system suitable for long-term in vitro ciliary beat frequency and ciliotoxicity studies. Preservation of CBF for a long period of time ensures availability of ciliated cells without the necessity of repeated and time-consuming cell isolations. Two culture systems, the CO_2 incubator and the perfusion system, have been tested for their ability to maintain stable and reactive CBF of human nasal epithelial cells in vitro. The perfusion culture system MinuCell (Minuth et al., 1992) has been successfully used for culturing renal collecting duct epithelial cells (Minuth et al., 1997, 1999; Strehl et al., 1997), gastric epithelium (Kloth et al., 1998), bovine oviduct epithelial cells (Reischl et al., 1999) and porcine retinal pigment epithelium (Framme et al., 2002). This system provides continuous supply of fresh medium and removal of secreted metabolites and allows long-term culture of epithelia, as well as the use of various cell-support materials (Minuth et al., 2000, 2001; Schumacher et al., 2002).

2. Materials and methods

2.1. Chemicals and materials

Pronase XIV, cholera toxin, glycocholate, and penicillin-streptomycin solution (10,000 IU/ml and 10,000 μg/ml, respectively) were purchased from

Sigma Chemical Co. Ltd. (St. Louis, MO, USA). DMEM-Ham's F12 1:1 medium, Ultroser G and NU-serum were obtained from Life Technologies Ltd. (Paisley, UK). Vitrogen was purchased from Nutacon B.V. (Leimuiden, The Netherlands), HEPES buffer from Cambrex Bio Science Verviers (Verviers, Belgium), chlorocresol from UCB (Leuven, Belgium), polyethylene terephthalate membrane with pore size of 3 μm from Cyclopore S.A. (Louvain-La-Neuve, Belgium). Agar 100 Resin, dodecenyl succinic anhydride, methyl nadic anhydride, and benzyldimethylamine were purchased from Agar Scientific Ltd. (Essex, UK). The MinuCell perfusion system container (holding 24 rings), 13 mm diameter cell-support material holding rings, a thermo plate (Medax, Nogel GmbH), and a IPC high precision multichannel dispenser (Ismatec SA, Labortechnik-Analytik, Glattbrugg-Zürich, Switzerland) were obtained from MinuCells and Minutisue Vertriebs GmbH (Bad Abbach, Germany). Tissue culture 6-well plates (Nunc) were obtained from International Medical Products (Brussels, Belgium), 12-well plates (Costar) from Elscolab (Kruibeke, Belgium), 25 cm^2 tissue culture flasks and a 70 μm nylon cell strainer from BD Falcon (Oxnard, CA).

2.2. Cell isolation

Human nasal epithelial cells were isolated from nasal biopsies as described previously (Dimova et al., 2003). Briefly, the human nasal epithelial tissues were enzymatically dissociated using 0.1% pronase solution in DMEM-Ham's F12 1:1 medium, supplemented with 50 IU/ml penicillin and 50 μg/ml streptomycin for a period of 24–44 h at 4 °C. The protease activity was inhibited with 10% NU-serum. The cells were washed three times in DMEM-Ham's F12 1:1 medium, supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin, 2% Ultroser G, and 10 ng/ml cholera toxin by centrifugation (800 rpm, 5 min, 4 °C) and incubated for 1 h in a 25 cm^2 plastic tissue culture flask in a CO_2 incubator (5% CO_2–95% air, 37 °C) to allow selective attachment of the contaminating fibroblasts and macrophages. After centrifugation (800 rpm, 5 min, 4 °C) the cells were resuspended in 3–5 ml medium (depending on the cell pellet size), and were filtered through a 70 μm pore size nylon filter in order to obtain a more homogenous cell suspension. The cell number was determined using a Bürker chamber; the cell viability, assessed by trypan blue exclusion, was 92 ± 3% (range 87–97).

2.3. Cell culture

Polyethylene terephthalate membrane with pore size of 3 μm, coated with glutaraldehyde-stabilized Vitrogen (1.2 mg/ml) was used as a cell-support material. The membranes were fixed in holding rings (MinuCell rings) with external and internal diameters of 13 and 9 mm, respectively (Fig. 1). In order to ensure high cell plating density, the holding rings were placed in cut 15 ml tubes. The autoclaved membranes were coated with neutralized Vitrogen (pH 7.5, 1.2 mg/ml)

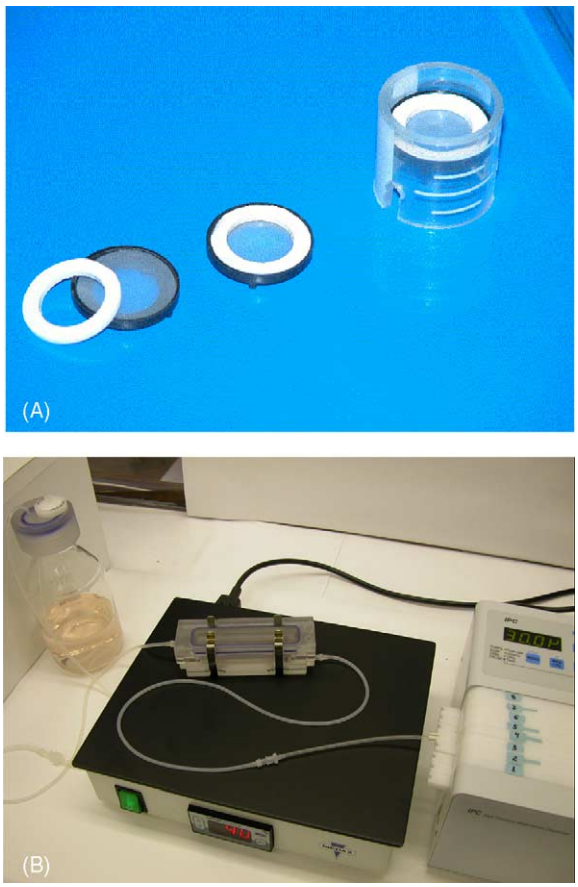

Fig. 1. (A) MinuCell setup rings used to hold the support filters and the tubes in which the rings were positioned. (B) The perfusion system.

and dried for 60–120 min at room temperature. The gels were stabilized by glutaraldehyde cross-linking (2.5% glutaraldehyde in PBS, 0.5 ml/ring, 10 min), rinsed five times with PBS, and preconditioned with culture medium for at least 30 min. The rings positioned in the "tubes" were placed in 6-well plates containing 11 ml medium at the basolateral compartment. The cells were plated at a density of $0.8–1 \times 10^6$ viable cells/cm^2 in 0.5 ml medium per ring. The effective growth surface of the support filter fixed in MinuCell rings is 0.636 cm^2.

2.3.1. CO_2 incubator cultures

The cells were cultured in DMEM-Ham's F12 1:1 medium, supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin, 2% Ultroser G and 10 ng/ml cholera toxin at 37 °C in an atmosphere of 5% CO_2–95% air. The medium in the apical compartment (0.5 ml) was changed six times/week, while 5 ml of the medium in the basolateral compartment was changed every other day. Human nasal epithelial cell cultures were cultured at CO_2 incubator conditions for 2–24 days and used for CBF measurements every other day after plating.

2.3.2. Perfusion system cultures

The cultures were transferred from the CO_2 incubator into the perfusion culture container on days 8–9 after plating. The container was placed onto a 40 °C thermo plate (Fig. 1B) including a cover lid. DMEM-Ham's F12 1:1 medium, supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 2% Ultroser G, 10 ng/ml cholera toxin and HEPES (35 mM final concentration) was continuously perfused at a rate of 30 μl/min using a peristaltic pump. HEPES was used to maintain constant pH at 7.4 in the perfusion system which runs outside a CO_2 incubator at laboratory room atmosphere. The medium used for the perfusion system was maintained at 4 °C. Human nasal epithelial cells were cultured at perfusion system conditions for 7 and 14 days with total culture time of 15–16 and 22–23 days, respectively.

2.4. Cell treatment

In order to assess the reactivity of the CBF of the cell culture systems, glycocholate and chlorocresol were used as reference cilio-stimulatory and cilio-inhibitory compounds, respectively. The effect of glycocholate (0.5%) and chlorocresol (0.005%), dissolved in the corresponding cell-culture medium, on the CBF was tested. The CO_2 incubator cultures were used on days 13–14, and the 7- and 14-days perfusion system cultures on days 15–16 and 22–23 after plating, respectively.

The cells were preconditioned for at least 30 min at 24 °C. CBF was measured between 10 and 30 min after submerging the cells in a solution of the test compounds (2 ml/well, 12-well plates). To investigate whether the effect on CBF is reversible after withdrawal of xenobiotic exposure, the CBF was also determined between 25 and 45 min after rinsing of the cells (3×2 ml medium in 3 min) and incubation with medium. The control cells were incubated with medium only and treated in the same way.

2.5. Ciliary beat frequency measurement

The ciliary beat frequency (CBF) was determined at 24.1 ± 0.8 °C by a computerized microscope photometry system consisting of Olympus IX70 microscope and FluarQuant photometry system (Applied Scientific Instrumentation Inc., Eugene, OR, USA) supplied by Omnilabo, Belgium. The signal from the fluctuations of light intensity, caused by beating cilia, was transduced to an electrical signal, amplified and transmitted to a personal computer. The signal was measured using 600× microscope magnification for a period of 1 min, with a sampling interval of 5 ms (FluarQuant software). The recorded signal was analysed by performing time spectral analysis using Fast Fourier Transformation on the waveform obtained (WinDaq software). Ten periods from the signal obtained for 1 min were analysed for each cell. The highest peak of the first harmonic within these time segments represented the mean beat frequency of the cilia. The CBF of 5–14 different cells was measured per group in a given experiment. The number of cells measured varied among groups in an experiment depending on the ease with which an acceptable signal was obtained during the defined time period and on the suitability to calculate CBF from the recorded signals afterwards. The interindividual, inter- and intracellular variability in CBF was calculated as previously described (Jorissen and Bessems, 1995a).

2.6. Morphology of the cultures

The morphology of the cultured cells was characterized by transmission electron microscopy. Perfusion system cultures (7 and 14 days at perfusion system culture conditions) and the corresponding CO_2 incubator cultures (15–16 and 22–23 days after plating) were removed from the 13 mm holding rings, fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) overnight at 4 °C, and maintained in 0.1 M sodium cacodylate buffer until further processing (4 °C). The samples were postfixed for 1 h in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4), and dehydrated in a graded series of ethanol (25, 50, 75% and absolute). After 1 h incubation in Epon:propylene oxide (1:1) the membranes were embedded in fresh Epon. The Epon was polymerised for 24 h in a 57 °C oven. Ultrathin sections were cut (Ultramicrotome System 2128, LKB), mounted on copper grids, stained with uranyl acetate and lead citrate, and examined with a Philips (CM 10) electron microscope.

2.7. Determination of the percentage of ciliated cells

The percentage of ciliated cells was estimated in some of the cell cultures by counting the number of the ciliated and non-ciliated cells in 100 areas on the screen of the photometry system (microscope magnification of 600×).

2.8. Data presentation and statistical analysis

The results are expressed in hertz as mean ± S.D. The interindividual variability represents the S.D. of the mean CBF of all individuals at the same time point in culture; the intercellular variability represents the S.D. of the mean CBF obtained from each measurement; and the intracellular variability represents the S.D. of the mean CBF determined in 10 consecutive periods for each cell (Jorissen and Bessems, 1995a). The data were statistically analysed by paired Student's *t*-test, one-way or repeated measures ANOVA followed by Bonferroni's multiple comparison test. $p < 0.05$ was considered as significant. The statistical method used in a given analysis is specified in the corresponding text, table or figure legend.

3. Results and discussion

Due to species differences, the use of primary human nasal epithelial cells in culture is a promising system enabling the prediction of the nasal toxic potential and its mechanism in humans. Because the human origin of the cells gives more direct clinical relevance of the studies performed, cultures of human nasal epithelial cells were used in this study. A primary goal in the development of an ideal in vitro cell culture systems is to maintain differentiated morphology and biochemical features, resembling the original tissue as closely as possible (Yamaya et al., 1992; Gruenert et al., 1995). A nasal epithelial cell culture suitable for CBF and cilio-toxicity studies requires ciliated cells with coordinated and reactive ciliary activity, CBF values in the physiological range (7–12 Hz), as well as preservation of these parameters for a relatively long in vitro period. Our preliminary results have shown a mean CBF value of 8.6 ± 0.1 Hz (three individuals) on day 12 at submerged CO_2 incubator conditions and 8.3 ± 0.7 Hz (five individuals) after 8–9 days in the perfusion system (days 14–17 after plating) in human nasal epithelial cells cultured on glutaraldehyde-stabilized Vitrogen-coated polyethylene terephthalate membranes. The main goal of this study was to explore the conditions of a perfusion system for culturing human nasal epithelial cells suitable for long-term in vitro ciliary beat frequency measurements and cilio-toxicity studies.

Because the time-related pattern of ciliary activity is more stable at 25 °C as compared to 33 and 37 °C (Phillips et al., 1990), the CBF was determined at a relatively constant room temperature of 24.1 ± 0.8 °C.

3.1. CBF with time in culture

3.1.1. CO_2 incubator cultures

The CBF of submerged CO_2 incubator cultures established from biopsy material obtained from three patients was followed from day 2 to day 24. Stable ciliary activity was expressed and maintained in human nasal epithelial cell cultures on Vitrogen-coated polyethylene terephthalate membranes from day 2 (8.3 ± 0.35 Hz) to day 24 (8.4 ± 0.50 Hz) after initiation of the cultures (Fig. 2). No significant differences in the mean CBF values, interindividual, and intercellular variability have been observed as a function of

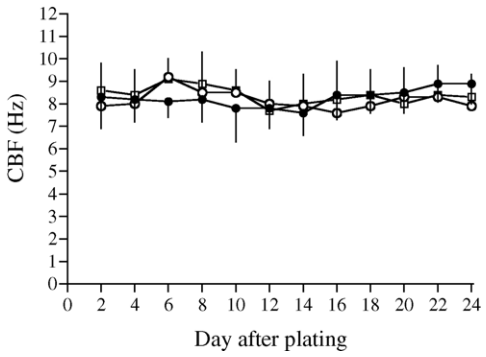

Fig. 2. CBF of human nasal epithelial cell cultures from three individuals as a function of time in culture. The cells were plated at a density of 0.8–1 × 10^6/cm^2 on Vitrogen-coated polyethylene terephthalate membranes, and cultured under submerged CO_2 incubator conditions. The results are expressed in hertz as mean ± S.D. (intercellular variability), n = 10–14 cells, except day 4 in one of the cultures n = 6 (-●-).

culture time (repeated measures ANOVA, Bonferroni's multiple comparison test).

The mean CBF determined in all measurements performed in this experiment was 8.3 ± 1.1 Hz (n = 412) with a 95% confidence interval (CI) from 8.2 to 8.4 Hz, and ranged between 5.3 and 12 Hz. These data agree with the range of ciliary frequency reported for in vitro conditions and determined at room temperature (Jorissen and Bessems, 1995a; Boek et al., 1999; Jorissen et al., 2000). The CBF observed on day 24 after initiation of the cultures was 8.4 ± 0.5 Hz, a value which is close to the frequency determined in human nasal epithelial cells cultured 3 weeks in suspension (8.6 ± 0.9 Hz) (Jorissen and Bessems, 1995a).

The interindividual variability (0.34 ± 0.13 Hz), was lower than that reported for biopsy material (1.6 Hz), monolayer culture (1.6–3.8 Hz), and suspension culture (1.0 Hz) (Jorissen and Bessems, 1995a) suggesting the high reproducibility and stability of the cell culture system used. The intercellular and intracellular variability for all measurements were 1.1 ± 0.26 Hz (n = 36) and 0.31 ± 0.21 Hz (n = 412), respectively and were similar to the same functional ciliary parameters reported for biopsy material, monolayer and suspension cultures (Jorissen and Bessems, 1995a).

The stable ciliary activity (up to 24 days) in human nasal epithelial cells on polyethylene terephthalate membranes (pore size 3 μm), coated with glutaraldehyde-stabilized Vitrogen, and cultured at submerged CO_2 incubator conditions suggests the potential of this cell-support material for long-term preservation of ciliary function in vitro.

3.1.2. *Perfusion system cultures*

The MinuCell perfusion culture system has been successfully used for culturing epithelial cells with different origin. The continuous slow-rate perfusion of the cultures guarantees constant supply of nutrients, prevents unphysiological accumulation of metabolic products, and mimics more closely the in vivo conditions (Minuth et al., 2000). This perfusion system offers the possibility for long-term cell culturing with reduced culture handling, leading to lower risk of contamination. The system is semi-automated, and those assembled under sterile conditions can run outside a CO_2 incubator in a not sterile laboratory environment.

Human nasal epithelial cells originating from four patients were cultured in the perfusion system for 7 and 14 days. The CBF was determined, and compared to this of the corresponding CO_2 incubator cultures (Table 1). No significant difference was observed in CBF between days 7 (8.4 ± 0.87) and 14 (8.8 ± 0.38), suggesting stable ciliary activity under perfusion system conditions. Although the CBF in perfusion system cultures was lower (significant at day 7 in perfusion system; days 15–16 after plating) compared to the corresponding CO_2 incubator cultures, the mean CBF values and frequency range (6.8–11.7 Hz) were similar to these reported for suspension cultures (Jorissen and Bessems, 1995a). The interindividual, intercellular and intracellular variability in the perfusion system cultures were comparable to the same functional parameters in the corresponding CO_2 incubator cultures. These results showed stable and reproducible ciliary activity in human nasal epithelial cells, cultured at perfusion system conditions.

In cultures of human nasal epithelial cells on uncoated polyethylene terephthalate filters, 5% ciliated cells on day 6 has been reported (Werner and Kissel, 1995), and disappearance of ciliated cells before day 21 has been observed in air–liquid interface cultures on collagen type I coated microporous membranes and CD-24 cellagen inserts (Agu et al., 2001). Although a decline of the number of ciliated cells may have occurred as a function of culture time, a sufficient number of ciliated cells for CBF measurement was present up

Table 1
CBF, interindividual, intercellular and intracellular variability (Hz) of human nasal epithelial cells cultured at CO_2 incubator and perfusion system conditions

Culture conditions	CO_2 incubator		Perfusion system	
Days after plating	15–16	22–23	15–16	22–23
Days in perfusion system			7	14
Number of cultures	4	4	4	4
CBF	9.5 ± 0.59	9.9 ± 0.96	$8.4 \pm 0.87^*$	8.8 ± 0.38
Interindividual variability	0.59	0.96	0.87	0.38
Intercellular variability	1.13 ± 0.22	1.4 ± 0.50	0.95 ± 0.40	0.85 ± 0.35
Intracellular variability	0.23 ± 0.10	0.23 ± 0.13	0.28 ± 0.05	0.23 ± 0.13
Percentage of ciliated cells	14 ± 4	13 ± 2	13 ± 2	12 ± 3

The human nasal epithelial cells were plated at a density of $0.8–1 \times 10^6/cm^2$ on polyethylene terephthalate membranes coated with glutaraldehyde-stabilized Vitrogen, and cultured in submerged conditions in a CO_2 incubator and/or a perfusion system. The results are presented in hertz as mean ± S.D.

* $p < 0.05$ vs. corresponding time-point CO_2 incubator value, paired Student's *t*-test.

to day 23 under CO_2 incubator and perfusion system conditions (Table 1). The mean percentage of ciliated cells on days 22–23 after plating in the two-cell culture systems tested was greater than 10%. No substantial differences in the percentage of ciliated cells were observed between perfusion system and CO_2 incubator culture conditions, and between days 15–16 and 22–23 after plating, suggesting long-time preservation of ciliary cell differentiation.

An in vitro system of human nasal epithelial cells suitable for cilio-toxicity studies requires the presence of ciliated cells with CBF values in the physiological range, as well as reactive cilia. To assess the CBF reactivity and the reliability of the cell culture systems used for cilio-toxicity studies, the effect of reference cilio-stimulatory (glycocholate) and cilio-inhibitory (chlorocresol) compounds was tested.

3.2. Reactivity of the ciliary function

Glycocholate and chlorocresol have been used with success as reference cilio-stimulatory and cilio-inhibitory compounds, respectively to validate the potential of human nasal epithelial cell cultures for cilio-toxicity studies (Agu et al., 1999; Dimova et al., 2003). The CBF was measured between 10 and 30 min after exposure to the model compounds and after rinsing and subsequent incubation (25–45 min) with medium only. The selection of these time points was based on the nasal clearance half-life in humans, which has been determined to be in the range of 15–30 min (Andersen and Proctor, 1983; Soane et al., 1999). For these experiments, CO_2 incubator cultures were used at days 13–14, and 7- and 14-days perfusion system cultures on days 15–16 and 22–23 after plating, respectively.

No significant differences in the mean control CBF values were observed between 10–30 min of incubation with medium and 25–45 min after rinsing, suggesting the stability of the ciliary activity during the experimental procedure in human nasal epithelial cells cultured under CO_2 incubator and perfusion system conditions (Fig. 3).

The absorption enhancer glycocholate (0.5%) showed a reversible cilio-stimulatory effect after 10–30 min of exposure in the two cell culture systems used. In submerged CO_2 incubator cultures, CBF increased by 23% (Fig. 3). A similar cilio-stimulatory effect of 26 and 21% was observed in human nasal epithelial cells cultured in a perfusion system for 7 and 14 days, respectively. These findings are in agreement with previously reported results concerning the effect of glycocholate on CBF of human nasal epithelial cells in monolayer and suspension cultures obtained with a Leitz MDV C2 photometry system (Agu et al., 1999; Dimova et al., 2003).

The lipophilic preservative chlorocresol (0.005%) exerted a reversible cilio-inhibitory effect of 36, 40 and 36% in human nasal epithelial cells cultured at CO_2 incubator conditions, and perfusion system conditions for 7 and 14 days, respectively (Fig. 3). A cilio-inhibitory effect of chlorocresol between 30 and 70% has been shown in chicken and rat tracheal explants and human nasal epithelial cells in vitro and its degree of inhibition

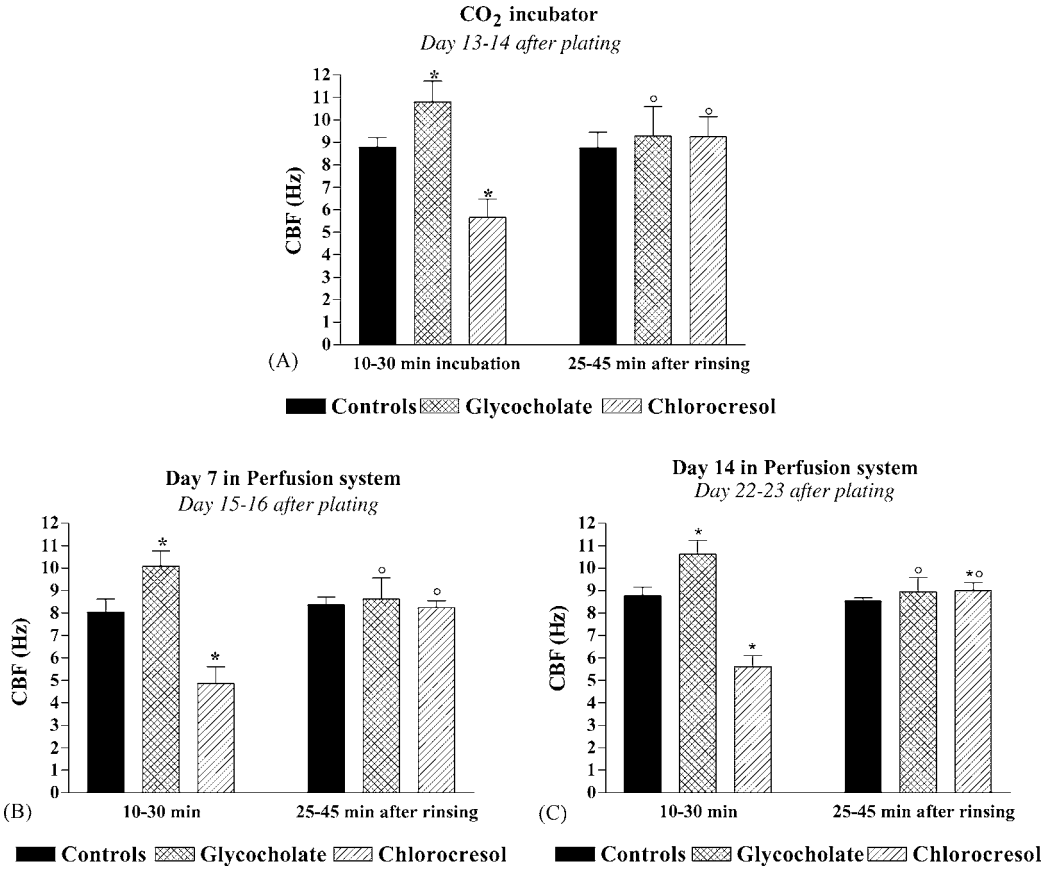

Fig. 3. Effect of 0.5% glycocholate (reference cilio-stimulatory compound) and 0.005% chlorocresol (reference cilio-inhibitory compound) on CBF of human nasal epithelial cells after 10–30 min exposure and 25–45 min after rinsing. (A) CO_2 incubator cultures on days 13–14 after initiation. (B) Perfusion system cultures on day 7 (days 15–16 after plating). (C) Perfusion system cultures on day 14 (days 22–23 after plating). The results are expressed as mean ± S.D. (interindividual variability, $n = 3$–4); each value represents the mean CBF of 5–11 cells. $^{*}p < 0.05$ vs. corresponding time-point control, $^{o}p < 0.05$ vs. corresponding 10–30 min; paired Student's *t*-test.

depends on the concentration(s) tested and the model used (Batts et al., 1990; Joki et al., 1996; Agu et al., 1999). The inhibitory effect observed in the cell culture systems used in this study was close to that described in suspension culture system (34%) (Agu et al., 1999).

The modulation of the ciliary activity by reference cilio-stimulatory and cilio-inhibitory compounds showed the reactivity of the cilia, and suggests the usefulness of human nasal epithelial cells cultured under immersed CO_2 incubator and perfusion system conditions for cilio-toxicity studies. The potential of perfusion system(s) for culturing human nasal epithelial cells for long-term CBF studies has been shown for the first time. The system is characterised by high reproducibility, stability, simplicity and reduced cell culture handling.

3.3. Morphology of the cultures

The morphology of the cultures was characterised by transmission electron microscopy. No substantial differences between the CO_2 incubator and perfusion system cultures, and between 15–16 and 22–23 days after plating have been observed. In the two-cell culture systems the cells remain polarized and revealed

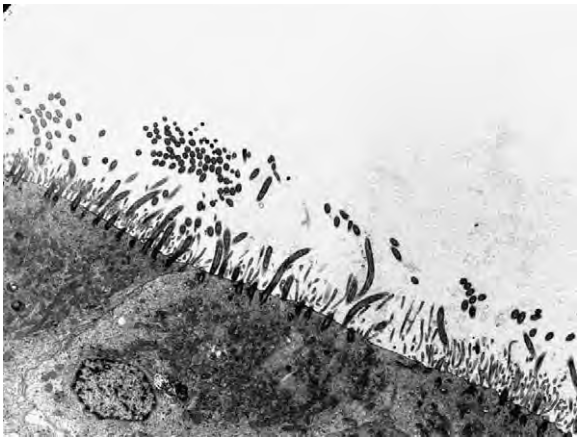

Fig. 4. Electron micrograph of human nasal epithelial cells cultured on Vitrogen-coated polyethylene terephthalate membrane, cultured for 14 days under perfusion system conditions (day 22 after plating). Original magnification: 3900×.

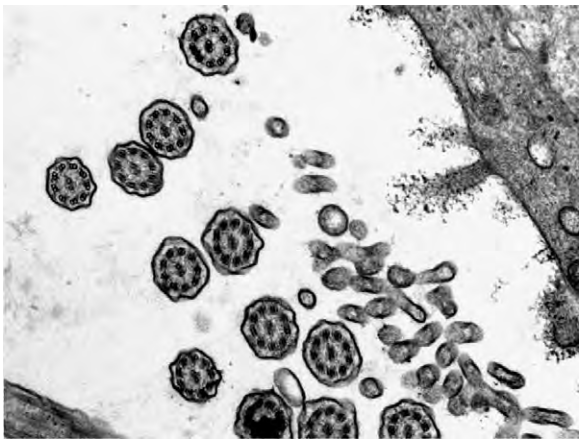

Fig. 5. Electron micrograph of cross-sections of cilia of human nasal epithelial cells cultured on Vitrogen-coated polyethylene terephthalate membrane, under perfusion system culture conditions for 7 days (day 15 after plating). Original magnification: 39000×. The cross-sections of the cilia revealed normal ciliary structure, and one-directional orientation of the central microtubular singlets, suggesting coordinated ciliary activity.

epithelial cell differentiation up to days 22–23 after plating.

The vertical sections showed that the epithelial layer was composed of one to three layers of cells. The upper cell layer consists of cuboidal to columnar cells, with microvilli and/or numerous cilia with basal bodies (Fig. 4). The cross-sections of the cilia revealed normal ciliary structure, and one-directional orientation of the central microtubular singlets suggesting coordinated ciliary activity (Fig. 5). The underlying cells were more flattened, with long plasma membrane extensions, and lysosomal granules, which were more abundant in 22–23 days-old cultures. Occasionally, single fibroblasts could be seen in some of the sections, but the presence of this cell type was not typical of the cultures.

In summary, human nasal epithelial cells on Vitrogen-coated polyethylene terephthalate membranes maintained polarized epithelial cuboidal to columnar apical layer morphology, with well-differentiated ciliated cells at CO_2 and perfusion system culture conditions (up to days 22–23 after plating; day 14 at perfusion system).

The reason for the maintenance of differentiated epithelial morphology, with a sufficient percentage of ciliated cells which allows easy selection during CBF-measurements and with stable and reactive CBF in relatively simple cell culture systems (submerged culturing conditions, medium supplemented with Ultroser G, cholera toxin and antibiotics only) is not yet clear but some speculative suggestions can be made. It has been shown that in monolayer cultures, different factors affect the maintenance of ciliated cell morphology: cell-support material, extracellular matrix, high plating density, air–liquid interface, medium and its supplements (Van Scott et al., 1986; Davenport and Nettesheim, 1996).

In the culture system tested the polyethylene terephthalate membranes were coated with glutaraldehyde-stabilized Vitrogen (collagen type I), the cells were plated at relatively high density ($0.8–1 \times 10^6$ cells/cm^2), and Ultroser G was used instead of serum. It has been established that cells grown on permeable supports show better preservation of morphology and function than cells grown on a plastic support (Van Scott et al., 1986). Collagen type I has shown the most promising results concerning the maintenance of ciliary cell differentiation in airway epithelial cell cultures (Davenport and Nettesheim, 1996; Agu et al., 2001), which could be related to the fact that the collagen fibers represent the main component of the respiratory basement membrane (Mygind and Dahl, 1998). It has been found that the collagen gels

increase attachment efficiency, cuboidal morphology, and are critical for ciliated cell differentiation of airway epithelial cells in vitro (Van Scott et al., 1988; Davenport and Nettesheim, 1996; Schmidt et al., 1996; Agu et al., 2001). Glutaraldehyde cross-linking of collagen and collagen-coated biomaterials has often been used to reduce the in vivo resorption rate and to improve the mechanical properties of the materials, as well as to stabilize collagen gels and films used as a cell-support material for cell cultures in vitro (Sung et al., 1997; Wissink, 1999). The mechanism consists in introducing cross-links between two ε-amino groups of lysine and/or hydroxylysine residues. It has been shown that residual glutaraldehyde in the gels completely inhibits in vitro fibroblast proliferation at concentrations as low as 3 ppm (Speer et al., 1980), which may contribute to obtaining purer cultures of epithelial cells. The synthetic serum substitute, Ultroser G has been used as medium supplement for human nasal epithelial cells in both immersion and air–liquid interface systems (Yamaya et al., 1992; Jorissen and Bessems, 1995a, 1995b; Hoefnagels-Schuermans et al., 1999; Agu et al., 2001, 2002). Human tracheal epithelial cells grown in Ultroser G-supplemented medium have higher probability of becoming electrically tight than cells grown in medium supplemented with serum or different growth factors (Yamaya et al., 1992). It has been shown that the supplementation of the culture medium with cholera toxin improves cell growth and inhibits the differentiation-inducing activity of serum (Lechner et al., 1984; Wu et al., 1985). The frequent (six times a week) changing of the medium at the apical compartment, performed in the CO_2 incubator cultures, and the perfusion system conditions, ensure removal of the metabolic products and supply with fresh nutrients, required for proper cell function. The replacement of the medium could also mechanically stimulate the cilia. All these factors cannot be ignored as possible contributors for the maintenance of stable ciliary activity in the cell culture systems tested.

In conclusion, culturing human nasal epithelial cells on Vitrogen-coated polyethylene terephthalate membranes (pore size 3 μm), at submerged conditions in a CO_2 incubator and a perfusion system offers the possibility for long-term preservation (up to 23–24 days) of stable and reactive ciliary activity in vitro. The possible advantages of the cell culture systems used include its simplicity, reproducibility, and potential for long-term investigations of ciliary function in vitro.

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